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PHARMACOLOGICAL STUDIES OF SOME PSYCHOACTIVE PHENYLALKYLAMINES: ENTACTOGENS, HALLUCINOGENS, AND ANORECTICS

by

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DEDICATION

This thesis is dedicated to:

My parents, *Vincent and Marion Cozzi*,

who nurtured my curiosity and gave me their love and support,

and

Professors *Frank Oliver* and *Alexander Shulgin*,

for inspiration, encouragement, and enthusiasm.

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TABLE OF CONTENTS

Dedication ii

Acknowledgements iii

Table of Contents iv

List of Figures viii

List of Tables x

Abstract xi

Chapter One: Introduction 1

Chapter Two: Methods 6

Chemical Syntheses and Analyses 7

(2,5-Dimethoxyphenyl)-2-nitroethene (1) 10

2-(2,5-Dimethoxyphenyl)-aminoethane (2C-H) 10

2-(4-Chloro-2,5-Dimethoxyphenyl)-aminoethane (2C-C) 12

2-(4-Bromo-2,5-Dimethoxyphenyl)-aminoethane (2C-B) 14

2-(2,5-Dimethoxy-4-iodophenyl)-aminoethane (2C-I) 15

Animals 16

Synaptosome Technique 17

Preparation 17

Static neurotransmitter uptake 18

Superfusion: 5-HT release 20

Brain Slice Technique 22

Preparation 22

Superfusion: GABA and glutamate release 23

Depletion of Endogenous Serotonin 28

Para-chlorophenylalanine (PCPA) 28

Para-chloroamphetamine (PCA) and PCPA 28

High Pressure Liquid Chromatography (HPLC)

Analysis of Serotonin 29

Mobile phase 29

HPLC system 29

Tissue preparation 30

Data analysis 30

Drug Discrimination Studies 31

Materials and apparatus 31

Training protocol 32

Testing protocol 33

Data analysis 33

Statistics 34

Chapter Three: Pharmacological Examination of Benzofuran, Indan, and Tetralin Analogs of (3,4-Methylenedioxy)amphetamine (MDA) 35

Abstract 36

Introduction 37

Pharmacology 41

Results 42

Discussion 45

Chapter Four: 5-HT_{2A} Modulation of Amino Acid Transmitter Release In Cerebral Cortex 50

Abstract 51

Introduction 53

Drugs 60

Pharmacology 60

Results 63

Discussion 76

Chapter Five: Effects of Side-chain Cyclization and 4-Substitution on Neurotransmitter Uptake in Analogs of 2-(2,5-Dimethoxy-4-substituted-phenyl)-aminopropanes . . . 85

Abstract . . . 86

Introduction . . . 88

Pharmacology . . . 92

Results . . . 93

Discussion . . . 97

Chapter Six: Pharmacological Properties of Indan Analogs of Fenfluramine and Norfenfluramine . . . 105

Abstract . . . 106

Introduction . . . 108

Pharmacology . . . 112

Results . . . 115

Discussion . . . 126

Chapter Seven: Conclusion . . . 133

References . . . 139

LIST OF FIGURES

Figure 1. Synthetic Scheme for 2-(2,5-Dimethoxy-4-halophenyl)-aminoethanes 9

Figure 2. Experimental Protocol for Brain Slice Superfusion 25

Figure 3. Method for Calculating S1 and S2 Based on Area Under the Curve 27

Figure 4. Chemical Structures of MDA and Related Compounds 38

Figure 5. 5-BA Inhibition of Synaptosomal [^{3}H]5-HT Accumulation . . 43

Figure 6. Hypothetical Bonding Interactions with Catecholamine Uptake Carrier Proteins 46

Figure 7. Effect of 2C-I on Spontaneous [^{3}H]GABA Release from Rat Cortical Slices 66

Figure 8. K^+-stimulated [^{3}H]GABA Release from Rat Cortical Slices 67

Figure 9. K^+-stimulated [^{3}H]glutamate Release from Rat Cortical Slices 70

Figure 10. HPLC-EC Analysis of 5-HT in Brain Tissue of Control Animals 72

Figure 11. HPLC-EC Analysis of 5-HT in Brain Tissue of PCPA-treated Animals 73

Figure 12. Chemical Structures of Some Phenylisopropylamines and Benzoxepins 91

Figure 13. TFMBOX Inhibition of Synaptosomal [^{3}H]5-HT Accumulation 95

Figure 14. Chemical Structures of Fenfluramine, Norfenfluramine, and Aminoindans 109

Figure 15. Norfenfluramine Inhibition of Synaptosomal [^{3}H]DA Accumulation 118

Figure 16. Effect of FEN on Synaptosomal [^{3}H]5-HT Release 120

Figure 17. Effect of ETAI on Synaptosomal [^{3}H]5-HT Release 121

Figure 18. Effects of FEN, norFEN, ETAI, and TAI on Synaptosomal [^{3}H]5-HT Release 122

Figure 19. Effects of FEN, norFEN, ETAI, and TAI on [^{3}H]Paroxetine Binding 123

Figure 20. Effects of FEN, norFEN, ETAI, and TAI on Body Weight 124

LIST OF TABLES

Table I. IC_{50} For Monoamine Uptake Inhibition. I. 44

Table II. Binding Profiles of Some 5-HT_{2A} Agents 59

Table III. S2/S1 Values for K^+-evoked
[^{3}H]GABA Release: 2C-I 68

Table IV. S2/S1 Values for K^+-evoked
[^{3}H]GABA Release: Antagonists 69

Table V. S2/S1 Values for K^+-evoked [^{3}H]Glutamate Release 71

Table VI. S2/S1 Values for K^+-evoked
[^{3}H]GABA Release: PCPA Pretreatment 74

Table VII. S2/S1 Values for K^+-evoked
[^{3}H]GABA Release: PCA and PCPA Pretreatment 75

Table VIII. IC_{50} for Monoamine Uptake Inhibition. II. 96

Table IX. IC_{50} for Monoamine Uptake Inhibition. III. 119

Table X. Drug Discrimination Data for ETAI and TAI 125

PHARMACOLOGICAL STUDIES OF SOME PSYCHOACTIVE PHENYLALKYLAMINES: ENTACTOGENS, HALLUCINOGENS, AND ANORECTICS

by

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Under the supervision of Professor Thomas A. Rudy

at the University of Wisconsin-Madison

ABSTRACT

The mechanisms of action of three classes of psychoactive drugs—entactogens, hallucinogens, and anorectics—were investigated at the molecular, cellular, and behavioral levels.

The selectivity of analogs of the entactogen (3,4-methylenedioxy)-amphetamine in interacting with monoamine neurotransmitter carrier mechanisms is governed by the position and orientation of the phenyl ring ether-linked oxygen unshared electrons. The catecholamine mechanisms display more rigorous structural requirements than the serotonin mechanism, and this

likely reflects a requirement for hydrogen bonding interactions with the catecholamine carrier proteins.

5-HT_{2A} receptors, a putative site of action of phenylalkylamine hallucinogens, are involved in the regulation of γ-aminobutyrate (GABA) release in the cerebral cortex. In particular, 5-HT_{2A} blockade by antagonists decreases GABA release under depolarizing conditions. However, the 5-HT_{2A}-selective hallucinogen 2-(2,5-dimethoxy-4-iodophenyl)-aminoethane (2C-I) is inert in enhancing cortical GABA release. When animals are depleted of endogenous serotonin, high concentration 2C-I tends to decrease evoked GABA release, and this effect is additive with the decrease produced by the 5-HT_{2A} antagonist ketanserin. This suggests that phenylalkylamine hallucinogens may be partial agonists at cortical 5-HT_{2A} receptors that modulate GABA release.

The conformation of the propylamine side-chain and/or the orientation of the *ortho*-methoxy oxygen unshared electrons in hallucinogenic 2-(2,5-dimethoxy-4-substituted-phenyl)-aminopropanes is more important than the nature of the 4-substituent in controlling interactions with serotonin carrier mechanisms. Thus, the benzoxepin structure, in fixing both the side-chain conformation and the *ortho*-methoxy oxygen unshared electrons' orientation,

abolishes the differential potencies exhibited by various 4-substituted phenylisopropylamine hallucinogens in inhibiting synaptosomal serotonin accumulation.

Indan analogs of the anorectic drugs fenfluramine and norfenfluramine retain the ability to cause the release of serotonin *in vitro* and to decrease body weight but are less effective than fenfluramine and norfenfluramine both in inducing catecholamine release and as neurotoxins. In drug discrimination studies, the indans substitute for training drugs that simultaneously activate serotonin and catecholamine function but not for drugs that activate catecholamine or serotonin function selectively. These results suggest a role for serotonin in body weight control and for catecholamines in fenfluramine-induced neurotoxicity.

Approved:________________________

Thomas A. Rudy, Ph.D.

Professor of Pharmacology

Introduction

Many of the drugs affecting central nervous system monoamine function have molecular structures which contain the phenethylamine pharmacophore. Certain psychostimulant, hallucinogenic, anorectic, and anti-parkinsonism drugs owe their distinct pharmacological properties to particular structural modifications of the phenethylamine skeleton. Substitutions on the benzene ring, the ethylene side chain, and the primary amine result in phenylalkylamines with different affinities for various neuronal receptors, leading to selective electrical or biochemical effects in diverse neuronal populations, and hence to specific psychoactive effects. In addition to modifying receptor affinity, the variable structural elements may also contribute to differential absorption, distribution, metabolism, and excretion of these drugs.

As expected, receptors for the endogenous monoamine neurotransmitters serotonin, dopamine, and norepinephrine are the primary targets for some of these drugs. Other sites of action include monoamine transporter proteins, neurotransmitter storage and release mechanisms, and biosynthetic or metabolic enzymes.

The purpose of this study was to investigate the mechanisms of action of some psychoactive phenylalkylamine drugs at the molecular, cellular, and

behavioral levels. The drugs used in this study share the ability to influence monoamine neurotransmission by one or more different mechanisms: inhibition of neurotransmitter reuptake from the synaptic cleft, induction of neurotransmitter release from presynaptic stores, or by direct action at monoamine receptors. The phenylalkylamines studied represent three psychoactive drug classes: entactogens, hallucinogens, and anorectics.

Entactogens, represented by (3,4-methylenedioxy)amphetamine (MDA), constitute a novel class of psychoactive drugs first defined by Nichols and colleagues.[1-3] The psychopharmacology of entactogens is characterized by feelings of serenity, emotional peace, empathy and enhanced communication with other individuals, and a sense of mental and physical well-being.[4-8] Antidepressant and anxiolytic properties have been reported, and these drugs are claimed to have potential use in psychotherapy.[8-10] Entactogens appear to produce their psychoactive effects primarily through drug-induced release and inhibition of reuptake of endogenous neurotransmitters, especially serotonin and catecholamines, rather than through direct receptor actions.[11-15] Chapter Three describes molecular-level studies using synaptosomes to investigate some of the structural features of entactogens that govern interactions with monoamine neurons.

Hallucinogens, for example 2-(2,5-dimethoxy-4-iodophenyl)-aminopropane (DOI), produce significant changes in perception, interpretation, cognition, and emotion. While retaining full consciousness, the subject experiences a kind of dream world, with sensory illusions, synesthesia, conscious awareness of subconscious material, and distortions of time and space.[16-19] The psychoactive properties of these drugs appear to depend upon direct 5-HT_{2A} receptor[20] activation, but may also involve other receptor types and drug-induced release of endogenous neurotransmitter.[21-24] Chapters Four and Five describe molecular- and cellular-level experiments using synaptosomes and brain slices to investigate hallucinogenic phenylalkylamine drug effects on amino acid and monoamine neurotransmitter function.

Anorectic phenylalkylamine drugs such as fenfluramine decrease feelings of hunger and increase feelings of satiety, and are therefore useful in the clinical treatment of obesity.[25-27] These drugs help patients maintain the decreased food intake required in a weight loss program. Fenfluramine is thought to exert its anorectic effects by causing an increase in the synaptic concentration of serotonin, since the drug is both a serotonin reuptake inhibitor and a serotonin releaser.[28-30] Fenfluramine is also potentially neurotoxic to serotonergic axons, and Chapter Six describes molecular,

cellular, and whole animal studies with indan analogs of fenfluramine which retain fenfluramine's ability to increase serotonergic function and reduce body weight, but which appear to have reduced neurotoxic potential.

To accomplish these studies, several novel substituted phenylalkylamines representing entactogens, hallucinogens, and anorectics were synthesized and tested for their effects on neurotransmitter uptake and release *in vitro* and for their behavioral properties in conscious animals.

Methods

Chemical Syntheses and Analyses

Several 2,5-dimethoxy-4-halophenethylamines, putative agonist ligands for the 5-HT_{2A} receptor, were synthesized as shown in Figure 1. These compounds were analyzed by melting point determination, proton nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, and high pressure liquid chromatography (HPLC). Melting points were determined on a Mettler TA 3000 thermal analysis system with DSC 20 cell. ^{1}H NMR spectra were recorded on either a Varian EM 360 (60 MHz) or Bruker AM 300 (300 MHz) instrument in $CDCl_3$ with 0.03% tetramethylsilane (TMS) as internal standard; chemical shifts are reported in parts per million (δ) relative to TMS. The following abbreviations are used to designate NMR signal patterns: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet. Electron impact mass spectra (EIMS) were determined on a Finnigan 4000 mass spectrometer at 40 eV, and are reported as *m/z* (relative abundance). HPLC was used to verify the purity of the compounds using the following system:

Mobile phase: The mobile phase consisted of HPLC grade MeOH (Baker) and H_2O (35:65) containing 0.1% triethylamine (Aldrich), pH adjusted to 7.5

with AcOH.

HPLC system: The HPLC system consisted of a reverse-phase Ultrasphere octadecylsilane analytical cartridge column (4.6 mm x 150 mm, 5 μm particle size; Beckman) supplied with mobile phase at 1.0 mL/min by a solvent delivery module (Beckman model 110B) and a UV (254 nm) absorbance detector (Beckman model 160) with data output to a chart recorder (Kipp & Zonen BD40). Samples were injected with an Altex 210A injection valve (20 μL; Beckman).

All compounds eluted as single peaks; retention times are listed as HPLC: R_t.

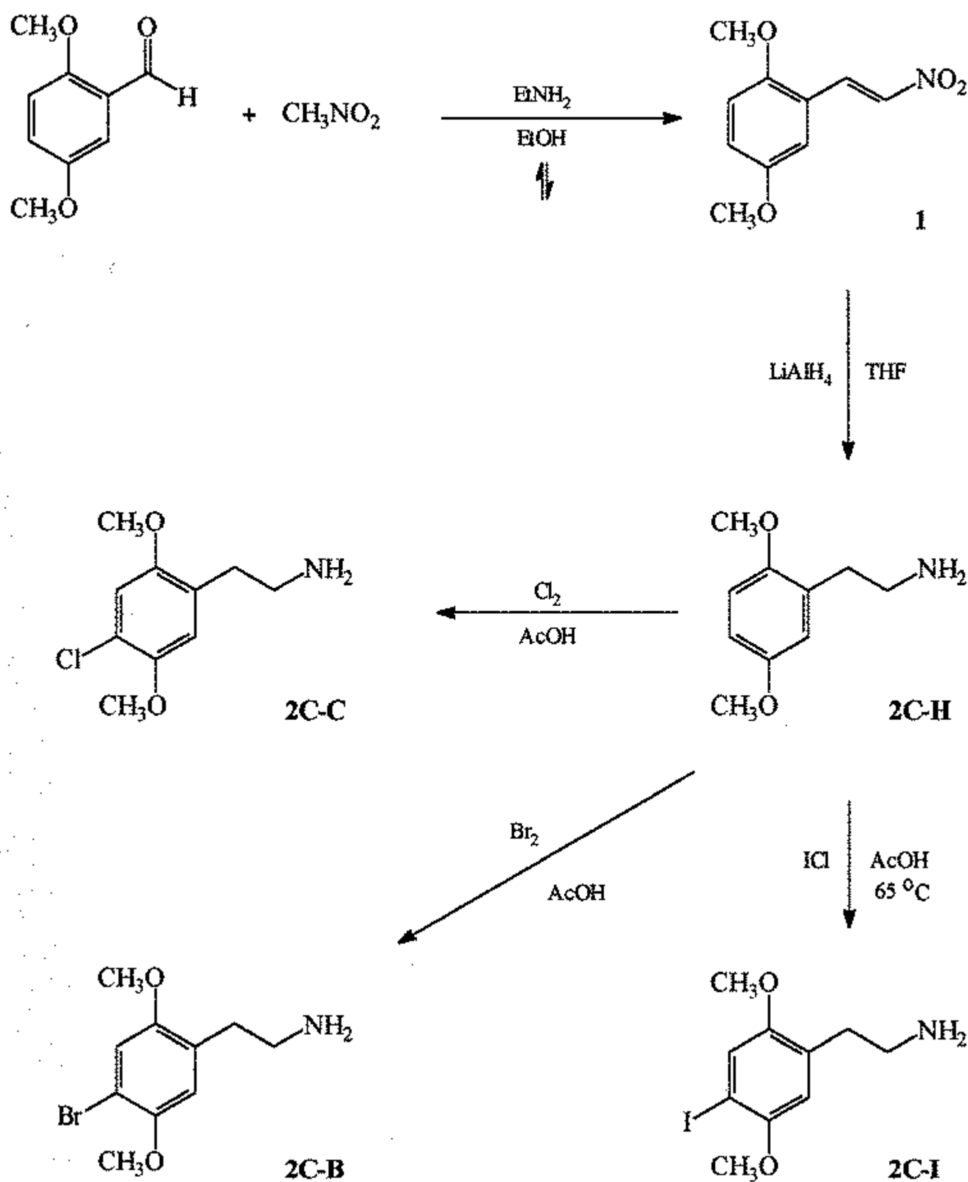


Figure 1. Synthetic Scheme for 2-(2,5-dimethoxy-4-halophenyl)-amino-ethanes.

(2,5-DIMETHOXYPHENYL)-2-NITROETHENE (1). A mixture of 2,5-dimethoxybenzaldehyde (100.0 g, 600 mmol) dissolved in EtOH (71 mL) and nitromethane (34.0 mL, 630 mmol) was added to a 500 mL roundbottom flask equipped with an electric heating mantle and a reflux condenser. A catalytic amount (5 mL) of 70% ethylamine was added and the wine-red mixture was heated to reflux. The mixture was refluxed for 1 h, then allowed to stand overnight. 25 mL EtOH was added and the mixture was again refluxed for 1 h, then allowed to cool. Upon standing, bright yellow solids precipitated. The solids were vacuum filtered and washed liberally with MeOH. The filter cake was then slurried with EtOH, with heating and stirring. The slurry was vacuum filtered, washed with EtOH, and the bright yellow crystals of (1) were allowed to air-dry. Yield: 52.1 g (41.4%), mp = 118.8 °C (lit.[31] mp = 118-119 °C). ^{1}H NMR (60 MHz): δ 8.20 (d, 1H, vinylic proton), 7.85 (d, 1H, vinylic proton), 7.00 (s, 3H, ArH), 3.90 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3).

2-(2,5-DIMETHOXYPHENYL)-AMINOETHANE (2C-H). A single neck 1 L roundbottom flask was fitted with a Claisen adapter which carried a 125 mL pressure-equalizing dropping funnel and a reflux condenser with a

$CaCO_3$ drying tube at the top. The roundbottom flask was placed in an electric heating mantle with a magnetic stirrer. The flask was charged with 100 mL tetrahydrofuran (THF), followed by lithium tetrahydridoaluminate (LAH) (25 g, 660 mmol), then an additional 170 mL THF. Nitroalkene (1) (51.7 g, 247 mmol) was dissolved in 400 mL THF with stirring, and the solution of the nitroalkene in THF was added dropwise to the LAH/THF solution at a rate sufficient to maintain reflux. The addition was completed in 70 min. 100 mL THF was added to the reaction mixture and heat was applied to maintain reflux for 3 h. The reaction mixture was allowed to stir at room temperature for 48 h, then 25 mL H_2O was cautiously added, followed by 25 mL 15% NaOH, and finally 75 mL H_2O. The white granular precipitate was removed by vacuum filtration, and the golden filtrate was concentrated by rotary evaporation. After the THF was stripped off, ca. 50 mL brown oily residue remained. The residue was vacuum fractionated; the product amine 2C-H distilled at 152-153 °C at aspirator pressure (lit.[31] bp = 110-120 °C, 0.05 mm Hg). Yield: 32.8 g (73.2%) of clear, colorless oil. ^1H NMR (300 MHz): δ 6.73 (m, 3H, ArH), 3.77 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 2.92 (t, 2H, CH_2), 2.73 (t, 2H, CH_2), 1.23 (br s, exchanges with D_2O, 2H, NH_2). The hydrochloride salt of 2C-H was prepared by dissolving

2.01 g of the free base in 25 mL *i*-PrOH and then acidifying with 12N HCl to pH ~ 2.5. The solution was then flooded with 175 mL Et_2O; white opalescent crystals appeared immediately. The crystals were collected by vacuum filtration and recrystallized from EtOH/Et_2O. After standing overnight, the 2C-H hydrochloride crystals were collected by vacuum filtration and washed with 3 x 25 mL Et_2O, then allowed to air-dry. Yield: 2.11 g (87.4%), mp = 139.0 °C (lit.[32] mp = 139.0 °C). EIMS: *m/z* 181 (58.79), 152 (100.00), 137 (100.00), 121 (87.77). HPLC: R_t = 7.6 min.

2-(4-CHLORO-2,5-DIMETHOXYPHENYL)-AMINOETHANE (2C-C).

