Yale University
EliScholar – A Digital Platform for Scholarly Publishing at Yale

Yale Medicine Thesis Digital Library | School of Medicine

1977

The interactions of cholinergic and anticholinergic drugs with nigro-neostriatal dopaminergic neurons

Robyn G. Young
Yale University

Follow this and additional works at: http://elischolar.library.yale.edu/ymtdl

Recommended Citation
Young, Robyn G., "The interactions of cholinergic and anticholinergic drugs with nigro-neostriatal dopaminergic neurons" (1977). *Yale Medicine Thesis Digital Library*. 3342.
http://elischolar.library.yale.edu/ymtdl/3342

This Open Access Thesis is brought to you for free and open access by the School of Medicine at EliScholar – A Digital Platform for Scholarly Publishing at Yale. It has been accepted for inclusion in Yale Medicine Thesis Digital Library by an authorized administrator of EliScholar – A Digital Platform for Scholarly Publishing at Yale. For more information, please contact elischolar@yale.edu.

YALE MEDICAL LIBRARY
3 9002 08676 2151

THE INTERACTIONS OF CHOLINERGIC AND ANTICHOLINERGIC DRUGS WITH NIGRO-NEOSTRIATAL DOPAMINERGIC NEURONS

Robyn G. Young

1977

YALE
MEDICAL LIBRARY

YALE
MEDICAL LIBRARY

Permission for photocopying or microfilming of "Interactions of Cholinergic + Anticholinergic Drugs -- etc."

(TITLE OF THESIS)

for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Robyn G. Young

Signature of Author

4/25/77

Date

Digitized by the Internet Archive
in 2017 with funding from
The National Endowment for the Humanities and the Arcadia Fund

https://archive.org/details/interactionsofch00youn

THE INTERACTIONS OF CHOLINERGIC AND ANTICHOLINERGIC DRUGS WITH NIGRO-NEOSTRIATAL DOPAMINERGIC NEURONS

by

Robyn G. Young

B.S., Stanford University, 1973

A Thesis Submitted to the Faculty of

the Yale University School of Medicine,

Department of Pharmacology

in partial fulfillment of the

requirements for the degree

Doctor of Medicine

1977

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DEDICATION

This thesis is dedicated to the loving memory of my grandfather, "Papadore", who felt that women only went to college to get married.

ACKNOWLEDGMENTS

I would sincerely like to thank Dr. Robert Roth for his help and guidance throughout the course of this thesis; Dr. Jonathan Pincus for his assistance, especially in the initiation of this project; Dr. Benjamin Shaywitz for the use of his 6-hydroxydopamine treated rats; Dr. Benjamin Bunney for reviewing this thesis; and Dr. David Cox for his unending support.

ACKNOWLEDGMENTS

I would sincerely like to thank Dr. Robert Bath for his
help and guidance throughout the course of this thesis.
Dr. Jonathan Tinnon for his assistance, especially in
the initiation of this project; Dr. Benjamin Shepperd for
the use of his 6-hydroxydopamine creatinine test; Dr. Benjamin
Bunney for reviewing this thesis; and Dr. David Cox for
his unending support.

INTRODUCTION

The purpose of this thesis was to further elucidate the effects and site of action of the cholinergic drugs on dopaminergic neurons in rat striatum with the use of certain biochemical parameters which provide an index of dopaminergic function. The short term accumulation of Dopa (3,4-dihydroxyphenylalanine), the immediate precursor of dopamine, following inhibition of Dopa decarboxylase was used as an in vivo estimate of tyrosine hydroxylase activity. Dopac (dihydroxyphenylacetic acid), a metabolite of dopamine, was measured to provide an index of changes in the physiological activity of nigro-neostriatal dopamine neurons (Fig. 1).

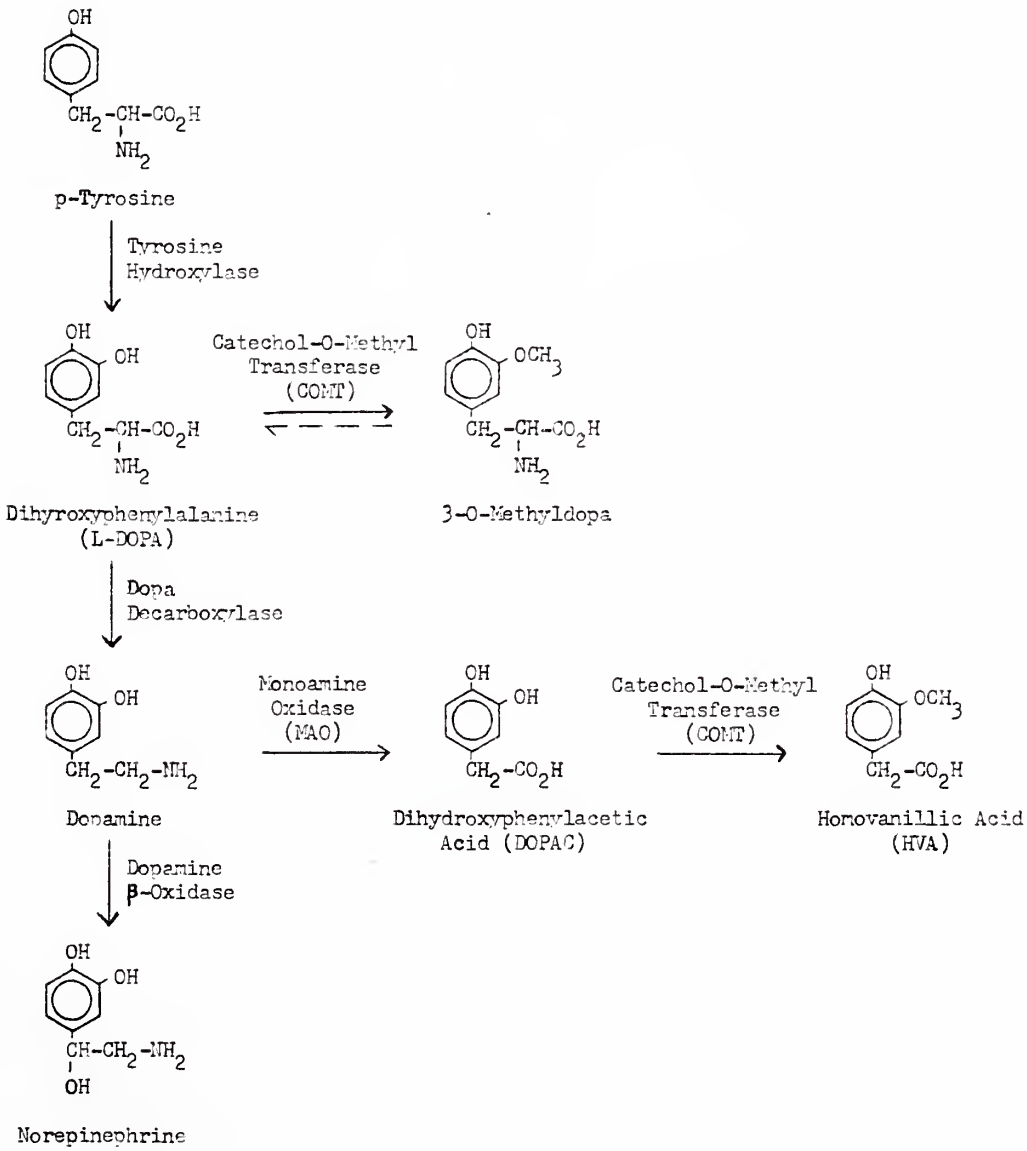
This study investigated:

(1) The effects of a variety of cholinergic and anticholinergic drugs on tyrosine hydroxylase activity, responsible for the conversion of tyrosine to Dopa in the dopamine synthetic pathway (Fig. 1).

(2) The possible site of action of these drugs, whether presynaptic or a more complex neuronal feedback mechanism (Fig. 2).

(3) The possibility that an experimental animal model of Parkinson's Disease might respond differently from normals to the same cholinergic and anticholinergic drugs.

FIGURE 1
DOPAMINE METABOLISM



The Extrapyramidal System:

The extrapyramidal system is responsible for control and fine tuning of motor activity. The basal ganglia, a primary component of this system, includes the corpus striatum (caudate nucleus, putamen, and globus pallidus), the first two known collectively as the neostriatum, and the amygdaloid nucleus.[11] The substantia nigra, the other major component of the extrapyramidal system, consists of the pars compacta and the zona reticulata. The nigrostriatal dopaminergic fiber system extends from the pars compacta of the substantia nigra to the ipsilateral striatum and appears to be topographically arranged. Each fiber has approximately 500,000 boutons to make synaptic contacts with striatal cells. Mid-brain lesions destroying the substantia nigra result in signs of degeneration in between 65% and 100% of the striatal boutons.[29] Stimulation of the substantia nigra increases release of striatal dopamine[29] and causes an increase in dopamine biosynthesis as a result of an activation of tyrosine hydroxylase.[46] Approximately 80% of the total dopamine (DA) and homovanillic acid (HVA), a metabolite of dopamine, in the mammalian brain is found in the caudate nucleus and putamen. The distribution of the dopamine synthesizing enzymes tyrosine hydroxylase and Dopa decarboxylase is similar.[29]

Drugs Affecting Striatal Dopaminergic Activity:[29]

Drugs which increase dopamine or dopamine effects at receptor sites in the striatum may act: (1) directly - as dopamine (or Dopa which is converted to dopamine) or apomorphine, a specific dopamine receptor agonist; (2) indirectly - as amphetamines and amantidine

which cause dopamine to be released from nerve terminals; and (3) as anticholinergics and antihistamines which may act by decreasing the cholinergic component of a dopaminergic - cholinergic balance in the striatum, or some may act by inhibiting the reuptake of dopamine into synaptosomes, making more dopamine available in the synaptic cleft.[14,20]

Drugs which decrease dopamine or dopamine effects at receptor sites may act by: (1) blocking dopamine receptor activity - as the butyrophenones, like haloperidol, and most phenothiazines; (2) inhibiting synthesis of dopamine - as α-methyl-p-tyrosine; (3) inhibiting dopamine storage and depleting synaptic vesicles - as reserpine; and (4) degeneration of the nigrostriatal pathway - as caused by lesions in the substantia nigra or nigrostriatal dopamine pathway, or by 6-hydroxydopamine.[62]