Chlorination of 2C-H at the 4-position was effected by an adaptation of the procedure of Aldous, *et al.*[33] Cl_2, generated by cautious addition of 12N HCl to 5.25% aqueous NaClO solution, was bubbled into 30 mL AcOH with stirring until 1.57 g (22.1 mmol) was taken up. The free base of 2C-H (4.01 g, 22.1 mmol) was dissolved in 20 mL AcOH, then the Cl_2/AcOH solution was added over an 8 min period, with stirring. The reaction mixture was stirred for 3 h at room temperature, then about half of the AcOH was stripped off with aspirator pressure. The mixture was allowed to stand overnight; white crystals precipitated. The crystals were collected by vacuum

filtration, and the mother liquor was concentrated (heat, vacuum) to produce a dark brown residue which solidified on cooling. The white crystals and brown solids were dissolved in 100 mL 10% $Na_2S_2O_4$ solution to remove residual chlorine. This solution was made basic (pH ~ 14.0) with concentrated NaOH and extracted with 3 x 40 mL Et_2O; the aqueous phase was discarded. The combined ether extracts were dried by shaking with saturated NaCl solution, then the ether was evaporated under a stream of dry N_2 to leave a dark brown oil. The hydrochloride salt of 2C-C was prepared as described for 2C-H. The crystals had a pink color which could not be removed by repeated recrystallizations from MeOH/Et_2O and from EtOH/Et_2O. The pink color was removed by dissolving the crystals in 1N NaOH, extracting with Et_2O, washing the ether extracts with saturated NaCl solution, and evaporating the ether under a stream of dry N_2 to leave a colorless oil. ^1H NMR (300 MHz) of the oil: δ 6.88 (s, 1H, ArH), 6.77 (s, 1H, ArH), 3.85 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 2.91 (t, 2H, CH_2), 2.73 (t, 2H, CH_2), 1.17 (br s, exchanges with D_2O, 2H, NH_2). White fibrous crystals were precipitated by acidifying the oil in *i*-PrOH with 12N HCl to pH ~ 2.0, then flooding with ether. Yield: 2.16 g (38.8%), mp = 222.1 °C (lit.[31] mp = 220-221 °C). EIMS: *m/z* 215 (4.28), 188 (21.51), 186 (69.82), 173 (8.68), 171 (29.34) 157

(4.68), 155 (12.57). HPLC: R_t = 14.0 min.

2-(4-BROMO-2,5-DIMETHOXYPHENYL)-AMINOETHANE (2C-B). 2C-B was synthesized according to the method of Shulgin and Carter.[34] The free base of 2C-H (4.00 g, 22.1 mmol) was dissolved in 20 mL AcOH. To this stirred solution was added a solution of Br_2 (3.53 g, 22.1 mmol) in 25 mL AcOH over a period of 7 min. The orange-red solution became yellow after 15 min and a voluminous precipitate formed; 75 mL AcOH was added to the reaction mixture to facilitate stirring. After stirring overnight, the reaction mixture became light tan-colored. The solids were collected by vacuum filtration and the filter cake was washed with 50 mL AcOH followed by 4 x 25 mL Et_2O. The filter cake was dissolved in 50 mL H_2O and made basic (pH ~ 14.0) with concentrated NaOH to generate the free base as a light pink oil. The aqueous layer was extracted with 4 x 30 mL ether and discarded. The combined ether extracts were dried with saturated NaCl washes, then the ether was evaporated under a stream of dry N_2 to leave a viscous residue. The amine hydrochloride of 2C-B was prepared as described for 2C-H. The *i*-PrOH/Et_2O solution with suspended 2C-B hydrochloride crystals was stirred for 2 h, then vacuum filtered. The filter cake was washed with 3 x 25 mL

Et_2O and vacuumed to dryness. Yield: 3.83 g (58.5%), mp = 237.5 °C (lit.[34] mp = 237-239 °C). ^1H NMR (300 MHz) of the free base: δ 7.03 (s, 1H, ArH), 6.75 (s, 1H, ArH), 3.85 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 2.91 (t, 2H, CH_2), 2.72 (t, 2H, CH_2), 1.25 (br s, exchanges with D_2O, 2H, NH_2). EIMS: *m/z* 261 (7.49), 259 (8.76), 232 (91.68), 230 (100.00), 217 (27.09), 215 (29.84), 201 (12.25), 199 (12.50). HPLC: R_t = 18.4 min.

2-(2,5-DIMETHOXY-4-IODOPHENYL)-AMINOETHANE (2C-I). 2C-H free base (2.57 g, 14.2 mmol) was dissolved in 40 mL AcOH. The 2C-H/AcOH solution was heated to 65 °C and then a solution of ICl (2.30 g, 14.2 mmol) in 10 mL AcOH was added in one portion. The reaction mixture was stirred for 15 min at 65 °C, then quenched by pouring it into 100 mL 20% $Na_2S_2O_4$ solution. The lemon-yellow mixture was made basic (pH ~ 14.0) with concentrated NaOH to generate the amine free base as a purple-brown oil. After cooling, the mixture was extracted with 4 x 50 mL ether and the aqueous layer was discarded. The combined ether extracts were dried with saturated NaCl solution washes, then the ether was evaporated under a stream of dry N_2 to leave an olive-colored oil. This oil was taken up in 50 mL *i*-PrOH and acidified (pH ~ 2.0) with 12N HCl. The 2C-I hydro-

chloride salt precipitated from the *i*-PrOH and was collected by vacuum filtration. For recrystallization, the filter cake was dissolved in boiling EtOH/*i*-PrOH (1:1); upon cooling, white fibrous crystals formed. The crystals were collected by vacuum filtration, washed with 3 x 25 mL Et_2O, and allowed to air-dry. Yield: 0.87 g (17.9%), mp = 245.8 °C (lit.[35] mp = 246-247 °C). ^1H NMR (300 MHz) of the free base: δ 7.22 (s, 1H, ArH), 6.67 (s, 1H, ArH), 3.83 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 2.91 (t, 2H, CH_2), 2.72 (t, 2H, CH_2), 1.30 (br s, exchanges with D_2O, 2H, NH_2). EIMS: *m/z* 307 (30.93), 278 (100.00), 263 (46.86), 247 (20.46), 232 (7.70). HPLC: R_t = 28.0 min.

Animals

Male Sprague Dawley rats (175-200 g initial weight; 46-49 days old) were obtained from Harlan Labs, Indianapolis, IN. The animals for *in vitro* experiments were group-housed under a 12 h light/dark schedule and received food (Lab Blox, Purina) and water *ad libitum*. Animals that were used for drug discrimination experiments were individually caged and received an amount of food sufficient to maintain them at approximately 80%

of their free feeding weight; water was available *ad libitum*.

Synaptosome Technique

Preparation. Crude synaptosomes were prepared according to the method of Gray and Whittaker,[36] with slight modifications. For static neurotransmitter uptake experiments, three rats were decapitated and their brains rapidly removed and dissected over ice. The cerebellums were removed and discarded and the remaining brain tissue (ca. 4 g, wet weight) was pooled, diced, and homogenized in 20 mL of ice-cold 0.32 M sucrose. Homogenizations were done in a prechilled Potter-Elvehjem tissue grinder with a motor-driven Teflon pestle at 0 °C, for two periods of 1 min each, 6 strokes/min, with a 15 s interval between periods. The tissue homogenate was poured into a plastic centrifuge tube and the tissue grinder was rinsed out with an additional 5 mL ice-cold isotonic sucrose, which was also added to the centrifuge tube. This mixture was subjected to centrifugation (Beckman J2-21 with JA-20 rotor; 4 °C) at 1090 x g for 10 min. The pellet was discarded and the supernatant was subjected to centrifugation at 17,400 x g for 30 min. The synaptosomal (P_2) pellet was resuspended with a polytron

(setting 5, 20 s; Kinematica) in 30-40 mL ice-cold, aerated (5% CO_2 in O_2) modified Krebs-Ringer bicarbonate (KR) buffer containing (mM): NaCl (124.3), KCl (2.95), $MgSO_4$ (1.30), KH_2PO_4 (1.25), $NaHCO_3$ (26.0), $CaCl_2$ (2.41), *d*-glucose (10.4), and Na ascorbate (0.06); pH 7.4-7.6. The synaptosomal suspension was stored on ice until use. For superfusion experiments, synaptosomes were prepared as described, except only one rat was used for each experiment, and the P_2 pellet was resuspended in 10 mL aerated (5% CO_2 in O_2) 37 °C KR buffer containing 100 μM pargyline HCl (Sigma) to block the metabolism of neurotransmitter and test drugs by monoamine oxidase.

Static neurotransmitter uptake. In experiments designed to test the interaction of drugs with monoamine carrier mechanisms, the ability of synaptosomes to accumulate tritiated serotonin (5-HT), dopamine (DA), and norepinephrine (NE) was measured in the absence and presence of various concentrations of test drugs as follows: a 200 μL aliquot of the synaptosomal suspension was added to test tubes containing 1.65 mL ice-cold KR buffer, 50 μL test drugs (dissolved in deionized water) or deionized water (for total and nonspecific determinations), and 50 μL pargyline HCl solution (final

concentration, 100 μM). The test tubes were preincubated in an aerated (5% CO_2 in O_2) 37 °C shaking water bath for 5 min. The tubes were then returned to the ice bath and chilled for 10-15 min. Tritiated neurotransmitter (New England Nuclear) was added (50 μL of stock solution; final concentration, 10 nM), giving a final incubation volume of 2 mL. All tubes except nonspecific tubes were returned to the aerated 37 °C shaking water bath for 5 min to initiate neurotransmitter uptake. Uptake was terminated by chilling the test tubes in an ice bath, then rapidly filtering them through glass fiber filters (Whatman GF/C) using a 24-well cell harvester (Brandel). Filters were washed with 2 x 3 mL ice-cold KR buffer, allowed to air dry for 10 min, and then placed in plastic liquid scintillation vials. Scintillation cocktail (10 mL Ecolite; ICN Biomedicals) was added, and the vials were sealed, vortexed, and allowed to stand overnight. Radioactivity was measured using liquid scintillation spectroscopy (Packard model 4430, 40% efficiency). Specific uptake was defined as uptake at 37 °C minus uptake at 0 °C, in the absence of drugs. Under these conditions, specific uptake represented greater than 74% of the total uptake of 5-HT, greater than 91% of the total uptake of DA, and greater than 85% of the total uptake of NE.

Superfusion: 5-HT release. In some experiments, synaptosomes were superfused with KR buffer in the absence and presence of various concentrations of test drugs to determine whether the drugs interfered with tritiated 5-HT accumulation by blocking neurotransmitter uptake, or whether they stimulated release of neurotransmitter. Since released neurotransmitter is continually swept away in a superfusion experiment, reuptake is minimized and any increase in radioactivity in the superfusate can be ascribed to evoked release, not uptake inhibition. A 10 mL P_2 suspension containing 100 μM pargyline was prepared as described above. [^{3}H]5-HT (1 μCi/mL, final concentration ca. 25 nM; New England Nuclear) was added to the synaptosomal suspension which was then incubated and aerated at 37 °C for 20 min to allow neurotransmitter uptake. After the labeling incubation, 250 μL aliquots of the synaptosomal suspension were transferred to each of twelve superfusion chambers of a superfusion apparatus (Brandel model SF12). The superfusion apparatus allows the synaptosomes to be continuously superfused with buffer supplied by a peristaltic pump and permits the collection of superfusate from each chamber. A glass fiber filter at the entrance and exit of each superfusion chamber prevents efflux of the synaptosomes but allows the superfusion buffer to flow through unimpeded. A solid-state temperature con-

troller maintains the superfusion buffer at 37 °C, and gas delivery probes allow the buffer to be continuously aerated. The synaptosomes were superfused with KR buffer (0.5 mL/min) for a 20 min washout period after the labeling incubation with [^{3}H]5-HT to achieve a basal level of spontaneous neurotransmitter release. Following the washout period, 10 serial 2 min superfusate fractions were collected directly into plastic scintillation vials. At the end of the experiment, the glass fiber filters containing the synaptosomes were also placed into scintillation vials containing 1.0 mL KR buffer. Scintillation cocktail (5 mL Ecolite; ICN Biomedicals) was added to all the vials, and the vials were sealed and vortexed. Radioactivity was measured using liquid scintillation spectroscopy (Packard model 4430, 40% efficiency). To test the effect of drugs on spontaneous release, various concentrations of drugs were introduced during fractions 3 and 4. The amount of tritium released was compared to the amount released in the absence of drugs and is expressed as percent released. Percent released for any fraction is calculated by dividing the amount of tritium released during that fraction by the total synaptosomal tritium present at the start of that fraction collection period and multiplying by 100. The synaptosomal tritium present at the start of a collection period is the sum of the tritium released during that collection

period, all subsequent collection periods, and the glass fiber filter tritium content at the end of the experiment.

Brain Slice Technique

Preparation. Following decapitation, the rat brain was rapidly removed and dissected over ice. A coronal cut through both hemispheres was made caudal to the neostriatum, and the hemispheres were separated by a midline sagittal cut. The frontal cortex was then trimmed of striatal, thalamic, and piriform cortex tissue. During the dissection, the tissue was regularly moistened with ice-cold, aerated KR buffer identical to that described previously, except sodium ascorbate was omitted from the buffers used in brain slice superfusion experiments. A McIlwain tissue chopper was used to prepare 10-15 350 μm coronal cortical slices from each hemisphere, beginning at the level of the caudal border of the neostriatum and proceeding rostrally to the prefrontal area. The cortical slices were then transferred to a holding bottle containing 100 mL aerated 37 °C KR buffer for a 1 h preincubation. The time from decapitation to incubation in warm buffer was approximately 5 min.

Superfusion: GABA and glutamate release. The GABA and glutamate neurotransmitter pools in the brain slices were labeled with exogenous [^{3}H]GABA or [^{3}H]glutamate (New England Nuclear) prior to the release experiments. Eighteen intact slices were selected from the preincubation bottle on the basis of visual appearance and were transferred to an incubation buffer containing 30 mL aerated 37 °C KR buffer and [^{3}H]GABA or [^{3}H]glutamate (1 μCi/mL, final concentration ca. 25 nM, along with 1 μM unlabeled neurotransmitter as a carrier) for a 30 min incubation period. In experiments in which endogenous serotonin was depleted by pretreatment with *para*-chlorophenylalanine, the slices that were not selected for superfusion were retained for HPLC analysis of serotonin content. These slices were placed in 1.5 mL Eppendorf tubes, weighed, and quickly frozen in liquid N_2. The frozen slices were then stored at -70 °C until HPLC analysis. For slices that were selected for superfusion, carboxy-methoxylamine (10 μM; Aldrich), a transaminase inhibitor, was present in the incubation buffer and all subsequent control and drug buffers to block the metabolism of the labeled neurotransmitters.[37,38] After the 30 min labeling incubation, 12 intact slices were transferred to each of 12 superfusion chambers in the superfusion apparatus described previously for a 30 min

washout period with buffer (0.5 mL/min) to achieve a basal level of spontaneous neurotransmitter release.

For spontaneous release control runs, 10 (for GABA release) or 15 (for glutamate release) serial 2-min fractions were collected after the 30 min washout period. To test the effect of agonist drugs on spontaneous GABA release, five spontaneous release fractions were collected (after washout), and then various concentrations of agonist were introduced into the buffer flow line and five additional release fractions were collected. When antagonists were tested, the drugs were introduced after four spontaneous release fractions were collected, so they were present prior to the introduction of agonists. In experiments involving glutamate release, the agonist was introduced at the beginning of fraction six, and nine additional release fractions were collected. The antagonist, when present, was introduced at the beginning of fraction four. Once drugs were introduced, they remained present for the remainder of all experiments. The experimental protocol is summarized in Figure 2. The amount of tritium released in the presence of drugs is compared to the amount released during control runs and is expressed as percent released, as previously described.

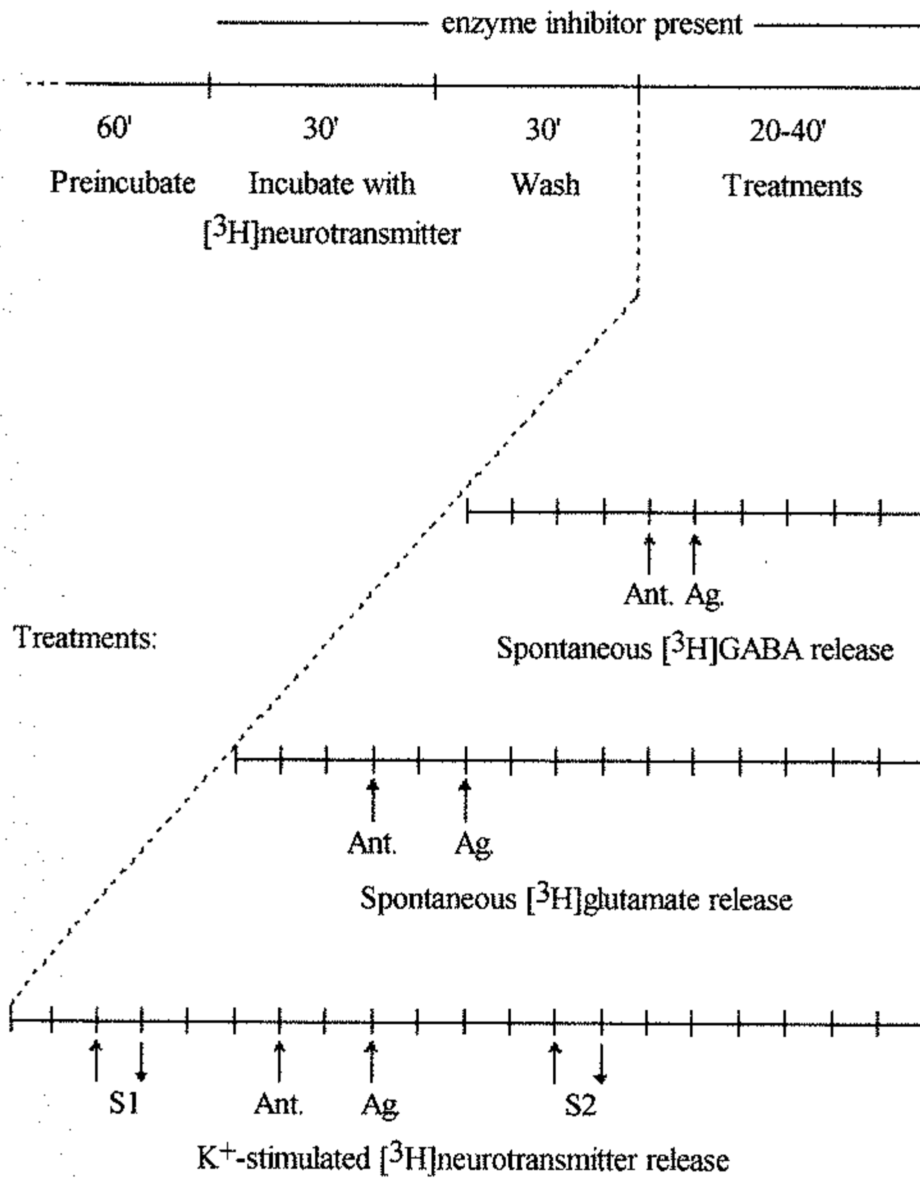


Figure 2. Experimental Protocol for Brain Slice Superfusion.

Drug effects were also determined under K^+-evoked release conditions. For K^+-stimulated release control runs, 20 2-min fractions were collected after the 30 min washout period. During fractions 3 and 13 (S1 and S2), 40 mM (for evoked GABA and glutamate release) or 55 mM (for evoked glutamate release only) K^+ KR buffer (composition identical to regular KR except KCl was increased to 40 mM or 55 mM and NaCl was reduced to 87 mM or 72 mM, respectively, to maintain iso-osmicity) was introduced into the buffer flow line (see Figure 2). The amount of evoked neurotransmitter release (over baseline) was calculated as the area under the stimulation curve (AUC) from fractions 3-8 for S1 and from fractions 13-18 for S2 (illustrated for GABA release, Figure 3). Baseline was defined as the least-squares regression line through fractions 1, 2, 9, 10, 11, 12, 19, and 20. To test the effects of drugs on K^+-stimulated release, the agonist was introduced to the slices at the beginning of fraction 9 and was present continuously throughout the rest of the experiment. Antagonists, when tested, were introduced at the beginning of fraction 7 and were present throughout the rest of the experiment (see Figure 2). To quantify drug effects, the S2/S1 ratio (AUC for fractions 13-18/AUC for fractions 3-8) was calculated and compared to the (control) ratio in the absence of drugs.

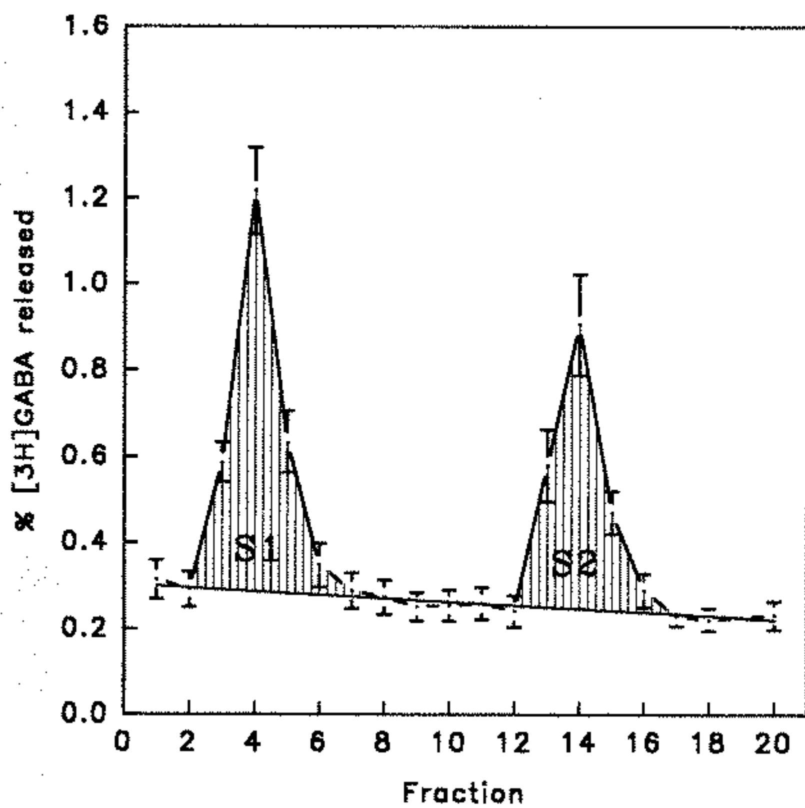


Figure 3. **Method for Calculating S1 and S2 Based on Area Under the Curve.**

Depletion of Endogenous Serotonin

Para-chlorophenylalanine (PCPA). Some rats were treated with the tryptophan hydroxylase inhibitor PCPA to deplete endogenous stores of serotonin.[39-40] PCPA methyl ester HCl (Sigma) was dissolved in 0.9% saline to a final concentration of 80 mg/mL. Rats were given two intraperitoneal injections of 200 mg/kg PCPA 12 h apart, for a total dose of 400 mg/kg. The animals were sacrificed 48 h after the last dose, and their brain tissue was used to prepare brain slices for superfusion experiments as previously described, and for analysis of serotonin content as described under High Pressure Liquid Chromatography (HPLC) Analysis of Serotonin.