Parkinson's Disease

It is now well established that the basic abnormality in Parkinson's Disease is a striatal dopamine deficiency. The most common etiologies are either idiopathic or post-encephalitic. The majority of the latter cases are thought to be a late sequela of encephalitis lethargica, a disease caused by Von Economo's virus during the 1918 to 1926 pandemic. Other possible causes include senile arteriosclerosis, chronic manganese poisoning, tumor, and a reversible Parkinsonian-like syndrome sometimes resulting from the use of neuroleptic drugs such as reserpine, most phenothiazines, and the butyrophenones. In all but the neuroleptic-induced syndrome, the primary pathological abnormality

is degeneration of the dopamine containing cells in the pars compacta of the substantia nigra projecting to the striatum.[29] In these patients, striatal levels of dopamine and HVA are markedly decreased,[29,31,56] as are the activities of the dopamine synthesizing enzymes, tyrosine hydroxylase and Dopa decarboxylase.[56] In addition, a positive correlation exists between the degree of nigral cell loss, the degree of striatal dopamine deficiency[56], and the severity of the Parkinsonian symptoms of akinesia, rigidity, and tremor.[29] That dopamine levels must fall by 50% or more to manifest the disease clinically, suggests that milder degrees of striatal dopamine deficiency can be compensated for functionally.[29] The reversible drug-induced Parkinson's-like syndrome produced to varying degrees by most of the antipsychotics is a manifestation of the dopamine receptor blocking ability of these drugs rather than of an absolute striatal dopamine deficiency. Consistent with this is the observation that treatment with L-Dopa, which is converted to dopamine by Dopa decarboxylase, produces marked symptomatic improvement in Parkinson's Disease where there is an absolute dopamine deficiency, but is of little value in treating the drug-induced Parkinsonian syndrome where symptoms are due to receptor blockade.[23] Optimal therapy for Parkinson's Disease at present involves the use of L-Dopa in conjunction with carbidopa, a peripheral Dopa decarboxylase inhibitor. With the combined use of these drugs less Dopa needs to be administered, since more enters the brain, and fewer side-effects are experienced from peripheral conversion of Dopa to dopamine.[15,23,45] Although L-Dopa acts by replacing the deficient neurotransmitter,

dopamine, it does not affect the course of Parkinson's Disease, in that there is continued degeneration of the nigrostriatal dopamine neurons.[29] Dopamine levels in the striatum of Parkinson's patients can be increased 5 to 8 times with L-Dopa treatment, and levels of its metabolite, HVA, are increased by even more.[30] HVA accumulates throughout the brain, wherever there is Dopa decarboxylase activity, but dopamine accumulates only in areas of dopaminergic innervation, such as the striatum.[29,31] This could be due to uptake and storage of the dopamine in the dopaminergic nerve terminals or to a depot source, such as 3-O-methyldopa, which also increases in L-Dopa treated patients.[29,31] The former, however, is more likely, as 3-O-methyldopa increases throughout the brain, like HVA, rather than locally, as with dopamine.[30]

Another phenomenon observed in Parkinson's patients and in animals with lesions destroying the nigrostriatal dopamine neurons may be the development of denervation supersensitivity, an increase in dopamine receptor sensitivity to the effects of dopamine.[29,61] Although patients on L-Dopa show no difference with respect to maximal therapeutic benefit, patients with severe akinesia react more sensitively to a single intravenous dose of L-Dopa than do patients with mild akinesia. Patients on high doses of L-Dopa and animals after injection of high doses of L-Dopa, develop abnormal choreiform movements due to the increased dopamine levels, which can be eliminated by decreasing the doses of L-Dopa.[29] During chronic treatment with L-Dopa, patients often develop an increased sensitivity to both the therapeutic effects and the choreiform side effects. This effect may also be due to

denervation supersensitivity, resulting from continued degeneration of the dopaminergic neurons.[29]

Effects of Increased Dopaminergic Activity:[29]

Increased dopaminergic activity manifests itself as locomotor hyperactivity and stereotyped motor behavior. This increased activity could be produced either (1) by facilitation of excitatory systems or (2) by removal of inhibitory influences upon such systems (disinhibition). The latter is most widely held to be correct. In unanesthetized animals, the spontaneous firing rate of most caudate cells is decreased by locally applied dopamine and by electrical stimulation of the substantia nigra which increases release of striatal dopamine. It is possible that the enhancement of motor activity by dopamine agonists is due to elimination of normal striatal inhibitory impulses which modify the "primitive motor patterns of the globus pallidus."[29]

Post-Synaptic Dopamine Receptor Activation and Neuronal Feedback:

Most of the antipsychotics, the phenothiazines like chlorpromazine and the butyrophenones like haloperidol, are potent dopamine receptor blockers. Their ability to increase the firing rate of dopamine neurons in the pars compacta of the substantia nigra[1] and to increase synthesis and turnover[63] of dopamine is presumed to be due to a neuronal feedback mechanism, since the effect is dependent upon impulse flow in dopamine neurons.[3] Dopamine agonists, direct and indirect, have the opposite effect upon dopamine neurons. The agonists

result in decreases in the synthesis and release of dopamine, an effect which can be blocked by the dopamine receptor blockers.[24]

Presynaptic Dopamine Receptors:

The firing of dopaminergic neurons, and the synthesis and release of dopamine, appears to be affected not only by a postsynaptic feedback mechanism, but also by dopamine receptors located on the terminals of the dopamine neuron itself. Support for this comes in the findings: (1) that dopamine receptors have been found on the cell body and dendrites of dopamine neurons and, therefore, may also be present along the axon and nerve terminals, and (2) that the presynaptic and the postsynaptic dopamine receptors appear to respond differently to some drugs.[10]

In normal impulse flow, the action of synaptic dopamine is terminated primarily by reuptake into nerve terminals rather than by degradation of the neurotransmitter. Increasing the firing rate of dopaminergic neurons, as in other types of nerves, results in a stimulus dependent increase in synthesis and release of dopamine. Unlike in other neurons, however, cessation of impulse flow in dopaminergic neurons, induced either pharmacologically or by axotomy, leads to a paradoxical increase in tyrosine hydroxylase activity and in dopamine synthesis.[10,34,48] Presynaptic dopamine receptors are postulated to be responsible, since the interruption of impulse flow excludes feedback neuronal regulation. The suggested mechanism is a decrease in the release of dopamine secondary to interruption of impulse flow, leading

to a decrease in synaptic cleft dopamine available to react with the presynaptic receptors.

The pool of dopamine in the nerve terminal is probably controlled by a balance of release, reuptake, and synthesis. Tyrosine hydroxylase activity appears to be subject to end-product inhibition by this neuronal dopamine pool, such that decreased utilization leads to an increased neuronal dopamine pool, which then inhibits tyrosine hydroxylase activity and dopamine synthesis. This end-product inhibition appears to be dependent upon calcium influx into the neuron, which in turn may depend upon stimulation of presynaptic dopamine receptors. Lesions of the dopamine neurons or pharmacological treatment with GBL (γ-butyrolactone), leads to cessation of impulse flow in the nigroneostriatal dopamine neurons, with a concomittant cessation of dopamine release and calcium influx. The result is an activation of tyrosine hydroxylase with an increase in synthesis and in the neuronal dopamine pool to which the tyrosine hydroxylase is no longer responsive, possibly because of the decreased influx of calcium. The postulated role of the presynaptic receptors is to allow calcium influx and return some of the end-product regulation by the neuronal dopamine pool.[48] Direct dopamine agonists, as dopamine and apomorphine, do in fact cause a partial inhibition of the lesion-induced increase in tyrosine hydroxylase activity, presumably by allowing calcium influx through their interaction with presynaptic dopamine receptors. That this inhibition of tyrosine hydroxylase activity is mediated via presynaptic dopamine receptors is supported by the ability of dopamine receptor

blockers, such as haloperidol, to block the apomorphine effect.[10,34,48] Dopamine and apomorphine have also been shown to inhibit the synthesis of dopamine in rat striatal synaptosomes, a medium of the synaptic terminals in which only local responses are measured.[43]

Further support for the role of presynaptic receptors in the regulation of dopamine synthesis comes from the observations (1) that the dopamine receptor blockade with haloperidol will increase tyrosine hydroxylase activity beyond that maximally obtained by stimulation alone, and (2) that iontophoretically applied dopamine to the nigrostriatal dopamine neurons in the pars compacta inhibits firing of the dopamine neurons, possibly by reacting with dopamine autoreceptors on the cell body, a similar decrease in activity to that obtained with dopamine agonists in presynaptic dopamine receptor studies.[48]

Cholinergic Contribution to the Extrapyramidal System:

Cholinergic neurons appear to play an important role in the extrapyramidal system. The highest concentrations in the brain of choline acetylase and acetylcholinesterase, the enzymes responsible for synthesis and degradation, respectively, of acetylcholine are found in the caudate and putamen.[6,36,37] Correspondingly, the caudate contains the highest number of muscarinic receptors, with the putamen and globus pallidus containing an intermediate amount.[26] Both afferent nigrostriatal cholinergic fibers[51] and efferent cholinergic striatopallidal and striatonigral fibers[42] have been described. Selective lesions disrupting these pathways, however, fail to significantly decrease choline acetylase or acetylcholinesterase in the striatum,

suggesting that the neostriatal cholinergic system is primarily contained within the neostriatum as interneurons.[36,37] Similarly, although lower levels of these enzymes are found in the substantia nigra, the levels are unchanged by lesions of the striatonigral pathway.[36] The possibility, however, of substantia nigral cholinergic interneurons or cholinergic input from other areas of the brain still exists.

The execution of purposeful movement appears to be dependent upon a dopmainergic - cholinergic balance within the striatum. Acetylcholine has been shown to increase, while dopamine decreases, the spontaneous firing rate of most cells in the caudate.[51] Local injections of carbachol or physostigmine into the striatum have been shown to decrease the circling induced in rats by dopamine.[22] Although the ability of antipsychotic drugs to induce Parkinsonian-like symptoms is related to their ability to block dopamine receptors, several antipsychotic drugs with potent dopamine receptor blocking ability have been found which show a much lower incidence of extrapyramidal side effects than would be expected. These drugs were found to have antimuscarinic properties. Subsequent studies demonstrated that the greater the antimuscarinic potencies of these antipsychotic drugs, the less the frequency of extrapyramidal side effects.[27,38,54] Anticholinergic drugs are not only very effective clinically in counteracting the extrapyramidal side effects of the antipsychotics, but are also of some benefit in Parkinson's patients and in animals with lesions of the dopaminergic nigrostriatal pathway[23], while cholinergic drugs, such as physostigmine, exacerbate Parkinsonian extrapyramidal

symptoms.[61]

There is a great deal of evidence to suggest that cholinergic nerves are not only involved in maintaining a balance with dopamine in the striatum, but also have either direct or indirect effects upon dopaminergic neurons themselves. Since dopaminergic activity within the striatum has been found to have an inhibitory effect upon cholinergic neurons, such that an increase in dopaminergic activity is associated with a decrease in cholinergic activity, and a decrease in dopaminergic activity with an increase in cholinergic activity[46], it is not surprising that cholinergic activity in turn may affect dopaminergic neurons. Carbachol infusion into the substantia nigra leads to similar changes as those caused by decreased impulse flow in the nigrostriatal pathway[46]; that is, an increase in striatal dopamine levels by decreasing turnover without changing synthesis.[32] Infusion of atropine in the substantia nigra has been shown to increase both turnover and synthesis of dopamine[32], as is found with an increase in impulse flow. Further studies demonstrated that neurons in the zona reticulata are stimulated by iontophoretic acetylcholine, an effect which can be specifically blocked by the anticholinergic, scopolamine. The enhanced firing of the zona reticulata cells is associated with a reduced firing of cells in the pars compacta. This, plus the fact that acetylcholine has no effect when administered directly into the pars compacta, suggests that acetylcholine may be exciting neurons in the zona reticulata, which then form inhibitory synapses on pars compacta cells.[46] Although these results suggest a possible role for

cholinergic influence in the substantia nigra, they are not consistent with results obtained with the systemic administration of the same cholinergic drugs. Physostigmine, oxotremorine, and arecoline, given systemically, have been shown to increase release and turnover of dopamine, as measured by HVA levels. This effect can be counteracted by the systemic administration of atropine.[40,44] Although some studies investigating the effect of anticholinergic drugs on turnover rate of dopamine have noted a significant decrease in HVA levels[41,44], most studies have found either no change or only a slight decrease (1) in the rate of disappearance of dopamine after α-methyl-p-tyrosine[2,12], (2) on the endogenous levels of dopamine[2,12,41,44], or (3) on HVA levels.[40] The results obtained from the systemic use of the cholinergic and anticholinergic drugs are more consistent with the effects of these drugs when given intrastriatally. Perfusion of the caudate with acetylcholine plus physostigmine was found to increase the release of dopamine[8], while microinjections of atropine in the caudate were found to have no effect on HVA levels.[40]

Substantial evidence has accumulated for the existence of presynaptic cholinergic receptors on adrenergic nerve terminals outside the central nervous system. Regulation of the release of norepinephrine from adrenergic nerves by inhibitory muscarinic and stimulatory nicotinic receptors have been demonstrated, the muscarinic receptors being approximately 2 to 3 times more sensitive to acetylcholine than the nicotinic receptors.[19] It is, therefore, possible that such cholinergic presynaptic receptors may be present on dopamine terminals,

affecting the release of dopamine. In support of this is the recent finding that in striatal slices acetylcholine inhibits the release of ^{3}H-dopamine induced by electrical or KCl stimulation.[60] These results are similar to those found in peripheral adrenergic nerves with regard to inhibitory muscarinic receptors. However, it has also been demonstrated that perfusion of the caudate with oxotremorine or acetylcholine plus physostigmine increases, rather than decreases, release of striatal dopamine.[8] Prior to this, benztropine and other anti-parkinsonian anticholinergic and antihistaminic drugs were shown to inhibit the reuptake of dopamine in striatal synaptosomes[14,20,52] and into dopaminergic neurons in vivo.[17] Since the ability to block reuptake of dopamine appeared to coincide with their relative clinical potencies in treating Parkinsonian extrapyramidal symptoms, it was suggested that the beneficial effect of these drugs was due to their ability to inhibit reuptake of dopamine, making more dopamine available in the synaptic cleft, rather than, or in addition to, their anticholinergic properties. This is not supported clinically, however, in that atropine and scopolamine, which are active anti-Parkinson's agents, demonstrate little or no ability to block dopamine reuptake[20,52], and in that atropine, as well as benztropine, has been shown to potentiate the Dopa and apomorphine induced increase in locomotion in rats.[20] Further biochemical studies also tend to support a feedback, as opposed to a local, cholinergic mechanism for the effects seen in dopaminergic neurons. Anticholinergics have been shown to reduce the phenothiazine or haloperidol induced increase in dopamine turnover,

as measured both by the rate of disappearance of dopamine from the striatum[2,12] and by the HVA levels.[9] Since this effect is seen in the face of dopamine receptor blockade, it is presumed to take place beyond the receptor and is postulated to involve an inhibition of the receptor blocker induced increase in impulse flow through dopaminergic neurons.[12]

Noradrenergic Neurons:

Although little norepinephrine is contained within the striatum, approximately 1/50th to 1/100th the concentration of dopamine, there is some feeling that this transmitter may play an important role. This is based upon the observations that (1) L-Dopa which is also a precursor of norepinephrine via dopamine is more effective in producing locomotor hyperactivity than apomorphine, a specific dopamine agonist, and (2) L-Dopa is not as effective if the formation of norepinephrine by dopamine β-hydroxylase is inhibited. It is therefore possible that the noradrenergic system, while not directly involved, is playing a regulatory role in determining the sensitivity of the extrapyramidal system to dopamine. More work is necessary in this area, however, before this can be established.[29]

GABA-ergic Neurons:

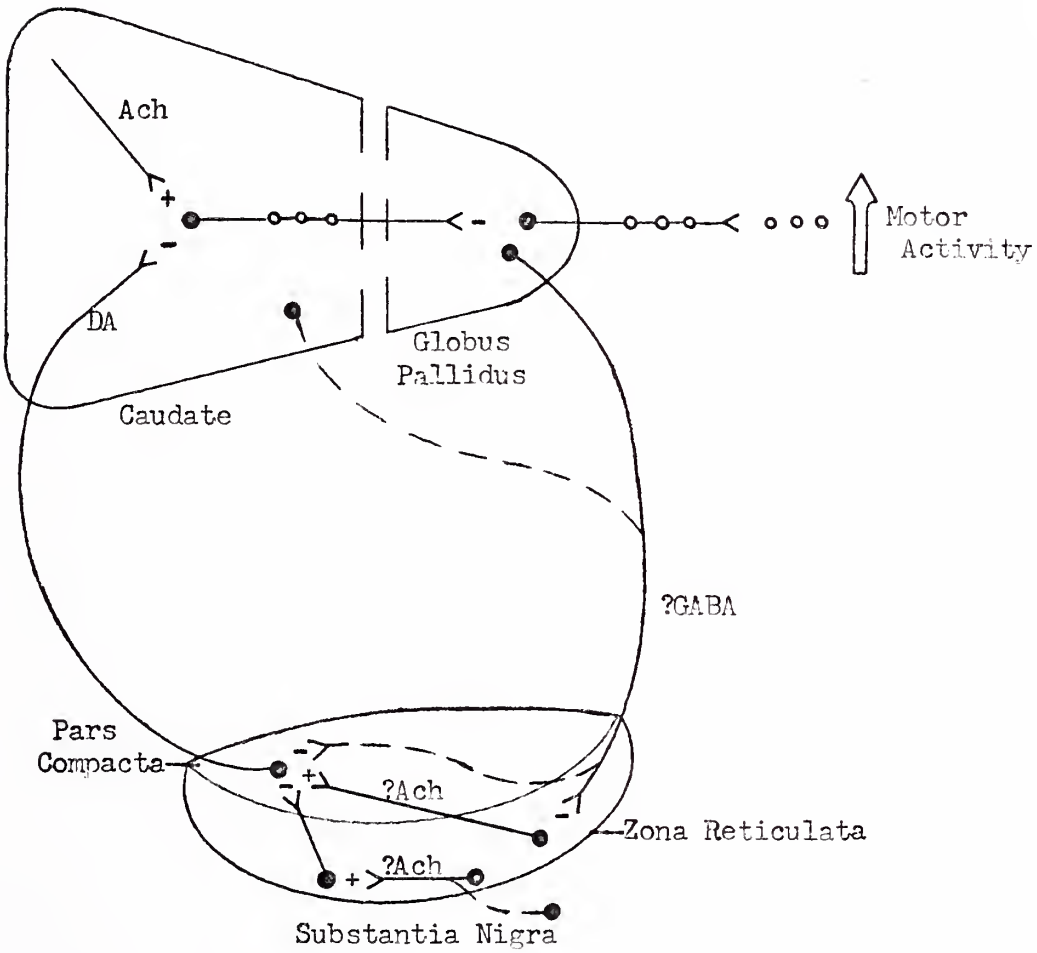
It appears likely that GABA-ergic (gamma amino butyric acid) neurons have an important inhibitory function in the substantia nigra and possibly also play a role in the striatum. Evidence for a GABA-ergic role in the substantia nigra includes: (1) in the brain, GABA

and its synthesizing enzyme, glutamic acid decarboxylase, are found in highest concentrations in the substantia nigra;[46] (2) lesions of the strionigral pathway markedly decrease both GABA and glutamic acid decarboxylase in the substantia nigra;[46] (3) electrical stimulation of the caudate blocks the firing rate of nigral units in the zona reticulata;[8] (4) iontophoretically applied GABA in the substantia nigra invokes the same response as electrical stimulation of the caudate, blocking the activity of cells in the zona reticulata of the substantia nigra;[8,46] and (5) intravenous or substantia nigral administration of biculline, a GABA receptor blocker, causes an increased release of striatal dopamine.[8] This all lends strong support for a strionigral GABA-ergic system inhibitory to nigrostriatal dopaminergic neurons.

There is some evidence that GABA may also play a role in the striatum. Labeled GABA is taken up by receptors in the striatum, and glutamic acid decarboxylase activity can be found in striatal synaptosomes, establishing the presence of GABA-ergic neurons in the striatum.[8] It has been demonstrated that GABA in the striatum causes a decrease in the release of dopamine and that the GABA receptor blockers, biculline and picrotoxin, cause an increase in the release of dopamine and are capable of counteracting the decrease in dopamine release induced by GABA.[8] Whether these represent feedback or presynaptic responses has yet to be determined.

FIGURE 2

MODEL OF POSSIBLE NIGROSTRIATAL NEURONAL INTERACTIONS



Ach = acetylcholine

DA = dopamine

GABA = -aminobutyric acid

+ = excitatory

- = inhibitory

o-o-o = Possible Multineuronal Pathway

EXPERIMENTAL DESIGN

Dopa Assay:

To determine the effects of cholinergic and anticholinergic drugs on tyrosine hydroxylase activity, the dopa decarboxylase inhibitor, Ro 4-4602, was given to block the conversion of Dopa to dopamine, and a modification of the method of Kehr, Carlsson, and Lindquist was used to assay for Dopa.[33] In the normal untreated rat brain, Dopa is rapidly converted to dopamine and endogenous Dopa levels are very low (< 10 ng/g).[33] After Ro 4-4602, there is a linear increase in Dopa for up to an hour.[59] Determining striatal Dopa levels in an Ro 4-4602 treated rat is, therefore, a reliable method for measuring the effects of drugs on tyrosine hydroxylase activity in vivo, and thus of assessing the effective rate of synthesis of dopamine.

Ro 4-4602 was given to all rats 30 min prior to kill in a dose of 800 mg/kg, which is known to block central dopa decarboxylase activity.[59]

Dopa Assay using 6-Hydroxydopamine Treated Rats:

Intracisternal 6-hydroxydopamine (6-OH DA) has been shown to cause selective degeneration of monoaminergic neurons. When a norepinephrine uptake blocking drug is given prior to the 6-OH DA to prevent its action on noradrenergic neurons, the degeneration is specific for dopaminergic neurons.[50,62] In order to determine the effects of physostigmine and benztropine on tyrosine hydroxylase activity in a dopamine deficient state, such as Parkinson's Disease, a pilot study was carried out using 6-OH DA treated rats as an experimental animal model. The rats were obtained at 5 weeks of age from Dr. B. Shaywitz, in whose lab, 2 litters of rats had been randomly divided at 5 days of age into treated and non-treated groups. The treated rats had received 100 µg 6-OH DA intracisternally with the norepinephrine uptake blocker, desmethylimipramine, 25 mg/kg intraperitoneally, being given 30 min prior to the 6-OH DA in order to protect the noradrenergic neurons. All rats used in this study were taken from these same 2 litters, including 3 untreated rats as controls.

The rats were treated identically to those used for the other Dopa assays, receiving 800 mg/kg Ro 4-4602 30 min prior to kill.

Dopa Assay with GBL:

Gamma butyrolactone (GBL) has been shown to selectively block impulse flow in central dopaminergic neurons and thus block the release of dopamine. The block of impulse flow produced by GBL administration causes approximately a 3-fold increase in tyrosine hydroxylase activity, as measured using Ro 4-4602 and the Dopa assay, results similar to that produced by axotomy.[59] By blocking impulse flow, any change in the rate of synthesis, outside of that caused by GBL itself, would have to be mediated locally via presynaptic receptors on the dopaminergic nerve terminal, rather than through a neuronal feedback mechanism. In order to help define the site of action of the cholinergic and anticholinergic drugs, GBL was administered and striatal Dopa assayed as before.