Para-chloroamphetamine (PCA) and PCPA. In some serotonin depletion experiments, animals were treated with both PCPA and the serotonergic neurotoxin PCA.[40-42] This was to insure that serotonin biosynthesis was inhibited and to deplete vesicular and nonvesicular stores of endogenous serotonin. PCPA was prepared as described above. PCA HCl (synthesized locally) was dissolved in 0.9% saline to a final concentration of 10 mg/mL. Rats were treated with two intraperitoneal injections of 10 mg/kg PCA 24 h

apart, for a total dose of 20 mg/kg. Approximately 6.5 days after the last dose of PCA, the rats were treated with 2 x 200 mg/kg PCPA 12 h apart for a total dose of 400 mg/kg PCPA. These animals were sacrificed 48 h after the last dose of PCPA, and their brain tissue was used for brain slice superfusion experiments as previously described.

High Pressure Liquid Chromatography (HPLC) Analysis of Serotonin

Mobile phase. The mobile phase consisted of (mM): NaH_2PO_4 (50), citric acid (30), disodium ethylenediaminetetraacetate (0.1), sodium octyl sulfate (1.46), and CH_3OH, 25% v/v; pH = 2.75.

HPLC system. The HPLC system consisted of a refrigerated autosampler (TosoHaas model TSK-6080), a reverse-phase Brownlee octadecylsilane analytical cartridge column (4.6 mm x 250 mm, 5 μm particle size; Anspec) supplied with mobile phase by a Rainin pump at a flow rate of 0.7 mL/min, and an electrochemical detector (EG & G Princeton Applied Research Corporation model 400) with a dual electrode potential set at E_1 = -200 mV and E_2 = 850 mV against a Ag/AgCl reference electrode. Automatic sample injection and data acquisition were controlled with an Apple Macintosh SE

computer running commercial software (Dynamax Method Manager; Rainin).

Tissue preparation. Frozen, weighed brain tissue, obtained from rats pretreated with *para*-chlorophenylalanine, was thawed and homogenized in 5 μL/mg HPLC mobile phase (minus sodium octyl sulfate) in 1.5 mL Eppendorf tubes with a motor-driven Teflon pestle at 0 °C. The homogenate was then centrifuged with a table-top centrifuge (American Scientific Products Biofuge A) at 13,000 rpm for 5 min. 50 μL aliquots of the supernatant were used for determination of serotonin content by HPLC with electrochemical detection (HPLC-EC).

Data analysis. 5-HT standard solutions (25, 50, 100 ng/mL) were prepared from a stock solution of serotonin creatinine sulfate (Aldrich) in HPLC mobile phase diluted to the appropriate concentration with additional mobile phase. The 5-HT standards were then analyzed by HPLC-EC and standard curves were generated by fitting the 5-HT peak areas (μV-sec) to a least-squares regression line. The derived regression equation was used to calculate 5-HT concentrations in tissue samples from saline-treated and PCPA-treated animals. The identity of the 5-HT peak in tissue extracts was

verified by spiking the samples with authentic 5-HT.

Drug Discrimination Studies

Materials and apparatus. Using the two-lever drug discrimination assay, animals can be trained to distinguish between injections of a psychoactive drug and saline.[43-45] The protocol for this method is as follows: four groups of rats (10-15 rats per group) were trained to discriminate four different psychoactive training drugs from 0.9% saline. Training drugs were (*S*)-*N*-methyl-1-(1,3-benzodioxol-5-yl)-2-aminobutane hydrochloride (*S*-MBDB, 1.75 mg/kg), 5-methoxy-6-methyl-2-aminoindan hydrochloride (MMAI, 1.71 mg/kg), (*S*)-amphetamine sulfate (AMP, 1 mg/kg), or *d*-lysergic acid diethylamide tartrate (LSD, 0.08 mg/kg). *S*-MBDB and MMAI were synthesized in Dr. David Nichols' laboratories at Purdue University. AMP was purchased from Smith, Kline & French Laboratories, Philadelphia, PA and LSD was obtained from the National Institute on Drug Abuse, Washington, DC. Six standard operant chambers with white noise sound masking (Coulbourn Instruments), enclosed in ventilated, sound-attenuated cubicles were used for drug discrimination training and testing. The

chambers contained a white light and a ventilating fan, and were equipped with two response levers separated by a food pellet (Bioserve 45 mg dustless pellets) delivery system; the levers and food delivery system were located 2.5 cm above the chamber floor. The chambers and levers were cleaned with a 10% ethanol solution between individual training or testing sessions to remove olfactory cues.[46] An 80486 central processing unit-based computer controlled reinforcement and data acquisition through digital-to-analog and analog-to-digital interfaces (Med Associates).

Training protocol. All animals were drug naive prior to training. All drugs were dissolved in 0.9% saline and were administered intraperitoneally in a volume of 1.0 mL/kg, 30 min prior to the start of a training session. A fixed ratio 50 schedule of food reinforcement in a two lever paradigm was used. To avoid positional preference, half of the rats were trained on drug-left, saline-right and the other half were trained on drug-right, saline-left. Training sessions (15 min) were conducted at the same time each day, and were continued until rats achieved an accuracy of 85% correct lever responses for eight of ten consecutive sessions.

<u>Testing protocol.</u> Test drug treatments were randomized among each group of rats tested. Test drugs were dissolved in 0.9% saline and were administered intraperitoneally in a volume of 1.0 mL/kg, 30 min prior to the start of a testing session. Test sessions were run once or twice a week, and were separated by at least one drug and one saline training session. In order to receive a test drug, animals were required to maintain the 85% correct response criterion on training days. Test data (less than 5%) were discarded if rats failed to attain 85% correct responses on the two training sessions following a test session. Test sessions were run under conditions of extinction; rats were removed from the operant chambers when either 50 presses were completed on one lever, or when 5 min had elapsed. If a rat did not produce 50 presses on either lever in 5 min, the test session was ended and the rat was scored as disrupted; these data were not used for subsequent calculations.

<u>Data analysis.</u> The lever upon which a rat first completed 50 responses during a test session was scored as the "selected" lever. The percentage of rats selecting the drug lever (SDL) was determined for each dose of test compound, and the degree to which a test drug substituted for a training drug

was determined by the maximum percentage of rats selecting the drug lever over all doses of the test drug. Thus, "no substitution" (NS) is defined as 50% or less SDL, "partial substitution" (PS) is 51-79% SDL, and "full substitution" (FS) is 80% or more SDL. If a dose of the test drug produced FS, the ED_{50} was calculated from quantal dose-response curves according to the method of Litchfield and Wilcoxon,[47] as incorporated in the computer program Pharmacologic Calculation System Version 4.[48] If 50% or more of the test animals were scored as disrupted, then no ED_{50} was calculated, regardless of the % SDL in non-disrupted animals.

Statistics

All data are expressed as the mean ± SEM, except drug discrimination data, which are expressed as the degree of substitution (defined above) and the ED_{50} value with 95% confidence limits. Multiple mean comparisons were made using one-way ANOVA, followed by a Student, Tukey-Kramer, Student-Newman-Keuls, or Dunnett *t* test, as appropriate, using the computer program INSTAT Version 2.0.[49]

Pharmacological Examination of Benzofuran, Indan, and Tetralin Analogs of (3,4-Methylenedioxy)amphetamine (MDA)

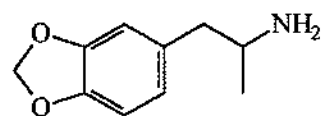
Abstract

Benzofuran, indan, and tetrahydronaphthalene analogs of (3,4-methylenedioxy)amphetamine (MDA) were prepared in order to examine the role of the dioxole ring oxygen atoms of MDA in interacting with the serotonin and catecholamine uptake carriers *in vitro*. The series of compounds was evaluated for the ability to inhibit uptake of [^{3}H]serotonin, [^{3}H]dopamine, and [^{3}H]norepinephrine into crude synaptosome preparations. The results of these studies indicate that selectivity for serotonin versus catecholamine uptake carriers may be modulated by the position and orientation of the ring oxygen atoms. However, the nonoxygenated indan isostere possessed high potency at all uptake sites examined. Enlargement of the saturated ring by one methylene unit to give the tetralin derivative resulted in a large (3- to 4-fold) reduction in activity at catecholamine sites.

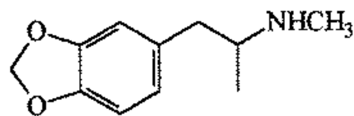
Introduction

In recent years, there has been much interest in compounds related to (3,4-methylenedioxy)amphetamine (MDA) and (3,4-methylenedioxy)methamphetamine (MDMA) (Figure 4). MDMA became popular as a recreational drug known as "ecstasy" and has human psychopharmacology and animal pharmacology that differs significantly from the pharmacology of psychostimulants such as amphetamine, or hallucinogens such as *d*-lysergic acid diethylamide (LSD) or the methoxylated amphetamines.[1-3,44,50-53] In particular, users of MDA and MDMA have reported that these substances induce greatly enhanced feelings of "closeness" and communication with other people, as well as a sense of calmness and physical and mental well-being.[4-8,54] It was proposed that MDMA and similar compounds belong to a new pharmacological class, named entactogens.[1-3]

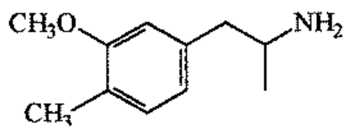
There have been reports by a number of psychiatrists that MDMA has potential as an adjunct to psychotherapy.[8-10] Clinical evaluation of this claim was hindered, however, by reports that MDMA produced a loss of serotonergic axons and terminals in rodent[55,56] and nonhuman primate brain.[57,58] Therefore, efforts were directed toward clarifying the neuropharmacology



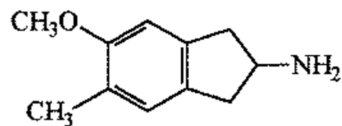
MDA



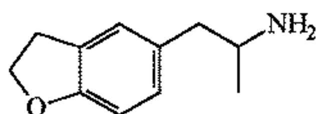
MDMA



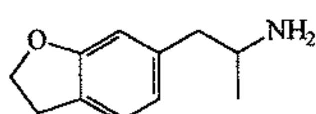
MMA



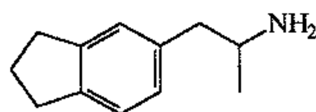
MMAI



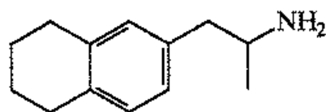
5-BA



6-BA



IA



TA

Figure 4. Chemical Structures of MDA and Related Compounds.

position of the oxygen atom in relation to the alkylamine side-chain, and by the orientation of the oxygen unshared electrons. Hence, the isomeric benzofuran compounds 6-(2-aminopropyl)-2,3-dihydrobenzofuran (6-BA) and 5-(2-aminopropyl)-2,3-dihydrobenzofuran (5-BA), where one of the dioxole ring oxygen atoms has been replaced with a methylene unit, were prepared to examine the pharmacology of these compounds (Figure 4). In addition, 5-(2-aminopropyl)-2,3-dihydro-1*H*-indene (IA) was prepared to examine the effect of replacing both oxygen atoms of MDA with methylene units, and 2-(2-aminopropyl)-5,6,7,8-tetrahydronaphthalene (TA) was prepared to study the effect of expanding the size of the fused ring (Figure 4). Thus, these compounds were synthesized and evaluated for their ability to inhibit the accumulation of [^{3}H]serotonin ([^{3}H]5-HT), [^{3}H]dopamine ([^{3}H]DA), and [^{3}H]norepinephrine ([^{3}H]NE) into crude synaptosomes.

Results

All of the test compounds exhibited smooth concentration-response curves for the *in vitro* inhibition of synaptosomal [^{3}H]neurotransmitter accumulation. Slope coefficients were not different from 1, indicating single-site interaction. Representative data for the inhibition of [^{3}H]5-HT uptake by 5-BA are illustrated in Figure 5. The *in vitro* pharmacology revealed clear differences among the compounds' abilities to inhibit synaptosomal accumulation of [^{3}H]5-HT, [^{3}H]DA, and [^{3}H]NE (Table I). While 5-BA, IA, and TA exhibited IC_{50}'s for 5-HT accumulation in the hundred nanomolar range, MDA and 6-BA had some three-fold lower activity in this assay. In the dopamine assay, 6-BA, IA, and MDA all had IC_{50}'s under 2 μM, while 5-BA and TA had IC_{50}'s of 6-7 μM. The rank order of IC_{50}'s in the norepinephrine assay paralleled those of the dopamine assay, but the compounds were all about twice as potent at inhibiting norepinephrine uptake compared to dopamine uptake (Table I). Thus, in the catecholamine assays, MDA, 6-BA, and IA showed a greater than three-fold higher potency at inhibiting uptake, relative to 5-BA and TA.

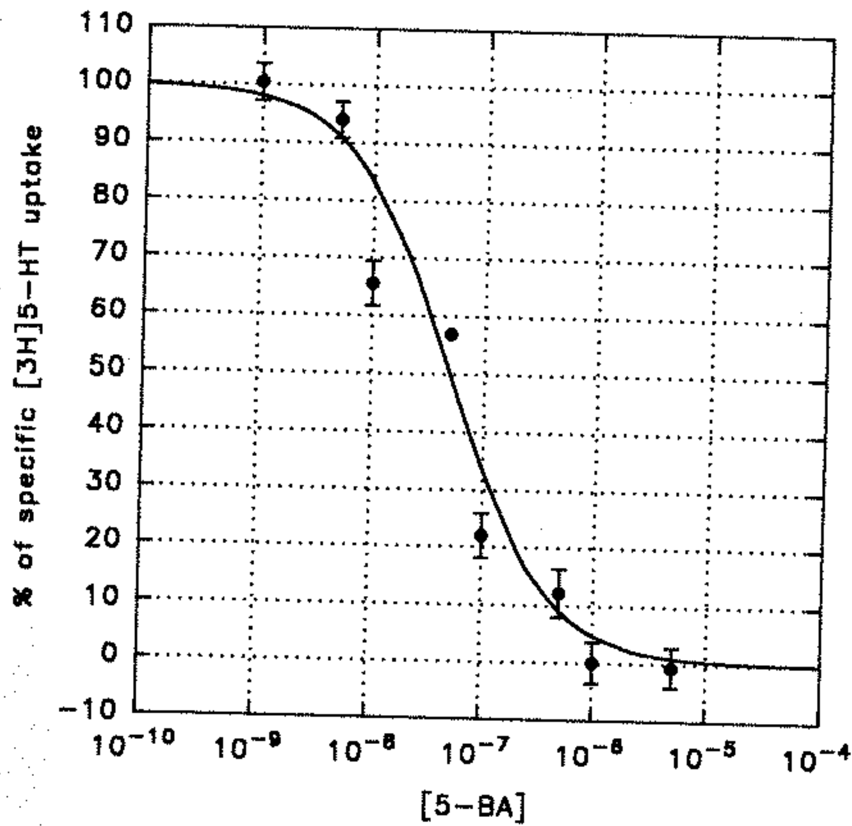
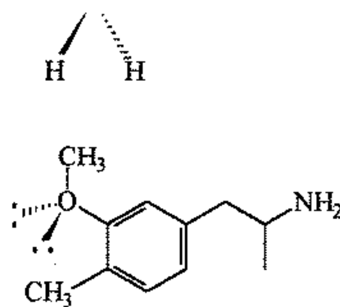


Figure 5. 5-BA Inhibition of Synaptosomal [^{3}H]5-HT Accumulation. The ability of 5-BA to inhibit uptake of [^{3}H]5-HT was examined in crude synaptosomes. Data are the mean ± SEM of three determinations, each run in triplicate. Data were fitted to a four-parameter logistic curve for IC_{50} determination and plotting.

Discussion

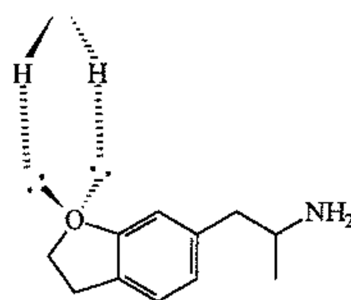
These results clearly support the hypothesis that serotonin/catecholamine selectivity can be controlled by the position and orientation of ring oxygen atoms. In an earlier study by Johnson, *et al.*,[61] it was reported that MMA was a highly selective 5-HT-releasing agent with no neurotoxic liability. This observation led to the hypothesis that serotonin and catecholamine selectivity could be modulated by the position of the oxygen atom in relation to the alkylamine side-chain, and by the orientation of the oxygen unshared electrons. For example, in MMA, the *O*-methyl is forced into an *anti* conformation relative to the 4-methyl group due to steric interactions between these two substituents. This causes the unshared electrons of the ether oxygen atom to be directed towards the 4-methyl group in a *syn* orientation (Figure 6). This orientation may be highly unfavorable for bonding interactions with the catecholamine carrier proteins, and thus leads to low affinity for the DA and NE uptake sites. Conversely, the 5-HT carrier does not appear to have such requirements for the *meta*-methoxy group, and the result is the observed high selectivity for the 5-HT uptake carrier relative to catecholamine uptake sites. This hypothesis allowed us to predict that

Hydrogen Bond Donor(s)



MMA

Hydrogen Bond Donor(s)



6-BA

Figure 6. Hypothetical Bonding Interactions with Catecholamine Uptake Carrier Proteins.

6-BA and 5-BA would show differential selectivity for the uptake sites, with 6-BA being more selective for catecholamine sites, and 5-BA being more selective for the 5-HT carrier.

A comparison of the IC_{50} values for 6-BA and 5-BA (Table I) shows that this is indeed the case. These results, taken with those for MMA,[61] clearly demonstrate that monoamine-releasing activity is facilitated by a proper orientation of the unshared electrons of the ring oxygen substituents. To achieve high-affinity interactions with the catecholamine carriers, it appears necessary to have the unshared electrons of the oxygen *meta* to the side chain directed away from the *para* position (in an *anti* orientation), as in 6-BA (Figure 6). Activity at catecholamine sites is abolished, however, if the orientation is *syn*, as illustrated by the pharmacological profile of MMA. Conversely, interactions with the 5-HT carrier protein appear to be favored by the presence of an oxygen atom *para* to the side-chain, as in 5-BA, although this is not an absolute requirement. Hence, the 5-HT site can tolerate either an *anti* or *syn* orientation of the *meta*-oxygen electron pairs with respect to the 4-position.

These arguments only apply, however, when there is an oxygen atom present at the 3- or 4-position of the aromatic ring. The nonoxygenated

compound IA showed activity quite similar to both MDA and 6-BA in the catecholamine assays, and was found to inhibit 5-HT accumulation at low concentration (Table I). Indeed, IA was the most potent compound in the series for inhibiting [^{3}H]5-HT uptake. Since IA is so potent at all of the monoamine uptake sites examined, one conclusion is that the ring oxygen atoms are not required for tight binding to these proteins. However, alternative mechanisms to account for these observations cannot be ruled out. For example, the monoamine uptake carriers have binding sites for Na^+ and Cl^- and transport these ions into the cytosol along with their respective neurotransmitters.[189,190] IA may interact with one of these other binding sites. TA, by comparison, shows a large reduction of *in vitro* activity at the catecholamine sites, but not the 5-HT site. This observation indicates a decreased degree of tolerance for steric bulk by the catecholamine uptake transporters.

To summarize, these findings indicate that, generally, the catecholamine-releasing agents have more rigorous structure-activity requirements than agents which affect 5-HT release. For example, in oxygenated compounds, it appears necessary that there be an oxygen atom *meta* to the side-chain with unshared electrons directed *anti* to the 4-position to attain a high

degree of potency (Figure 6). An additional requirement for catecholamine activity is that there be limited steric bulk around the 3,4-positions of the aromatic ring. Within the series of compounds examined in this study, serotonin-releasing activity was reduced only in agents that possessed a *meta*-oxygen atom. This may be because the *anti*-directed, *meta*-oxygen lone-pair electrons, as in MDA and 6-BA, interfere in some way with the 5-HT release mechanism. In racemic MMA, however, the orientation of the *meta*-oxygen lone pairs is *syn*, and this compound retains high activity as a selective 5-HT-releasing agent.[61]

$5\text{-}HT_{2A}$ Modulation of Amino Acid Transmitter Release

In Cerebral Cortex

Abstract

The putative 5-HT_{2A} agonist 2-(2,5-dimethoxy-4-iodophenyl)-aminoethane (2C-I) and several 5-HT_{2A} antagonists were tested for their effects on spontaneous and K^+-evoked [^{3}H]γ-aminobutyrate (GABA) and [^{3}H]glutamate release from superfused slices of rat frontal cortex. 2C-I, in concentrations of 1 nM to 10 μM, had no effect on spontaneous or K^+-stimulated release of GABA or glutamate. 10 μM ketanserin had no effect on the spontaneous release of GABA or glutamate, but did depress K^+-stimulated GABA release by 26.8%. K^+-stimulated glutamate release appeared depressed, but this did not reach significance. Other 5-HT_{2A} antagonists, namely *R*-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenethyl)]-4-piperidinemethanol (MDL 100,907), ritanserin, and spiperone were tested for their effects on K^+-evoked GABA release. MDL 100,907, at 1 μM and 10 μM, reduced GABA release by 11.9% and 30.8%, respectively. Ritanserin and spiperone, at 10 μM, also had an inhibiting effect, decreasing GABA release by 16.4% and 22.0%, respectively.