All rats were given 750 mg/kg GBL at 35 min and 800 mg/kg Ro 4-4602 at 30 min prior to kill.

Dopac Assay:

To determine the effects of cholinergic and anticholinergic drugs on impulse flow in dopaminergic neurons, a modification of the method of Murphy, Robinson, and Sharman was used to measure 3,4-dihydroxyphenylacetic acid (DOPAC) in rat striatum.[39] The Dopac assay was selected rather than the measurement of HVA levels, which has been used to measure turnover of dopamine in previous studies, because of the more rapid and consistent response of Dopac to changes in dopamine degradation, and because it closely reflects changes in impulse flow in the nigrostriatal dopamine neurons.[47]

MATERIALS AND METHODS

Male Sprague Dawley (Charles River Co., Inc.) albino rats weighing between 150-350 gm were used. All drugs were given by intraperitoneal injection at times noted as minutes prior to kill. For the Dopa assay, all rats received Ro 4-4602 at 30 min prior to kill. The rats were killed by decapitation and the neostriatum (caudate and putamen) was dissected out rapidly and placed on dry ice. Estimations of Dopa were made on the bilateral striata of each rat. For the Dopac assay, estimations were also made on the bilateral striata of each rat.

Control rats in the Dopa experiments received Ro 4-4602 and in the Dopa experiments with GBL received Ro 4-4602 and GBL.

Drugs used in Dopa and Dopac Experiments:

Drugs	Pharmaceutical Companies
Anticholinergics:	
Atropine sulfate	Merck & Co.
Atropine methyl nitrite	K & K Laboratories
Scopolamine·HBr trihydrate	Regis Chem. Co.
Benztropine Mesylate	Merck, Sharp, & Dohme
Artane	Lederle
Cholinergics:	
Physostigmine·SO_4	Calif. Corp. for Biochem. Research
Pilocaprine·HCl	Merck & Co.
Oxotremorine Sesquifumarate	Aldrich Chem. Co.
Arecoline·HBr	Regis Chem. Co.
DA Impulse Flow Blocker:	
Gamma-butyrolactone (GBL)	Matheson, Coleman & Bell
Dopa decarboxylase Inhibitor:	
Ro 4-4602 (seryl-trihydroxy-benzyl hydrazine)	Hoffmann-LaRoche, Inc.

Dopa Assay:

Sample Preparation:

The caudate pairs were weighed and homogenized in 2 ml (+ 2 ml more to wash the pestle and tube) of .4N PCA soln. containing - 50 mg $Na_2S_2O_5$ + 2 ml 10% EDTA + 10 ml 4N Perchloric acid - made up to 100 ml with distilled H_2O (made fresh for each experiment). For each tissue blank and recovery, half the cerebellum from an untreated rat was prepared in the same way. 100 ng of Dopa was added to the tissue recovery tubes. The homogenates were centrifuged at 11,000 RPM and supernatants were brought to pH 2 with KOH (5N, 1N, & .1N). Samples were then frozen overnight at -20°C.

Column Procedure:

Preparation of Dowex 50W, X-4, 200-400 mesh, H^+: 100 g of Dowex was washed with 200 ml of 2N NaOH (containing 1% EDTA), stirring for 10 min. After allowing to separate, the liquid was poured off and the wash repeated. The Dowex was then rinsed with 1000 ml distilled H_2O in the same manner, repeating until approximately the pH of H_2O. The Dowex was washed 3 times with 100 ml 2N HCl and rinsed again with 1000 ml distilled H_2O until approximately the pH of H_2O. It was then kept stored in the refrigerator with 200 ml of H_2O above the Dowex.

Column preparation: A 4 cm column of Dowex was made in a 50 ml column containing a glass wool plug. The sides of the column were washed with H_2O. Twenty ml of 0.1N sodium phosphate buffer (pH 6.5) with 0.1% EDTA was run through the column which was then rinsed with 5 ml of H_2O.

Elution: The samples were thawed, centrifuged at 11,000 RPM for 10 min, and poured over the columns. The following solutions were run through the column and the eluates discarded: (1) 20 ml 60% aqueous methanol, (2) 10 ml H_2O, (3) 10 ml 0.1M sodium citrate phosphate buffer (pH 2.5), (4) 10 ml H_2O, and (5) 1 ml 0.1M sodium citrate buffer (pH 4.5). The Dopa was then eluted with 3 ml of the sodium citrate buffer.

Fluorometric Determination of Dopa:

Amino settings: Activating peak - 360 nm

Fluorescence peak - 480 nm

Slits: 1 - slit 4 mm at position 2

2 - 3 mm

3 - 4 mm

4 - 4 mm

5 - 3 mm

6 - slit 4 mm at position 2

7 - position 3

Meter multiplier - .003

Treatment of samples: Samples were run in duplicate with a set of standards (0, 10, 25, 50, & 75 ng Dopa). The following solutions were placed in tubes, vortexing between additions: (1) 0.5 ml 0.15M Na_2HPO_4, (2) the appropriate amount of Dopa to the standards plus 0.5 ml of 0.1M sodium citrate, (3) 0.5 ml sample in the appropriate tubes, (4) 0.2 ml 95% ethanol, (5) 0.05 ml 0.2% $ZnSO_4$, (6) 0.05 ml 0.1% $K_3Fe(CN)_6$ - addition timed so that 6 min later 0.5 ml of 5N NaOH with 1% ascorbic acid (made fresh just prior to step #6) was added. The samples were read between 3 min and 30 min after adding the NaOH.

Treatment of Data:

Using the % Transmission (T) obtained from the standards (subtracting the blank from the standards), a graph of % T vs. ng Dopa was plotted. The % T of the tissue blanks (approximately 1% T greater than the standard blanks) was subtracted from that of the samples and the amount of Dopa in the sample determined from the graph. The ng Dopa/g tissue was obtained by multiplying by 6 (to find the total amount of Dopa eluted), and dividing by the % recovery (usually around 60%) and by the weight of the caudates.

Dopac Assay:

Sample preparation:

The caudate pairs were weighed and homogenized in 2 ml (+ 1 ml to rinse the pestle and tube) of cold 0.1N HCl. Tissue blanks and recoveries (containing 100 ng Dopac/tube) were prepared from half a cerebellum/tube. 0.12 ml conc. PCA (perchloric acid) and approximately 1.4 g KCl was added to the tubes, which were then centrifuged at 10,000 RPM for 10 min. The supernatant was poured into prechilled tubes containing 7 ml butylacetate (Baker's Superior Grade - redistilled) and approximately 0.4 g KCl. After capping and shaking in the cold room for 10 min, the tubes were centrifuged in the International Centrifuge at 15 RPM for 5 min. Six ml of butyl-acetate was drawn off the samples and added to tubes containing 2 ml Soln. A (see below). Four tubes for the standard curve were set up containing 2 ml butyl acetate and 2 ml Soln. A. After capping and shaking for 6 min in the cold room, the tubes were centrifuged for 5 min at 15 in the International Centrifuge.

Soln. A: 35 ml H_2O, 1 ml 2N HCl, 1.5 ml ethylenediamine (twice distilled)

B: 6 ml H_2O, 6 ml conc. HCl

C: 18 ml H_2O, 2 ml ethylenediamine (EDA, twice distilled)

Assay:

Two tubes for use in setting the amino were prepared with

.85 ml Soln. A and 50 ng & 100 ng Dopac, respectively. Eight tubes for use as standards were prepared with .85 ml of the butylacetate-Soln. A mixtures made in the previous section (2 blanks, 2 with 25 ng Dopac, 2 with 50 ng Dopac, 1 with 75 ng Dopac, and 1 with 100 ng Dopac). Samples were run in duplicate, .85 ml/tube. Marbles were used to cover the mouths of the tubes, which were then placed in a water bath at 60°C with a tight lid for 20 min. The tubes were placed in ice and the bucket covered. Twenty-five ml of Soln. B was added to each of the tubes, which were then vortexed, and the bucket recovered. After approximately 10 min, 25 ml of Soln. C was added, the tubes vortexed, and the bucket recovered. The samples were read in a dark room in the Aminco: activating peak - 385 nm; fluorescence peak - 455 nm; using a Corning filter # 3389 (CS3-73, 1.52 min); slit settings were the same as for the Dopa Assay: Meter multiplier - .03.

Treatment of the Data:

Using the % Transmission (T) obtained from the standards (subtracting the blanks from the standards), a graph of % T vs. ng Dopac was plotted, the % T of the tissue blanks was subtracted from that of the samples, and the amount of Dopac assayed determined from the graph. The ng Dopac/g tissue was obtained by dividing the ng Dopac assayed by the fraction of sample used for the assay (36.4%), the % recovery (usually around 50%), and the weight of the caudates.

RESULTS

Data was analyzed using the Standard Estimate of the Mean (SEM) and a two-tailed "t" test. $p < .05$ is considered a significant difference, while $p > .05$ is not a significant difference.

Dopa Assay: (Tables 1 & 2; Figures 3-6)

Benztropine was found to be a potent and dose related inhibitor of Dopa accumulation. While artane, atropine, and scopolamine showed only slight, if any, inhibitory properties. All of the cholinergic drugs tested - physostigmine, pilocarpine, oxotremorine and arecoline - significantly increased accumulation of Dopa. A better response was found when the drugs were given at 40 min as opposed to 45 min prior to kill, probably due to their shorter duration of action. All the anticholinergic drugs, including those having only minimal effects when used alone, successfully prevented or significantly inhibited the physostigmine induced increase in Dopa accumulation. The central action of these drugs was confirmed by using atropine methylnitrite (AMN), a peripheral anticholinergic with no central effects, which was shown to be ineffective in preventing the physostigmine induced increase in Dopa accumulation. Neither atropine nor benztropine were able to significantly affect the pilocarpine induced increase in Dopa accummation.

TABLE 1

DOPA ASSAY - EFFECTS OF CHOLINERGIC AND ANTICHOLINERGIC DRUGS USED INDIVIDUALLY

DRUG*	DOSE (mg/kg)	t**	n	DOPA(ng/g ± SEM)***	p+	%Δ++
Control			29	1033 ± 34		
Atropine	50	60	17	928 ± 31	.05	-10
Scopolamine	25	60	3	1042 ± 79	N.S.[a]	
"	50	60	4	895 ± 91	N.S.	
Benztropine	15	60	4	779 ± 44	.01	-25
"	30	60	5	504 ± 61	<.01	-51
"	50	60	3	387 ± 33	<.01	-63
Artane	50	60	6	863 ± 86	.05	-16
Physostigmine	1	45	11	1661 ± 77	<.01	+61
"	1	40	7	1898 ± 142	<.01	+84
Pilocarpine	100	45	4	1271 ± 152	.04	+23
"	100	40	8	1861 ± 140	<.01	+80
Oxotremorine	3	40	3	1423 ± 37	<.01	+38
Arecoline	50	40	3	1411 ± 126	<.01	+37

* All rats received 800 mg/kg Ro 4-4602 at 30 min prior to kill.
** min prior to kill
*** Dopa measured in ng/g of tissue.
\+ Compared with the control.
++ Percent change in Dopa accumulation from control
Increase = + decrease = -
a N.S. = Not significant at the .05 level.