Rats were treated with the serotonin depleting agent *para*-chlorophenylalanine (PCPA) alone and in combination with the serotonergic neurotoxin

para-chloroamphetamine (PCA), in an attempt to unmask an agonist effect of 2C-I and to reverse the ketanserin-induced suppression of stimulated GABA release. Despite the absence of detectable serotonin by HPLC-EC in cortical tissue from PCPA treated animals, 2C-I at 10 μM did not affect K^+-evoked GABA release, and stimulated GABA release in the presence of 10 μM ketanserin was still depressed 22.5% from control. In animals treated with both PCPA and PCA, 10 μM ketanserin alone or 10 μM 2C-I alone each appeared to decrease stimulated GABA release (by 21.6% and 9.4%, respect-ively) but this did not reach significance. However, when ketanserin and 2C-I were simultaneously present in the superfusion buffer, an additive inhibitory effect was observed, and GABA release was decreased 29.5% relative to control. These results suggest that serotonin facilitates GABA release in frontal cortex via 5-HT_{2A} receptors, and that 2C-I may be a partial agonist at 5-HT_{2A} receptors. Furthermore, the functional efficiency of this serotonergic system is highly resistant to serotonin depletion.

Introduction

Pharmacological studies, including radioligand binding data,[21-24,65-67] drug discrimination assays,[43,68-71] electrophysiological experiments,[72-81] and neurochemical assays[82-89] implicate the 5-HT_{2A} receptor as a site of action of hallucinogenic phenylalkylamines. Prototypical hallucinogenic phenylalkyl-amines such as 2-(2,5-dimethoxy-4-bromophenyl)-aminopropane (DOB) and 2-(2,5-dimethoxy-4-iodophenyl)-aminopropane (DOI) show high affinity and biochemical potency at 5-HT_{2A} sites and relatively low activity at other serotonin and monoamine neurotransmitter sites. These drugs produce characteristic psychological effects in humans, including alteration of sensory perception in all sense modalities, conscious access to subconscious material, dream-like eyes-closed imagery, greatly exaggerated or greatly depressed emotional responses, and changes in the way in which symbols or events are interpreted.[16-19] Evidently, 5-HT_{2A} receptor sites are involved in the regulation or activation of these mental processes.

Autoradiographic studies in rodents using radiolabeled DOB or DOI have revealed high densities of 5-HT_{2A} sites in olfactory bulb, claustrum, and layer IV/Va of the cerebral cortex.[21,90-94] In primates, the highest density of

5-HT_{2A} specific binding is in layer IIIb/IV of the neocortex.[95-98] In both rodents and primates, layer IV of the cerebral cortex is the primary terminal projection layer for specific sensory afferents relayed by the thalamus and contains large populations of relay neurons whose neuronal processes do not leave the cortex, but instead make extensive local connections.[99,100] These interneurons regulate the signals entering and leaving the so-called cortical modules.[101,102] The cortical modules are discrete anatomical elements of the cerebral cortex comprising specific thalamocortical afferent fibers, cortico-cortical interconnective fibers, pyramidal output cells, and the aforementioned interneurons.[100,103] Layer IV interneurons in cortical modules receive sensory input signals from specific thalamic nuclei and also receive processed information from other cortical modules. These neurons, in turn, excite or inhibit other interneurons and the output pyramidal cells of their own and adjacent modules.[99,101,102,104] The interneurons thus have a central role in relaying sensory signals from the periphery and in processing signals relayed from other cortical areas. The anatomical correspondence between these cells and 5-HT_{2A} receptors, and the cells' functional role in information processing makes them likely targets for hallucinogenic phenylalkylamine drugs. However, it is not known whether 5-HT_{2A} receptors are involved in modulat-

ing these interneurons.

The cortical interneurons utilize a variety of neurotransmitters to convey information, but two neurotransmitters of major importance are γ-aminobutyrate (GABA) and glutamate.[105-110] Based on the arguments given above, it was hypothesized that hallucinogenic phenylalkylamine drugs, acting as agonists at 5-HT_{2A} receptors on GABAergic and/or glutaminergic modulatory interneurons in layer IV of the cerebral cortex, alter the responsiveness of these interneurons to their input signals. This alteration would be functionally manifested by changes in the amount of GABA or glutamate these cells release, either spontaneously or under stimulation conditions. This hypothesis thus proposes a cellular-level functional basis for the psychopharmacology of hallucinogenic drugs, with activity changes in cortical sensory information-processing neurons contributing to or leading to the characteristic hallucinogenic effects of these drugs. 5-HT_{2A} antagonists, on the other hand, would be expected to block the effects of serotonin or exogenous agonists, leading to a reversal of any effects on GABA or glutamate release engendered by the agonists, and a reversal of the psychoactive effects of 5-HT_{2A} hallucinogens.

Predicting the direction of change of GABA or glutamate release, *i.e.*

whether a 5-HT_{2A} agonist would increase or decrease neurotransmitter release, is problematic. Davies, *et al.* showed that in layer IV of somatosensory cortex, application of serotonin to the area of the apical dendrite tree of identified pyramidal cells resulted in a depolarization in almost 70% of the neurons studied, probably by decreasing a resting K^+ conductance.[79] This depolarization was blocked by 5-HT_{2A} antagonists. Araneda and Andrade reported that layer V pyramidal cells in slices of rat association cortex were depolarized by bath application of the hallucinogen DOB; this effect was reversed by ketanserin and low concentrations of spiperone.[80] Rahman and Neuman reported that activation of 5-HT_{2A} receptors enhanced *N*-methyl-*d*-aspartate (NMDA) induced depolarization of cortical neurons.[111] These studies suggest that 5-HT_{2A} receptor activation increases the activity of cortical pyramidal cells through one or several mechanisms. These mechanisms may involve direct, facilitory effects on the pyramidal cells, or indirect effects mediated by local cells which synapse on and modulate the pyramidal cells, or some combination of these. One would predict, at least, an increase in glutamate release following 5-HT_{2A} activation of pyramidal cells, since these cells are known to use glutamate as a neurotransmitter.[106-108] One might also expect to see decreased GABA release from local inter-

neurons.[105,112-116]

Sheldon and Aghajanian, however, report 5-HT_{2A} activation of inter-neurons in piriform cortex layer II/III *increases* bicuculline-sensitive inhibitory postsynaptic potentials in piriform cortex layer II pyramidal cells,[81] and Maura, *et al.* report a presynaptic 5-HT_{2A}-mediated *decrease* in K^+-evoked glutamate release from cerebellar mossy fiber terminals.[82,83] Based on these studies, one would predict the opposite effects on glutamate and GABA release from those described in the preceding paragraph.

The results of 5-HT_{2A} receptor activation therefore appear to be specific to particular brain areas, and one cannot predict *a priori* whether 5-HT_{2A} activation would lead to an increase or decrease in GABA or glutamate release in frontal cortex. This study was designed to test the hypothesis that 5-HT_{2A} receptors modulate GABA or glutamate release in frontal cortex, and to determine whether this modulation results in an increase or decrease in neurotransmitter release in cortex under resting and stimulation conditions. Thus, the hallucinogen 2-(2,5-dimethoxy-4-iodophenyl)-aminoethane (2C-I)[117] was tested for its potential 5-HT_{2A} agonist effects, and ketanserin, MDL 100,907, ritanserin, and spiperone were tested for their 5-HT_{2A} antagonist effects on GABA release from slices of rat frontal cortex. In addition, 2C-I

and ketanserin were tested for their effects on spontaneous and K^+-evoked glutamate release from cortical slices. These drugs were chosen for their high affinity and selectivity for $5\text{-}HT_{2A}$ sites (Table II).

Table II. Binding Profiles of Some 5-HT_{2A} Agents.[a]

Receptor	Compound				
	2C-I[b]	Ketanserin	MDL 100,907[c]	Ritanserin[d,e]	Spiperone
5-HT_{2A}	1.52	0.39[f]	0.36	0.9	0.53[g]
5-HT_{2C}	--	100[h]	105	--	4,800[i]
5-HT_{1A}	1548	1900[h]	> 10,000	> 1000 vs	130[i]
5-HT_{1B}	68.7	1900[h]	--	5-HT_1 sites	47,000[i]
5-HT_{1D}	--	--	> 10,000		--
5-HT_3	--	--	> 1,000	--	--
Histamine	> 10,000	16[d,e]	1,100[e]	35	> 954[g]
σ	--	--	67	--	--
α_1	> 10,000	31[d,e]	545[e]	97	10[g]
α_2	> 10,000	> 1000[d,e]	16,000[e]	150	> 954[g]
Dopamine	> 10,000	620[d,e,j]	> 75,000[e,j]	70[j]	0.20[g,j]
Glycine	--	--	> 100,000[e]	--	--
$GABA_A$	--	--	> 100,000[e]	--	--
Muscarinic[k]	--	--	51,000[e]	--	--

[a]K_I values (nM), except as noted. [b]Values are for displacement of [^{125}I]2C-I by various ligands, ref. 118. [c]Ref. 119. [d]Ref. 120. [e]IC_{50} (nM). [f]Ref. 121. [g]Ref. 122. [h]Ref. 123. [i]Ref. 124. [j]D_2 sites. [k]3-quinuclidinyl benzilate sites.

Drugs

Several 5-HT_{2A} receptor-selective 2-(2,5-dimethoxy-4-halophenyl)-aminoethanes were synthesized for these studies. See **Methods** and Figure 1 for synthetic and analytical details. One of these agents, the hallucinogen 2C-I,[117] was selected as a representative 5-HT_{2A} agonist. The 5-HT_{2A} antagonists ketanserin, ritanserin, and spiperone were obtained from a commercial source (Research Biomedicals, Inc), and *R*-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenethyl)]-4-piperidinemethanol (MDL 100,907) was a gift from Marion Merrell Dow.

Pharmacology

In vitro studies were performed to assess the effect of 5-HT_{2A} manipulation on the release of GABA and glutamate from slices of rat frontal cortex. Coronal slices of rat frontal cortex were preloaded with [^{3}H]GABA or [^{3}H]glutamate and then exposed, by superfusion, to the various agonist and antagonist drugs under resting or stimulated conditions. Slices were prepared and superfused as described under **Methods** (Brain Slice Technique).

For spontaneous release experiments with ketanserin, and spontaneous and K^+-stimulated release experiments involving multiple concentrations of 2C-I, individual slices were exposed to single concentrations of test drug, and each "n" represents one slice from one animal. Stimulated release experiments with other antagonists, in PCPA and PCA/PCPA treated animals, and for [^{3}H]glutamate release, were performed in triplicate, and the mean of these three determinations represents one "n". 40 mM K^+ KR buffer was used to evoke GABA release, and 40 mM and 55 mM K^+ KR buffers were used to evoke glutamate release. The concentration of NaCl in these buffers was reduced to 87 mM and 72 mM, respectively, to maintain iso-osmotic conditions.

Some rats were pretreated with the tryptophan hydroxylase inhibitor PCPA as described under **Methods** (Depletion of Endogenous Serotonin). This drug will cause massive (> 90%) depletion of brain serotonin within 48 hours of dosing, and this depletion will persist for about one week.[39,40] Another group of animals was pretreated with the serotonergic neurotoxin PCA, followed by PCPA (see **Methods**). PCA causes fine serotonergic axons, originating in the dorsal raphe nuclei, to degenerate within one week.[40-42] These axons innervate precisely those areas of the cerebral cortex that

contain the highest densities of 5-HT_{2A} receptors.[41,93,125,126,132] These two treatments were designed to reduce or abolish endogenous serotonin function in order to amplify any potential agonist drug effects that might be obscured by the presence of endogenous serotonin. The treatments would also be expected to reverse any antagonist drug effects.

Data from the release experiments were transformed from dpm to percent tritium released, and, for stimulated release experiments, the S2/S1 value was calculated and used to compare drug effects, as described under **Methods** (Brain Slice Technique). For GABA release, multiple comparisons of the various 2C-I concentrations against controls in drug-naive rats were performed using one-way ANOVA followed by Dunnett's *t* test; pairwise comparisons of the different antagonists to controls were made using Student's *t* test. Pairwise comparisons of drug effects to controls in PCPA and PCA/PCPA treated rats were made using Student's *t* test. For glutamate release, drug treatments were compared to controls using Dunnett's *t* test.

The 5-HT content of control and PCPA-treated animals was assessed using HPLC-EC, as described under **Methods** (High Pressure Liquid Chromatography (HPLC) Analysis of Serotonin).

Results

2C-I, tested at concentrations from 1 nM to 10 μM, had no effect on GABA release under resting conditions. 2C-I, at 10 μM, was tested for its effect on spontaneous glutamate release, and likewise had no effect. Ketanserin, at 10 μM, also failed to affect the spontaneous release of either neurotransmitter. Representative data for the effect of 2C-I on spontaneous [^{3}H]GABA efflux are shown in Figure 7.

Stimulated GABA release, evoked by 40 mM K^+ KR, was robust and readily reproducible (Figure 8). Under K^+ stimulation, 2C-I again had no effect on GABA release (Table III). Ketanserin, however, showed a depressant effect on GABA release, with a 10 μM concentration decreasing release by 26.8% ($P < 0.005$) relative to controls (Table IV). The three other 5-HT_{2A} antagonists, MDL 100,907, ritanserin, and spiperone, also significantly reduced K^+-stimulated GABA release (Table IV). MDL 100,907, tested at 1 μM and 10 μM, reduced GABA release by 11.9% ($P < 0.05$) and 30.8% ($P < 0.0001$), respectively. Ritanserin, at 10 μM, depressed GABA release by 16.4% ($P < 0.01$), and spiperone, also tested at 10 μM, reduced GABA efflux by 22.0% ($P < 0.005$).

Stimulated glutamate release was very weak with 40 mM K^+ KR; the data from these experiments were discarded, and 55 mM K^+ KR was used instead (Figure 9). 2C-I and ketanserin, alone or in combination, at 10 μM, were ineffective in modifying glutamate release under these conditions (Table V). 55 mM K^+ KR-evoked glutamate release, while stronger than that achieved with 40 mM K^+ KR, was highly variable and still not as robust as evoked GABA release (see Figures 8 and 9). This produced large standard errors of the means and made data interpretation difficult. Therefore, further experiments were restricted to the study of GABA release only.

HPLC-EC analysis of the 5-HT level in frontal cortex from a control (saline-treated) rat revealed a 5-HT content of 217.8 pg/mg wet weight, in excellent agreement with previously reported results[127] (Figure 10). Treatment of rats with PCPA two days prior to sacrifice resulted in the complete loss of detectable serotonin by HPLC-EC in all animals. A representative chromatogram is shown in Figure 11. The lowest concentration of 5-HT used to generate standard curves was 25 ng/mL (= 125 pg/mg wet weight; see **Methods**), and it is apparent that the amount of 5-HT present in tissues from PCPA-treated animals was much less than 125 pg/mg wet weight (Figure 11). Despite this loss of endogenous 5-HT, 10 μM 2C-I alone still

had no effect on stimulated GABA release (Table VI). The depressing effect of 10 μM ketanserin was not reversed by the PCPA treatment, as the S2/S1 ratio was still 22.5% lower than controls ($P < 0.05$); 1 μM ketanserin was without effect (Table VI). When 2C-I and ketanserin (both at 10 μM) were present simultaneously, the reduction in GABA release was not quite significant ($P < 0.052$).

PCPA treatment preceded by PCA treatment 6.5 days previously (see **Methods**) did reverse the depression in stimulated GABA release produced by 10 μM ketanserin (Table VII); release was still reduced, but this was no longer significantly different from controls. There was also a small, nonsignificant reduction in GABA release in the presence of 10 μM 2C-I. When both drugs were simultaneously present in the superfusion buffer, GABA release was again significantly depressed by 29.5% ($P < 0.05$) relative to controls (Table VII).

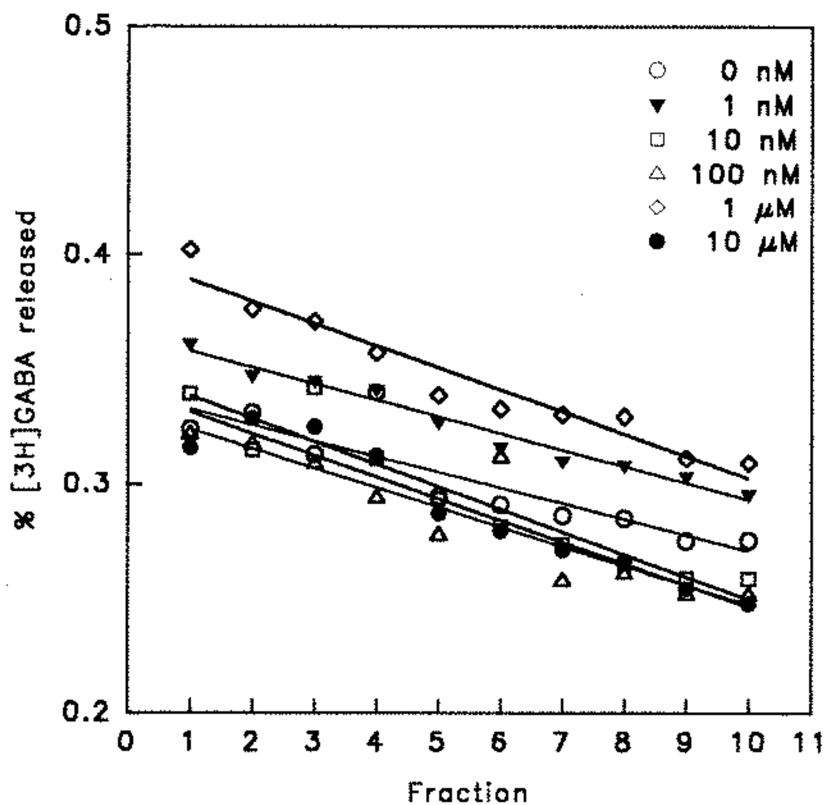


Figure 7. Effect of 2C-I on Spontaneous [^{3}H]GABA Release from Rat Cortical Slices. The effect of various concentrations of 2C-I on spontaneous [^{3}H]GABA release was examined in superfused slices of rat frontal cortex (see **Methods**). Concentrations were assessed using 4 or 5 singular determinations. SEM ~ 15% of mean, not shown for clarity. Data were fitted to computer-generated least-squares regression lines.

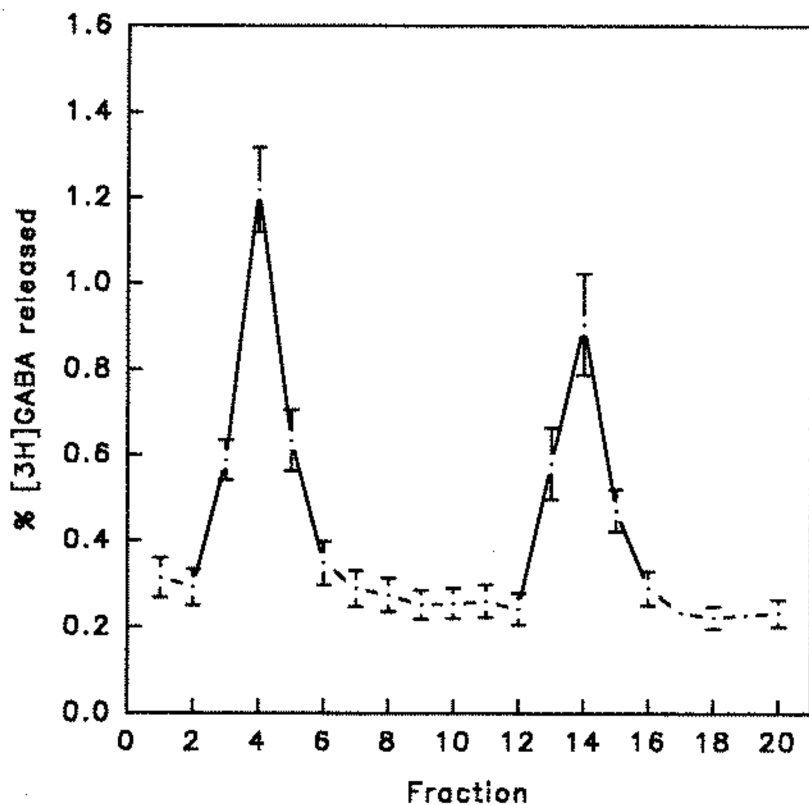


Figure 8. K$^+$-stimulated [^{3}H]GABA Release from Rat Cortical Slices. [^{3}H]GABA release was evoked from superfused slices of rat frontal cortex by replacing normal KR buffer with 40 mM K$^+$ KR during fractions 3 and 13 (see **Methods**). Data are the mean ± SEM of six singular determinations.

Table III. S2/S1 Values for K^+-evoked [^{3}H]GABA Release: 2C-I.[a]

[2C-I]	S2/S1	n[b]
0 (control)	0.867 ± 0.023	13
1 nM	0.830 ± 0.098	5
10 nM	0.899 ± 0.086	6
100 nM	0.830 ± 0.111	6
1 μM	0.914 ± 0.078	6
10 μM	1.078 ± 0.209	7

[a]The ability of various concentrations of 2C-I to affect K^+-stimulated [^{3}H]GABA release was examined in slices of rat frontal cortex. S2/S1 values ± SEM were calculated based on areas under the stimulation curves and were compared to controls using Dunnett's *t* test (see **Methods**). None of the values are different from controls at $P < 0.05$. [b]n is the number of animals assessed (singular determinations) at each concentration of drug.

Table IV. S2/S1 Values for K^+-evoked [^{3}H]GABA Release: Antagonists.[a]

Treatment	S2/S1	n[b]
Control	0.867 ± 0.023	13
Ketanserin, 100 nM	0.893	1
Ketanserin, 1 μM	0.833	1
Ketanserin, 10 μM	0.634 ± 0.064[e]	9
MDL 100,907, 1 μM	0.764 ± 0.036[c]	5
MDL 100,907, 10 μM	0.600 ± 0.022[f]	5
Ritanserin, 10 μM	0.725 ± 0.023[d]	4
Spiperone, 10 μM	0.676 ± 0.010[e]	3

[a]The ability of various antagonists to affect K^+-stimulated [^{3}H]GABA release was examined in slices of rat frontal cortex. S2/S1 values ± SEM were calculated based on areas under the stimulation curves and pairwise comparisons to controls were made using Student's *t* test (see **Methods**). [b]n is the number of animals assessed (triplicate determinations) at each concentration of drug. [c]$P < 0.05$. [d]$P < 0.01$. [e]$P < 0.005$. [f]$P < 0.0001$.

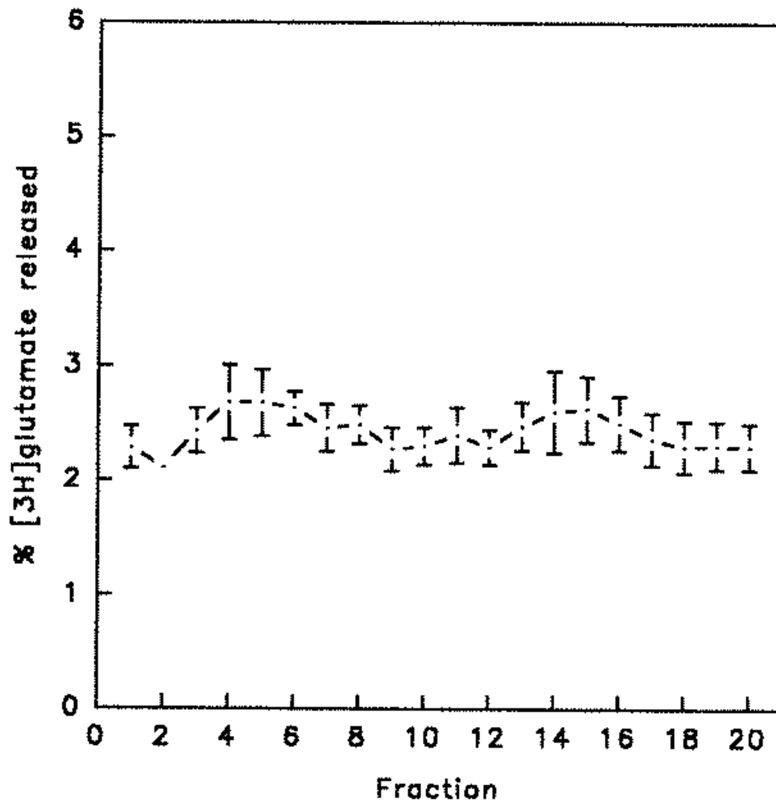


Figure 9. K^+-stimulated [^{3}H]glutamate Release from Rat Cortical Slices. [^{3}H]glutamate release was evoked from superfused slices of rat frontal cortex by replacing normal KR buffer with 55 mM K^+ KR during fractions 3 and 13 (see **Methods**). Data are the mean ± SEM of three triplicate determinations.

Table V. S2/S1 Values for K^+-evoked [^{3}H]Glutamate Release.[a]

Treatment	S2/S1	n[b]
Control	0.789 ± 0.201	3
2C-I, 10 μM	0.812 ± 0.100	3
Ketanserin, 10 μM	0.538 ± 0.087	3
2C-I + ketanserin, 10 μM	0.508 ± 0.147	3

[a]The ability of 2C-I and ketanserin to affect K^+-stimulated [^{3}H]glutamate release was examined in slices of rat frontal cortex. 55 mM K^+ KR was used to evoke release and S2/S1 values ± SEM were calculated based on areas under the stimulation curves and were compared to controls using one-way ANOVA followed by Dunnett's *t* test (see **Methods**). None of the values are different from controls at $P < 0.05$. [b]n is the number of animals assessed (triplicate determinations) at each concentration of drug.

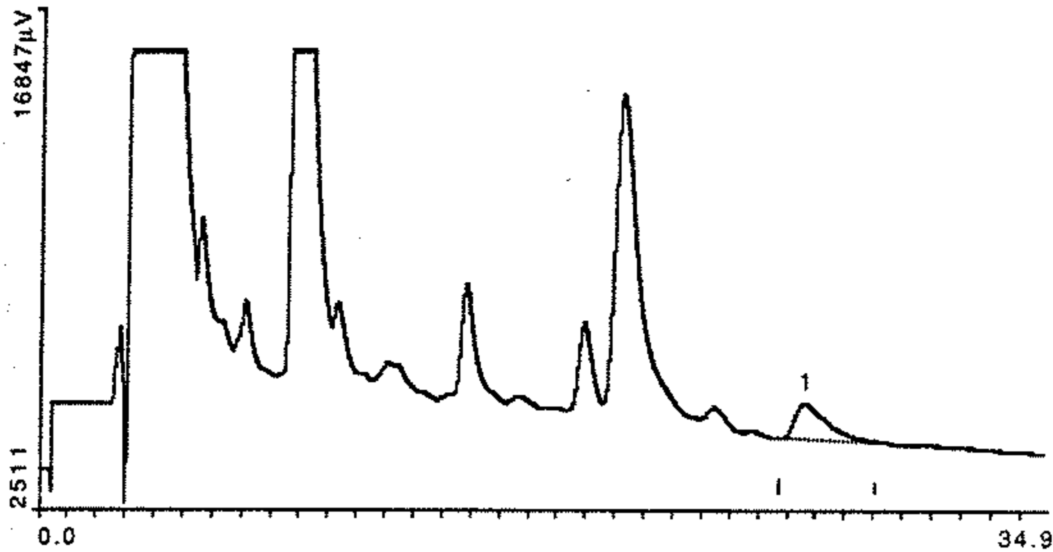


Figure 10. HPLC-EC Analysis of 5-HT in Brain Tissue of Control Animals. The 5-HT content of cortical tissue harvested from a saline-treated rat was assessed using HPLC-EC (see **Methods**). The peak marked "1" is the 5-HT peak (retention time = 26.6 min). The peak area was used to calculate 5-HT concentration (= 43.6 ng/mL, 217.8 pg/mg wet weight) from standard curves.

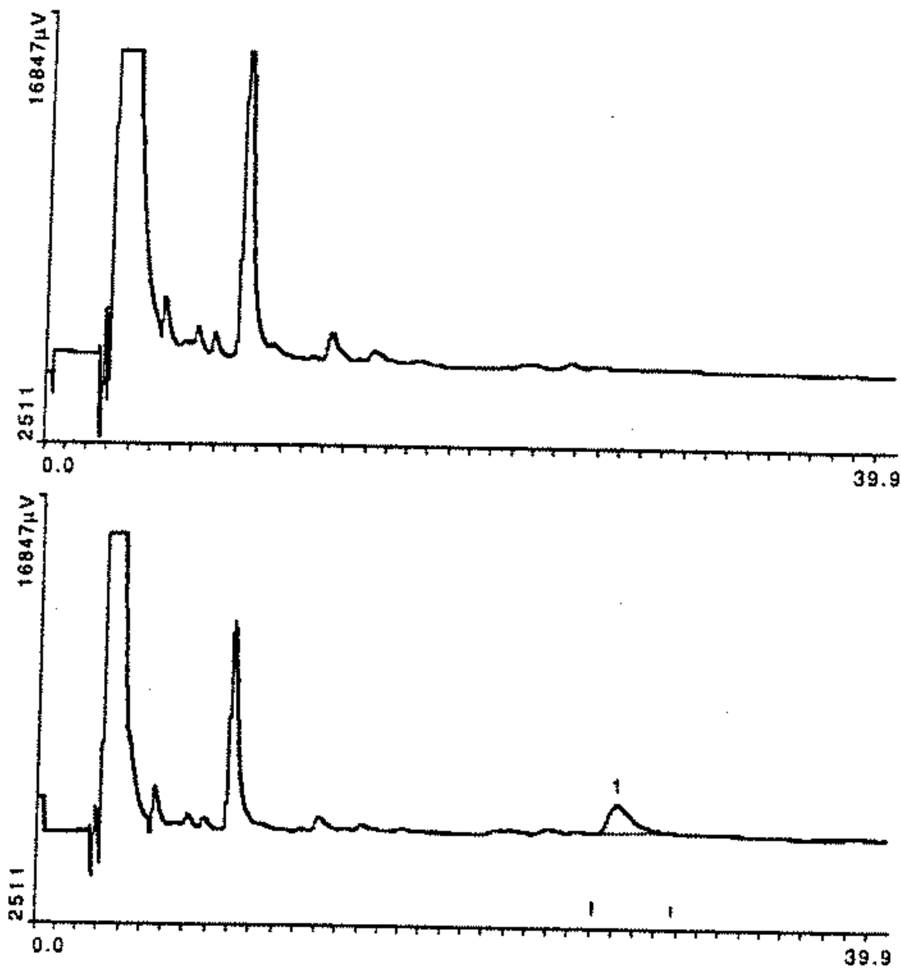


Figure 11. HPLC-EC Analysis of 5-HT in Brain Tissue of PCPA-treated Animals. The 5-HT content of cortical tissue harvested from a PCPA-treated rat was assessed using HPLC-EC (see **Methods**). **Upper Plot:** Note the absence of the 5-HT peak at 27 min. **Lower Plot:** Sample in upper plot spiked with authentic 5-HT (= 25 ng/mL; peak "1").

Table VI. S2/S1 Values for K^+-evoked [^{3}H]GABA Release: PCPA Pretreatment.[a]

Treatment	S2/S1	n[b]
Control	0.773 ± 0.042	6
2C-I, 10 μM	0.809 ± 0.025	3
Ketanserin, 1 μM	0.777 ± 0.055	3
Ketanserin, 10 μM	0.599 ± 0.041[c]	3
2C-I + ketanserin, 10 μM	0.625 ± 0.018	3

[a]The ability of 2C-I and ketanserin to affect K^+-stimulated [^{3}H]GABA release was examined in slices of rat frontal cortex harvested from animals that had been treated with PCPA 48 h previously. S2/S1 values ± SEM were calculated based on areas under the stimulation curves and pairwise comparisons to controls were made using Student's *t* test (see **Methods**). [b]n is the number of animals assessed (triplicate determinations) at each concentration of drug. [c]$P < 0.05$ compared to controls.

Table VII. S2/S1 Values for K^+-evoked [^{3}H]GABA Release: PCA and PCPA Pretreatment.[a]

Treatment	S2/S1	n[b]
Control	0.805 ± 0.033	3
2C-I, 10 μM	0.729 ± 0.012	3
Ketanserin, 10 μM	0.631 ± 0.029	3
2C-I + ketanserin, 10 μM	0.567 ± 0.076[c]	3

[a]The ability of 2C-I and ketanserin to affect K^+-stimulated [^{3}H]GABA release was examined in slices of rat frontal cortex harvested from animals that had been treated with PCA 8.5 days previously and PCPA 48 h previously. S2/S1 values ± SEM were calculated based on areas under the stimulation curves and were compared to controls using one-way ANOVA followed by Dunnett's *t* test (see **Methods**). [b]n is the number of animals assessed (triplicate determinations) at each concentration of drug. [c]$P < 0.05$ compared to controls.

Discussion

The antagonist data from drug-naive rats support the hypothesis that serotonin regulates GABA function through 5-HT_{2A} receptors in cerebral cortex. The regulation by serotonin is facilitory, since receptor blockade results in decreased GABA release. All four putative 5-HT_{2A} antagonists significantly depressed K^+-evoked GABA release, with MDL 100,907 exhibiting dose-dependent effects (Table IV). While none of these drugs is absolutely selective for the 5-HT_{2A} receptor, the 5-HT_{2A} receptor is the only known receptor for which all of these drugs share high affinity (Table II). The fact that these drugs have different chemical structures makes it improbable that they share some other common molecular recognition site. However, the possibility that these drugs are blocking some other element in the neurotransmitter release mechanism, for example, synaptic voltage-sensitive calcium channels, cannot be ruled out. It is also unlikely that at the concentrations used these drugs are causing nonspecific anesthetic-like depression of neuronal function. For example, the thermodynamic activities[128] of the antagonists used in this study are on the order of 10^{-4}; nonspecific drugs generally have higher thermodynamic activities, between

0.01 and 1.[188]

The failure of PCPA pretreatment to reverse the ketanserin-induced depression of GABA release (Table VI), even though 5-HT levels were undetectable (Figure 11), is puzzling. However, there is evidence for a large functional reserve in the serotonergic system. For example, Kuhn, *et al.* were able to elicit serotonin-mediated behaviors (head weaving, reciprocal forepaw treading, hindlimb abduction, salivation, wet-dog shakes) after reducing brain 5-HT levels by 90-95% with reserpine.[129] Chaput, *et al.* showed that even when 5-HT content in the dorsal hippocampus was reduced by 90% with PCPA treatment, the efficiency of serotonergic neurotransmission to CA3 pyramidal cells was unaltered in most animals tested, as assessed electrophysiologically.[130] Postsynaptic receptor supersensitivity[131] or increases in 5-HT_{2A}receptor numbers following PCPA treatment[133,134] may provide mechanisms for maintaining normal function.

To address the possibility that residual stores of 5-HT were able to furnish enough 5-HT to activate 5-HT_{2A} receptors, which could be antagonized by ketanserin, some rats were treated with both PCA and PCPA. Since PCA causes serotonergic axonal degeneration,[41,125] this treatment should eliminate hypothetical 5-HT stores that are unaffected by PCPA alone.

While the degree of axonal degeneration was not specifically tested in these experiments, numerous studies have shown that the treatment protocol used here is an effective means of lesioning 5-HT axons.[40-42,126,132] With this treatment, the ketanserin-induced inhibition of GABA release was no longer significantly different from controls (Table VII). This result indirectly supports the hypothesis that 5-HT increases GABA release via 5-HT_{2A} receptors in rat cerebral cortex, and, examined in the light of the PCPA results, also suggests that this serotonergic system requires aggressive manipulations to decrease its efficiency.[129,130]

The supposed agonist property of the hallucinogen 2C-I is called into question by these experiments; no concentration of 2C-I affected spontaneous or evoked GABA release. Based on the antagonist data, one would expect an agonist to increase GABA release under K^+ stimulation, but this was not observed. There are several possibilities to explain this finding.

One possibility is that 2C-I is not an agonist at 5-HT_{2A} receptors, but is instead an antagonist. This seems untenable for several reasons: 2C-I did not exhibit the inhibiting effect on GABA release in drug-naive animals that was seen with the other antagonists used in this study; the chemical structure of 2C-I is quite similar to the structures of other known 5-HT_{2A} agonists

such as DOI, which is simply α-methyl 2C-I; 2C-I has high affinity and selectivity for the 5-HT_{2A} receptor, and this affinity and selectivity is almost identical with that displayed by the more active isomers of drugs such as DOI;[118] 5-HT_{2A} antagonists are not hallucinogenic;[135-139] finally, the psychological effects of 2C-I are known to be qualitatively similar to the effects of other hallucinogenic 5-HT_{2A} agonists like DOI.[117]

Another possibility is that 2C-I is a partial agonist. If 2C-I is a partial agonist only, any drug-induced effect could be eclipsed by the presumably larger pool of non5-HT_{2A}-sensitive neurons. That is, of the total population of GABAergic neurons in the cerebral cortex, only a subset might express 5-HT_{2A} receptors. This subset could be small enough such that if 2C-I is only a partial agonist, the increase in the amount of GABA released is hidden by the larger amount of GABA released (under nonspecific K^+ stimulation) by the population of neurons that do not express 5-HT_{2A} receptors. At the same time, the 5-HT_{2A} subset of neurons is large enough to allow the depression in GABA release induced by antagonists to be expressed. The possibility that 2C-I is a partial agonist is strengthened by research reported from other laboratories. Pierce and Peroutka[140] and Sanders-Bush, *et al.*[141] showed that the hallucinogens DOB, DOI, and (2,5-dimethoxy-4-methylphenyl)-2-amino-

propane, which are structurally similar to 2C-I, exhibited efficacies lower than serotonin itself at stimulating phosphatidylinositol hydrolysis in rat cortex. Also, Seggel and coworkers reported the partial agonist effect of DOB and DOI on platelet aggregation,[142] and others have reported partial agonist activity of *d*-lysergic acid diethylamide in behavioral tests.[143-146]

Related to the putative partial agonist activity of 2C-I is the possibly confounding effect of endogenous serotonin; this was addressed in the serotonin depletion experiments. The full-efficacy agonist effect of endogenous 5-HT, which is also released under K^+ stimulation and which possibly reaches millimolar concentrations in the synapse, might obscure any agonist effect of 2C-I, especially if 2C-I is of low efficacy. In experiments conducted with cortical slices harvested from rats that were pretreated with both PCA and PCPA (Table VII), 10 μM ketanserin or 2C-I alone each depressed GABA release (21.6% and 9.4%, respectively) but this depression did not reach significance. This suggests that 5-HT function has been impaired, but still retains some residual capacity. When both drugs at the same concentrations were simultaneously present in the superfusion buffer, an additive inhibitory effect was observed, and the S2/S1 ratio was 29.5% less than the control ratio (Table VII). Thus, after lesioning serotonergic axons and

depleting serotonin, 10 μM 2C-I "decreased" GABA release, and this effect was additive with the "decrease" produced by ketanserin. This is exactly the behavior one would expect of a partial agonist; at high concentrations or in the presence of a full-efficacy agonist, the partial agonist appears to be an antagonist. Implicit in this analysis is the assumption that some residual 5-HT function remains in the tissue, even after rather extreme pharmacological manipulations. This assumption is not unreasonable in the light of other evidence reported elsewhere.[129-134]

The proposed cellular functional model for the mechanism of action of 5-HT_{2A} hallucinogens receives some support from these studies. However, it is not entirely clear how the putative small increase in GABA release elicited by partial 5-HT_{2A} agonists would lead to the psychological effects of hallucinogens. One could describe a mechanism whereby enhanced inhibition of cortical pyramidal cells by GABA leads to lower peaks of neuronal activity within cortical receptive fields, effectively neutralizing the effects of lateral or surround inhibition, and leading, in turn, to compromised integrity of sensory signaling pathways.[102,113,147,148] On the other hand, one could envision an alternate mechanism for hallucinogenesis in which *decreased* GABA release leads to ineffectual surround inhibition. This, too, would

result in compromised signal pathways (although 5-HT_{2A} antagonists, which decreased GABA release in this study, are not hallucinogenic[135-139]). In any case, the model must remain speculative until it can be shown (perhaps electrophysiologically) that hallucinogens alter the sensitivity and selectivity of cortical pyramidal cells and their respective receptive fields and that alterations in receptive field properties leads to hallucinogenic effects. On this note, Dykes and colleagues[114,115] and Sillito[116] have shown that blockade of GABA receptors with bicuculline increases the size of somatosensory receptive fields and reduces or abolishes the selectivity of responses of visual cortex neurons to the orientation, length, and direction of movement of visual stimuli.

In summary, the ability of chemically distinct antagonist compounds with similar affinities for the 5-HT_{2A} receptor to depress GABA release suggests that serotonin, acting on 5-HT_{2A} receptors, facilitates GABA release in the cerebral cortex. Other common sites of action for the antagonist drugs cannot be ruled out, however. In addition, the serotonin system that regulates GABA release was seen to be very resistant to treatments designed to impair its function, such as PCPA- and PCA/PCPA-induced serotonin depletion.

2C-I, a presumptive 5-HT_{2A} agonist, did not have the expected facilitory effect on either spontaneous or evoked GABA release. This may be because 2C-I is only a partial agonist at 5-HT_{2A} receptors, and its effect is obscured by the much larger effect of endogenous serotonin. Partial agonist activity of 2C-I is a more reasonable idea than the notion that the drug is an antagonist, for a variety of reasons already discussed. Also, in serotonin-deficient brain tissue, 2C-I exhibited behavior consistent with that of a partial agonist, although in drug-naive animals, no effect was observed. More sensitive studies, *e.g.* single unit electrophysiological recordings, may be required to answer this question definitively.

Regarding glutamate, the failure of both 2C-I and ketanserin to affect its release suggests that 5-HT_{2A} receptors do not modulate this neurotransmitter, in conflict with theoretical considerations and with the results reported by Maura, *et al.*[82,83] However, the unreliability of either 40 mM or 55 mM K^+ buffer in evoking glutamate release prevents drawing any conclusions. Also, as noted earlier, the effects of 5-HT_{2A} activation appear to be tissue-specific, and this may explain why Maura and colleagues were able to observe 5-HT_{2A}-mediated effects on glutamate release in cerebellum; in cerebral cortex, these receptors may not be expressed on glutaminergic

neurons. Other experimental methods, such as immunocytochemical double labeling studies for 5-HT_{2A} receptors and glutamate or its biosynthetic enzymes, or electrophysiological experiments, may resolve this question.