TABLE 2

DOPA ASSAY - INHIBITION OF THE CHOLINERGIC-INDUCED INCREASE IN DOPA ACCUMULATION BY ANTICHOLINERGIC DRUGS

DRUGS*	DOSES (mg/kg)	t's**	n	DOPA(ng/g ± SEM)***	p+	p++
Physostigmine	1	45	11	1661 ± 77		
"	1	40	7	1898 ± 142		
AMN+++ + Physostigmine	25/1	60/40	3	2174 ± 190	N.S.	
Atropine + Physostigmine	50/1	60/45	15	822 ± 41	<.01	.05
"	50/1	60/40	4	1099 ± 48	<.01	.03
Scopolamine + Physostigmine	25/1	60/45	3	823 ± 85	<.01	N.S.
Artane + Physostigmine	50/1	60/45	3	683 ± 29	<.01	N.S.
Benztropine + Physostigmine	15/1	60/40	3	1075 ± 173	.02	N.S.
"	30/1	60/45	5	708 ± 83	<.01	N.S.
Pilocarpine	100	40	8	1861 ± 140		
Atropine + Pilocarpine	50/100	60/40	3	1809 ± 73	N.S.	
Benztropine + Pilocarpine	15/100	60/40	3	1728 ± 31	N.S.	

* All rats received 800 mg/kg Ro4-4602 at 30 min prior to kill.
** min prior to kill
*** Dopa measured in ng/g of tissue.
\+ Compared with the cholinergic drug (physostigmine or pilocarpine).
++ Compared with the anticholinergic drug (atropine, scopolamine, artane, or benztropine).
+++ Atropine methylnitrite, a peripheral anticholinergic which does not enter the CNS.

FIGURE 3

DOPA ASSAY - EFFECTS OF ANTICHOLINERGIC DRUGS

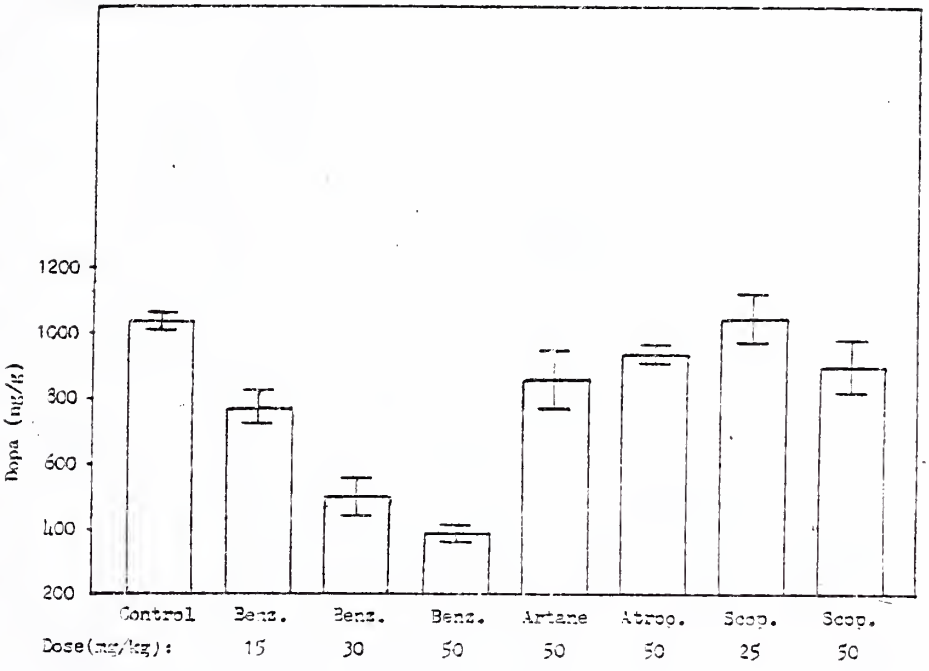


FIGURE 4

DOPA ASSAY - EFFECTS OF CHOLINERGIC DRUGS

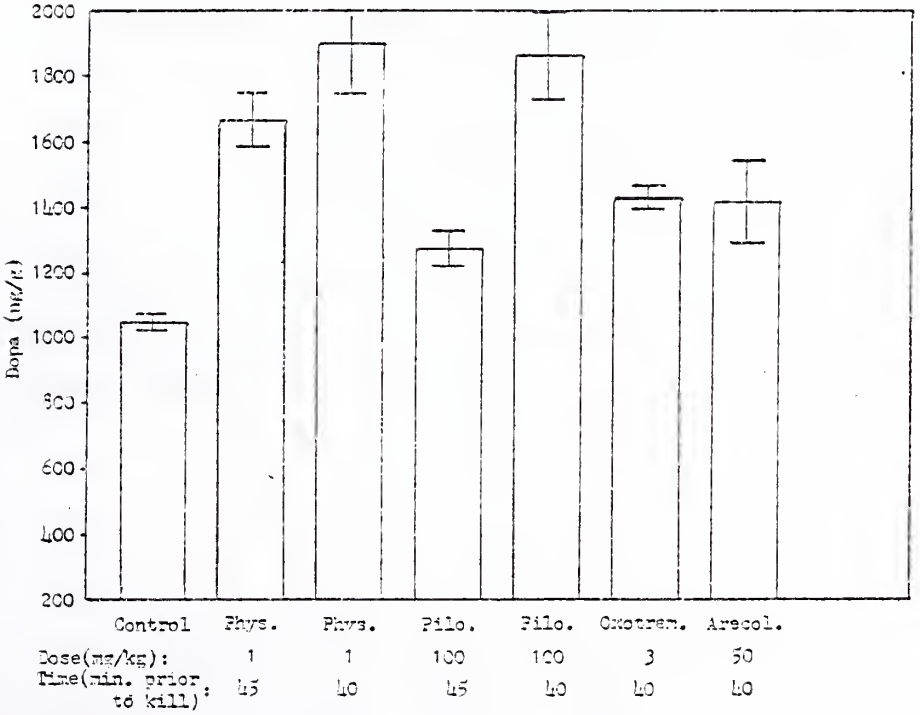


FIGURE 5

DOPA ASSAY - INHIBITION OF PHYSOSTIGMINE-INDUCED INCREASE IN DOPA ACCUMULATION BY ANTICHOLINERGIC DRUGS

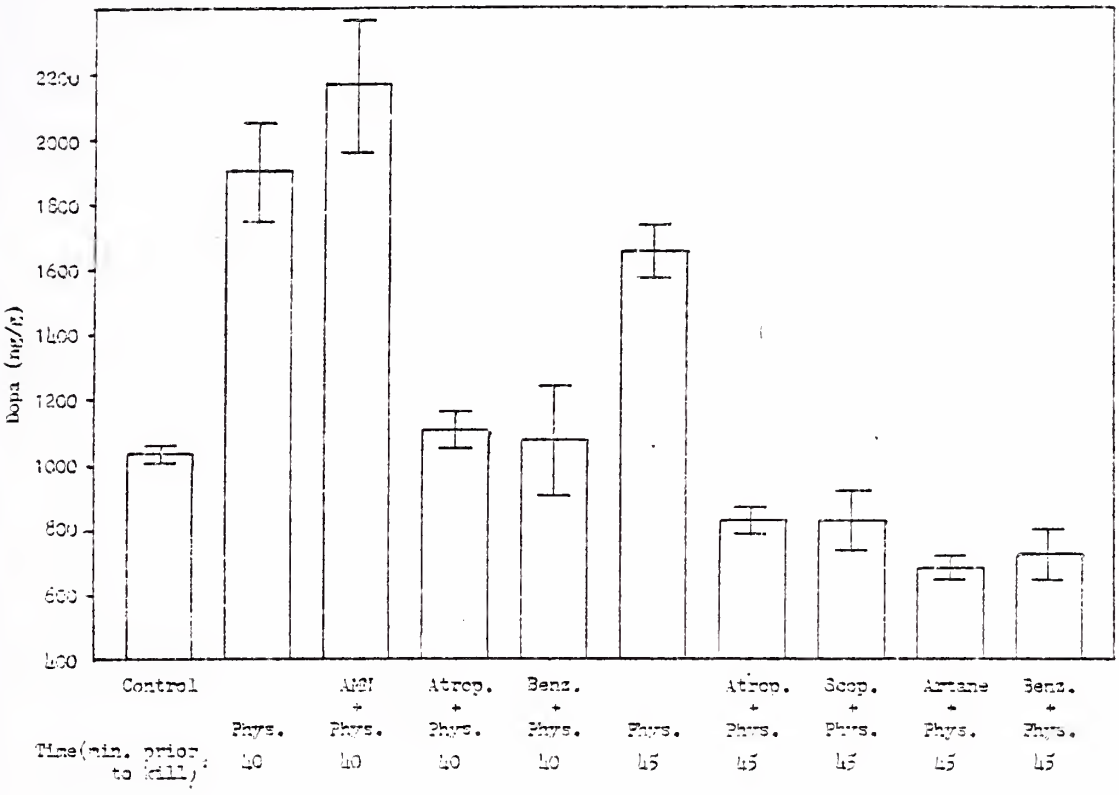
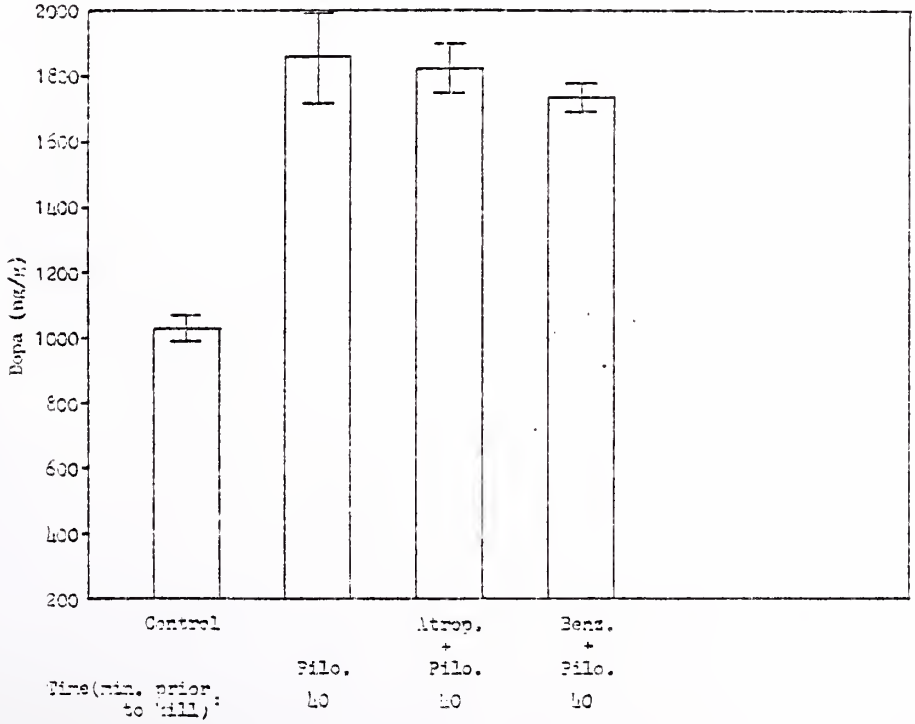


FIGURE 6

DOPA ASSAY - ANTICHOLINERGIC DRUGS WITH PILOCARPINE



Dopa Assay Using 6-OH DA Treated Rats: (Table 3; Figure 7)

In this pilot study, it was found that the Dopa accumulation in the 6-OH DA treated control rats was only about 50% that of their untreated littermates. With regard to drug effects in the 6-OH DA treated rats, benztropine produced a significant decrease, while physostigmine was found to produce no significant change in Dopa accumulation from that of the 6-OH DA control rats.

TABLE 3

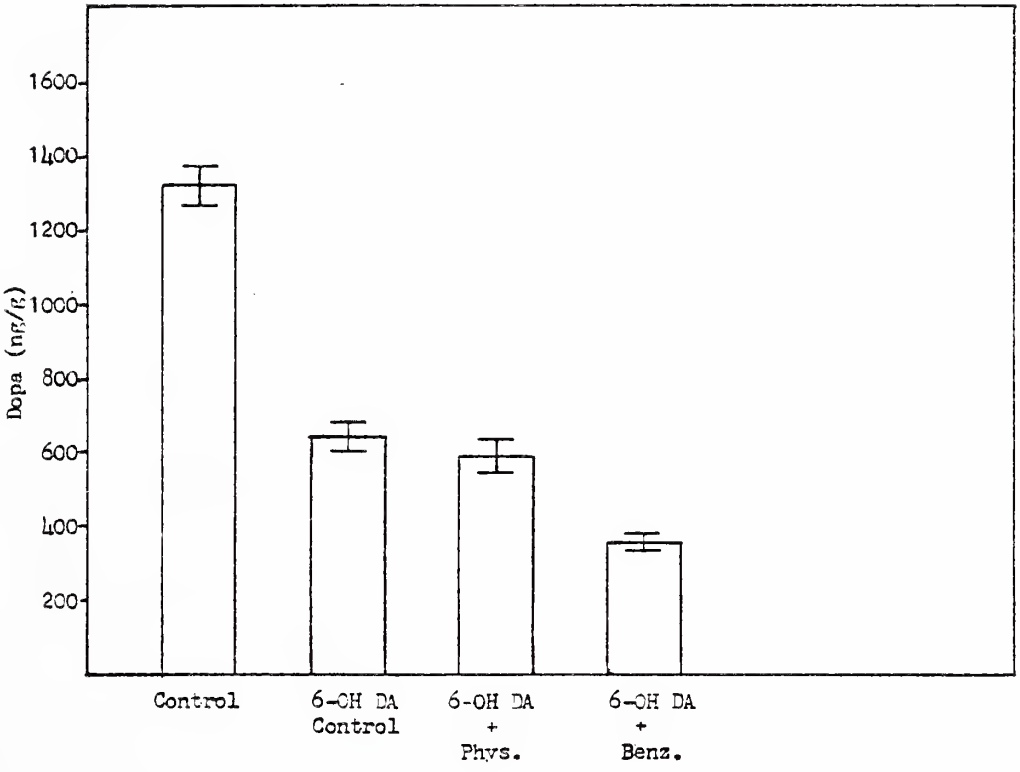
DOPA ASSAY - USING 6-OH DA TREATED RATS

DRUG*	DOSE (mg/kg)	t**	n	DOPA(ng/g ± SEM)***	p+	p++	%Δ+++
Control			3	1313 ± 44			
6-OH DA Control			3	638 ± 35	<.01		
6-OH DA/Phys.	1	45	3	590 ± 35		N.S.	
6-OH DA/Benz.	10	60	3	364 ± 16		<.01	-43

* All rats received 800 mg/kg Ro4-4602 at 30 min prior to kill.
** min prior to kill
*** Dopa measured in ng/g of tissue.
\+ Compared with control.
++ Compared with 6-OH DA control.
+++ Percent change in Dopa accumulation from 6-OH DA.
Decrease = -

FIGURE 7

DOPA ASSAY USING 6-OH DA TREATED RATS



Dopa Assay with GBL: (Table 4)

After stopping impulse flow through the dopaminergic nigrostriatal neurons with GBL, no change in the accumulation of Dopa could be produced with either the anticholinergic drugs, atropine and benztropine, or the cholinergic drugs, physostigmine and pilocarpine.

TABLE 4

DOPA ASSAY - AFTER TREATMENT WITH GBL

DRUG*	DOSE (mg/kg)	t**	n	DOPA(ng/g ± SEM)***	p+
GBL Control			6	2548 ± 104	
Atropine	50	60	6	2294 ± 189	N.S.
Benztropine	15	60	6	2677 ± 176	N.S.
Physostigmine	1	40	6	2457 ± 293	N.S.
Pilocarpine	100	40	3	2547 ± 230	N.S.

* All rats received 750 mg/kg GBL at 35 min and 800 mg/kg Ro4-4602 at 30 min prior to kill.
** min prior to kill
*** Dopa measured in ng/g of tissue.
\+ N.S. - Not significant at the .05 level as compared with the GBL control.

Dopac Assay: (Table 5; Figure 8)

The cholinergic drugs, physostigmine and pilocarpine, caused a significant increase in Dopac levels in the striatum, while the anticholinergic drugs, atropine and benztropine, had no significant effect on Dopac levels. From the graph it appeared that benztropine was more effective in countering the physostigmine induced increase in Dopac, while atropine was more effective in countering the pilocarpine induced increase in Dopac; however, after statistical analysis, all that could be said was that the physostigmine, but not the pilocarpine, induced increase in Dopac could be inhibited by benztropine. Atropine did not produce a statistically significant inhibition of either the physostigmine or the pilocarpine induced increase in Dopac.

FIGURE 8

DOPAC ASSAY

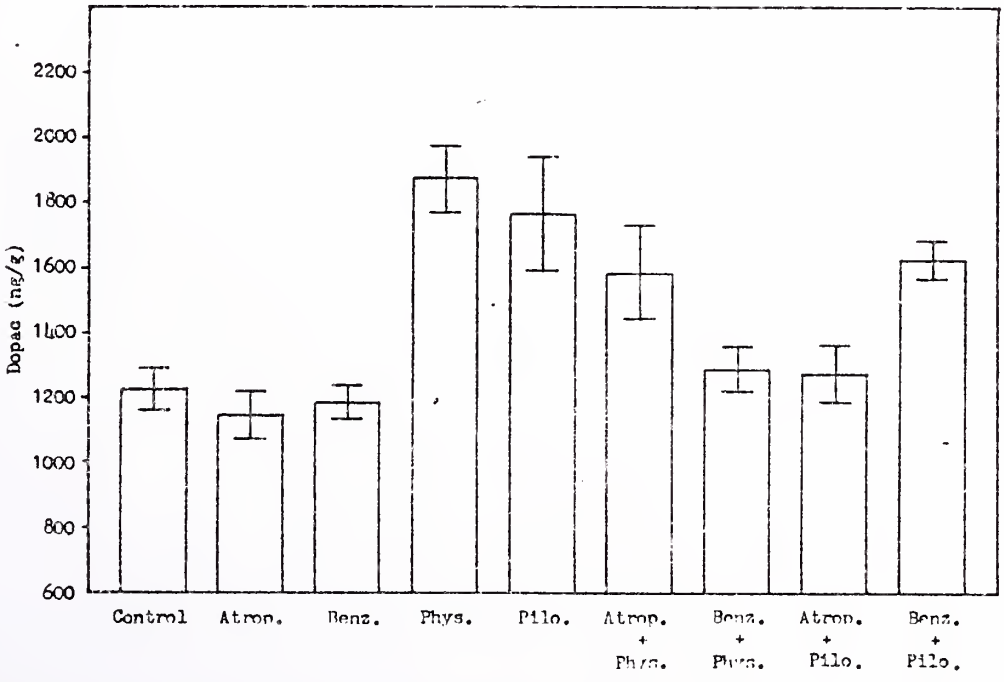


TABLE 5

DOPAC ASSAY

DRUGS*	DOSES (mg/kg)	n	DOPAC(ng/g ± SEM)**	p+	p++	p+++	%Δ†
Control		6	1218 ± 66				
Atropine	50	4	1148 ± 64	N.S.			
Benztropine	20	8	1192 ± 37	N.S.			
Physostigmine	1	8	1879 ± 92	<.01			+54
Pilocarpine	100	3	1770 ± 173	.02			+45
Atropine + Physostigmine	50/1	4	1588 ± 141		N.S.	.05	
Benztropine + Physostigmine	20/1	4	1294 ± 77		<.01	N.S.	
Atropine + Pilocarpine	50/100	4	1285 ± 84		N.S.	N.S.	
Benztropine + Pilocarpine	20/100	3	1614 ± 58		N.S.	<.01	

* The anticholinergic drugs (atropine and benztropine) were given at 60 min prior to kill, and the cholinergic drugs (physostigmine and pilocarpine) were given at 40 min prior to kill.
** Dopac was measured in ng/g of tissue.
\+ Compared with control.
++ Compared with the cholinergic drug.
+++ Compared with the anticholinergic drug.
† Percent change in Dopac accumulation from control. Increase = +

DISCUSSION

The administration of a variety of cholinergic drugs, including physostigmine, pilocarpine, arecoline, and oxotremorine, consistently produced an increase in dopaminergic activity. This stimulation of dopaminergic activity by cholinergic drugs involved both an increase in tyrosine hydroxylase activity,as measured by Dopa accumulation, and an increase in impulse flow,as measured by Dopac accumulation (with physostigmine and pilocarpine). The increase in synthesis demonstrated in this study is consistent with the recent observation by Ulus and Wurtman, Dec. 1976, that choline administration, which increases acetylcholine levels, results in an increase in tyrosine hydroxylase activity in rat striata.[58]

While the anticholinergic drugs, atropine, scopolamine, and artane, had either no effect or caused only a slight decrease in tyrosine hydroxylase activity when used alone, they all significantly inhibited the physostigmine-induced increase in tyrosine hydroxylase activity. Since this effect is seen with several anticholinergics, including atropine, which is a specific muscarinic blocking agent, the mechanism of action is most likely due to a blocking of the effects of the increased muscarinic cholinergic activity produced by physostigmine.

The failure of these anticholinergic drugs to decrease tyrosine hydroxylase activity significantly in the absence of an initial cholinergic stimulus to increase tyrosine hydroxylase activity, suggests a normally low cholinergic component to the striatal dopaminergic - cholinergic balance. If the cholinergic contribution is normally low,

the anticholinergic drugs would be expected to have little or no effect. This is also consistent with the observation that anticholinergic drugs appear to inhibit the increase in dopaminergic activity induced by antipsychotic drugs.[2,9,12] Since dopamine has been shown to inhibit striatal cholinergic activity[46], blockade of dopamine receptors by the antipsychotic drugs would release the cholinergic neurons from inhibition and lead to an increase in cholinergic activity. This increase in cholinergic activity would result in, or at least contribute to, the increase in dopaminergic activity seen following the administration of the antipsychotic drugs. By blocking this increase in cholinergic effects, the anticholinergic drugs would, therefore, also be expected to inhibit the antipsychotic-induced increase in dopaminergic activity. This concept of a dopaminergic - cholinergic balance such that dopamine plays a primary tonic inhibitory role is also supported by the behavioral observation that the dyskinesia associated with intrastriatal dopamine is counteracted by cholinergic drugs, but is not made more severe by pretreatment with atropine.[13]

There are two possible explanations for the ability of benztropine, but not the other anticholinergics, to decrease tyrosine hydroxylase activity. First, benztropine may be a more potent anticholinergic[23], and thus higher doses of the other anticholinergics would be needed to display the same inhibitory effect produced by benztropine. The other, and more likely, possibility is that benztropine affects tyrosine hydroxylase activity by a direct effect, independent of its cholinergic properties. This was suggested by Coyle and Snyder (1969) in their

study of the effects of benztropine on reuptake of dopamine into striatal synaptosomes.[14] It is still, therefore, possible that this effect is being mediated through benztropine's ability to decrease reuptake of dopamine, making more dopamine available at the postsynaptic receptor. This additional action of benztropine may also explain why benztropine is clinically more effective than the other anticholinergic drugs in counteracting Parkinsonian extrapyramidal symptoms.