Effects of Side-chain Cyclization and 4-Substitution on Neurotransmitter Uptake in Analogs of 2-(2,5-Dimethoxy-4-substituted-phenyl)-aminopropanes

Abstract

The potent hallucinogens 2-(2,5-dimethoxy-4-bromophenyl)-aminopropane, 2-(2,5-dimethoxy-4-iodophenyl)-aminopropane, and 2-(2,5-dimethoxy-4-trifluoromethylphenyl)-aminopropane and their respective benzoxepin analogs were tested and compared with 2-(4-methylthiophenyl)-aminopropane (MTA) for their abilities to inhibit the accumulation of [^{3}H]serotonin (5-HT), [^{3}H]dopamine, and [^{3}H]norepinephrine into crude synaptosomes. Among the phenylisopropylamine drugs, the presence of methoxy substituents at the 2- and 5-positions attenuated activity at 5-HT sites by 100-fold and at catecholamine sites by 10- to 20-fold, suggesting unfavorable electrostatic or steric interactions around those parts of the molecule. When methoxy groups were present, the substituent at the 4-position controlled potency at 5-HT carrier sites; the least electronegative substituents were the most effective at inhibiting uptake. The benzoxepin analogs, regardless of the substituent at the 4-position, exhibited similar IC_{50}'s for 5-HT uptake inhibition. These results indicate that the 5-HT carrier has at least two dissimilar binding sites for these compounds and that activity at serotonin carrier sites may be independently modulated by the

conformation of the isopropylamine side-chain and the nature of the 4-substituent, with the side-chain conformation making a greater contribution.

Introduction

2-(2,5-dimethoxy-4-substituted-phenyl)-aminopropanes are among the most potent hallucinogens known.[18,117,149-151,153] Only *d*-lysergic acid diethylamide and several related lysergamides are more potent. The potency of the phenylalkylamine hallucinogens depends strongly on the methoxy substituents at the 2- and 5-positions on the phenyl ring, with the 2-position making a greater contribution.[18,117,149] For example, among the trimethoxy-substituted phenylisopropylamines, 2,4,5-trimethoxyphenylisopropylamine is the most potent, with a human effective dose[153] of 20-40 mg; shifting the 5-methoxy one position to yield 2,4,6-trimethoxyphenylisopropylamine results in only a small diminution of potency (human effective dose 25-50 mg). If the 2-methoxy is moved over one position, however, to give 3,4,5-trimethoxyphenylisopropylamine, there is a 5-10 fold reduction in potency (human effective dose 100-250 mg). The presence of a substituent at the ring 4-position is also required, and the human psychopharmacology suggests that this substituent can modify both the potency of these drugs and their qualitative aspects.[18,117]

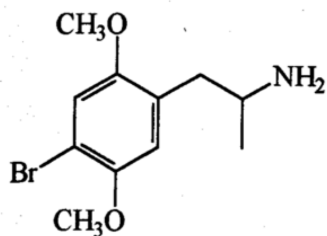
The presence of an α-methyl moiety on the ethylamine side-chain of

psychoactive phenylalkylamines results in an approximate 10-fold increase in potency. As an example, the phenethylamine 2C-I (Figure 1) is active at doses of 15-30 mg, while the α-methyl homolog DOI (Figure 12) is active in the 1-3 mg range.[117] Part of this increase in potency is thought to result from the ability of the α-methyl group to inhibit metabolism by monoamine oxidase.

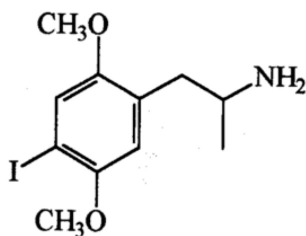
While there is strong evidence that hallucinogenic phenylalkylamines interact with 5-HT_{2A} receptors,[21-24,65-89] they may also interact with other monoamine receptors or storage and release mechanisms, as these compounds may reach brain concentrations ranging from 100 nM to 10 μM at clinical doses. Sargent, *et al.*, using [^{77}Br]- and [^{82}Br]-labeled 2-(2,5-dimethoxy-4-bromophenyl)-aminopropane (DOB, Figure 12) to study the human pharmacodynamics and pharmacokinetics of DOB, reported that none of the radioactivity bound to human plasma proteins, and that brain concentrations reached 3% of the administered dose.[152] Since the human effective dose is 1-3 mg,[117] this represents a brain concentration of approximately 100-200 nM. If compounds such as 2C-B (Figure 1), with a human effective dose of 12-30 mg[117] exhibit similar biopharmaceutics (a reasonable supposition), one would expect their brain concentrations to reach low micromolar concentra-

tions. At these concentrations, 5-HT_{2A} receptor sites are saturated, and other monoamine binding sites may begin to play an important role in the mechanism of hallucinogenic activity.

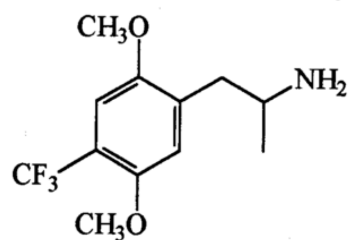
This study was designed to test the hypothesis that phenylalkylamine hallucinogens interact with monoamine carrier mechanisms, and to examine the role of the alkylamine side-chain and the substituent at the ring 4-position in modulating these interactions. Therefore, DOB, DOI, 2-(2,5-dimethoxy-4-trifluoromethylphenyl)-aminopropane (DOTFM), 4-amino-8-bromo-7-methoxy-2,3,4,5-tetrahydro-1-benzoxepin (BBOX), 4-amino-8-iodo-7-methoxy-2,3,4,5-tetrahydro-1-benzoxepin (IBOX), and 4-amino-7-methoxy-8-trifluoromethyl-2,3,4,5-tetrahydro-1-benzoxepin (TFMBOX) were tested for their ability to inhibit the accumulation of [^{3}H]serotonin ([^{3}H]5-HT), [^{3}H]dopamine ([^{3}H]DA), and [^{3}H]norepinephrine ([^{3}H]NE) into crude synaptosomes. 2-(4-methylthiophenyl)-aminopropane (MTA), a potent serotonin releasing agent, was included for comparison with previous reports.[154] See Figure 12 for chemical structures.



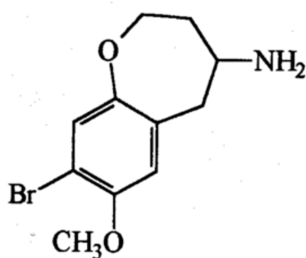
DOB



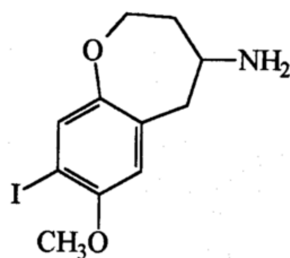
DOI



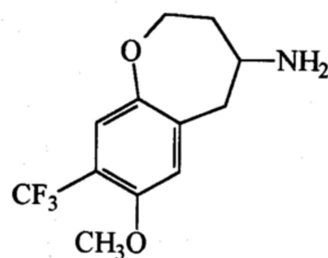
DOTFM



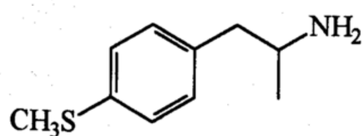
BBOX



IBOX



TFMBOX



MTA

Figure 12. **Chemical Structures of Some Phenylisopropylamines and Benzoxepins.**

Pharmacology

The ability of the benzoxepins and ring-substituted phenylisopropylamines to inhibit the accumulation of $[^3H]$5-HT, $[^3H]$DA and $[^3H]$NE into crude synaptosomes was examined to determine whether these compounds interact with monoamine uptake/release mechanisms and to provide information regarding the relative contributions of the side-chain conformation and the substituent at the 4-position in these interactions. Synaptosomes were prepared, and neurotransmitter uptake was assayed, as described under **Methods** (Synaptosome Technique). Compounds were screened at concentrations of 25, 50, and 100 μM; IC_{50} values were determined only if a compound inhibited neurotransmitter accumulation by at least 50% at 25 μM. *In vitro* data were transformed from dpm to percent inhibition of specific uptake and fitted to a four-parameter logistic curve (see page 41) using the computer program EBDA,[64] from which IC_{50} values were calculated. Multiple IC_{50} comparisons were performed as described under **Methods** (Statistics), using one-way ANOVA and a *post hoc* Student-Newman-Keuls *t* test.

Results

All drugs were effective at inhibiting [^{3}H]5-HT uptake at concentrations of less than 25 μM, and exhibited smooth dose-response curves with slope coefficients not different from 1, indicating that the drugs interacted with a single site. Figure 13 is a representative plot of the data for the inhibition of [^{3}H]5-HT accumulation by TFMBOX. The hallucinogenic phenylisopropylamines and the benzoxepins suppressed 5-HT uptake with IC_{50}'s in the 7-20 μM range (Table VIII). Among the hallucinogenic phenylisopropylamines, the substituent at the 4-position exerted a distinct influence on 5-HT uptake inhibition. Thus, DOI was about twice as potent as DOB, and three times as potent as DOTFM in this assay (Table VIII). Constraining the alkylamine side-chain and 2-methoxy orientation, as in the benzoxepin compounds, completely abolished the differences in IC_{50}values displayed among the phenylisopropylamines and also tended to increase their potency at 5-HT uptake inhibition (Table VIII). MTA was the most potent drug in the 5-HT assay, with an IC_{50} in excellent agreement with the results from an earlier study.[154]

With the exception of MTA, none of the test compounds was very

effective at inhibiting catecholamine accumulation (Table VIII); drug IC_{50} values were therefore not determined for these neurotransmitters. During the initial screening experiments, it was established that the IC_{50}'s for MTA in the catecholamine uptake inhibition assays were in agreement with previously reported values;[154] for purposes of comparison, these values are included in Table VIII.

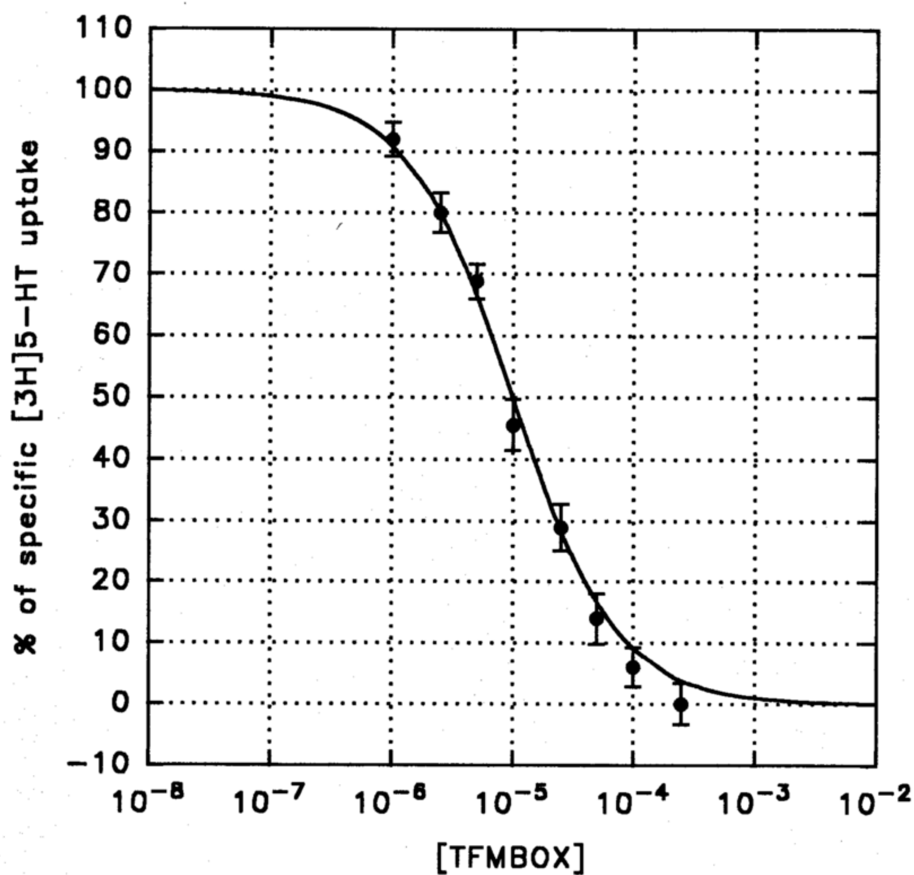


Figure 13. TFMBOX Inhibition of Synaptosomal [^{3}H]5-HT Accumulation. The ability of TFMBOX to inhibit uptake of [^{3}H]5-HT was examined in crude synaptosomes. Data are the mean ± SEM of three determinations, each run in triplicate. Data were fitted to a four-parameter logistic curve for IC_{50} determination and plotting.

Table VIII. IC_{50} for Monoamine Uptake Inhibition. II.[a]

	IC_{50} (μM)		
Compound	[^{3}H]5-HT	[^{3}H]DA	[^{3}H]NE
BBOX	10.3 ± 1.2[b,f]	> 50	> 25
IBOX	10.9 ± 2.5[b,f]	> 50	> 25
TFMBOX	9.9 ± 1.4[b,f]	> 100	> 50
DOB	14.1 ± 1.9[b,g]	> 100	> 50
DOI	7.60 ± 0.04[c,d,e]	> 50	> 50
DOTFM	19.9 ± 0.9[b]	> 100	> 50
MTA	0.085 ± 0.02	3.07 ± 0.41[h]	2.38 ± 0.12[h]

[a]The ability of the test compounds to inhibit accumulation of monoamines was examined in crude synaptosomes. Compounds were examined at eight concentrations, each run in triplicate. The data from three experiments were combined and the IC_{50} values ± SEM were calculated by curve fitting; multiple comparisons were made using ANOVA followed by a Student-Newman-Keuls *t* test (see **Methods**). [b]$P < 0.001$ vs **MTA**. [c]$P < 0.01$ vs **MTA**. [d]$P < 0.05$ vs **DOB**. [e]$P < 0.001$ vs **DOTFM**. [f]$P < 0.01$ vs **DOTFM**. [g]$P < 0.05$ vs **DOTFM**. [h]Ref. 154.

Discussion

The structural permutations of the drugs used in this study provide molecular information about the serotonin and catecholamine carrier mechanisms. For example, when 4-substituted phenylisopropylamines also carry methoxy substituents at the 2- and 5-positions, activity at all the monoamine sites is greatly reduced (compare MTA with the other phenylisopropylamines, Table VIII, and compare DOI with *p*-iodophenylisopropylamine: IC_{50}'s for uptake inhibition = 82 nM [5-HT][183], 589 nM [DA][60], 993 nM [NE][60]). This suggests unfavorable steric or electrostatic interactions with the 5-HT carrier protein or release mechanism around these positions. The catecholamine sites are also less tolerant of structural modifications to phenylalkylamines than are the 5-HT sites, confirming a conclusion reached in Chapter Three.

A more detailed analysis of the IC_{50}'s and molecular structures among the 2-(2,5-dimethoxy-4-substituted-phenyl)-aminopropanes and the 4-amino-7-methoxy-8-substituted-2,3,4,5-tetrahydro-1-benzoxepins reveals further information about the binding interactions with the 5-HT carrier. The differences in IC_{50} values observed among the phenylisopropylamines imply

that the 4-substituent is involved in binding interactions with the carrier protein. These differences in IC_{50} are not likely due to unfavorable steric interactions. The iodine atom has a larger van der Waals radius than bromine and is comparable in size to the trifluoromethyl group,[155,156] and iodine also has a longer carbon sp^2 bond length than either bromine or the trifluoromethyl group,[157-160] yet DOI exhibited a smaller IC_{50} than DOB or DOTFM (Table VIII). The IC_{50} values are, however, inversely correlated ($r = 0.991$) with the logarithm of the electronegativity of the 4-substituent.[161,162] In this series of drugs, the more electronegative a 4-substituent is, the less potent is the substituted phenylisopropylamine at inhibiting 5-HT uptake. This suggests that the binding site contains a negatively charged group in the vicinity of the 4-substituent or that the benzene ring participates in a charge-transfer complex as an electron donor when the ligand is bound.

The side-chain amine is seen to be involved in a separate binding interaction. Comparing DOTFM and TFMBOX (Table VIII), it is apparent that the conformation of the side-chain is a key determinant in ligand binding. In this example, constraining the alkylamine side-chain in a benzoxepin structure resulted in a doubling of potency in inhibiting 5-HT uptake. Further, the change in free energy due to the amine binding appears to

dominate the contribution made by the 4-substituent: the benzoxepin structure increases the potency of DOTFM in inhibiting 5-HT uptake and abolishes the differences among the IC_{50}'s exhibited by the 4-substituted phenylisopropylamines.

Taken together, these results indicate that there are at least two distinct ligand binding sites on the 5-HT carrier, one in the vicinity of the 4-substituent, and one in the vicinity of the amine nitrogen. If there were a common binding site which interacted with either the amine function or the 4-substituent, one would observe differences among the various 4-substituted benzoxepins, as were observed among the analogous phenylisopropylamines. Since this was not the case, one must conclude that there are at least two dissimilar sites. With two binding sites, there are two pathways possible in the binding mechanism. One pathway involves sequential binding of the 4-substituent followed by the amine moiety. The other pathway involves initial binding of the amine, followed by binding of the 4-substituent. The second binding step in either pathway can be assumed to be rapid relative to the first binding step, since the ligand is favorably positioned and the major loss of entropy has already occurred. Therefore, among the drugs DOB, DOI, and DOTFM, the order of binding is 4-substituent followed by amine binding,

with the different 4-substituents controlling the rate of the first binding step and giving rise to the observed differences in IC_{50}. The benzoxepins, on the other hand, must bind in the reverse order, since no differences were observed among their IC_{50} values, *i.e.* the conformation of the side-chain controls the rate of the first binding step, which would be identical for BBOX, IBOX, and TFMBOX. This analysis implies that the potential energy barrier that must be overcome for amine binding is greater than the potential energy barrier for 4-substituent binding. Hence, the benzoxepin structure reduces the amine potential energy barrier below that of the 4-substituent and thus shifts the binding order to the second pathway described above.

There are several caveats to be kept in mind regarding this analysis. The benzoxepin structure, in addition to fixing the side-chain amine conformation, fixes the orientation of the unpaired oxygen electrons in the "2-methoxy" isostere. In the substituted phenylisopropylamines, the methyl moiety of the 2-methoxy group assumes an *anti* orientation with respect to the side-chain, due to steric interactions. This, in turn, presents the oxygen unshared electrons toward the 1-position, in a *syn* orientation with respect to the side-chain. In the benzoxepins, the unshared oxygen electrons are positioned away from the 1-position, in an *anti* orientation. It is possible that

this different spatial arrangement of electrons contributes to or even dominates the binding reaction and leads to the increased potency observed in the benzoxepins. Another consideration is that the mere presence of a methoxy substituent at the 2-position of the phenylisopropylamines interferes with activity (compare with MTA, Table VIII). Since the benzoxepin structure implicitly incorporates a "2-methoxy" moiety, the loss of differential potencies among the various 4-substituents may only be apparent, *i.e.* the benzoxepin structure, while favorably positioning the amine for binding, does so at the expense of introducing unfavorable steric or electrostatic interactions around the 1- or 2-positions. This may limit the ability of the molecule to interact with the uptake carrier/release mechanism regardless of the substituent at the 4-position. Finally, the rigidity of the benzoxepin structure may orient the 4-substituent such that carrier interactions with the 4-substituent are diminished or prevented.

In summary, this study demonstrates that if hallucinogenic phenylalkylamine derivatives have effective psychoactive doses in humans of about 15 mg or greater, there may be an influence on serotonin function beyond direct action at 5-HT_{2A} receptors at clinically relevant brain concentrations. Apparently, increased synaptic 5-HT concentration could be a contributing

factor in the mechanism of psychoactive effects of these compounds. Increased synaptic 5-HT concentration is *not* likely to contribute to the effects of the hallucinogenic phenylisopropylamines (DOB, DOI, DOTFM) included in this study; the IC_{50} values of these compounds are 50 to 100-fold higher than their expected psychoactive brain concentrations.[152] It should therefore be possible to dissociate the 5-HT-releasing component from the direct 5-HT_{2A} receptor-activating component of the mechanism of action using behavioral studies. For example, 5-HT_{2A} antagonists should not block (or at most, partially block) the interoceptive cue in animals trained to discriminate serotonin releasing agents from saline, but should be very effective at blocking the discriminative cue of DOI. Serotonin depletion, on the other hand, should abolish or attenuate the discriminative cue of serotonin releasing agents, but should have no effect on the cue produced by DOI.

It is evident from this study that the catecholamine uptake/release mechanisms have more rigorous structural requirements than the 5-HT site (Table VIII). This was proposed in Chapter Three, and the present results strengthen this conclusion.

Certain structural features, specifically the nature of the 4-substituent, the conformation of the alkylamine side-chain, and the presence of substi-

tuents at the 2- and 5-positions of 4-substituted phenylisopropylamines play important roles in interactions with the monoamine uptake or release mechanisms. Methoxy substitutions at the 2- and 5-positions attenuate the activity of these drugs at all monoamine uptake/release sites. Among the (2,5-dimethoxy)phenylisopropylamines, a more electronegative substituent at the 4-position was associated with decreased potency in inhibiting 5-HT uptake. Experiments using compounds with 4-substituents having higher or lower electronegativities (*e.g.* F, CBr_3) would be useful in testing this hypothesis.

Comparing the phenylisopropylamines with their benzoxepin analogs suggests the possibility of two separate interactions in the binding pocket of the serotonin carrier, with the conformation of the alkylamine side-chain controlling the binding order at these two sites. However, interference with binding caused by steric bulk or unfavorable electrostatic interactions around the 1- or 2-positions of the benzoxepins may have obscured the "real" gain in potency resulting from side-chain constraint. Serotonin uptake studies using substituted naphthalenes or isoquinolines (*e.g.* 5-methoxy-6-substituted-1,2,3,4-tetrahydronaphth-1-yl-methylamine and 7-methoxy-8-substituted-hexa-hydro-benzo[h]isoquinoline), in which the alkylamine geometry is fixed without introducing steric or electrostatic liability around the "2" position

would help answer this question.