That neither atropine nor benztropine was able to inhibit the pilocarpine induced increase in tyrosine hydroxylase activity implies either a direct pilocarpine effect, independent of its cholinergic properties, or a more potent cholinergic effect, which was not counteracted by the anticholinergics in the doses used in this study. Although, peripherally, pilocarpine has been found to have a complex mixture of nicotinic as well as muscarinic responses[23], it is doubtful that the effects in this study were being produced by stimulation of nicotinic receptors. If nicotinic receptors played a role in the stimulation of tyrosine hydroxylase activity, physostigmine, which increases acetylcholine at the receptor site by inhibiting acetylcholinesterase, would also be expected to have both muscarinic and nicotinic effects. Since atropine, a specific muscarinic receptor blocker, was able to totally block the physostigmine-induced increase in tyrosine hydroxylase activity, stimulation of nicotinic receptors was apparently not of major importance. Pilocarpine does seem to have a cholinergic action which is not countered by atropine administration, since the activity of cholinergic neurons remains depressed even after atropine

administration.[25] In addition to its central cholinergic effects, such as cortical arousal in cats, which can be blocked by atropine, pilocarpine has been found to have some strychnine-like effects.[23] It is uncertain, however, what part, if any, this plays in the response seen in this study.

The absence of any effect on tyrosine hydroxylase activity with the cholinergic and anticholinergic drugs after treatment with GBL to block impulse flow through the dopaminergic neurons makes the possibility of a major presynaptic site of action for these drugs unlikely, at least when they are given systemically. This is supported by the results of the Dopac assay. The cholinergic drugs, physostigmine and pilocarpine, which had produced increases in tyrosine hydroxylase activity, also produced increases in impulse flow in the dopamine neurons. Atropine had no significant effect on either impulse flow or on tyrosine hydroxylase activity. These results suggest that the stimulation of tyrosine hydroxylase activity by cholinergic drugs involves the intact dopamine neuron and is via increased impulse flow rather than through presynaptic receptors.

It has been demonstrated that microinjections of carbachol into the substantia nigra inhibit dopamine turnover and have no effect on synthesis, while atropine in the substantia nigra increases turnover.[32] These results are opposite to those found following the systemic use of the same drugs.[40,44] It is therefore unlikely that the substantia nigra is the major site of action of the systemically administered cholinergic and anticholinergic drugs. On the other hand, perfusion

of the caudate with acetylcholine plus physostigmine or with oxotremorine alone was found to increase the release of dopamine[8] and microinjections of atropine into the caudate were found to have no effect on HVA levels.[40] These results are more consistent with the effects following the systematic use of these drugs. Although these findings suggest that the major site of action of the cholinergic and anticholinergic drugs is in the striatum with a feedback loop to the dopamine neurons in the substantia nigra, an extra-striatal site of action for these drugs cannot be ruled out. It is also still possible that local acetylcholine in both the striatum and the substantia nigra may play a role in normal regulation of dopaminergic activity, since the effects of systemic cholinergic and anticholinergic drugs may have a lower affinity for these regions of the brain, or may have more difficulty gaining access to these sites as the result of local blood supply or diffusion.

The results of the Dopac experiments in which the cholinergic drugs, physostigmine and pilocarpine, were given in conjunction with the anticholinergic drugs, atropine and benztropine, are difficult to interpret. The expected result was that the anticholinergic drugs would at least counteract the increase in Dopac seen with physostigmine. This was, in fact, the case with benztropine, which did cause a statistically significant inhibition of the physostigmine induced increase in Dopac. However, because of the relatively greater range of error encountered in the Dopac experiments, as compared to the Dopa assays, no statistically significant change from the cholinergic-induced increase in Dopac could be seen after the addition of atropine to

physostigmine or either atropine or benztropine to pilocarpine treatment.

The pilot study using rats which had been treated with intracisternal 6-hydroxydopamine in conjunction with intraperitoneal desmethylimipramine, to cause a selective degeneration of dopaminergic neurons, showed a decrease of approximately 50% in Dopa accumulation as compared with normal rats. These rats showed a further decrease in Dopa accumulation when treated with benztropine, a similar decrease to that seen when treating normal rats with benztropine. The 6-hydroxydopamine treated rats, however, were unable to increase tyrosine hydroxylase activity when treated with physostigmine, whereas normal rats could. This may be a clue to the mechanism by which cholinergic drugs are able to exacerbate Parkinson's Disease and Parkinsonian-like movement disorders induced by neuroleptic drugs[61], while normal untreated animals and people are able to compensate for these cholinergic drug effects without the development of Parkinsonian symptoms. In Parkinson's Disease, where there is a degeneration of a large part of the dopaminergic neurons, the remaining neurons may already be producing dopamine optimally and be unable to increase synthesis further to compensate for the increase in cholinergic activity. In the neuroleptic induced Parkinsonian-like syndrome, where there is already an increase in dopaminergic activity in order to compensate for the receptor blockade[1], it may be that the addition of cholinergic drugs is unable to further stimulate dopamine production, and the balance would thus be tipped even more in the direction of increased cholinergic versus dopaminergic activity in the striatum. This hypothesis remains to be tested experimentally.

SUMMARY

1. Using an assay for Dopa after inhibition of Dopa decarboxylase as a measurement of tyrosine hydroxylase activity and an assay for Dopac as an indicator of changes in impulse flow in nigro-neostriatal dopamine neurons, this study has shown that the systemic administration of cholinergic drugs stimulates dopaminergic activity. The systemically administered cholinergic drugs, physostigmine, pilocarpine, arecoline, and oxotremorine, all significantly increased tyrosine hydroxylase activity. Although all of the anticholinergic drugs tested counteracted the physostigmine-induced increase in tyrosine hydroxylase activity, atropine, scopolamine, and artane, used alone, had either no effect or only slightly decreased tyrosine hydroxylase activity. This observation was postulated to be due to a tonic inhibitory action of dopamine neurons on cholinergic neurons, such that the normal cholinergic contribution in the striatum would be relatively low as compared to that of dopamine. Benztropine was the only anticholinergic drug which, when used alone, caused a significant and dose related decrease in tyrosine hydroxylase activity. This effect was felt to be due to benztropine's ability to inhibit reuptake of dopamine into nerve terminals, making more dopamine available at the postsynaptic receptor. The possibility, however, that benztropine was simply a much more potent anticholinergic than the others tested could not be ruled out on the basis of this study. The pilocarpine induced increase in tyrosine hydroxylase activity was not inhibited by either atropine or benztropine. This pilocarpine effect was thought

to be due possibly to a direct cholinergic effect of pilocarpine not subject to atropine or benztropine inhibition.

2. The mode of action of the cholinergic and anticholinergic drugs was shown to be through a neuronal feedback mechanism involving the entire dopamine neuron rather than through a presynaptic mechanism, since the actions of these drugs was dependent upon impulse flow in the nigrostriatal dopamine neurons. This conclusion was reached after the cholinergic drugs, physostigmine and pilocarpine, and the anticholinergic drugs, atropine and benztropine, failed to affect tyrosine hydroxylase activity after the cessation of impulse flow in the dopaminergic neurons with GBL, and after physostigmine and pilocarpine were shown to increase the accumulation of Dopac in the striatum, an indication of increased impulse flow in the nigro-neostriatal dopamine neurons. Based on previous work with intrastriatal injections of cholinergic and anticholinergic drugs, which showed similar changes to those found with the systemic use of these drugs in this study, it was postulated that the site of action may be within the striatum and mediated by a neuronal feedback to the dopaminergic nerve cell bodies in the substantia nigra. The possibility, however, of an extra-striatal site of action for these drugs with a neuronal input system to the substantia nigra cannot be ruled out by this study.

3. 6-Hydroxydopamine treated rats, used as a possible animal model of Parkinson's Disease, responded in the same way as normal rats to

benztropine, by decreasing tyrosine hydroxylase activity; however, they were unable to increase tyrosine hydroxylase activity after treatment with physostigmine, as normal rats did. This inability to increase dopaminergic activity in order to compensate for increased cholinergic input was suggested as the explanation for the ability of cholinergic drugs to exacerbate Parkinsonian extrapyramidal symptoms.

It therefore appears that there is a balance of dopaminergic - cholinergic activity with respect to striatal impulse outflow, with each of the systems not only under feedback regulation of their own neurotransmitters, but also under the influence of each other's activity. The cholinergic neurons are influenced by tonic inhibitory dopaminergic activity, while the dopamine neurons are, in turn, affected by stimulatory cholinergic activity, with all systems working to maintain the proper dopaminergic - cholinergic balance within the striatum.

BIBLIOGRAPHY

1. Andén, N.E., "Antipsychotic Drugs and Catecholamine Synapses - Summary", J. Psychiat. Res. (1974), 11:97-104

2. Andén, N.E. and Bédard, P., "Influences of Cholinergic Mechanisms on the Function and Turnover of Brain Dopamine", J. Pharm. Pharmacol. (1971) 23:6:460-462

3. Andén, N.E., Corrodi, H., Fuxe, K., and Ungerstedt, U., "Importance of Nervous Impulse Flow for the Neuroleptic Induced Increase in Amine Turnover in Central Dopamine Neurons", Eur. J. Pharmacol. (1971) 15:193-199

4. Andén, N.E., Dahlström, A., Fuxe, K., and Larsson, K., "Functional Role of the Nigro-Neostriatal Dopamine Neurons", Acta Pharmacol. et Toxicol. (1966) 24:263-274

5. Arnfred, T. and Randrup, A., "Cholinergic Mechanism in Brain Inhibiting Amphetamine-Induced Stereotyped Behavior", Acta Pharmacol. et Toxicol. (1968) 26:384-394

6. Barbeau, André, "Biochemistry of Huntington's Chorea", Advances in Neurology Vol. 1 (1973) Raven Press, N.Y., p. 473-516

7. Bartholini, G. and Pletscher, A., "Atropine-Induced Changes of Cerebral Dopamine Turnover", Experientia (1971) 27:11:1302-1303

8. Bartholini, G. and Stadler, H., "Cholinergic and GABA-ergic Influence on the Dopamine Release in Extrapyramidal Centers", Chemical Tools in Catecholamine Research Vol. 2 (1975) North-Holland/American Elsevier Publ. Co., p. 235-242

9. Bowers, M.B. and Roth, R.H., "Interaction of Atropine-Like Drugs with Dopamine-Containing Neurones in Rat Brain", Brit. J. Pharmacol. (1972) 44:2:301-306

10. Carlsson, A., "Dopaminergic Autoreceptors", Brit. J. Pharmacol. (1974) 50:219-225

11. Carpenter, Malcolm B., Core Text of Neuroanatomy, (1974) Williams & Wilkins Co., Baltimore, Md., p. 181-194

12. Corrodi, H., Fuxe, K., and Lidbrink, P., "Interaction between Cholinergic and Catecholaminergic Neurones in Rat Brain", Brain Res. (1972) 43:2:397-416

13. Costall, B. and Naylor, R.J., "Cholinergic Modification of Abnormal Involuntary Movements Induced in the Guinea Pig by Intrastriatal Dopamine", J. Pharm. Pharmacol. (1975) 27:273-275

14. Coyle, J.T. and Snyder, S.H., "Antiparkinsonian Drugs: Inhibition of Dopamine Uptake in the Corpus Striatum as a Possible Mechanism of Action", Science (1969) 166:907:899-901

15. De la Torre, J.C., "Localization of Dopamine in Brain Following Administration of L-DOPA and a Decarboxylase Inhibitor", Adv. in Neurol. Vol. 2 (1973) Raven Press, N.Y., p. 59-77

16. Dilbey, S., Catzias, G.C., Steck, A., and Papavasilion, P.S., "Apomorphine Versus L-DOPA in Parkinsonism", Fed. Proc. (1971) 30:216

17. Farnebo, L.O., Fuxe, K., Hamberger, B., and Ljungdahl, H., "Effect of Some Antiparkinsonian Drugs on Catecholamine Neurons", J. Pharm. Pharmacol. (1970) 22:10:733-737

18. Frigyesi, T.L. and Purpura, D.P., "Electrophysiological Analysis of Reciprocal Caudato-Nigral Relations", Brain Res. (1967) 6:440-456

19. Furchgott, R.F., Steinsland, O.S., and Wakade, T.D., "Studies on Prejunctional Muscarinic and Nicotinic Receptors", Chemical Tools in Catecholamine Research Vol. 2 (1975) North Holland/ American Elsevier Publ. Co., p. 167-174

20. Fuxe, K., Goldstein, M., and Ljungdahl, A., "Antiparkinsonian Drugs and Central Dopamine Neurons", Life Sci. (1970) 9:1:811-824.