Pharmacological Properties of Indan Analogs of Fenfluramine and Norfenfluramine

Abstract

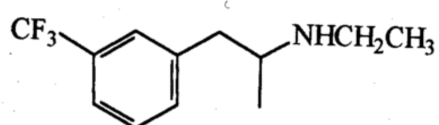
N-ethyl-5-trifluoromethyl-2-aminoindan (ETAI) and 5-trifluoromethyl-2-aminoindan (TAI) were used to study the effects of side-chain cyclization on the pharmacological properties of the anorectic drugs fenfluramine (FEN) and norfenfluramine (norFEN). It was hypothesized that the indan compounds would have reduced neurotoxic liability compared to FEN and norFEN due to decreased activity at catecholamine uptake sites, but would retain the anorectic effects of FEN and norFEN by retaining activity at serotonin (5-HT) sites. The hypothesis was supported by the following results: ETAI and TAI selectively inhibited the ability of synaptosomes to accumulate 5-HT, but were threefold less potent than FEN and norFEN at inhibiting catecholamine uptake. Superfusion experiments revealed that FEN, norFEN, and TAI induced release of 5-HT, whereas ETAI did not, except at high concentrations. ETAI is therefore a 5-HT uptake blocker and TAI is both an uptake blocker and releaser. Rats treated with ETAI or TAI lost 10-15% of their pretreatment body weight in four days, as did rats treated with FEN and norFEN; control animals gained 8% over the same period. Decreases in the density of 5-HT uptake sites induced by ETAI and TAI were about 50% less

than the decreases induced by FEN and norFEN. In drug discrimination assays, ETAI and TAI substituted for drugs that simultaneously activate serotonin and catecholamine function, but did not substitute for drugs that activate serotonin or catecholamine function selectively. Taken together, these results indicate that ETAI and TAI may be useful anorectic drugs with reduced neurotoxic liability, that 5-HT is involved in body weight regulation, and that catecholamines are involved in neurotoxicity.

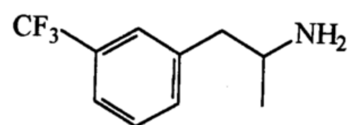
Introduction

Fenfluramine is a substituted phenylisopropylamine that is used as an adjunct to dietary restrictions for the clinical treatment of obesity.[25-27] Fenfluramine is largely metabolized to its *N*-dealkylated derivative, norfenfluramine, which is also active[15,163,164] (Figure 14). The mechanism of anorexia apparently involves decreased neuronal activity in the "feeding center" of the brain, located in the lateral hypothalamus, and increased neuronal activity in the "satiety center", located in the ventromedial hypothalamus.[165-169] These hypothalamic actions are thought to result from increased serotonin (5-HT) release from central neurons.[28-30,170]

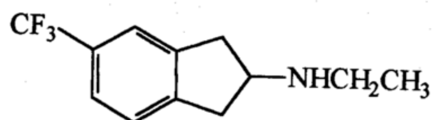
As early as 1967, it was reported that fenfluramine caused long-lasting decreases in 5-HT brain levels in rats.[171] This was later confirmed and extended when researchers showed that in addition to decreases in 5-HT, there were also decreases in serotonin's major metabolite, 5-hydroxyindole acetic acid, and in the number of 5-HT uptake sites labeled with [^{3}H]paroxetine.[172-175] Tryptophan hydroxylase activity was also significantly depressed.[176,177] Since the plasma half-life of fenfluramine in rats is about 4 hours, and that of norfenfluramine is about 24 hours,[178] and since the



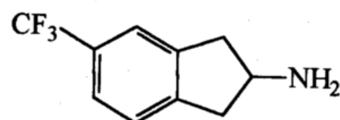
FEN



norFEN



ETAI



TAI

Figure 14. Chemical Structures of Fenfluramine, Norfenfluramine, and Aminoindans.

decreases in 5-HT markers persisted for many months,[174,175,177] the possibility was raised that fenfluramine and norfenfluramine were toxic to serotonergic neurons.

Anatomic studies of rodents and non-human primates treated acutely with fenfluramine revealed massive losses of 5-HT immunoreactivity, as well as morphological changes to serotonergic axons, including swelling, breakage, and degeneration.[126,179,180] Fine serotonergic fibers originating in the dorsal raphe nuclei and projecting to the cerebral cortex are specifically targeted by these drugs. The mechanism of neurotoxicity is unknown, but the pattern of degeneration is similar to that seen with other neurotoxic phenylisopropylamines such as PCA, MDA, and MDMA.[180]

Previous work by Fuller, *et al.*[181,182] and Nichols, *et al.*[63,183] demonstrated that cyclization of the alkylamine side-chain of phenylisopropylamine-based neurotoxins such as PCA and MDA abolished serotonergic neurotoxicity with no diminution in behavioral effects. These indans and tetralins also had a decreased ability to inhibit [^{3}H]dopamine ([^{3}H]DA) uptake into synaptosomes or to stimulate [^{3}H]DA release.[11,13,60-62] This suggested that increased DA function is somehow involved in the mechanism of serotonergic neurotoxicity, and that this effect can be selectively attenuated.

It was therefore hypothesized that the cyclized analog of fenfluramine, *N*-ethyl-5-trifluoromethyl-2-aminoindan (ETAI), and its *N*-dealkylated derivative, 5-trifluoromethyl-2-aminoindan (TAI) (Figure 14), would retain the anorectic effects and 5-HT releasing properties of fenfluramine and nor-fenfluramine, but would have decreased neurotoxic potential due to decreased interactions with catecholaminergic neurons. Thus, ETAI, TAI, fenfluramine (FEN), and norfenfluramine (norFEN) were examined for their effects on monoamine uptake and release in synaptosome preparations, and for their discriminative cue properties in rats trained to discriminate various mono-amine-activating drugs from saline. The test drugs were also evaluated for their effects on [^{3}H]paroxetine binding site density and for their effects on body weight.

Pharmacology

Crude synaptosomes were prepared and used for static neurotransmitter uptake studies and superfusion release experiments. Drugs were screened for static [^{3}H]5-HT, [^{3}H]DA and [^{3}H]norepinephrine ([^{3}H]NE) uptake inhibition at 25 μM. If a drug displayed greater than 50% inhibition of neurotransmitter accumulation at 25 μM, the IC_{50} was determined from displacement curves using eight concentrations of the drug. IC_{50} values were calculated from at least three experiments, each done in triplicate, as described under **Methods** (Synaptosome Technique). Multiple IC_{50} comparisons were made using one-way ANOVA and a *post hoc* Tukey-Kramer *t* test (see **Methods**).

ETAI, TAI, FEN, and norFEN were also tested for their ability to stimulate release of [^{3}H]5-HT from superfused synaptosomes using three concentrations, each done in triplicate (see **Methods**). This method, together with the results of a static uptake experiment, distinguishes whether a drug interferes with synaptosomal neurotransmitter accumulation by blocking neurotransmitter uptake or whether the drug stimulates release of the neurotransmitter.

Some animals were treated with ETAI, TAI, FEN, and norFEN to

determine the effects of these drugs on body weight and on the density of [^{3}H]paroxetine binding sites (a putative marker for serotonergic axon terminals). Rats were randomly assigned to one of five treatment groups, with eight animals per group, and were treated intraperitoneally with 10 mg/kg of test drugs or saline twice a day for four days, and their weights were taken on the fourth day. Two weeks later, these animals were sacrificed and various brain tissues were assayed for specific [^{3}H]paroxetine binding using a single-point determination at a saturating (1 nM) concentration of [^{3}H]paroxetine. Specific binding was defined with 1 μM fluoxetine. Data from these experiments were analyzed using one-way ANOVA followed by a Dunnett's *t* test.

Drug discrimination studies were conducted (see **Methods**) to determine whether the test drugs substituted for drugs known to activate monoamine function. Drugs were evaluated in rats trained to discriminate a selective 5-HT releaser/uptake blocker, 5-methoxy-6-methyl-2-aminoindan (MMAI);[60,61] a mixed 5-HT and NE releaser/uptake blocker, *(S)*-1-(1,3-benzodioxol-5-yl)-2-butanamine (*S*-MBDB);[1,11,13] a DA and NE releaser/uptake blocker, *d*-amphetamine (AMP);[13] and a mixed 5-HT/catecholamine receptor agonist, *d*-lysergic acid diethylamide (LSD),[24,141,186,187] from saline.

If a test drug produced full substitution (FS) for a training drug at any dose, the ED_{50} and 95% confidence interval were calculated using the method of Litchfield and Wilcoxon as incorporated in the computer program Pharmacologic Calculation System Version 4 by Tallarida and Murray.[48] See **Methods** (Drug Discrimination Studies) for details.

Results

The test compounds exhibited single-site displacement curves for monoamine uptake, with slope coefficients not different from 1. Figure 15 is a representative plot showing the concentration-dependent inhibition of $[^3H]$DA uptake by norFEN. All of the test drugs were potent inhibitors of $[^3H]$5-HT accumulation into synaptosomes using the static uptake assay (Table IX). FEN, norFEN, and TAI inhibited $[^3H]$5-HT uptake with similar IC_{50}'s, in the range of 500-600 nM. ETAI was 4- to 5-fold less potent, with an IC_{50} of 2.6 μM.

The indan compounds were about 3-fold less potent at inhibiting $[^3H]$DA and $[^3H]$NE uptake than FEN and norFEN, and the presence of an *N*-ethyl moiety decreased potency 3- to 4-fold relative to the primary amine compounds (Table IX).

Experiments using superfused synaptosomes were performed to further study the mechanism of 5-HT uptake inhibition. These experiments revealed that TAI, FEN, and norFEN depressed 5-HT accumulation mainly by evoking the release of 5-HT whereas ETAI functioned as a 5-HT uptake blocker, except at high concentration (100 μM), where ETAI also stimulated release

of the neurotransmitter. Figure 16 illustrates the effect of FEN on [^{3}H]5-HT release, and Figure 17 shows the effect of ETAI on [^{3}H]5-HT release. The mean percent tritium released during fractions 3-5 was calculated, normalized to controls, and used to construct dose-response curves (Figure 18). This plot shows the dose-dependent 5-HT releasing properties of FEN, norFEN and TAI, and the rightward shift of the curves for ETAI and TAI.

The results of the [^{3}H]paroxetine binding experiments revealed that all of the test drugs caused some reduction in the number of binding sites, but that FEN and norFEN were the most potent compounds. FEN and norFEN each reduced the density of binding sites by 60-70% ($P < 0.01$) in cortex, hippocampus, and neostriatum (Figure 19). ETAI and TAI were about half as effective at reducing the number of binding sites in cortex and hippocampus on a mg/kg basis than were FEN and norFEN, producing only 30-35% decreases ($P < 0.01$) in binding site density (Figure 19). There were also regional differences observed: in striatal tissue, although the density of [^{3}H]paroxetine binding appeared to be less than controls for ETAI and TAI, this decrease was not significant (Figure 19).

All of the test compounds were equally effective at reducing body weight over a four day period compared to saline-treated controls (Figure

20). In animals treated with the test drugs, there was a 10-15% reduction in body weight over the four day period. In contrast, saline-treated control animals gained about 8% of their beginning weight over the same time.

In drug discrimination studies, TAI fully substituted, and ETAI partially substituted, for *S*-MBDB, a drug that simultaneously activates 5-HT and NE function indirectly, by causing release of these neurotransmitters[1,11,13] (Table X). TAI had an ED_{50} of 0.54 mg/kg (95% confidence interval = 0.38-0.79 mg/kg) in this assay. Both ETAI and TAI also partially substituted for LSD, a partial 5-HT agonist which also has high affinity for catecholamine sites.[24,141,186,187] At the lowest dose tested (0.5 mg/kg) ETAI partially substituted for the 5-HT releaser MMAI.[60,61] However, higher doses of ETAI failed to substitute for MMAI, and TAI did not substitute for MMAI at any dose tested. Both drugs also failed to substitute for AMP, a catecholamine releaser/uptake blocker[13] (Table X). In these animals, many disruptions were observed.

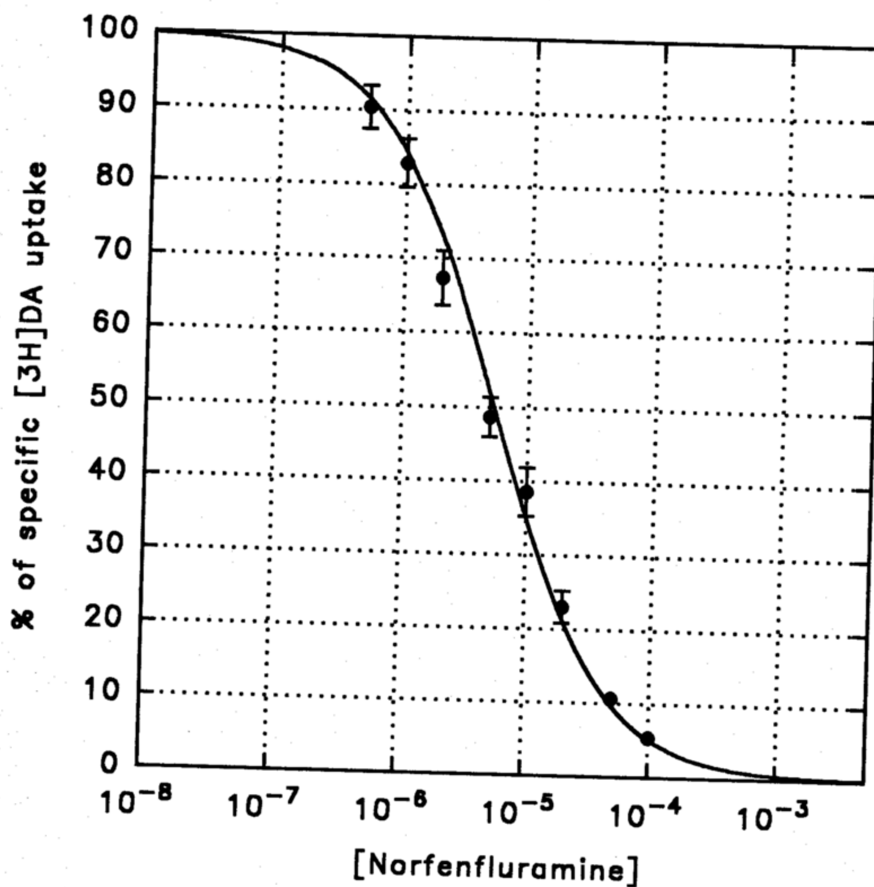


Figure 15. Norfenfluramine Inhibition of Synaptosomal [^{3}H]DA Accumulation. The ability of norFEN to inhibit uptake of [^{3}H]DA was examined in crude synaptosomes. Data are the mean ± SEM of three determinations, each run in triplicate. Data were fitted to a four-parameter logistic curve for IC_{50} determination and plotting.

Table IX. IC_{50} for Monoamine Uptake Inhibition. III.[a]

Compound	IC_{50} (μM)		
	[^{3}H]5-HT	[^{3}H]DA	[^{3}H]NE
FEN	0.490 ± 0.017	21.5 ± 0.85	8.28 ± 1.4
NorFEN	0.590 ± 0.062	5.41 ± 0.92[c]	2.75 ± 0.22[e]
ETAI	2.61 ± 0.34[b]	> 25	> 25
TAI	0.604 ± 0.066	17.8 ± 0.041[d]	7.41 ± 0.96

[a]The ability of the test compounds to inhibit accumulation of monoamines was examined in crude synaptosomes. For IC_{50} determination, compounds were examined at eight concentrations, each run in triplicate. The data from three experiments were combined and fitted to a four-parameter logistic curve from which IC_{50} values ± SEM were calculated. Multiple comparisons were made using ANOVA followed by a Tukey-Kramer *t* test (see **Methods**). [b]$P < 0.001$ vs **FEN, norFEN, TAI**. [c]$P < 0.001$ vs **FEN, TAI**. [d]$P < 0.05$ vs **FEN**. [e]$P < 0.05$ vs **FEN, TAI**.

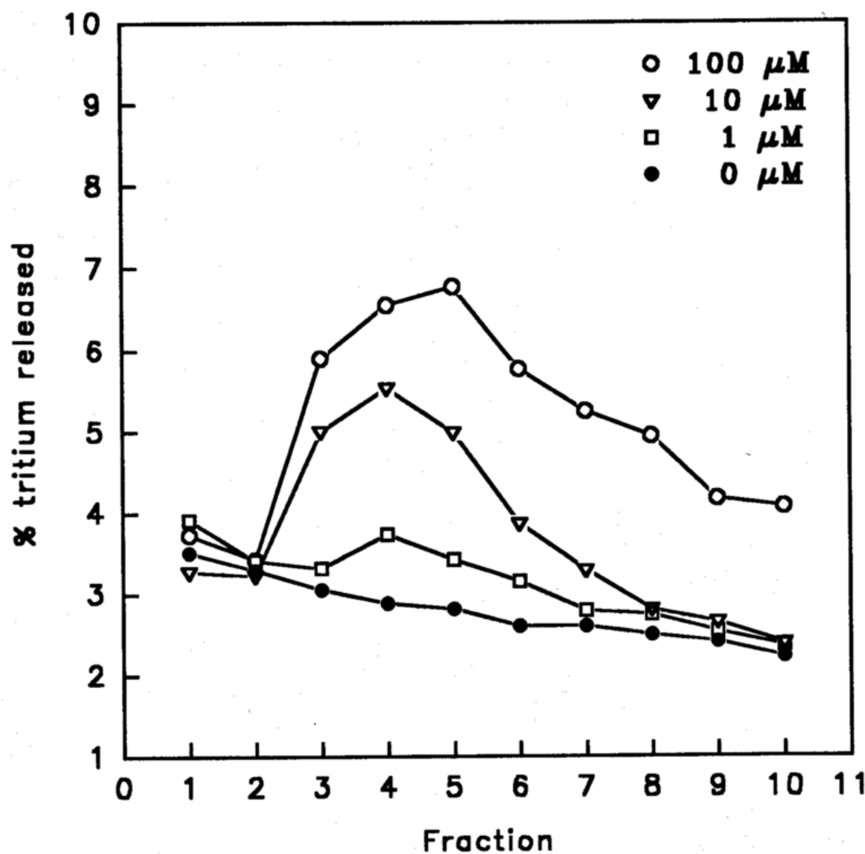


Figure 16. Effect of FEN on Synaptosomal [^{3}H]5-HT Release. Crude synaptosomes were prepared and superfused with various concentrations of fenfluramine during fractions 3 and 4 (see **Methods**). Data were transformed from dpm to percent tritium released; each point is the mean of three determinations, each run in triplicate. SEM not shown for clarity, ~ 10%.

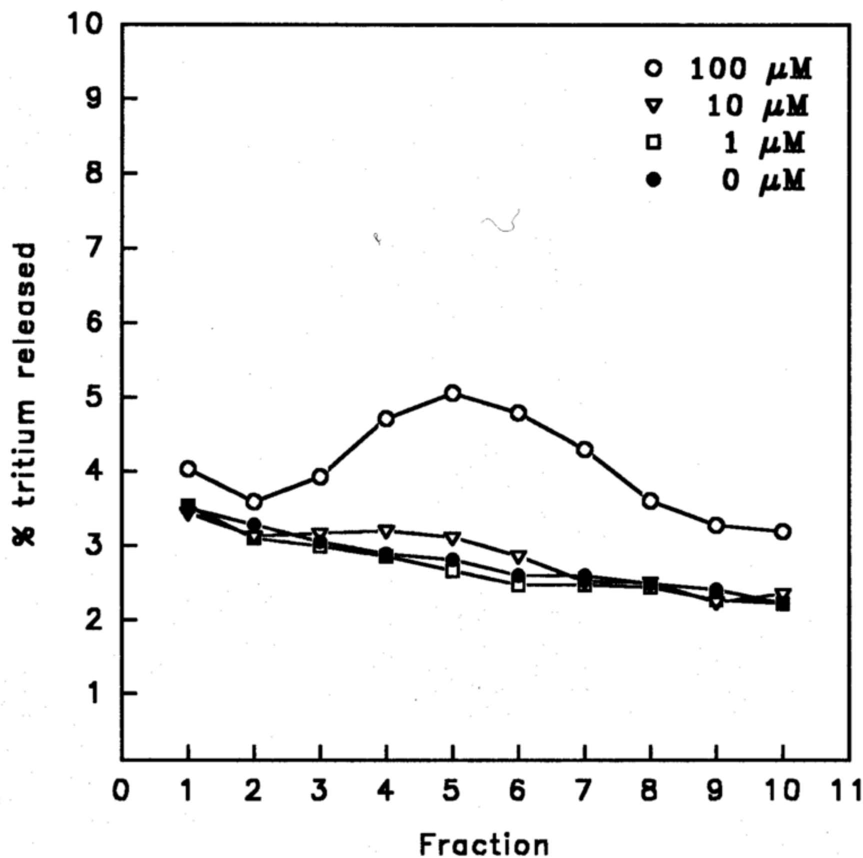


Figure 17. Effect of ETAI on Synaptosomal [^{3}H]5-HT Release. Crude synaptosomes were prepared and superfused with various concentrations of ETAI during fractions 3 and 4 (see **Methods**). Data were transformed from dpm to percent tritium released; each point is the mean of three determinations, each run in triplicate. SEM not shown for clarity, ~ 10%.

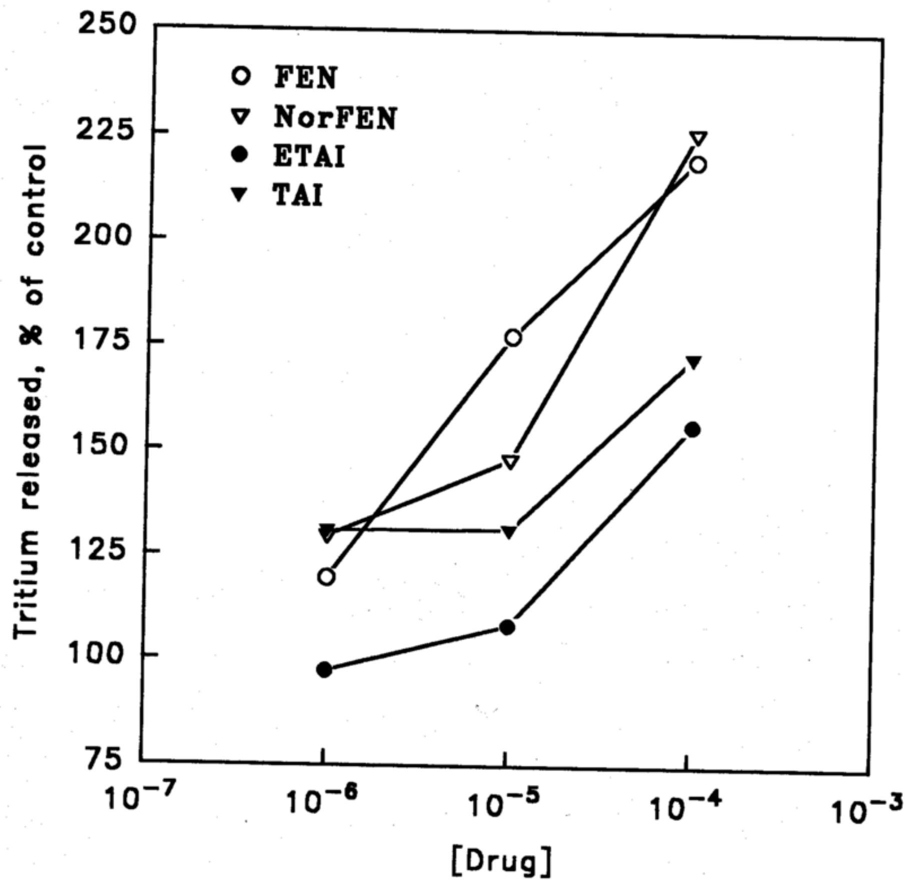


Figure 18. Effect of FEN, norFEN, ETAI, and TAI on Synaptosomal [^{3}H]5-HT Release. Data from superfusion experiments were used to generate dose-response curves. The mean percent tritium released during fractions 3-5 was calculated, normalized to release in the absence of drugs, and plotted as a function of drug concentration. SEM not shown for clarity, ~ 10%.

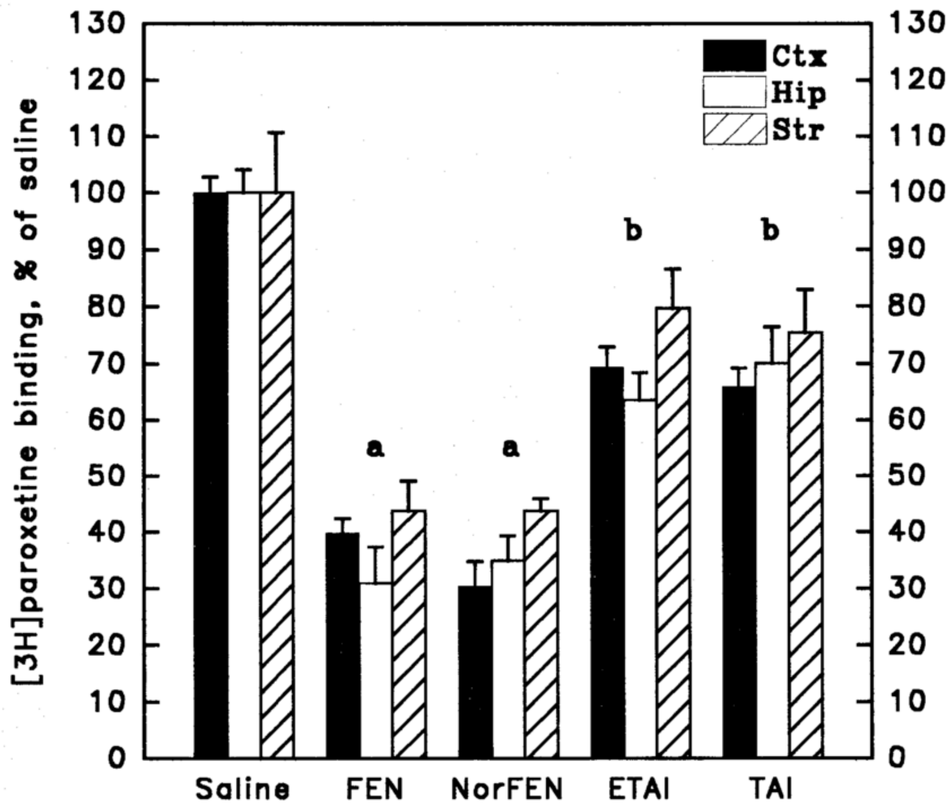


Figure 19. Effect of FEN, norFEN, ETAI, and TAI on [^{3}H]Paroxetine Binding. The density of specific [^{3}H]paroxetine binding sites in cortex, hippocampus, and neostriatum was assessed 10 days after a 4 day regimen of drug (2 x 10 mg/kg/day) or saline treatment. Densities were normalized to saline-treated controls. N = 8 per treatment. ^{a}P < 0.01 vs saline in all tissues. ^{b}P < 0.01 vs saline in cortex and hippocampus only.

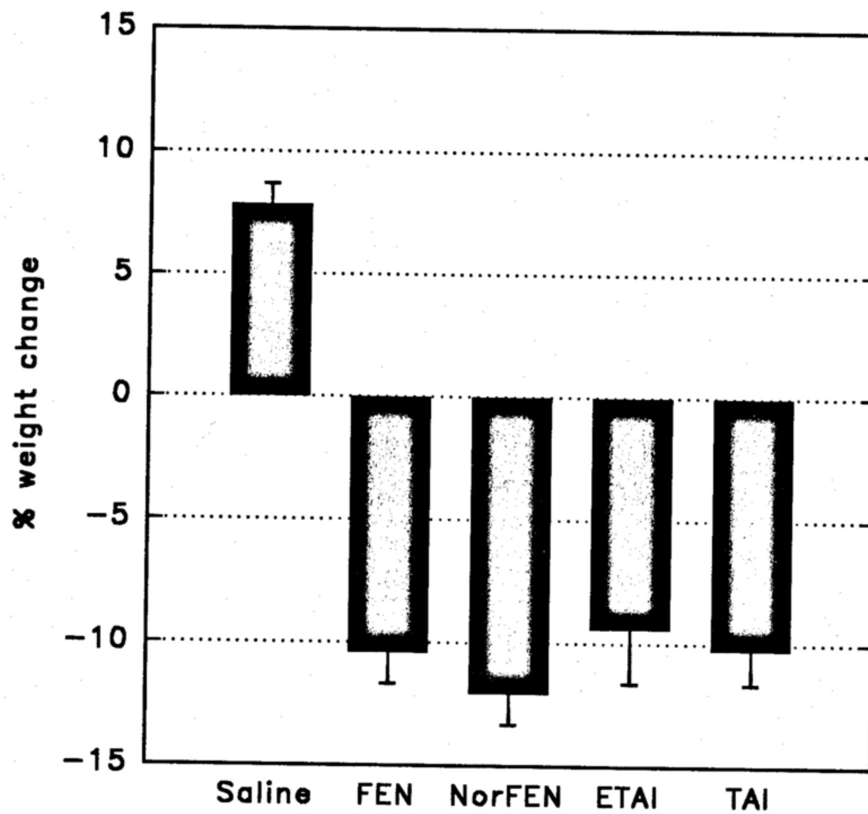


Figure 20. Effect of FEN, norFEN, ETAI, and TAI on Body Weight. Rat body weights were taken after a 4 day regimen of drug (2 x 10 mg/kg/day) or saline treatment. Data are expressed as percent change from pretreatment weight. N = 8 per treatment.

Table X. Drug Discrimination Data for ETAI and TAI[a]

Training Drug	Test Drug	Dose, mg/kg	N	% SDL[b]	% disrupted	Level of Substitution[c]
MMAI	**ETAI**	0.5	5	60	0	PS
		1.0	6	33	0	
		2.0	4	0	25	
	TAI	0.5	8	14	12.5	NS
		1.0	9	29	22	
		2.0	9	0	77	
***S*-MBDB**	**ETAI**	0.5	11	44	18	PS
		1.0	15	31	13	
		2.0	14	70	29	
		4.0	5	50	60	
	TAI	0.25	17	20	11	FS
		0.5	16	50	12.5	
		1.0	16	64	12.5	ED_{50} =
		2.0	11	100	27	0.54 mg/kg
AMP	**ETAI**	0.5	3	0	0	NS
		1.0	3	0	0	
		2.0	6	0	33	
		4.0	2	0	50	
	TAI	0.5	3	0	0	NS
		1.0	6	0	66	
		2.0	2	0	50	
		4.0	2	0	100	
LSD	**ETAI**	0.5	5	60	0	PS
		1.0	12	45	8	
		2.0	12	62.5	33	
	TAI	0.5	13	38	0	PS
		1.0	7	60	29	
		2.0	9	33	67	

[a]Rats were trained to discriminate training drugs from saline. ETAI and TAI were administered to test whether they substituted for training drugs (see **Methods**). [b]Percent selecting drug lever. [c]See **Methods** for level criteria.

Discussion

These studies demonstrate that selectivity of drug interactions with serotonergic and catecholaminergic neurons can be modulated by constraining the geometry of the alkylamine side-chain of phenylisopropylamine drugs. This confirms and extends previous work with various substituted amino-indans by Nichols, *et al.*[61,63,183] When the side-chains of FEN and norFEN were cyclized into the indan structure, as in ETAI and TAI, potency at inhibiting catecholamine uptake was decreased to about one-third of that observed for the analogous phenylisopropylamines, with activity at NE sites about twice that observed at DA sites (Table IX). ETAI was less effective than TAI at inhibiting 5-HT uptake. This indicates that the 5-HT carrier mechanism does not tolerate a bulky amine substituent in an aminoindan, but that such substituents are tolerated in a phenylisopropylamine. FEN, by virtue of its flexible side-chain, is able to adopt a favorable binding conformation despite the presence of an *N*-ethyl substituent.

The superfusion studies revealed that the test compounds inhibited serotonin accumulation through at least two different mechanisms. FEN, norFEN, and TAI all were effective at inducing the release of 5-HT at

concentrations near their IC_{50} values for uptake inhibition, whereas ETAI was ineffective even at five times its IC_{50} (Figures 15-17). ETAI did stimulate 5-HT release, but only at high (100 μM) concentration. This indicates that some of the 5-HT uptake-inhibiting effects of FEN, norFEN, and TAI observed in the static uptake experiments are due to the drugs' ability to evoke 5-HT release, superimposed on their ability to act as competitive substrates for 5-HT at the uptake carrier. This conclusion is consistent with work reported by Gobbi, *et al.*,[184] which showed that FEN at 500 nM induced synaptosomal 5-HT release through an exocytotic mechanism dependent upon extracellular Ca^{++}, and with work by Garattini, *et al.*,[185] which suggested that FEN uses the 5-HT uptake carrier to enter synaptosomes. ETAI, on the other hand, acts as a "pure" uptake blocker at its IC_{50}.

Analysis of [^{3}H]paroxetine binding following treatment with the test drugs revealed additional differences between the phenylisopropylamines and the indans. While FEN and norFEN both reduced the number of binding sites by about 60-70% in cortex, hippocampus, and neostriatum, ETAI and TAI reduced the number of binding sites in cortex and hippocampus by only 30-35% and did not significantly reduce striatal binding (Figure 19). These

results support the hypothesis that increased synaptic catecholamine concentrations are necessary for serotonergic neurotoxicity: ETAI and TAI were less effective than FEN and norFEN at inhibiting NE and DA accumulation *in vitro* (Table IX), and they were less effective at decreasing the number of 5-HT uptake sites (Figure 19). Interestingly, the patterns of [^{3}H]paroxetine binding decreases across the different brain regions were similar for drugs with *N*-ethyl substituents (FEN and ETAI) and for those with primary amines (norFEN and TAI) (Figure 19).

All of the test compounds were equally effective in causing a reduction in body weight following a 4 day treatment. This supports the position that brain serotonin is involved in body weight regulation, since the test drugs share the ability to increase synaptic 5-HT at low concentrations but are 10- to 30-fold less potent at increasing synaptic catecholamine concentrations (Table IX). It is known that FEN and norFEN decrease food intake,[164] but it is possible that ETAI and TAI decrease body weight through some other, nonanorectic mechanism. For example, these compounds may elevate basal metabolism through a nonserotonergic mechanism, or may interact with serotonergic neurons in the gut to change bowel transit times or other parameters to interfere with nutrient absorption. Feeding studies in which the

animals' food intake is monitored, metabolic studies in which O_2 uptake and CO_2 production are monitored, and experiments in which the gut transit time of latex beads is examined in the absence and presence of these drugs would resolve these questions.

The drug discrimination results largely paralleled the results of the *in vitro* pharmacology. Based on the monoamine IC_{50} values (Table IX), one would predict that ETAI and TAI would not substitute for drugs that activate catecholamine function (AMP), but would substitute for drugs that activate serotonin *and* catecholamine function (*S*-MBDB, LSD), or serotonin function only (MMAI). The data show that neither ETAI nor TAI substituted for AMP (Table X). ETAI partially substituted, and TAI fully substituted, for *S*-MBDB. The ED_{50} value for TAI (Table X) suggests that TAI is perhaps slightly more potent than *S*-MBDB (training dose = 1.75 mg/kg) at eliciting the discriminative cue. Both ETAI and TAI partially substituted for LSD. However, only the lowest dose of ETAI produced partial substitution for MMAI, and TAI did not substitute for MMAI at any dose tested (Table X).

This last result is puzzling, since ETAI and TAI are 10- to 30-fold selective for 5-HT neurons *in vitro* (Table IX), and TAI evokes 5-HT release (Figure 18), while MMAI is 50- to 100-fold selective for 5-HT neurons,[62]

and also evokes release.[60] The partial substitution of ETAI for MMAI must be viewed suspiciously, since it only occurred at low frequency at one dose; also, TAI is the more potent 5-HT releaser/uptake blocker *in vitro*. The lack of substitution at higher doses of ETAI and at all doses of TAI indicates that the discriminative cue produced by these drugs includes another, probably catecholaminergic component (which is picked up by the animals trained to discriminate the less selective drugs *S*-MBDB and LSD) that is not activated by the MMAI training dose. The 3- to 5-fold higher selectivity of MMAI compared to ETAI and TAI for 5-HT neurons *in vitro* is thus reflected by its stricter requirements for substitution in drug discrimination: the indans' catecholaminergic properties interfere with the serotonergic cue. Parenthetically, the 5-HT selectivity of MMAI suggests that it should also have anorectic properties, if the 5-HT hypothesis of feeding behavior is correct.

It should be kept in mind that in the drug discrimination studies, the test compounds are administered intraperitoneally 30 min prior to testing. This route of administration allows for peripheral metabolism and opens the possibility that ETAI and TAI are extensively transformed, probably via *N*-dealkylation or deamination, during the course of a drug discrimination experiment. The *in vitro* protocols eliminate the latter metabolic route by

including pargyline in the incubation buffers to inactivate monoamine oxidase (MAO). Also, in the superfusion experiments, fresh drug is continuously presented to the synaptosomes and potential metabolites are washed away. The behavioral pharmacology possibly represents the expression of a mixture of ETAI and TAI (or other metabolite) psychoactivity then, even when ETAI is administered alone.

In summary, these studies demonstrate that the side-chain conformation and *N*-alkylation of phenylisopropylamines are important determinants for interactions with monoamine-releasing neurons, with catecholaminergic cells exhibiting more rigorous structural requirements for uptake inhibition than serotonergic cells. This is consistent with conclusions reached in Chapters Three and Five. The indan structure changes the mechanism of 5-HT accumulation inhibition from one that is a combination of uptake blocking and release-evoking properties (FEN) to one that is essentially all uptake blocking (ETAI), and the primary amine (TAI) is apparently more easily transported into the cytosol by the 5-HT uptake carrier than is the *N*-alkylated compound (ETAI).

The indan-based drugs also reduced the neurotoxic potential of substituted phenylisopropylamines as assessed by changes in the density of

$[^3H]$paroxetine binding sites. These results, together with the data from the monoamine uptake and release experiments, implicate catecholamines in the mechanism of neurotoxicity at serotonergic axons and terminals.

ETAI and TAI were as effective as FEN and norFEN in decreasing body weight. Since these drugs share the ability to selectively increase synaptic 5-HT concentrations, 5-HT is implicated in body weight regulation. However, other mechanisms, including metabolic effects, cannot be ruled out.

The results of the drug discrimination studies generally supported predictions made on the basis of *in vitro* experiments regarding similarities between ETAI and TAI and training drugs. ETAI and TAI either fully or partially substituted for training drugs that simultaneously activate both 5-HT and catecholamine function, but not for drugs that are "purely" catecholaminergic or serotonergic.

Together, these results support the hypothesis proposed for this study, and suggest that ETAI and TAI may be useful anorectic drugs with reduced neurotoxic liability; further studies are indicated to assess this potential.

Conclusion

This thesis has examined the molecular, neurochemical, and behavioral pharmacology of a number of different phenylalkylamine derived drugs. It is evident from published reports that minor modifications to the drugs' molecular structures can result in large differences in the psychopharmacologies exhibited by these substances. To understand the psychopharmacology, one must include an understanding of the ways in which different molecular physico-chemical properties and structural permutations lead to diverse interactions with the elements of the central nervous system. This has been one goal of the present work.

Among entactogens, it was shown that interactions with monoamine neurons can be modulated by the position and orientation of the unshared electrons present on phenyl ring ether-linked oxygen atoms. This, in turn, has suggested a model for the binding site on serotonin and catecholamine carrier proteins, and suggests how new monoamine-selective drugs may be designed to exploit these binding site interactions.

It is generally accepted that hallucinogenic phenylalkylamines owe their psychoactivity to specific interactions with 5-HT_{2A} receptors. Some researchers have gone further, and demonstrated post-receptor biochemical or electrophysiological effects due to these drugs. In this thesis, evidence was

presented that 5-HT_{2A} receptors modulate GABA function, and a model was proposed to relate altered GABA function to hallucinogenic activity. The model may be useful in understanding brain functions such as sensory perception, integration, and interpretation, and further experiments are suggested to test the model's utility.

It was also shown that nonspecific serotonin activation may contribute to the psychoactivity of some hallucinogenic phenylalkylamines. Drugs which require effective doses in the 20-100 mg range very likely interact with multiple receptor sites or monoamine transport proteins, and such interactions probably give rise to the subtleties in psychoactive effects seen with these drugs. The combination of direct receptor interactions with effects on serotonin or other monoamine carrier proteins can be manipulated by varying certain molecular fragments, such as the substituent at the 4-position, or the conformation of the alkylamine side-chain in these drugs.

The psychopharmacology of anorexia often involves central stimulation and hyperexcitability. With fenfluramine, these unwanted effects have been largely eliminated, but other concerns, namely neurotoxicity, have been raised. It was shown that weight loss may be isolated from potential neurotoxicity with judicious molecular modification, and these two physiological

effects were related to changes in synaptic serotonin and catecholamine concentrations, respectively. In this process, two potentially useful therapeutic agents were discovered.

If one considers the data on phenylalkylamine interactions with monoamine neurotransmitters presented in this thesis as a whole, one fact becomes obvious: the catecholamine carriers have much more rigorous structural requirements for ligands than does the 5-HT carrier. This is reflected in the uniformly larger IC_{50} values at catecholamine sites compared to 5-HT sites across all drugs examined in these studies. While interactions at all of the monoamine carriers can be manipulated by certain structural modifications, the selectivity for 5-HT carriers is often achieved because of the catecholamine carriers' intolerance to deviations from an "ideal" ligand: a phenylalkylamine with a hydrogen bond-capable moiety at the ring 3-position, no substitutions at the ring 2- or 5-positions, and a flexible alkylamine side-chain (or perhaps a properly constrained side-chain), with no amine substitutions. When these conditions are not met, one obtains 5-HT-selective compounds by default. It is tempting to speculate that the differences in ligand structural specificity arise because 5-HT and the 5-HT carrier evolved earlier than did the catecholamines and their carriers. In this scene, the

catecholamine carriers would have had to discriminate among several monoamine neurotransmitters, whereas the 5-HT carrier, predating the appearance of the catecholamines, would not. Consistent with this idea, it is noted that there are many more known serotonin receptor subtypes than there are known subtypes of catecholamine receptors.

Gross changes in neurotransmitter function, as observed and measured *in vitro*, do not go far enough to explain or describe the very different psychological effects of the various classes of drugs studied here. It is very difficult to explain how a drug such as MDMA elicits entactogenic effects and why a drug such as fenfluramine does not; both drugs potently and selectively increase serotonin function *in vitro*. Even sophisticated animal behavioral assays such as drug discrimination fall short of being able to predict the unique human psychopharmacology of these compounds. For example, rats will completely substitute MDMA for fenfluramine in such assays, yet it is known from other reports that these drugs produce very different effects in humans. Ultimately, it will be possible to relate the molecular, cellular, and behavioral pharmacology to the human psychopharmacology only if clinical studies are performed to complement the *in vitro* and animal behavioral work.

Most of the clinical studies with hallucinogens were performed over 25 years ago, before the discovery of the receptor-selective agents now available. And there is very little in the scientific literature regarding the human psychopharmacology of entactogens. Most of the current knowledge we have about these compounds comes from anecdotal reports or sensational essays in the news media and popular press. Such accounts are obviously of limited scientific value.

It must be remembered that the major goal of this research is to understand drug effects on *human* brain function, with the possibility of providing new pharmacological strategies for the treatment of *human* disease. To the extent that animal studies provide direction to future work in humans, it is hoped that the present efforts have contributed to this goal.

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