21. Fuxe, K. and Ungerstedt, U., "Antiparkinsonian Drugs and Dopaminergic Neostriatal Mechanisms", Pharmacol. & Therap. Part B (1976) 2:1:41-47

22. Fuxe, K. and Ungerstedt, U., "Studies on the Cholinergic and Dopaminergic Innervation of the Neostriatum with the Help of Intraneostriatal Injections of Drugs", Pharmacol. & Therap. Part B (1976) 2:1:29-36

23. Goodman, L.S. and Gilman, A., The Pharmacological Basis of Therapeutics, 5th ed. (1975) MacMillan Publ. Co., Inc., N.Y., pp. 227-239, 467-476, 514-532

24. Harris, J.E., Baldessarini, R.J.,and Roth, R.H., "Amphetamine Induced Inhibition of Tyrosine Hydroxylation in Homogenates of Rat Corpus Striatum", Neuropharm. (1975) 14:457-471

25. Haubrich, D.R. and Reid, W.D., "Effects of Pilocarpine or Arecoline Administration on Acetylcholine Levels and Serotonin Turnover in Rat Brain", J. Pharmacol. Exp. Therap. (1972) 181:1:19-27

26. Hiley, C.R. and Burgen, A.S.V., "The Distribution of Muscarinic Receptor Sites in the Nervous System of the Dog", J. Neurochem. (1974) 22:159-162

27. Hiley, C.R. and Miller, R.J., "Antimuscarinic Properties of Neuroleptics and Drug-Induced Parkinsonism", Nature (1974) 248:596-597

28. Holz, R.W. and Coyle, J.T., "The Effects of Various Salts, Temperature, and the Alkaloids Veratridine and Batrachotoxin on the Uptake of ^{3}H-Dopamine into Synaptosomes from Rat Striatum", Molec. Pharmacol. (1974) 10:746-758

29. Hornykiewicz, O., "Dopamine in the Basal Ganglia - It's Role and Therapeutic Implications", Brit. Med. Bull. (1973) 29:172-178

30. Hornykiewicz, O., "Mechanisms of Action of L-DOPA in Parkinsonism", Adv. in Neurol. Vol. 2 (1973) Raven Press, N.Y., p. 1-11

31. Hornykiewicz, O., "Parkinson's Disease: From Brain Homogenate to Treatment", Fed. Proc. (1973) 32:2:183-190

32. Javoy, F., Agid, Y., Bovet, D., and Glowinski, J., "Changes in Neostriatal Dopamine Metabolism after Carbachol or Atropine Microinjections into the Substantia Nigra", Brain Res. (1974) 68:253-260

33. Kehr, W., Carlsson, A., and Lindqvist, M., "A Method for the Determination of 3,4-Dihydroxyphenylalanine (Dopa) in Brain", Naunyn-Schmiedeberg's Arch. Pharmacol. (1972) 274:273-280

34. Kehr, W., Carlsson, A., Lindqvist, M., Magnusson, T., Attack, C., "Evidence for a Receptor-Mediated Feedback Control of Striatal Tyrosine Hydroxylase Activity", J. Pharm. Pharmacol. (1972) 24:744-747

35. Kelly, P.H. and Miller, R.J., "The Interaction of Neuroleptic and Muscarinic Agents with Central Dopaminergic Systems", Brit. J. Pharmacol. (1975) 54:1:115-121

36. McGeer, E.G., Fibiger, H.C., McGeer, P.L., and Brooke, S., "Temporal Changes in Amine Synthesizing Enzymes of Rat Extrapyramidal Structures after Hemitransections or 6-hydroxydopamine Administration", Brain Res. (1973) 52:289-300

37. McGeer, P.L., McGeer, E.G., Fibiger, H.C., and Wickson, V., "Neostriatal Choline acetylase and Cholinesterase following Selective Brain Lesions", Brain Res. (1971) 35:308-314

38. Miller, R.J. and Hiley, R., "Antimuscarinic Actions of Neuroleptic Drugs", Adv. Neurol. (1975) 9:141-154

39. Murphy, G.F., Robinson, D., and Sharman, D.F., "The Effect of Tropolone on the Formation of 3,4-dihydroxyphenylacetic acid in the Brain of the Mouse", Brit. J. Pharmacol. (1969) 36:107-115

40. Nose, T. and Takemoto, H., "Effect of Oxotremorine on HVA Concentration in the Striatum of the Rat", Eur. J. Pharmacol. (1974) 25:1:51-55

41. O'Keefe, R., Sharman, D.F., and Vogt, M., "Effect of Drugs used in Psychoses on Cerebral Dopamine Metabolism", Brit. J. Pharmacol. (1970) 38:287-304

42. Olivier, A., Parent, A., Simard, H., and Poirer, L.J., "Cholinesterase Striatopallidal and Striatonigral Efferents in the Cat and the Monkey", Brain Res. (1970) 18:273-282

43. Patrick, R.L., Snyder, T.E., and Barchas, J.D., "Regulation of Dopamine Synthesis in Rat Brain Striatal Synaptosomes", Molec. Pharmacol. (1974) 11:621-631

44. Perez-Cruet, J., Gessa, G., Tagliamonte, A., and Tagliamonte, P., "Evidence for a Balance in the Basal Ganglia between Cholinergic and Dopaminergic Activity", Fed. Proc. (1971) 30:216

45. Porter, C.C., "Inhibitors of Aromatic Amino Acid Decarboxylase", Adv. in Neurol. Vol. 2 (1973) Raven Press, N.Y., p. 37-58

46. Roth, R.H. and Bunney, B.S., "Interaction of Cholinergic Neurons with other Chemically Defined Neuronal Systems in the Central Nervous System", Biology of Cholinergic Function (1976) Raven Press, N.Y., p. 379-394

47. Roth, R.H., Murrin, L.C., and Walters, J.R., "Central Dopaminergic Neurons: Effects of Alterations in Impulse Flow on Accumulation of Dihydroxyphenylacetic Acid", Europ. J. Pharmacol. (1976) 36:1:163-171

48. Roth, R.H., Walters, J.R., Murrin, L.C., and Morgenroth, V.H., "Dopamine Neurons: Role of Impulse Flow and Pre-Synaptic Receptors in the Regulation of Tyrosine Hydroxylase", In Pre- and Postsynaptic Receptors (1975) Marcel Dekker, Inc., p. 5-48

49. Schechter, M.D. and Rosecrans, J.A., "Behavioral Evidence for Two Types of Cholinergic Receptors in the Central Nervous System", Europ. J. Pharmacol. (1971) 15:375-378

50. Shaywitz, B.A., Yager,R.D., and Klopper, J.H., "Selective Brain Dopamine Depletion in Developing Rats: An Experimental Model of Minimal Brain Dysfunction", Science (1976) 191:305-308

51. Shute, C.C.D. and Lewis, P.R., "The Ascending Cholinergic Reticular System: Neocortical, Olfactory, and Subcortical Projections", Brain (1967) 90:497-520

52. Snyder, S.H., "Actions of Anticholinergic Drugs on Striatal Dopamine", Pharmacol. & Therap. Part B (1976) 2:1:65-70

53. Snyder, S.H. and Coyle, J.T., "Regional Differences in ^{3}H-Dopamine Uptake into Rat Brain Homogenates", J. Pharmacol. Exp. Therap. (1969) 165:1:78-86

54. Snyder, S.H., Greenberg, D., and Yamumura, H.I., "Antischizophrenic Drugs: Affinity for Muscarinic Cholinergic Receptor Sites in the Brain Predicts Extrapyramidal Effects", J. Psychiat. Res. (1974) 11:91-95

55. Sourkes, T.L., "Enzymology and Sites of Action of Monoamines in the Central Nervous System", Adv. in Neurol. Vol. 2 (1973) Raven Press, N.Y., p. 13-36

56. Sourkes, T.L., "Parkinson's Disease and Other Disorders of the Basal Ganglia", Med. Neurochem. (1972) p. 565-578

57. Trabucchi, M., Cheney, K.L., Hanin, I., and Costa, E., "Application of Principles of Steady-State Kinetics to the Estimation of Brain Acetylcholine Turnover Rate: Effects of Oxotremorine and Physostigmine", J. Pharmacol. Exp. Therap. (1975) 194:1:57-64

58. Ulus, I.H. and Wurtman, R.J., "Choline Administration: Activation of Tyrosine Hydroxylase in Dopaminergic Neurons of Rat Brain", Science (1976) 194:1060-1061

59. Walters, J.R. and Roth, R.H., "Dopaminergic Neurons: Drug-Induced Antagonism of the Increase in Tyrosine Hydroxylase Activity Produced by Cessation of Impulse Flow", J. Pharmacol. Exp. Therap. (1974) 191:1:82-91

60. Westfall, T.C., "Effect of Muscarinic Agonists on the Release of ^{3}H-Norepinephrine and ^{3}H-Dopamine by Potassium and Electrical Stimulation from Rat Brain Slices", Life Sci. (1974) 14:9:1641-1652

61. Van Woert, M.H., Ambani, L., and Bowers, M.B., "Levodopa and Cholinergic Hypersensitivity in Parkinson's Disease", Neurol. Suppl. (May 1972) p. 86-93

62. Ziegler, H., Del Basso, P., and Longo, V.G., "Influence of 6-Hydroxydopamine and of α-methyl-p-tyrosine on the Effects of Some Centrally Acting Agents", Physiol. Behav. (1972) 8:3:391-396

63. Zivkovic, B., Guidolti, A., Revuelta, A., and Costa, E., "Effect of Thioridazine, Clozapine, and Other Antipsychotics on the Kinetic State of Tyrosine Hydroxylase and on the Turnover Rate of Dopamine in Striatum and Nucleus Accumbens", J. Pharmacol. Exp. Therap. (1975) 194:1:37-46

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE