


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TITLE OF THESIS/TITRE DE LA THÈSE Effects of Prenatal Haloperidol on Brain Dopamine Development

UNIVERSITY/UNIVERSITÉ McMaster University

DEGREE FOR WHICH THESIS WAS PRESENTED/ GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE Ph.D.

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE DEGRÉ 1983

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EFFECTS OF PRENATAL HALOPERIDOL ON
BRAIN DOPAMINE DEVELOPMENT

By

© NADIA RAMONA PLACH, Hons.B.A.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

1982

EFFECTS OF PRENATAL HALOPERIDOL ON
BRAIN DOPAMINE DEVELOPMENT

DOCTOR OF PHILOSOPHY (1982)
Medical Sciences (Neuroscience)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Effects of Prenatal Haloperidol on
Brain Dopamine Development

AUTHOR: Nadia Ramona Plach, Hons.B.A., (Laurentian University)

SUPERVISOR: Michel P. Rathbone, Assistant Professor

NUMBER OF PAGES: ix, 223

ABSTRACT

Little is known about the potential toxicity of neuroleptics administered during pregnancy, although they are known to cross the placenta and reach the fetal brain. The neuroleptic, haloperidol, is often the drug of choice for treatment of psychotic and affective disorders and sometimes as an adjunct during labour. Hence it is frequently administered to women of childbearing age and during pregnancy. Haloperidol is a potent antagonist of dopamine (DA) receptors in the brain. The question raised is "Does the administration of haloperidol during the period of DA neuron synaptogenesis (third trimester in the rat) interfere with the normal development of DA neurons and postsynaptic receptors? If so, does this alter subsequent brain DA function as reflected in behaviour and hormonal regulation in the offspring?"

Haloperidol or vehicle (control) were administered to timed-pregnant rats on gestation days 15 to 21. At birth, all offspring were fostered by untreated lactating dams. The effects of this treatment on three DA systems, (nigrostriatal, mesolimbic and tuberoinfundibular systems) in the brains of offspring were assessed on postnatal day 25 using morphological, pharmacological, biochemical and behavioural analyses.

The results indicated that haloperidol treatment during the third trimester produced a deficit in the growth of nigrostriatal DA neurons and decreased postsynaptic DA receptors without affecting the postsynaptic enzyme, adenylate cyclase. Offspring of haloperidol rats

showed locomotor hyperactivity even after reaching adulthood. They also showed an abnormal increase in prolactin secretion when challenged with haloperidol on postnatal day 25. There was a significant sex difference in behavioural and hormonal responses to haloperidol, with female offspring showing greater sensitivity.

These data demonstrate that treatment with haloperidol for even a short period during pregnancy can produce long term, perhaps even permanent alterations in morphological, pharmacological, behavioural and hormonal development of brain DA neurons, in the absence of overt teratogenic effects. Subtle defects in offspring brain development may predispose them to later difficulties associated with abnormal DA function, especially in situations of stress. These findings emphasize the need for further investigation of the potential toxicity of neuroleptics, especially during prenatal development.

ACKNOWLEDGEMENTS

I wish to express my gratitude to the members of my supervisory committee: Drs. R.J. Racine, J.M. Cleghorn, R.M. Epand and G.M. Brown for their valuable feedback during the course of this research; to Drs. L.J. Grotta and J. Seggie for numerous helpful discussions and support of part of this work; to M. Holmes and C. Ikeson for their expert technical assistance.

Special thanks to my supervisor, Dr. M.P. Rathbone for his advice and continued support throughout the work.

Finally, I would like to express my sincere appreciation to my husband, George J. Plach, and my parents Bill and Marlene Bezzubyk, Karol and Aniela Plach for their unending patience and encouragement.

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LIST OF ABBREVIATIONS

α -MD	alpha-methyl-dopa
α -MPT	alpha-methyl-para-tyrosine
cyclic AMP	adenosine 3'5'-cyclic monophosphate
ATP	adenosine triphosphate.
Bmax	number of binding sites (Scatchard plot)
COMT	catechol-o-methyl-transferase
con	control
DA	dopamine
Dopa	dihydroxyphenylalanine
l-Dopa	levo-dopa
dpm	disintegrations per minute
Flu	fluphenazine
^3H	tritiated-ligand
Hal	haloperidol
KD	binding affinity (Scatchard plot)
MAO	monoamine oxidase
n	number
obj	objective
p	probability value
r	correlation coefficient
s.c.	subcutaneous
sem	standard error of the mean
SPG	sucrose, phosphate, glyoxylate solution
\bar{x}	mean

INTRODUCTION

A - DOPAMINE AS A TROPHIC AGENT IN PRENATAL DEVELOPMENT

The mammalian brain is highly specialized for the processing and transfer of information based on a very complex but precise organization of its neurons (McGeer et al., 1978). The term "neural specificity" has been used to describe this predictability in structure of brain neurons and their connections with appropriate target cells (Lund, 1978). The establishment and maintenance of this precise organization is an important component in the maintenance of normal function within the central nervous system (Sperry, 1971; McGeer et al., 1978). The mechanisms by which these precise connections are established and maintained, however, are not yet fully understood (Lund, 1978).

In the adult animal, it is known that neurotransmitters play an important role in the maintenance of pre- and post-synaptic viability, an effect that is referred to as trophic influence (Guth, 1968; Smith and Kreutzberg, 1976; Dismukes and Daly, 1976). Some brain neurotransmitters, such as dopamine (DA) are detectable early in prenatal development, before axons have started to develop (Loizou, 1971; Coyle, 1974). An intriguing question is whether DA, present at this early stage in development, subserves a function that is different from its role as a transmitter later in development. For example, does it initiate communication with target cells and form a link within a feedback system that ultimately leads to the establishment of appropriate neural connections? Further, is the maintenance of the postsynaptic target dependent on presynaptic innervation,

and on receiving a dopaminergic signal at the appropriate time in development?

This thesis study addresses itself to the dynamic interactions that take place between developing nerve terminals and their target sites during the prenatal period of development when pre- and post-synaptic connections are first being established (Loizou, 1972; Coyle and Henry, 1973). The specific questions raised are:

1. Does the administration of a specific antagonist of postsynaptic sites alter the normal growth of presynaptic neurons?
2. If presynaptic neural development is abnormal, does this alter the normal development of postsynaptic target sites?
3. Do abnormalities in pre- and postsynaptic development lead to significant alterations in the subsequent activity of that neural system?

In order to answer these questions, it is necessary to obtain a suitable experimental model. See Figure 1. In particular, one requires a neural system that has been relatively well studied so that sufficient information about its normal pattern of development and function is available. Secondly, one must use a potent antagonist of postsynaptic receptor sites so that the natural transmitter's interactions with postsynaptic sites is blocked. Thirdly, there should be a variety of accurate measures available to detect morphological and biochemical changes in the normal development of that neural system. And finally, there should be a reliable means of determining whether alterations in the development of that neural system become expressed as functional deficits.

One system that fulfills all these criteria is the central

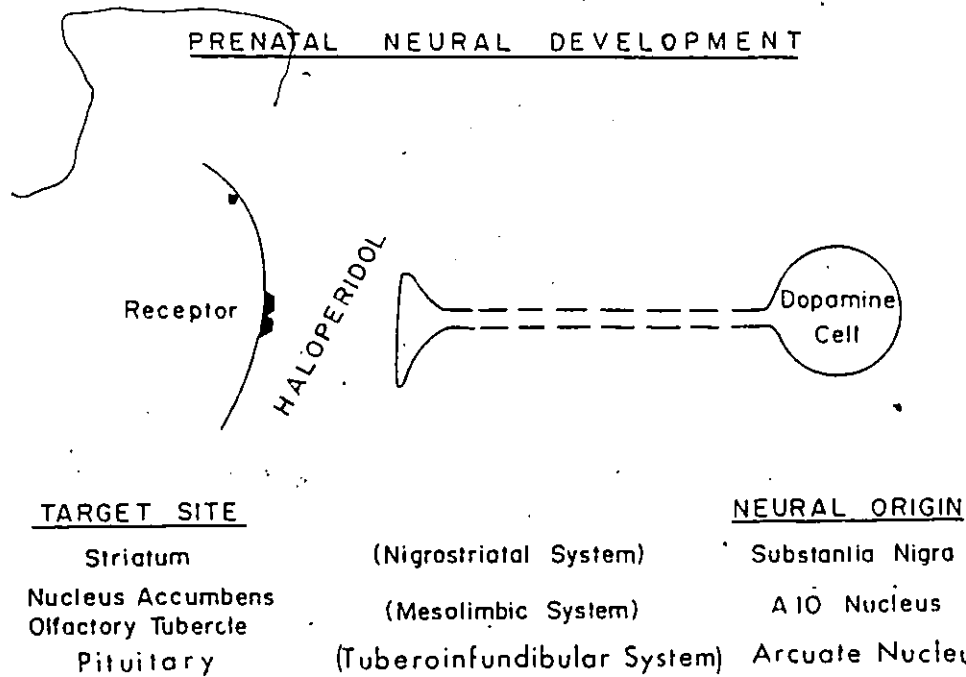


Figure 1: Schematic of a neural cell (dopamine) whose axon is growing towards target sites during prenatal development of the brain. The antagonist, haloperidol, is shown to block interactions between the presynaptic neuron and its postsynaptic receptors. Below the model are listed the 3 major brain dopamine systems including their origins and terminal areas.

dopamine system of the rat. The anatomy and function of brain DA tracts is fairly well documented relative to other neural systems (Introduction, part B). Sensitive techniques are available to analyze the morphological development of DA neurons, for example, glyoxylate fluorescence microscopy (de la Torre and Surgeon, 1976) that can be used to visualize presynaptic terminal development. There are well established markers of postsynaptic components within brain DA systems. These can be readily measured using a specific ligand of the postsynaptic DA receptor, ^3H -spiroperidol (Seeman et al., 1975; 1976) and by analyzing the activity of the enzyme, adenylate cyclase, that is highly sensitive to DA and is thought to be coupled to postsynaptic DA receptors (Mishra et al., 1974; Greengard, 1976). A very potent and relatively specific antagonist of postsynaptic DA receptors is the neuroleptic haloperidol, whose effects have been well studied in the adult system (Introduction, part E). Hence, there already exists a body of information with which to compare the results of this study:

Finally, it is well known that DA plays a major role in the control of motor, as well as emotional behaviour in the rat that can be measured using an open field chamber (Denny-Brown and Yanagisawa, 1976; Stevens, 1979; Stoof et al., 1978). It is also known that DA plays an important role in the regulation of pituitary hormones, especially prolactin (MacLeod, 1976). Hence changes in DA neural development and function, subsequent to treatment with haloperidol, would likely lead to significant changes in behaviour and prolactin secretion that can be readily measured.

By means of a multi-disciplinary approach incorporating each of

the parameters just described, the present study is designed to determine whether haloperidol, if administered during the prenatal period of initial DA synaptogenesis, will alter the normal development and function of DA neurons in the brains of rat offspring. A multi-disciplinary approach was used so that correlations between the morphological, biochemical and functional development of DA neurons could be investigated, since this would provide insight into the significance of their interrelationships. The prenatal period of development was of interest since almost no information exists regarding the effects of DA antagonism at this stage. The results of this study can be compared to those of previous studies using adult animals so that the similarities and/or differences between the developing and the mature DA system in terms of response to postsynaptic blockade can be defined. These data will provide important information about the plasticity of brain DA neurons, that is, capacity to compensate for an interference with transmitter interaction at postsynaptic sites (Quarton et al., 1967; Dismukes and Daly, 1976). These data may also be applicable to central neural systems in general. In addition, since haloperidol is used extensively in the treatment of psychoses and as an adjunct to analgesics, the results of this study may have significant clinical applications, especially when this drug is administered to women of childbearing age and during pregnancy (Breese et al., 1978; Goldberg and DiMascio, 1978).

In the subsequent sections of this introduction, a brief historical account of the discovery of DA neurons will be presented. This will be followed by a description of the major DA systems in the brain (Part B) and their development (Parts C and D). Finally, the consequences

of neuroleptic administration to adult animals (Part E) and what is known of their effects in developing animals will be reviewed (Part F).

Historical Perspective

Dopamine (DA) is a member of the naturally occurring family of monoamines collectively known as the catecholamines. The structure of DA, also known as β -3,4-dihydroxyphenylethylamine or 3'-hydroxytyramine, is comprised of a dihydroxyphenyl (catechol) ring coupled to an amine side-chain (Figure 2). DA, along with the other catecholamines, is synthesized from the amino acid tyrosine by a sequence of enzymatic steps first postulated by Blaschko in 1939 and finally confirmed by Nagatsu and coworkers in 1964. In mammals, tyrosine can be derived from dietary phenylalanine and both amino acids are present in a free form in the mammalian brain (Cooper et al., 1975).

The immediate precursor of DA is dihydroxyphenylalanine (Dopa), that is converted to DA by Dopa decarboxylase (Figure 2). Dopamine can then be converted to norepinephrine (noradrenaline) and epinephrine (adrenaline) by two further enzymatic steps (Figure 2). The rate-limiting step in the synthesis of DA and the catecholamines is that of tyrosine hydroxylase that converts tyrosine to l-Dopa (Udenfriend, 1966). Dopamine-synthesis can be inhibited by blocking this enzyme with alpha-methyl-p-tyrosine (a MPT) and by inhibiting the enzyme dopa decarboxylase with alpha-methyl dopa (Cooper et al., 1975). Both of these compounds have been used frequently in studies of DA synthesis and turnover (Ibid).

Dopamine was the last of the catecholamines to be identified and isolated from body tissues. In 1911, Funk synthesized its precursor

BIOSYNTHETIC PATHWAY OF DOPAMINE AND THE CATECHOLAMINES

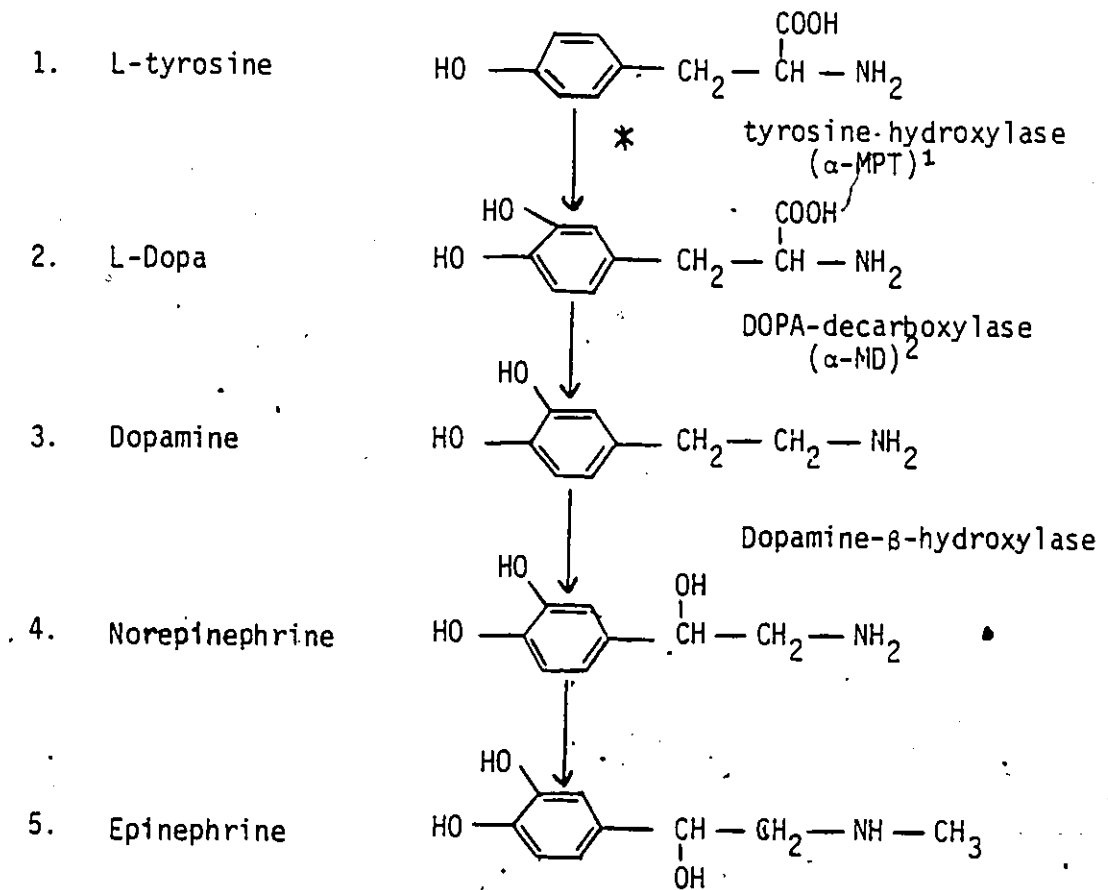


Figure 2: Shown is the sequence of steps in the biosynthesis of dopamine and the catecholamines including the rate limiting step (*) that is dependent on the activity of the enzyme tyrosine hydroxylase. Also shown are two inhibitors of dopamine synthesis that are antagonists of the enzymes tyrosine hydroxylase and Dopa-decarboxylase: alpha-methyl-para-tyrosine (α -MPT)¹ and alpha-methyl-dopa (α -MD)², respectively.

Dopa hoping to reveal the route of catecholamine synthesis. But it was not until 1939 that the critical experiment linking Dopa to the catecholamines was performed by Holtz. He incubated an extract from guinea pig kidney, which contained dopa decarboxylase, with its substrate (Holtz, 1939). He then injected the incubate intravenously, and produced a strong rise in blood pressure. He proposed that the active principal producing this biological effect was DA. Later in 1947, Holtz and his coworkers substantiated their hypothesis by injecting themselves with l-Dopa intravenously and isolating DA from their urine. It was not until 1951, however, that Goodall finally demonstrated that DA actually existed within mammalian tissues.

These studies established the existence of DA in peripheral tissues but its existence in the brain was not yet suspected. The major breakthrough occurred in 1954 when Vogt measured the "sympathin" content of brain. She discovered that both adrenaline and noradrenaline were present in amounts and distribution that could not be accounted for on the basis of brain vascularity (Vogt, 1954).

A series of highly significant discoveries then followed in rapid succession. The Rauwolfia alkaloid, reserpine, an ancient Indian remedy with an unknown physiological basis for its tranquilizing properties, was introduced clinically (McGeer et al., 1978). It produced an unexpected side-effect, that of a Parkinsonian-like rigidity, which was noted by several clinical groups using the drug. Carlsson and his coworkers then developed a chemical assay for DA and showed that its distribution differed from that of the other catecholamines (Carlsson, 1959). Very high concentrations of DA were found in the corpus striatum of the fore-

brain and, like other monoamines, its levels were reduced following treatment with reserpine. The correlation between high DA content in the striatum, its depletion by reserpine and the accompanying Parkinsonian-like side-effects lead Carlsson to propose DA played an important role in the control of extrapyramidal motor function (Carlsson, 1959).

Birkmayer and Hornykiewicz (1961) tested this hypothesis by measuring DA levels in the autopsied brains of a series of Parkinsonian patients. They found that the levels of DA were sharply decreased. Since it had been previously demonstrated that L-Dopa is converted to DA in mammalian tissues, clinical trials attempting a "replacement" therapy were soon initiated. The first significant clinical results were reported by Cotzias and coworkers who administered large doses of L-Dopa to their patients over a period of several weeks and found that it relieved the akinesia and rigidity associated with Parkinson's disease (Cotzias et al., 1969).

Although the importance of DA and the other catecholamines in the mammalian brain was established by the early 1960s, the questions of brain biosynthesis and cellular localization were still to be answered. McGeer et al. (1963) was the first to demonstrate that labelled tyrosine injected directly into the striatum was converted to catecholamines. Udenfriend (1966) then isolated and purified tyrosine hydroxylase and demonstrated that it is the rate-limiting enzyme in catecholamine synthesis.

The climatic event in the localization of DA in the brain came with the application of histofluorescent techniques for visualizing catecholamines in neural tissue. Eranko (1955) observed that adrenal

medullary tissue fixed in formaldehyde emitted a bright yellow colour when viewed with a fluorescence microscope. Several years later, Falck and Torp modified this technique to allow detection of catecholamines in nervous tissue (Falck et al., 1962). Shortly thereafter, Dahlstrom and Fuxe. (1964a; 1964b) using the Falck technique succeeded in mapping out DA along with other monoamine pathways in the brain.

B - ANATOMICAL AND FUNCTIONAL OVERVIEW

There are three major dopamine (DA) systems in the brain: the nigrostriatal, mesolimbic and tuberoinfundibular systems (Figure 3). Each of these is described in the following subsections, including a brief outline of their neuroanatomical features, regulation and function. Emphasis is placed on the important similarities and differences among these three DA systems, particularly with regard to the roles they play in regulating different behaviours as well as hormonal secretion.

Nigrostriatal DA System

Neuroanatomy: The cell bodies of the nigrostriatal DA neurons originate in the substantia nigra, areas A-8 and A-9 in the rat brain (Dahlstrom and Fuxe, 1964) and project their axons toward the forebrain to terminate in the striatum (Figure 3). The axons are characteristically very fine and only lightly myelinated (Hattori et al., 1973). Upon entering the striatum, each axon branches widely to form a diffuse network of very fine terminal axons characterized by varicosities along their lengths (Fuxe, 1965).

Anden et al. (1966) attempted to quantitate the number of cell bodies and axonal distribution of the nigrostriatal system in the rat. The results of their study demonstrated the great divergency of these DA neurons. For example, the zona compacta of the substantia nigra contains approximately 3500 DA cells. Each cell body gives rise to a single axon that branches profusely and contains about 500,000 varicosities (nerve endings or boutons "en passant") by the time it reaches the striatum.

MAJOR DOPAMINE TRACTS in the RAT BRAIN

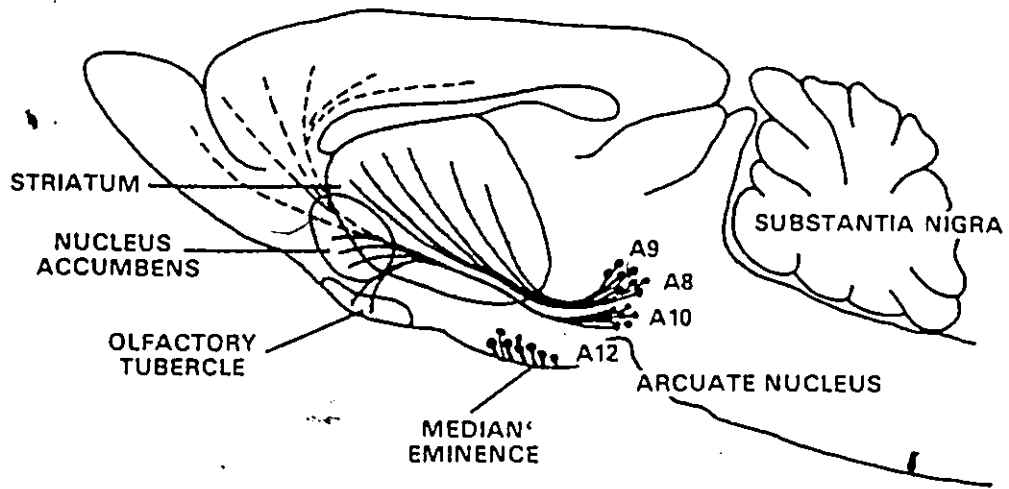


Figure 3: The three major dopamine systems in the rat brain include: (1) Nigrostriatal tract, originating in the substantia nigra (areas A-8 and A-9), terminating in the striatum; (2) Mesolimbic system originating in area A-10, terminating mainly in nucleus accumbens and olfactory tubercle, with some fibres continuing in the forebrain; and (3) Tubero-infundibular system originating in the arcuate nucleus (A-12) and terminating in the median eminence.

Since there are roughly 4 million neurons in the rat striatum, each striatal neuron could receive as many as 440 DA contacts assuming an equal distribution of synapses (Anden et al., 1966). This indicates that dopaminergic input to the striatum is quite massive and of a field-type distribution.

Nigrostriatal fibres form synaptic contacts with a variety of different types of cells in the striatum. One type of target cell includes small interneurons that have only intrinsic connections within the striatum (Hassler, 1979). These interneurons are referred to as "spiny" cells since they have numerous dendritic spines on which the vast majority of synaptic contacts with presynaptic terminals are made (Fox et al., 1971). These striatal interneurons are thought to contain acetylcholine (McLennan and York, 1966; McGeer et al., 1971) and gamma-aminobutyric acid (GABA) (McGeer and McGeer, 1975).

The remaining "aspiny" striatal neurons receive input via axosomatic and axodendritic contacts (Fox et al., 1971). The transmitters released by these neurons have not yet been fully detailed although the large and giant projection cells are thought to contain GABA (Kim et al., 1971) and the peptide, substance P (Hokfelt et al., 1975). Nigrostriatal neurons may also form synaptic contacts with other striatal cells containing serotonin (Holman and Vogt, 1972) and enkephalin (Kuhar et al., 1973). The relative levels of the latter transmitters, however, are only a fraction of the amount of DA, acetylcholine and GABA concentrated within the striatum. A schematic summary of the neuronal circuitry within the nigrostriatal system is presented in Figure 4.

At the time of printing of this thesis, a few points clarifying the current understanding of striatal circuitry were provided by Dr. T. Hattori (personal communication, 1982). Most of the medium spiny neurons in the striatum have been established to be projection neurons. Some of the giant aspiny neurons in the striatum are cholinergic interneurons. ~~GABA-~~containing cells are mostly medium sized projection neurons, which have collaterals terminating inside the striatal boundaries (Poirier et al. 1977, J. Neurol. Sciences 31:181-198).

BIOCHEMICAL INTERCONNECTIONS OF THE BASAL GANGLIA

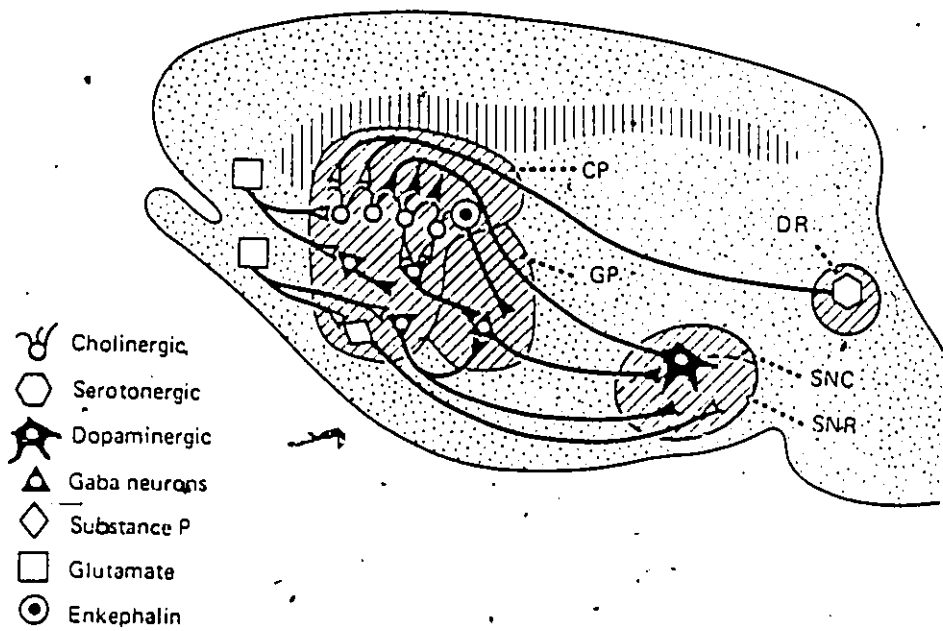


Figure 4: Schematic of the neuronal circuitry within the basal ganglia of the rat brain showing the interconnections between the nigrostriatal dopamine system and a variety of other neurons. CP, caudate putamen; GP, globus pallidus; DR, dorsal raphe; SNC, substantia nigra-pars compacta; SNR, substantia nigra pars reticulata. Adapted from McGeer et al., 1978.

Regulation of Nigrostriatal Function: The function of the nigro-striatal DA system is regulated by a multiplicity of inputs from many different brain areas that impinge either at striatal terminal sites or on DA cell bodies in the substantia nigra. For example, the striatum receives important afferents from the cortex (Kemp and Powell, 1971) thalamus (Mehler and Nauta, 1974) and midbrain (Fibiger et al., 1972; Hattori et al., 1973). The substantia nigra receives afferents from other areas of the midbrain (Fibiger and Miller, 1977), the cerebellum (Snider et al., 1976) and most importantly, the striatum (Bunney and Aghajanian, 1976). The way in which these afferents influence nigro-striatal function is as yet unknown.

Although the relationships between the neurons within the striatum and substantia nigra are exceedingly complex, it appears that at least on anatomic grounds, there exists a neuronal feedback loop between these two brain areas (Moore and Wuertheler, 1979). It has been estimated that approximately 50% of striatal neurons project back to the substantia nigra via a striato-nigral efferent system (Bunney and Aghajanian, 1976). There is pharmacological and biochemical evidence to indicate that altering DA stimulation of receptors in the striatum influences the activity of cell bodies in the substantia nigra via these striato-nigral efferents (Carlsson and Lindquist, 1963). This, in turn alters the subsequent release of DA from neurons in the striatum. The issue of whether these interactions constitute a positive or negative feedback system, or perhaps a combination has not yet been resolved (Groves et al., 1975).

The relative importance of this nigro-striatal-nigral feedback system has recently come into question. Chemical, electrolytic or mechanical lesions of the striato-nigral pathways do not attenuate the modulation of DA metabolism induced by dopaminergic agonists and antagonists (Moore and Wuerthele, 1979). This suggests that some alternate means for autoregulation must exist independent of this feedback system. Geffen et al. (1976) recently reported that dendrites of DA cell bodies in the substantia nigra release DA. Hence, DA neurons by means of dendrodendritic synapses may regulate the activity of their own cell bodies in the brainstem. In addition, they may also regulate the activity of other afferents to the substantia nigra, including the GABA and substance P projections from the striatum (Bartholini et al., 1976; Gale et al., 1977; Guidotti and Gale, 1977). Much work remains to be done before the details of these complex interrelationships are worked out.

Functions of the Nigrostriatal System: Despite the complexity of nigrostriatal circuitry, it is clear that this system plays a major role in the control of motor movement (Guyton, 1972; Denny-Brown and Yanagisawa, 1976). This is well exemplified by the clinical pathologies of Parkinson's and Huntington's diseases, both of which are movement disorders associated with degeneration of neurons in the substantia nigra and striatum, respectively. In Parkinson's disease, the degeneration of nigral cells leads to a reduction of DA input to the striatum. This produces the characteristic symptoms of akinesia and rigidity that are a consequence of a loss in the ability to initiate and carry out normal motor movement (Hornykiewicz, 1973). Comparable motor deficits can be

produced in animals by lesions (Denny-Brown and Yanagisawa, 1976) or pharmacological blockade (Muller and Seeman, 1978) of the nigrostriatal pathway.

In Huntington's disease there is a degeneration of GABA-containing cells (Perry et al., 1973) as well as ACh (Bird and Iversen, 1974) and substance P (Kanazawa et al., 1977) containing cells in the striatum. While Parkinson's disease is associated with an inability to initiate motor movement, Huntington's disease involves an inability to control motor movement. The patient presents with uncontrollable choreiform movements and postural abnormalities. A similar syndrome can be produced in animals by selective destruction of GABA and ACh neurons with local injections of kainic acid into the striatum (McGeer and McGeer, 1976).

The treatment for both Parkinson's and Huntington's diseases is based primarily on an attempt to normalize DA function. Parkinson's patients are treated with l-Dopa, precursor of DA (Figure 1) or other centrally acting drugs that mimic DA (Hornykiewicz, 1972; 1973). This is essentially a replacement therapy designed to restore the loss of normal dopaminergic input to the striatum following degeneration of the nigrostriatal tract. In Huntington's disease, the treatment of choice is neuroleptic therapy (Bird, 1978). The administration of DA antagonists, such as haloperidol, protect the postsynaptic DA receptors from excessive stimulation by nigral cells that have been released from tonic inhibition by the degeneration of striato-nigral efferents (McGeer and McGeer, 1976). Hence, the restoration of normal motor movement is dependent on normalized function of the nigrostriatal DA system.

Mesolimbic DA System

Neuroanatomy: The cell bodies of the mesolimbic DA neurons are found in the midbrain ventrosegmental area A-10 (Dahlstrom and Fuxe, 1964) just medial and superior to the substantia nigra (Figure 3). Their axons ascend in a rostral direction following a course that is medial to the nigrostriatal tract and terminate in the limbic area of the forebrain, including the nucleus accumbens and olfactory tubercle. Some of the fibres running along the medial aspect of the nucleus accumbens separate into a number of branches, the most abundant ones continuing rostrally to innervate the deep layers of the frontal cortex. A second branch turns dorsally along the corpus callosum and moves caudally to innervate the anterior limbic cortex (Lindvall and Bjorklund, 1974).

The mesolimbic system is similar to the nigrostriatal system in that it also serves as a bridge between DA cell bodies in the midbrain and terminal areas of the forebrain (Bjorklund and Lindvall, 1978). There is a slight overlap in their areas of termination (Nauta and Domesick, 1978). In addition, there is a crude topographic relationship with regard to the areas they innervate (Figure 3). For example, the laterally situated A-10 nuclei project to the more ventrally placed forebrain structures including nucleus accumbens, and the ventromedial aspects of the striatum while the A-8 and A-9 nuclei send projections to the remaining mass of the striatum (Domesick et al., 1976; Nauta and Domesick, 1977).

Regulation of Mesolimbic Function: Another parallel between the mesolimbic and nigrostriatal tracts is the multiplicity of afferents impinging on the system. There is a massive convergence of afferents from

the hippocampus, amygdala and allocortex onto the limbic forebrain nuclei that, in turn, send out a narrowing funnel of efferents back to the midbrain (Stevens, 1979). This neuroanatomical pattern of organization indicates that there must be a marked compression and integration of incoming information to the mesolimbic system, similar to that of the nigrostriatal system (Ibid).

The mesolimbic efferents terminate in various midbrain areas including the pars compacta of the substantia nigra (A-9) as well as in A-8 and A-10 (Nauta et al., 1978). Although there is a relative lack of information regarding the nature of the synapses involved, there is some evidence to suggest that there may also be a "feedback" pathway between the nucleus accumbens and mesencephalic projections (Nauta et al., 1978). This anatomical proximity between mesolimbic and nigrostriatal feedback loops also offers the potential of interactions between the mesolimbic and nigrostriatal DA cells. It is interesting to note that both mesolimbic and nigrostriatal neurons are under tonic inhibitory influence of GABA neurons, suggesting a close functional relationship in the activities of these two dopaminergic systems (Wolf et al., 1978; Stevens et al., 1974).

Functions of the Mesolimbic System: Aside from this very generalized similarity between the mesolimbic and the nigrostriatal DA systems, there is pharmacological, biochemical and behavioural evidence indicating that the functions of these systems are quite different. A particularly interesting feature that distinguishes the two DA systems is their difference in response to neuroleptic treatment. This is true for both acute and chronic treatment with neuroleptics such as haloperidol

(Scatton et al., 1975, 1976; Julou et al., 1977). For example, treatment with long acting neuroleptics produces a tolerance effect on the rate of DA synthesis in the nigrostriatal system, but not in the mesolimbic system (Asper et al., 1973; Bowers et al., 1974). Hence the administration of a given neuroleptic can have a very different effect on these two systems, despite their both being dopaminergic.

One postulate that may account for these differences in responsivity may be a variation in the nature of DA receptors within these two systems. Preliminary evidence indicates that the distribution of DA receptors in the mesolimbic system is different from that of the nigrostriatal system. For example, binding studies in primate brains in which the displacement potency of various dopaminergic agents on ³H-Spiroperidol binding was compared showed that certain drugs had a higher affinity for mesolimbic sites than nigrostriatal areas (Thal et al., 1978). The same observation was reported in similar studies using rats (Howard et al., 1978).

The results of behavioural studies provide further indications that there are basic differences in the functions carried out by the mesolimbic and the nigrostriatal systems. For example, Ungerstedt and Ljungberg (1977) with the help of a computer-linked test cage were able to "separate" behaviours controlled by the nucleus accumbens from those controlled by the striatum. They demonstrated that intracranial injections of DA into the striatum of rats induced stereotyped sniffing, licking and gnawing. In contrast, intracranial injections of the same amount of DA into the nucleus accumbens produced excessive locomotor activity. Similar findings were obtained in other studies using a

variety of more simple behavioural tests (Kelly et al., 1975).

The mesolimbic system has long been implicated in the control of emotional behaviour (Stevens, 1973; Powell and Hines, 1974). Recent interest in this system is centred around the possibility that a disturbance in the function of mesolimbic neurons, particularly DA neurons, may be involved in psychosis such as that of schizophrenia (Stevens, 1979; Manschreck, 1981). Treatment with DA antagonists, such as the neuroleptics alleviate the severe thought and emotional disturbances that characterize this illness (Snyder, 1973). Examination of postmortem schizophrenic brains indicate that there is an increased number of DA receptors in the limbic forebrain (Crow et al., 1978) even in the absence of prior treatment with neuroleptics that might secondarily increase their numbers (Lee et al., 1978). Much work remains to be done before the significance of these findings in terms of understanding mesolimbic functions is established.

In summary the mesolimbic DA system differs from the nigrostriatal system in a number of important ways: (1) the distribution of its neurons is to the more rostral and ventral forebrain areas; (2) it shows a differential response to neuroleptic treatment as well as a difference in receptor distribution; and (3) it regulates different behavioural responses and may be involved in emotional behaviour and psychosis.

Tuberoinfundibular DA System

Neuroanatomy: The tuberoinfundibular system is a short neuronal pathway contained entirely within the hypothalamus. The cell bodies of

these DA neurons are located within the mediobasal hypothalamus area A₁₂ in the rat (Dahlstrom and Fuxe, 1964). The axons project ventrally to terminate in all layers of the median eminence at the base of the hypothalamus (Figure 3). The density of these axons is greatest in the external layer of the median eminence where there is a rich vascular network that runs between the hypothalamus and pituitary, the hypothalamic-hypophyseal portal system (Oliver et al., 1977; Bergland and Page, 1978). The terminals of the tuberoinfundibular DA neurons form a dense neural plexus in the perivascular space of these portal vessels (Hokfelt, 1967). Gibbs and Neill (1978) have recently been able to detect physiological levels of DA in portal blood, indicating that tuberoinfundibular terminals may secrete DA directly into this portal system. The levels of DA are higher in portal plasma than in systemic arterial plasma (Ben-Jonathan et al., 1977). Hence, it appears that the tuberoinfundibular DA neurons form part of a neurovascular system at the base of the hypothalamus (Porter et al., 1972; Bergland and Page, 1979).

DA Regulation of Pituitary Prolactin Secretion: In contrast to the nigrostriatal and mesolimbic DA neurons, whose targets are other neurons, the target for tuberoinfundibular neurons are hormone-secreting cells in the pituitary (MacLeod and Lehmeyer, 1972). The pituitary is connected to the hypothalamus via the infundibular stalk (Figure 5). The anterior pituitary, where most hormone secreting cells are located, does not receive any direct neural input (Green, 1966). Nor does it receive any systemic blood (Bergland and Page, 1979). Since its major blood supply is that of the hypothalamic-hypophyseal portal system, it appears that the anterior pituitary is under neurovascular regulation via

Tuberoinfundibular System

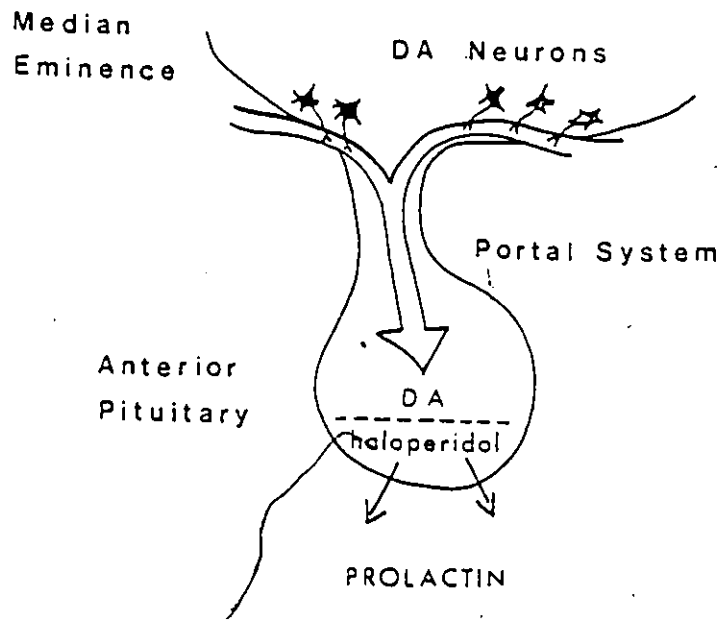


Figure 5: Schematic of the tuberoinfundibular system showing dopamine neurons terminating in the median eminence of the hypothalamus and releasing their transmitter into the portal system that carries it down to the anterior pituitary. Haloperidol is shown to block the tonic inhibitory effect of dopamine (DA) on the anterior pituitary, thereby producing an increased release of prolactin.

this portal system (Porter et al., 1972). Isolation of the pituitary from the hypothalamus by stalk-sectioning (Diefenback et al., 1976), pituitary transplantation (Everett, 1954) or destruction of the median eminence (Chen et al., 1970) all lead to an increase in pituitary prolactin secretion. Infusion of physiological amounts of DA into rats whose median eminence was destroyed produced a significant decrease in plasma prolactin levels (Gibbs and Neill, 1978). Hence, it was proposed that pituitary prolactin secretion is under the tonic inhibitory influence of tuberoinfundibular neurons (MacLeod, 1976).

The question as to whether tuberoinfundibular DA neurons exert a tonic inhibitory influence on pituitary prolactin secretion via a direct or indirect mechanism has been much debated. One proposal is that hypothalamic DA neurons stimulate the release of another substance, perhaps a polypeptide called prolactin-inhibiting factor or PIF (Greibrokk et al., 1974; 1975). Some DA terminals form presynaptic junctions with the axons of hormone-releasing neurons in the hypothalamus (Knigge and Scott, 1970). Tuberoinfundibular neurons may thereby regulate the release of PIF that is secreted into the hypothalamic-hypophyseal portal system and inhibit prolactin-secreting cells in the anterior pituitary. In support of this hypothesis, Ojeda et al. (1974) reported that a non-DA PIF is released from rat hypothalamus incubated in vitro and that the activity of this PIF cannot be blocked by DA antagonists. No one as yet, however, has been able to isolate and purify this PIF.

There is much evidence to support the alternate hypothesis that tuberoinfundibular DA neurons exert a direct tonic inhibitory influence on pituitary prolactin secretion (Meites, 1977). For example, pituitary

prolactin synthesis is inhibited by infusion of DA into the hypothalamic-hypophyseal portal system (Takahara et al., 1974) and by direct application to rat pituitaries in vitro (Koch et al., 1970; Ojeda et al., 1974). This inhibitory effect is mimicked by DA precursor l-Dopa (Friesen et al., 1972; Donoso et al., 1974) as well as DA agonists such as apomorphine (Martin et al., 1974). In contrast, prolactin levels increase when DA synthesis is inhibited by alpha-methyl-para-tyrosine (Donoso et al., 1971) or methyl-dopa (Steiner et al., 1976). Neuroleptics that block post-synaptic DA receptors (Burt et al., 1975; Seeman et al., 1975) also produce an increase in serum prolactin levels (Ben-David et al., 1970; Lu et al., 1970).

It was not clear at first whether DA antagonists exerted their effects at the level of the hypothalamus or directly on the prolactin-secreting cells of the pituitary (MacLeod, 1976). Brown et al., 1976 then reported the existence of DA-binding sites within the anterior pituitary of the rat. No DA receptors were found in the median eminence. Specific binding sites for DA and dopaminergic agents have also been found in the pituitaries of several other species of animals (Creese et al., 1977; Keabian and Calne, 1979; Cronin and Weiner, 1979). These data indicate that DA and neuroleptics exert a direct effect on prolactin secretion at the level of the anterior pituitary.

Regulation of Tuberoinfundibular System: In contrast to the nigro-striatal and mesolimbic DA systems, that are regulated by neuronal afferents, the tuberoinfundibular system appears to be regulated primarily by hormonal influences, especially prolactin (Moore and Wuerthele, 1979). Fuxe et al. (1969a; 1969b) found that the number of cells in

the hypothalamus and their DA content was increased in female rats during pregnancy, pseudopregnancy and lactation. DA turnover at these times was enhanced in the median eminence but unchanged in the nigrostriatal system. This led them to propose that tuberoinfundibular DA neurons were selectively activated during these states and that this activation was due to specific hormonal changes.

It was subsequently demonstrated that chronic administration of prolactin enhanced the rate of DA turnover in the median eminence (Hokfelt and Fuxe, 1972). Treatment of rats with DA antagonist haloperidol that increases serum prolactin also enhances DA turnover in the median eminence (Gudelsky and Moore, 1977). Similar effects are produced by other DA antagonists as well (Ibid). Removal of the pituitary abolishes the observed effects on DA turnover in the median eminence, whereas hypophysectomy without removal of the pituitary does not (Gudelsky et al., 1978). These data indicate that pituitary prolactin exerts a negative feedback on tuberoinfundibular DA neurons in the hypothalamus (Weiner and Ganong, 1978). Since the observed effects of prolactin and DA antagonists on DA turnover in the median eminence are not immediate and occur only after a latent period of a few hours, there may be another intermediary mechanism within this feedback system, although the specific details are not yet known (Wiesel et al., 1978).

There is also some neuroanatomical evidence that tuberoinfundibular DA neurons receive direct neuronal afferents which may influence their function. GABA-containing nerve terminals appear to synapse in the arcuate and ventromedial nuclei of the hypothalamus that give rise to the tuberoinfundibular system (Tappaz and Brownstein, 1977). In addition,

there exists a tuberoinfundibular GABA pathway that projects to the median eminence in a way that is analagous to the DA pathway (Tappaz and Brownstein, 1977). The results of recent experiments indicate that these neurons may influence DA regulation of prolactin secretion: in the first case, by inhibiting the release of DA from tuberoinfundibular neurons thereby increasing prolactin secretion (Enjalbert et al., 1979; Grandison and Guidotti, 1979; Racagni et al., 1979) and in the second case, by direct secretion independent of DA action (Grandison and Guidotti, 1979).

In summary, the tuberoinfundibular DA system is a neuroendocrine system that exerts a tonic inhibitory influence on pituitary prolactin secretion. Unlike the nigrostriatal and mesolimbic DA systems that form synapses with their postsynaptic target neurons, the tuberoinfundibular neurons release DA into the hypothalamic-hypophyseal portal system that transports it to the anterior pituitary where it acts on prolactin-secreting cells. Also, unlike the other two systems, that are regulated by neuronal afferents, tuberoinfundibular DA neurons appear to be regulated mainly by prolactin via a short negative feedback system. Despite these fundamental differences in neuroanatomy, regulation and function, postsynaptic DA receptors of the tuberoinfundibular system that are located in the anterior pituitary, show a similar responsiveness to DA agonists and antagonists. This can be measured as changes in prolactin secretion.

C - THE DEVELOPMENT OF CENTRAL DOPAMINE NEURONS

The major nuclei of the nigrostriatal and mesolimbic systems are formed well before birth in the rat brain (Coyle, 1974; Loizou, 1971; 1972) while development of the tuberoinfundibular system occurs mainly during the early postnatal period (Smith and Simpson, 1970; Loizou, 1971). The general direction of dopamine neuron development follows a caudal to rostral pattern. Once the appropriate number of cell bodies is established, there is a progressive outgrowth of DA fibres towards their respective target areas. This is followed by a period of axonal proliferation that continues until the end of the fourth postnatal week. Details of the morphological, biochemical and functional development of central DA systems are outlined in the following.

Nigrostriatal and Mesolimbic Systems

DA nuclei in the midbrain attain their full complement of cell bodies a week before birth in the rat (Coyle, 1974). Presumptive DA neuroblasts in the substantia nigra exhibit a brief period of cell division between 12 and 14 days of gestation, then divide no more (Coyle, 1974). By fetal day 15, these neuroblasts differentiate into DA cells and acquire a DA-induced fluorescence which grows in intensity during the subsequent week. During this time there is a centrifugal outgrowth of very fine DA fibres towards terminal areas of the forebrain. Many of these axons reach their target sites prior to birth. For example, the nigrostriatal tract can be traced in its entirety from the midbrain to the striatum as early as gestational day 17 in the rat (Olson et al.,

1972]. A similar pattern of DA neuron differentiation and outgrowth has been demonstrated in other species, including mice (Golden, 1972) and rabbits (Tennyson et al., 1972).

Biochemical studies indicate that DA synthesis in the rat brain begins early in gestation. DA and its biosynthetic enzymes, tyrosine hydroxylase and dopa decarboxylase (Figure 1), are present by prenatal day 15 (Coyle and Axelrod, 1972a, b; Lamprecht and Coyle, 1972). This coincides closely with the observations made in histofluorescence studies (Coyle, 1974). The level of DA in fetal brains at 15 days of gestation is 2 percent of adult levels while the biosynthetic enzymes have specific activities of about 10 percent of the adult brain (Coyle and Henry, 1973). During the subsequent week of development, the levels of DA increase till they reach 30 percent of the adult level by birth (Coyle and Henry, 1973).

As development progresses, there is a centrifugal movement of DA and its synthesizing enzymes from the regions of the cell bodies towards terminal areas, such as the striatum (Loizou, 1972; Coyle and Henry, 1973; Coyle, 1974). This regional translocation corresponds closely with the outgrowth of nigrostriatal DA axons demonstrated by histofluorescence techniques (Loizou, 1972). In addition, the activity of these enzymes increases more substantially in terminal areas than in areas containing the cell bodies. For example, the specific activity of tyrosine hydroxylase in the midbrain increases only moderately compared to a 12-fold increase in the striatum at maturity. The greatest increase occurs during the last week of gestation (Coyle, 1974; Coyle and Axelrod, 1972b). Concurrently, there is a change in the subcellular distribution of enzyme

activity from the soluble fraction in the fetal rat brain, to the synaptosomal fraction in the adult brain. Hence, there is a subcellular as well as regional translocation of enzyme activity during development (Coyle, 1974; Coyle and Axelrod, 1972b).

Scattered islands of DA terminals in the striatum, nucleus accumbens and olfactory tubercle are already established by birth (Loizou, 1969 and 1972). These nigrostriatal and mesolimbic terminals proliferate gradually until the 4th postnatal week. This pattern of development contrasts with that of other catecholamines which are comparatively immature at birth. For example, norepinephrine terminals do not develop until after birth, primarily during the first two postnatal weeks. Thereafter, their fibre density and norepinephrine content increases rapidly until an adult pattern is attained by the 4th to 5th week of life (Loizou, 1969; Coyle, 1974). Hence, DA neurons appear earlier in development but reach maturity at about the same time as other catecholamine neurons (Agrawal et al., 1966a; Breese and Traylor, 1972; Loizou and Salt, 1970).

Combined histochemical and ontogenic experiments in rats indicate that the nigrostriatal and mesolimbic systems contain two histochemically distinct types of DA terminals: (1) structures marked by circumscribed islands of a moderate to strong dotted DA fluorescence; and (2) structures marked by diffuse (green) DA fluorescence (Fuxe et al., 1974; Olson et al., 1972). The dotted fluorescence is localized mainly in the nucleus accumbens and olfactory tubercle and is only scattered intermittently within the striatum. The diffuse fluorescence, on the other hand, has

its highest density in the medial part of the striatum and is only lightly distributed through the limbic areas (Olson et al., 1972). Similar patterns of distribution have also been observed in the rabbit brain (Tennyson et al., 1972). The ontogenic appearance and biochemical properties of these two types of DA terminals differ, as well. For example, the dotted DA structures appear at an earlier stage in development than the diffuse structures and their rate of DA turnover is comparatively slower (Olson et al., 1972). It is not known whether the dotted and diffuse DA terminals originate from different cell bodies of the midbrain nuclei, although it is clear that nigral DA cell bodies contribute very little to the innervation of the nucleus accumbens and olfactory tubercle (Fuxe et al., 1979).

Neuronal Activity Prior to Morphological Maturity

Central DA neurons become biochemically differentiated and show neuronal activity very early in development even before birth when morphological development is not yet completed. For example, the catecholamine precursor, tyrosine, occurs in higher concentrations in the brain of newborn rats than in adults (Agrawal et al., 1966a). Hydroxylation of this precursor by tyrosine hydroxylase (Figure 1) begins as early as the third week of gestation, although the rate of hydroxylation is slower than that of adults (Coyle, 1974). Inhibition of tyrosine hydroxylase with α -methyl-para-tyrosine causes a significant decrease in DA levels at 18 days of gestation (Coyle and Henry, 1973). Since in the adult brain, the fall of catecholamines after inhibition of tyrosine hydroxylase is related to neuronal activity in catecholaminergic neurons

(Anden, 1967), these results indicate that dopaminergic neurons may be releasing DA as early as fetal day 18.

Monoamine oxidase (MAO), that plays a major role in the catabolism of DA (Figure 1), is already present in DA neurons at birth. Administration of a MAO inhibitor, nialamide increases the fluorescence intensity of DA-containing cell bodies, processes and terminals in the newborn rat brain (Loizou, 1971). This effect can be seen from 18 days of gestation up to 4 weeks of age. This indicates that DA metabolism in the fetal neuron is similar to that of the mature neuron. Little information is available on the other DA-degrading enzyme, catechol-O-methyl-transferase (COMT) except that its activity is apparently very low during development (Agrawal et al., 1966).

Pharmacological studies on fetal rat brain indicate that immature DA neurons have many properties in common with the mature neuron. Both show DA depletion after reserpine, that blocks the uptake-storage mechanism of monoamine-containing granules (Loizou, 1972; Olson et al., 1972). This effect is apparent as early as 18 days of gestation (Coyle and Henry, 1973). The neonate, however, does show a greater sensitivity to reserpine. This could be due to a number of reasons including a prolonged presence of reserpine in the brain, or a difference in the properties of terminal storage particles, or a difference in rate of production of new particles, or any combination of these factors (Kulkarni and Shideman, 1966; Mueller and Shideman, 1968). Nevertheless, since reserpine depletion is dependent on impulse transmission (Anden, 1967) it is clear that dopaminergic fibres show neuronal activity prior to birth.

Behavioural studies also indicate that functional DA synapses

have developed in the rat brain by birth. The administration of α -amphetamine, that acts presynaptically to release catecholamines (Carlsson, 1970) to a 1 day old rat produces gnawing responses, a stereotypic behaviour characteristic of excessive DA function in the striatum (Fog et al., 1967; Ernst, 1967). Unilateral lesions of the striatum at 2 days of age produce an ipsilateral rotation (Van Hartesveldt and Lindquist, 1978; White and Tapp, 1977), similar to the behaviour seen in adults after amphetamine injection (Pycock, 1980). Rotational behaviour can also be produced in 2 day old rats by direct injection of DA into terminal areas. Ormond and Van Hartesveldt (1979) have demonstrated that rotational behaviour can be dissected, on a neural basis, into two components: postural deviation likely mediated by the dorsal striatum, and locomotion mediated by limbic structures. This latter finding is also in accord with work showing that bilateral infusion of DA into mesolimbic structures of adult rats produce increased locomotion (Pijnenburg et al., 1976). Hence, even at this early stage of morphological immaturity, it appears that the nigrostriatal and mesolimbic DA systems not only show physiological activity but also differ in the behavioural responses they regulate, as is observed in the adult.

Tuberoinfundibular Dopamine System

In contrast to the nigrostriatal and mesolimbic systems, the tuberoinfundibular DA neurons develop mainly during the early postnatal period in the rat (Loizou, 1971; Smith and Simpson, 1970; Hyppa, 1969). It is not until postnatal day 2 that a few weakly fluorescing cells appear in the anterior part of the arcuate nucleus of the hypothalamus

(Loizou, 1971).

Both the number and fluorescence intensity of these cell bodies increase progressively until by two weeks, they form compact bodies of cells filling the whole extent of the arcuate nucleus and anterior periventricular nucleus. The adult pattern of cell distribution and fluorescence intensity is achieved between 3 to 5 weeks postnatally.

Similarly, it is not until postnatal day 2 or 3 that a few weakly fluorescent varicosities appear in the terminal region of the median eminence (Loizou, 1971). These increase in number and intensity during the first week until by day 6 or 7, numerous terminals can be seen in the external zone of the median eminence. By day 14, the fluorescent fibres have formed a dense plexus around the capillary loops of the portal system in the external zone of the median eminence. In the Wistar rat, the adult pattern of distribution of fiber terminals is attained by the 3rd week of postnatal development (Loizou, 1971, 1972)..

It is interesting to note that the ontogeny of tuberoinfundibular DA neurons coincides with the development of portal vessels in this region. Portal vessels appear 3 days before birth and the primary capillary plexus in the external zone of the median eminence, 5 days after birth (Bergland and Page, 1979). This correlates with the early establishment of fluorescent nerve plexuses around the primary capillary bed beginning the 1st postnatal week (Loizou, 1971). Hence, the potential for the release of DA directly into portal circulation to be transported to the pituitary and regulate hormonal secretion may already exist during the first postnatal week of development.

Onset of Dopamine Regulation of Prolactin Secretion

In support of an early role of DA in the regulation of pituitary hormones, Ojeda and McCann (1974) reported that a prolactin response is first evident by day 3 after birth. If prolactin secretion is under tonic inhibitory control by tuberoinfundibular DA neurons, then blockade of the DA influence with a DA antagonist should produce a rise in prolactin secretion. This has been demonstrated using the potent DA blocking agent, pimozide. The administration of pimozide to rat pups at 3 days of age produced a significant increase in serum prolactin. This neuroleptic was completely ineffective when administered at birth, but it produced a progressive increase in prolactin response from 3 to 35 days of age. Presumably, pimozide's ineffectiveness at birth reflects a lack of dopaminergic control of prolactin secretion at that age (Ojeda and McCann, 1974). Hence the onset of the dopaminergic inhibitory control begins at postnatal day 3, in the rat.

Measurement of basal prolactin titers during ontogeny gives further indication that DA establishes an inhibitory influence on prolactin secretion very early during postnatal life. Basal plasma prolactin levels in the newborn rat are higher than those at postnatal days 3 and 6 (Ojeda and McCann, 1974). Plasma prolactin titers in 21-day old fetuses are also higher than postnatal day 3 levels. After day 3, there is a drop in plasma prolactin until by postnatal day 6, the basal titers are essentially undetectable. Subsequently there is a progressive increase in basal levels of prolactin until 35 days of age which is attributable to the development of stimulatory influences in the pituitary (Ojeda and McCann, 1974; Dohier and Wuttke, 1974). If, however, adult female rats

are hypophysectomized, their plasma prolactin titers drop to neonatal levels, presumably due to the elimination of the hypothalamic DA inhibitory influence on the pituitary (Ojeda and McCann, 1974).

Similar data have been reported in other species, as well. For example, plasma prolactin titers increase in the last trimester of gestation in ovine, bovine and human fetuses (Oxender et al., 1972; Moger and Geschwind, 1971; Aubert et al., 1972). Titters then decrease shortly after birth in the bovine (Oxender et al., 1972) and human fetus (Aubert et al., 1973) again indicating the initiation of a hypothalamic inhibitory influence (Aubert et al., 1973). Hence, basal serum prolactin titers and the response to pimozide correlate in time sequence with the histochemical observations that tuberoinfundibular neurons are first detectable in the median eminence around postnatal day 3 (Hyppa, 1969; Loizou, 1972) and exert their inhibitory influence on prolactin secretion from this age onwards (Ojeda and McCann, 1974).

Neuronal Activity Prior to Morphological Maturity

Reminiscent of the nigrostriatal and mesolimbic systems, it appears that tuberoinfundibular DA neurons also exhibit neuronal activity early in their development. The correlations between the appearance of DA terminals in the median eminence and the simultaneous responsivity of pituitary prolactin system have just been described. In addition, plasma prolactin levels show diurnal fluctuations that are inversely related to circadian variations in brain catecholamines. For example, Cocchi et al. (1976) found that infant rats, like adults, show diurnal peaks of plasma prolactin that coincide with troughs of brain catecholamine levels.

Although these may not be causally related, it has been suggested that periodic reduction in catecholamine levels may intermittently reduce their inhibitory influence on prolactin secretion (Cocchi et al., 1976). This again, indicates the competence of catecholamines including DA, in controlling prolactin secretion during the early postnatal stage.

Developing tuberoinfundibular DA neurons also share other properties in common with the adult system. Treatment with nialamide (MAO inhibitor) leads to an increase in the intensity of fluorescent terminals in the median eminence in neonates up to 3 weeks of age, indicating the presence of intraneuronal MAO before morphological development is complete (Loizou, 1971). Treatment with reserpine caused a depletion of fluorescence in tuberoinfundibular neurons starting a few days after birth (Loizou, 1971). Since reserpine blocks the uptake-storage mechanisms of storage particles, this depletion indicates that catecholamines are held within storage particles, similar to those of the adult neuron, from the earliest time fluorescence is observed (Dahlstrom et al., 1967). In the adult animal, treatment with H44/69 (inhibitor of tyrosine hydroxylase) produces a depletion of catecholamines that is highly dependent on impulse flow (Anden et al., 1966). Similar depletion is induced in the neonate tuberoinfundibular system indicating that these neurons conduct impulses from the earliest time they acquire their first intracellular catecholamine content. Hence, tuberoinfundibular neurons show neuronal activity prior to attaining full morphological development.

Summary

Tuberoinfundibular DA neurons resemble those of the nigrostriatal

and mesolimbic systems in that they demonstrate neuronal activity before their morphological development is complete and share many properties in common with the adult system. They differ, however, in terms of function. Tuberoinfundibular neurons are primarily involved in hormonal regulation, especially prolactin (Brown et al., 1979), whereas the nigrostriatal system is involved in the control of motor movement (Guyton, 1972) and the mesolimbic system is involved in the regulation of emotional and locomotor behaviour (Stevens et al., 1979; Pijnenburg et al., 1976). Furthermore, the latter systems are differentiated prior to birth (Loizou 1972; Coyle, 1974) while the development of tuberoinfundibular DA neurons occurs mainly during the early postnatal period (Loizou, 1971; Hyppa, 1969; Smith and Simpson, 1970).

D - POSTSYNAPTIC COMPONENTS OF CENTRAL DOPAMINE SYSTEMS AND THEIR ONTOGENY

One of the major concerns in developmental neurobiology is the interrelationship between the growth of the presynaptic nerve terminals and the appearance of postsynaptic receptor sites. The development of the presynaptic elements of dopamine (DA) systems was discussed in the previous section of this thesis (Part C). This section will deal briefly with postsynaptic components of DA systems and their development. The discussion is centered around two components that have served as useful markers of postsynaptic sites. These are 1) the enzyme adenylylate cyclase that is specifically sensitive to DA and 2) the postsynaptic DA receptors. Both the enzyme and DA receptors are associated with the postsynaptic membranes of DA target cells, although the target cells are neurons in the case of the nigrostriatal and mesolimbic DA systems, and prolactin-secreting cells of the pituitary glands in the case of the tuberoinfundibular DA system (Brown et al., 1979).

Dopamine-Sensitive Adenylylate Cyclase

During the last decade, much work has been directed towards characterizing the biochemical properties of the postsynaptic DA receptor. This interest stemmed from a realization of the importance of DA as a neurotransmitter in the mammalian brain (Carlsson and Lindquist, 1963; Ungerstedt, 1971; Anden, 1974; Vogt, 1973). There is much evidence indicating that abnormalities of dopaminergic transmission in the brain are of clinical importance, particularly in Parkinson's Disease (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1973) and schizophrenia (Matthysse, 1973; Snyder et al., 1974). A major advancement was the discovery that

activation of DA receptors in the brain resulted in the increased formation of adenosine 3', 5' -monophosphate (cyclic AMP) (Nathanson, 1977).

The involvement of DA-sensitive adenylate cyclase in synaptic transmission was first suggested by the results of experiments by Greengard and colleagues using sympathetic ganglia as a model system (Greengard et al., 1972; McAfee et al., 1971; Keibian and Greengard, 1971). They found that the hyperpolarization of ganglionic neurons following pre-ganglionic nerve stimulation was due to the release of DA from small DA-containing interneurons, and that this inhibitory effect of DA appeared to be mediated by a DA-sensitive adenylate cyclase. On the basis of their experimental results, Greengard and coworkers proposed a model by which transmitter stimulation of adenylate cyclase could influence membrane permeability or properties of the postsynaptic neuron. (Figure 6).

In brief, the stimulation of adenylate cyclase activity by DA increases the intracellular formation of cyclic AMP which in turn functions as a "second messenger" for the effects of the transmitter on the postsynaptic cell. Numerous recent reviews provide detailed discussions on the role of cyclic AMP as a second messenger (Greengard et al., 1972; Greengard and Keibian, 1974; Nathanson, 1977). The transmitter-induced changes in intracellular cyclic AMP regulate the activity of cyclic AMP-dependent protein kinases. These kinases then phosphorylate specific proteins in the plasma membrane thereby modifying the permeability properties of the postsynaptic membrane that leads to changes in membrane conductance and the activity of electrogenic pumps (Figure 6). Hence the role of the DA-sensitive adenylate cyclase in this model is to couple the

MODEL OF DA-SENSITIVE ADENYLATE CYCLASE-CYCLIC AMP SYSTEM

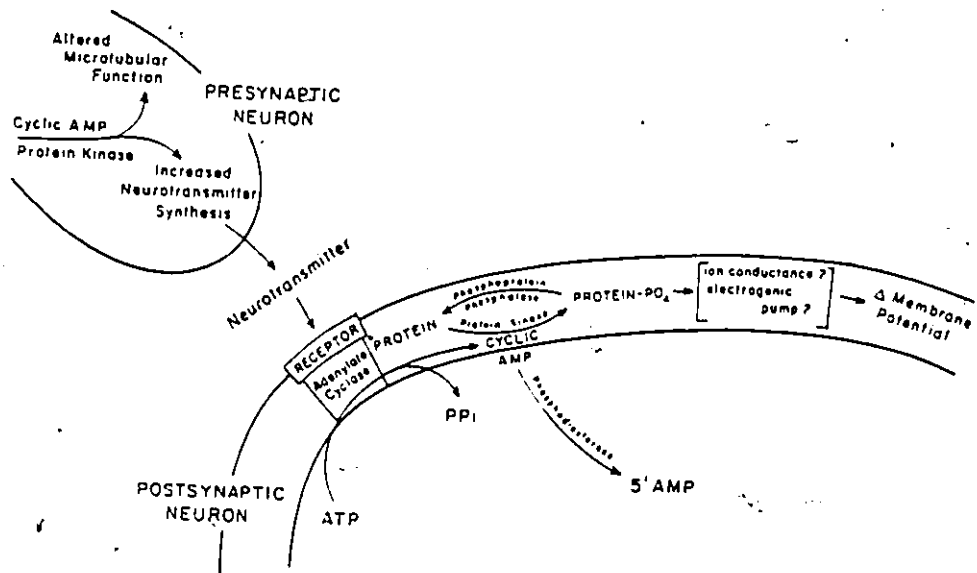


Figure 6: Proposed roles for cAMP and consequent protein phosphorylation in neuronal function. The most important proposed role is that of a second messenger for dopamine. Through a protein kinase system this second messenger affects the membrane potential of the cell. Other proposed roles include altered microtubular function and increased neurotransmitter synthesis. These are depicted as taking place in the presynaptic neuron. (From Greengard, 1976)

binding of DA to its receptor on the external surface of the postsynaptic membrane with the initiation of intracellular cyclic AMP-mediated events (Greengard and Keibarian, 1974).

Soon after, it was postulated that this cyclic AMP system might also play a role in the postsynaptic actions of DA in the brain (Keibarian et al., 1972; Brown and Makman, 1972). Adenylate cyclase activity and the concentration of cyclic AMP are higher in the brain than in any other organ (Daly et al., 1972; Iversen, 1975; Bloom, 1975; Bartfai, 1980). The first evidence supporting a role for a DA-sensitive adenylate cyclase in the CNS was reported by Brown and Makman (1972) who were studying the DA-containing amacrine cells of the retina and by Keibarian et al., (1972) who were studying the rat striatum since it contains the highest levels of DA in the brain. Before long, DA-sensitive adenylate cyclase was discovered in other DA terminal areas of the brain including the mesolimbic structures: olfactory tubercle and nucleus accumbens (Horn et al., 1974). The results of comparative studies indicated that the properties of this enzyme are similar in a variety of different species, including man (Clement-Cormier et al., 1974).

A unique feature of the adenylate cyclase that is found in the striatum and mesolimbic structures is its high sensitivity to DA (Perkins, 1973). This DA-sensitive adenylate cyclase is absent in all other areas of the brain where DA dopamine terminals are not found. The pharmacological properties of this enzyme clearly distinguish it from other brain adenylate cyclases for example, those sensitive to the catecholamine, norepinephrine (Brown and Makman, 1972; Keibarian et al., 1972). Pharmacokinetic studies indicate that there is a high degree of structural

specificity of DA analogs that will specifically stimulate the DA-sensitive adenylylase in striatal tissue (Miller et al., 1974; Makman et al., 1975). Compounds that lack the catechol hydroxyl groups or the two-carbon side chain of DA are without activity (Miller et al., 1974; Figure 2).

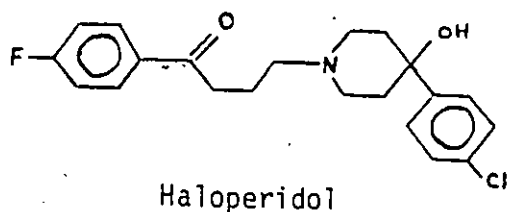
Another important property of the DA-sensitive adenylylase in the mammalian brain is its sensitivity to inhibition by certain neuroleptics, particularly the phenothiazines such as chlorpromazine (Figure 7). These drugs inhibit DA-stimulated adenylylase activity in a dose-dependent manner (Iversen, 1975). Kinetic analysis shows that this inhibition is competitive with DA (Miller et al., 1974). There is also evidence for an important structure-activity relationship between DA-sensitive adenylylase and the thioxanthene group of neuroleptics (Miller et al., 1974). For example, drugs such as flupenthixol exhibit a geometric isomerism in their structure. When tested for its potency in antagonizing adenylylase activity, only the cis-isomer, α -flupenthixol that has a potent antipsychotic action, is effective while the trans-isomer β -flupenthixol that has no antipsychotic action is without effect, even at relatively high concentrations (Miller et al., 1974).

Although most neuroleptics block the activity of DA-sensitive adenylylase in vitro, their relative potency as inhibitors does not correlate well with their known potencies in vivo (Seeman et al., 1978). For example, the butyrophenones, such as haloperidol and spiroperidol, are among the most potent antipsychotic drugs known (Seeman and Lee, 1975). They are effective clinically and in animal tests at doses many times lower than those of the phenothiazines (Seeman, 1977; Seeman et al.,

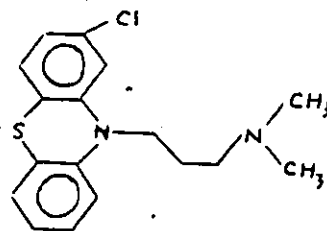
MOLECULAR STRUCTURES OF NEUROLEPTIC DRUGS

BUTYROPHENONES

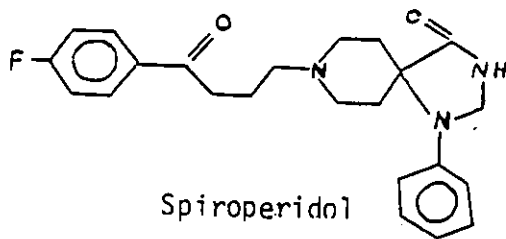
PHENOTHIAZINES



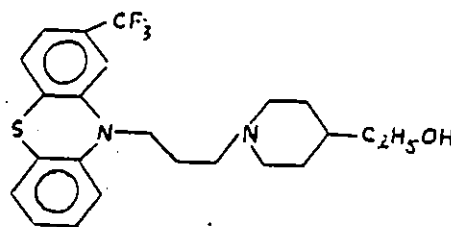
Haloperidol



Chlorpromazine



Spiroperidol



Fluphenazine

Figure 7: The molecular structures of two classes of neuroleptics, the butyrophenones and the phenothiazines are shown. Note the similarity in structures within the groups and the difference in structures between the groups. The butyrophenones have a high affinity for the D-2 dopamine receptors while the phenothiazines have greater affinity for the D-1 receptors, as described in Introduction, Part D.

1978). Yet, haloperidol and spiroperidol, are weaker inhibitors of DA-sensitive adenylate cyclase of rat brain than the phenothiazines (Miller et al., 1974; Iverson, 1975; Seeman et al., 1978). Despite their difference in potency as compared to other neuroleptics, kinetic studies indicate that the butyrophenones, like the phenothiazines inhibit DA-sensitive adenylate cyclase in a simple competitive manner (Miller et al., 1974; Iversen, 1975;). Factors such as absorption, distribution and metabolism of butyrophenones in vivo compared to that of other classes of neuroleptics may contribute to this differential potency of action (Usdin, 1978). Current evidence, however, indicates the possibility of an alternate explanation. Butyrophenones may act at postsynaptic DA receptors that are not coupled to the DA-sensitive adenylate cyclase (Creese et al., 1976; Seeman et al., 1975; Snyder et al., 1975; 1976). In fact, there may be a number of different classes of DA receptors, only one of which is coupled to the postsynaptic DA-sensitive adenylate cyclase (Seeman et al., 1975; Creese et al., 1976). The evidence for multiple classes of DA receptors is briefly summarized in the following.

Brain-Dopamine Receptors

The results of recent studies indicate that there is more than one class of DA receptors in the brain (Creese et al., 1975; Cools, and Van Rossum 1976; Seeman et al., 1978; Titeler et al., 1978). For example, Seeman and colleagues have proposed that there are at least 3 different populations of DA receptors which can be distinguished on the basis of their pharmacological binding properties (Titeler et al., 1978). These receptors are referred to as D-1, D-2 and D-3 receptors (Kebabian and

Calne 1979; Titeler et al., 1979). The D-1 and D-2 receptors appear to be associated with postsynaptic membranes while D-3 is more closely associated with presynaptic terminals (Titeler et al., 1978; 1979; Nagy et al., 1978).

The D-1 receptor appears to be the postsynaptic receptor that is most closely associated with DA-sensitive adenylate cyclase (Hyttel, 1978; 1980). The results of binding studies indicate that the ligand ^3H -Cis(Z) flupenthixol (^3H -FPT) is a useful marker for this type of DA receptor (Hyttel 1980). There is a high correlation between ^3H -FPT binding and DA-sensitive adenylate cyclase activity in terms of regional distribution in the brain and density of binding sites, both being highest in the striatum and olfactory tubercle (Hyttel, 1978). This differs from the binding patterns observed with other DA receptor ligands including ^3H -haloperidol (Hyttel 1978; Garau et al., 1978). Selective destruction of neural cells intrinsic to the rat striatum with kainic acid was found to completely eliminate DA-sensitive adenylate cyclase activity while ^3H -haloperidol and ^3H -spiroperidol binding was still present (Garau et al., 1978; Schwarcz et al., 1978). These data indicated that there must be at least 2 distinct populations of DA receptors in the striatum and that their distribution differed.

The results of competitive binding studies investigating the relative potencies of DA agonists and antagonists in displacing ^3H -FPT and ^3H -haloperidol binding in striatal homogenates indicated that the D-1 receptor has a lower affinity for DA (micropolar range) and its agonists than do other classes of DA receptors (Hyttel, 1980). Since dopaminergic agents, including the clinically potent butyrophenones show a

relatively low affinity for the D-1 receptor, the function of this population of DA receptors is not well understood. It has been postulated that rather than a direct involvement in the immediate events of synaptic transmission, the D-1 receptor, by virtue of its coupling to the DA-sensitive adenylate cyclase system, may regulate the longer term metabolic and trophic functions that involve cyclic AMP-mediated changes in post-synaptic target cells (McGeer et al., 1978).

The D-2 receptor represents the population of postsynaptic DA receptors that is most closely associated with the known neurotransmitter functions of central DA neurons (Seeman, 1977; 1981). The butyrophenone neuroleptics, for example ^3H -haloperidol and ^3H -spiroperidol, show a very high affinity (nanomolar range) for this population of receptors (Creese et al., 1977; Seeman et al., 1975; Leysen et al., 1978). The binding is stereospecific and can be distinguished from nonspecific binding by the use of (+) and (-) enantiomers of butaclamol (Seeman et al., 1975; 1976; Leysen et al., 1978). ^3H -butyrophenone binding sites are found in all DA terminal areas of the brain, including striatum nucleus accumbens and olfactory tubercle (Muller and Seeman, 1977; Burt et al., 1977; Lauduron et al., 1978). D-2 binding sites with saturation and kinetic properties very similar to those of the striatum have also been found in the pituitary (Brown et al., 1976; Seeman et al., 1976; Creese et al., 1977).

There is a strong positive correlation between ^3H -butyrophenone binding and a wide range of clinical, pharmacological and behavioural parameters associated with DA function (Muller and Seeman, 1978; Creese

et al., 1976; Seeman et al., 1976). For example, all clinically effective antipsychotic agents block the stereospecific binding of ^3H -haloperidol at concentrations that correlate directly with their clinical potencies as well as their plasma levels in patients undergoing neuroleptic therapy (Seeman et al., 1978; Seeman et al., 1976). Numerous animal studies demonstrate that prolonged administration of butyrophenones produce long term changes in DA-mediated behaviours such as locomotion and stereotypy, as well as ^3H -haloperidol and ^3H -spiroperidol binding and DA turnover in striatal and mesolimbic areas of the brain (Muller and Seeman, 1978, Plach et al., 1979). These data will be described in more detail in the subsequent section of this thesis (Part E). With regard to DA receptors in the pituitary, since very low doses of butyrophenones, for example haloperidol, effectively produce a significant increase in prolactin secretion, it is likely that this effect, like those observed in the brain, is mediated via the D-2 receptor (MacLeod, 1976; Meltzer et al., 1979; Plach et al., 1980a).

A third class of DA receptors, the D-3 receptor has very recently been described by Titeler et al. (1979). This population of receptors exhibits the highest affinity for DA and DA agonists such as apomorphine and N-propylnorapomorphine (NPA) (Titeler and Seeman, 1979; List et al., 1980) and is thought to be associated with presynaptic DA terminals (Titeler et al., 1980, 1981). In lesion studies, it was found that 80% of binding to the D-3 receptor disappears following destruction of the

nigrostriatal tract, leaving postsynaptic striatal cells intact (Nagy et al., 1978). In contrast, only 20% of this binding disappears following kainic acid lesions that destroy striatal cells leaving presynaptic terminals intact (Lee et al., 1978; Nagy et al., 1978). While there is some disagreement regarding the exact proportion of D-3 receptor binding that is associated with pre- and post-synaptic elements in the nigrostriatal system (Creese et al., 1979; Creese and Snyder, 1979; Lee et al., 1978; Nagy et al., 1978) it has been proposed that the D-3 receptor is likely involved in autoregulation of DA neurons (Titeler et al., 1980; Lee et al., 1981).

For completeness sake, it should be mentioned that other classifications of DA receptors, different from the foregoing description have also been proposed. For example, Cools and Van Rossum (1976) have proposed the existence of two populations of DA receptors: excitation mediating (DA_e) and inhibition-mediating (DA_i) receptors based on behavioural and pharmacological experiments (Cools 1977; 1978; Van Rossum, 1978). Other laboratories have proposed that the DA receptor exists in an agonist and antagonist state (Creese et al., 1975; Snyder et al., 1976) although the evidence for this has not been substantiated. Further details of these alternate classifications are provided in the stated references and will not be discussed here since the work in this thesis deals specifically with the DA-sensitive adenylyate cyclase and the spiroperidol-labelled postsynaptic receptor as best described by the D-1 and D-2 classification, already discussed.

Ontogeny of DA-Sensitive Adenylate Cyclase and DA Receptors

The development of the presynaptic elements of DA systems, including differentiation, DA metabolism and preterminal growth have already been discussed (Part C). Studies on the development of postsynaptic elements, including DA-sensitive adenylate cyclase and DA-receptors have only very recently been initiated (Coyle and Campochiaro, 1976; Pardo et al., 1977; Enjalbert et al., 1978; Plach et al., 1979). With regard to the enzyme, most studies investigated the ontogeny of catecholamine-sensitive adenylate cyclases using whole brain preparations (Schmidt et al., 1970; Kohrman, 1973) or large sections of brain (Weiss, 1971; Von Hungen et al., 1973; Perkins and Moore, 1973) rather than discrete terminal areas. In a review on the role of cyclic AMP during fetal and postnatal development, Weiss and Strada (1973) point out that the ontogeny of adenylate cyclases vary depending on the specific area of the brain being examined. Hence, factors that influence the development of postsynaptic adenylate cyclases may also vary from area to area, depending on the particular system being examined. The following discussion focusses particularly on the characteristics of the DA-sensitive adenylate cyclase in rat striatum and its relation with striatal DA receptors.

DA-sensitive adenylate cyclase activity is present in rat striatum at birth (Coyle and Campochiaro, 1976; Pardo et al., 1977; Enjalbert et al., 1978; Plach et al., 1980). The addition of DA (50 μ M) to cell-free homogenates prepared from the newborn striatum produces a significant stimulation of cyclic AMP formation which can be completely inhibited by low doses of the DA-antagonist, fluphenazine (Coyle and Campochiaro, 1976). The absolute amount of cyclic AMP formed is about 20% of that

produced in the adult although the relative stimulation by DA above basal cyclase activity is twice as high in the neonatal compared to the adult striatum (Coyle and Campochiaro, 1976). The specific activity of the DA-sensitive adenylate cyclase increases rapidly during the first two post-natal weeks. Whereas the DA-stimulated cyclase activity increases 5-fold from birth to adulthood, the basal activity increases 10-fold (Coyle and Campochiaro, 1976). This accounts for the observation that striatal adenylate cyclase is more sensitive to DA-stimulation at birth than in adulthood (Coyle and Campochiaro, 1976; Enjalbert et al., 1978; own observations: Results, Part B). Adult levels of activity are achieved during the third to fourth week after birth (Coyle and Campochiaro, 1976; Pardo et al., 1977; Enjalbert et al., 1978; Results, Part B).

The developmental rise in the activity of DA-sensitive adenylate cyclase parallels, but clearly precedes, the developmental rise in the three presynaptic markers for dopaminergic terminals: tyrosine hydroxylase activity, endogenous DA levels and synaptosomal uptake of $^3\text{H-DA}$ (Coyle and Campochiaro, 1976; Pardo et al., 1977). The ontogeny of these biochemical markers is closely associated with the progressive outgrowth of preterminal DA fibres and their innervation of the striatum in the rat as seen in histofluorescence studies (Loizou, 1972; Olson et al., 1972; Introduction, Part C). Hence, the development of DA-sensitive adenylate cyclase in the striatum precedes, and therefore may be independent of the ingrowth of dopaminergic terminals. A similar observation has been made in studies of the chick retina where DA-sensitive adenylate cyclase appears several days before the dopaminergic neurons begin to differentiate (Coyle and Campochiaro, 1976).

Snyder and coworkers were the first to study the ontogeny of the second postsynaptic component the DA receptor in the striatum, (Pardo et al., 1977). They used ^3H -haloperidol to label these receptors. The results of their studies indicate that specific ^3H -haloperidol binding is localized to regions of the brain rich in DA synapses (Burt et al., 1976; Snyder et al., 1975). At birth, ^3H -haloperidol binding is between 10-15% of adult levels and shows little change until 7 days postnatally (Pardo et al., 1977). Between 7 and 14 days, the binding more than doubles and then by 28 days it triples again. There is little change in binding between 28 days and young adult values. Striatal membranes of aged rats (greater than 180 days old) demonstrate a reduction in binding to about 65% of young adult levels.

There is some correlation between the development of presynaptic markers of DA neurons, for example synaptosomal ^3H -DA uptake and DA content, and the development of postsynaptic ^3H -haloperidol binding (Coyle and Campochiaro, 1976; Pardo et al., 1977). Both presynaptic markers exhibit a linear increase from birth to 28 days at which time they are 70% of adult levels (Coyle and Campochiaro, 1976). Specific ^3H -haloperidol binding also increases significantly during this period, although not as rapidly as during the first postnatal week, and reaches adult levels by the 28th day (Pardo et al., 1977). Unlike ^3H -haloperidol binding, however, ^3H -DA uptake and DA content continue to increase after day 28 (Coyle and Campochiaro, 1976). These similarities and differences between the ontogeny of presynaptic markers and ^3H -haloperidol binding indicate that the relationship between the development of presynaptic DA terminals and postsynaptic receptors is complex. The relative

synchrony in their ontogeny, however, suggests that they may be inter-related, particularly during the first few weeks after birth.

A comparison of the ontogeny of ^3H -haloperidol binding, and DA-sensitive adenylate cyclase activity in the striatum reveals that there are significant differences in their rates of development (Pardo et al., 1977). While there is little change in ^3H -haloperidol binding between birth and 7 days, the cyclase activity more than doubles during this period. Between 21 and 28 days, specific ^3H -haloperidol binding doubles, while there is only a 21% increase in cyclase activity. Hence the earlier development of the DA-sensitive adenylate cyclase indicates that the postsynaptic DA receptor as labelled by ^3H -haloperidol is not coupled to the adenylate cyclase at this time (Pardo et al., 1977).

To summarize, very little is known about the factors that regulate the development of postsynaptic DA receptors and the DA-sensitive adenylate cyclase. On the basis of the few studies that have been done, it appears that the pattern of DA receptor development is different, and perhaps independent of the DA-sensitive adenylate cyclase. In terms of a relationship between presynaptic DA neurons and postsynaptic development, it appears that the ontogeny of DA receptors is more closely correlated with the maturation of some presynaptic events including ^3H -DA uptake and DA content than is the DA-sensitive adenylate cyclase, but the significance of these correlations is unclear. Further studies are required to evaluate these interrelationships.

This thesis investigation attempted to define the interactions between the presynaptic and postsynaptic components of the DA system during early development by examining the effects of prenatal blockade

with the neuroleptic, haloperidol during the third trimester.. Since most studies have investigated the effects of chronic neuroleptic blockade in the adult animal, the relevant findings from these studies are briefly summarized in the following section (Part E).



E - THE EFFECTS OF CHRONIC NEUROLEPTICS IN THE ADULT

Neuroleptics have proven to be a major advance in the treatment of psychiatric disorders such as schizophrenia (Iversen, 1975). Their principle mode of action, as described previously, is blockade of central DA receptors (Snyder et al., 1974; Iversen, 1975). Despite their efficacy in alleviating psychoses, however, long term treatment with neuroleptics can produce side effects, including pseudo-Parkinsonism and the tardive dyskinesias (Hornykiewicz, 1973; Crane, 1973). Pseudo-Parkinsonism is produced by a temporary blockade of nigrostriatal DA receptors that leads to a paucity of motor movement, akinesia, rigidity and abnormal posturing (Hornykiewicz, 1973). These symptoms disappear when the neuroleptic is withdrawn. The tardive dyskinesias, in contrast, are uncontrolled motor movements (dyskinesias) that in many cases are irreversible (Crane, 1973). The appearance of these symptoms is attributed to the prolonged administration of neuroleptics (months to years) that produces a "pharmacological denervation" of the striatal DA system and leads to the development of supersensitivity (Sayers et al., 1975; Curran et al., 1975).

The implication of the hypothesized development of supersensitivity following chronic neuroleptic blockade is that neurons in the adult brain maintain a capacity to compensate for a reduction in their input. Dismukes and Daly (1976) have reviewed the evidence that chronic blockade of neurotransmitter availability to the postsynaptic receptor sites leads to a compensatory increase in postsynaptic sensitivity while the reverse occurs with chronic over-stimulation of these sites. This ability to

compensate for an alteration in postsynaptic input is one example of synaptic plasticity (Quarson et al., 1967). Studies investigating the mechanisms underlying synaptic plasticity in the brain have only recently been initiated. Chronic treatment with neuroleptics has proved to be useful in the study of plasticity since the morphological, biochemical and behavioural parameters of central DA neurons have been relatively well defined. That chronic neuroleptic treatment produces supersensitivity of postsynaptic DA sites is indicated by the following behavioural, biochemical and pharmacological data.

Behavioural Evidence of DA Supersensitivity: Chronic administration of neuroleptics to rats produces a variety of abnormal DA-mediated behaviours including locomotor hyperactivity, stereotypy and catalepsy (Muller and Seeman, 1978). For example, chronic administration of haloperidol leads to an increase in both spontaneous (Smith and Davis, 1976; Gianutsos and Lal, 1976; Sahakian et al., 1976) and DA agonist-induced locomotor hyperactivity (Von Voigtlander et al., 1975; Smith and Davis, 1976; Gianutsos and Moore, 1977; Dustan and Jackson, 1976). Since increased locomotor activity is associated with excess DA function, these data indicate that chronic blockade with haloperidol produces a dopaminergic supersensitivity. The issue of which of the DA systems is involved in producing locomotor hyperactivity has not yet been resolved since some evidence indicates that locomotor activity is regulated by the nigro-striatal system (Denny-Brown and Yanagisawa, 1976) while other evidence indicates that it is mediated by the nucleus accumbens of the mesolimbic system (Kelly et al., 1975; Pijnenburg et al., 1976). Both systems may be involved, however, since at least on anatomical grounds there is some

overlap in DA neurons to these two terminal areas (Ungerstedt, 1974).

Another example of DA supersensitivity following chronic treatment with haloperidol is the occurrence of stereotypic behaviour (excessive sniffing, licking and gnawing) in response to DA agonists, for example apomorphine, at doses that are normally subthreshold (Tarsy and Baldessarini, 1974; Gianutsos et al., 1974; Gnegy et al., 1977; Smith and Davis, 1976). The appearance of these behaviours suggests that treatment with postsynaptic DA antagonists for a week or longer produces an increase in the sensitivity, in this case of the nigrostriatal system, to DA agonists. Numerous other behavioural studies using these and other behavioural measures such as catalepsy and rotation following unilateral treatment have led to the same conclusion (Muller and Seeman, 1978). That is, decreased input to postsynaptic DA sites caused by neuroleptic blockade, leads to a compensatory increase in their sensitivity that is ultimately expressed as excessive DA-mediated behavioural responses.

Behavioural studies can provide only indirect evidence of altered function in brain DA systems. More direct evidence for neuroleptic-induced DA supersensitivity comes from biochemical and pharmacological studies, in particular, studies of DA-sensitive adenylate cyclase activity and most recently, DA receptors.

DA-Sensitive Adenylate Cyclase and Supersensitivity: Several studies report a significant increase in the activity of DA-sensitive adenylate cyclase in the striatal homogenates of animals after chronic treatment with haloperidol (Burkard and Bartholini, 1974; Iwatsubo and Clouet, 1975; Gnegy et al., 1977) or other drugs interfering with DA trans-

mission (Seeber and Kuchinsky, 1976; Gnegy et al., 1977). This was taken to indicate that postsynaptic DA receptors became supersensitive after deprivation of normal DA input. Other investigators, however, found no change in striatal DA-sensitive adenylate cyclase activity following chronic neuroleptic treatment (Talwaker et al., 1963; Rotrosen et al., 1975; Von Voigtlander et al., 1975; Heal et al., 1976; Roufogalis et al., 1976; Hyttel, 1978). Taken together, these contradictory results have left unresolved the issue of whether there is an increase in DA-sensitive adenylate cyclase activity following chronic neuroleptic administration. Furthermore, they have called into question the relationship between the enzyme and the postsynaptic DA receptor, particularly with regard to their role in the development of DA supersensitivity. Direct investigation of these issues and the basis of DA supersensitivity became possible with the development of high affinity ligands for the postsynaptic DA receptor, as described next.

Supersensitivity of Postsynaptic DA Receptors: The results of receptor binding studies indicate that chronic neuroleptic treatment, that is known to produce behavioural supersensitivity, also produces an increase in the number of DA receptors within DA terminal areas of the brain, including the striatum (Burt et al., 1977; Chen et al., 1970; Friedhoff et al., 1977; Kobayashi et al., 1978; Muller and Seeman, 1977; Ebstein et al., 1979; own observations; Results, Part C) mesolimbic areas (Muller and Seeman, 1977) and pituitary gland (Friend et al., 1978). There are no changes in receptor binding in nondopaminergic terminal areas of the brain, for example, the hippocampus (Kobayashi et al., 1978). The results of most studies in adult rats indicate that there is no change in the

affinity of the binding sites except after extremely long periods, for example several months to a year of neuroleptic treatment (Ebstein et al., 1979). In that case, there is a decrease in the affinity of receptor binding which may be a function of permanent changes in cell function associated with particularly prolonged periods of neuroleptic blockade (Ebstein et al., 1979).

In summary, there is behavioural, biochemical and pharmacological evidence indicating that chronic neuroleptic administration leads to the development of supersensitivity of postsynaptic DA receptors in the adult brain (Muller and Seeman, 1977). These data indicate that the adult nervous system maintains a fair degree of plasticity, that is, capacity to compensate for an interference with transmitter interaction at postsynaptic sites (Quarton et al., 1967; Dismukes and Daly, 1976). It is clear that interactions between presynaptic neural endings and postsynaptic target sites are essential for normal DA function. An important question raised by this thesis is whether blockade of these interactions with neuroleptics early in development will produce permanent changes in the subsequent function of the DA system. What is known of neuroleptic effects on the developing DA system is described next.



F - EFFECTS OF NEUROLEPTICS ON THE DEVELOPING DA SYSTEM

There is a scarcity of information on the effects of neuroleptics on the developing DA system, particularly during the prenatal period when the interrelationships between presynaptic terminals and postsynaptic receptor sites are first being established (Loizou, 1971). In view of the profound effects neuroleptics can have on DA function in the adult system (Muller and Seeman, 1978) and since the period of greatest vulnerability to environmental influences is that of early development (Langman et al., 1975; Spyker, 1975) one might predict that the administration of neuroleptics during prenatal development will produce long term changes in the subsequent development and function of central DA neurons. Preliminary evidence in support of this hypothesis is described next.

Behavioural alterations in offspring that were exposed to psychotropic drugs during early development are being noted with increasing frequency, both clinically (Goldberg and DiMascio, 1978; Breese et al., 1978; Hutchings, 1978) and experimentally (Spyker, 1975; Vorhees et al., 1979; Kellogg et al., 1980). For example, clinically, women who are taking neuroleptics to control psychoses usually have to continue treatment even through pregnancy (Personal communication: Dr. J. Cleghorn, Dept. of Psychiatry, McMaster University). In addition, neuroleptics may be administered a few days prior to delivery, or as a labour adjunct to reduce emotional tension, control nausea, or to potentiate analgesia (Goldberg and DiMascio, 1978). Neuroleptics can easily cross the placental barrier and detectable concentrations have been reported within the

circulation of fetal liver and brain (Mirkin, 1973; Goldberg and DiMascio, 1978). A number of reports indicate that the offspring of these mothers show behavioural abnormalities including extrapyramidal reactions and Parkinson-like symptoms during the early postnatal period (Hill et al., 1966; Tamer et al., 1969; Levy and Wisniewski, 1974). Other dysfunctions include lethargy and hypotonicity (Hill et al., 1966; Hammond and Toseland, 1970) as well as abnormal spontaneous motor movements and reflex responses (Hammond and Toseland, 1970). Some neuroleptics, such as chlorpromazine, are known to accumulate selectively in the eye and can cause neonatal retinopathies (Lindquist et al., 1970; Mirkin, 1973). When given during the last trimester, neuroleptics can cause neonatal jaundice and hyperbilirubinemia, as well as melanin deposits in the eyes (Goldberg and DiMascio, 1978). While many of these side effects may be due to non-specific metabolic effects related to the inability of the fetus to metabolize and excrete these drugs efficiently, at least some of the side effects, particularly the extrapyramidal reactions and retinopathies, may be related to more specific effects on central DA systems (Breese et al., 1978). The results of perinatal animal studies tend to support this suggestion, as described next.

Beginning with pioneering work of Werboff and colleagues (Werboff and Dembichi, 1962; Werboff and Hoylena, 1962) there has been a continuous accumulation of evidence that perinatal treatment with neuroleptics produce adverse effects on subsequent development of offspring (Kornetsky, 1970; Thornburg and Moore, 1976). For example, daily maternal ingestion of chlorpromazine during pregnancy causes a dose-related reduction in litter size and weight of offspring (Ordy et al., 1966; Thornburg and

Moore, 1976). The 60-day survival of these offspring was significantly reduced compared to untreated controls. Chlorpromazine offspring also exhibited less spontaneous exploratory behaviour (Ordy et al., 1966).

Other behavioural deficits associated with perinatal neuroleptic treatment include abnormalities in offspring motor development. For example, the locomotor activity of rat pups treated with chlorpromazine during gestational days 12-15 was significantly higher than that of controls when tested 18 days after birth (Clark et al., 1970). The administration of other neuroleptics such as haloperidol to nursing-rabbits was found to temporarily retard offspring motor development (Lundborg, 1972). Haloperidol also reduced offspring sensitivity to DA agonist apomorphine in eliciting stereotyped responses when administered during the entire period of gestation (Rosengarten and Friedhoff, 1979).

Biochemical evidence for an alteration in DA development following perinatal neuroleptic treatment is provided by studies of catecholamine metabolism in the brains of the offspring. For example, Tonge (1973) reported permanent changes in brain monoamine metabolism nine months following the administration of psychotropic drugs, including chlorpromazine. More recently, Engel and Lundborg (1974; 1976) found that perinatal neuroleptic treatment produced a significant deficit in DA synthesis and turnover in the mesolimbic system of 4 week old offspring. There was a selective and pronounced deficit in the amount of dopa accumulated after inhibition of its degradative enzyme aromatic amino acid decarboxylase with NSD 1015 (Engel and Lundborg, 1974). In addition, there was a pronounced reduction in the rate of DA depletion in 4-week old

neuroleptic offspring when tested with α -methyl-paratyrosine (α MT). Since α MT inhibits tyrosine hydroxylase and is dependent on impulse flow, these findings were interpreted as indicating that the perinatal neuroleptic treatment produced a long term disturbance in normal dopaminergic turnover and feedback mechanisms in the offspring (Engel and Lundborg, 1976).

A particularly interesting finding in these studies is that the biochemical changes observed appeared to be localized in the mesolimbic system while the striatum showed no changes (Engel and Lundborg, 1974; 1976). Why striatal DA neurons remained intact while function in mesolimbic DA neurons was disturbed is not understood. It has been suggested that the ontogeny of these two DA systems may be slightly asynchronous, hence they may exhibit a difference in vulnerability at the time of neuroleptic administration (Engel and Lundborg, 1976). Further, more detailed, studies are required to determine the validity of this postulate.

In summary, preliminary data indicate that the administration of neuroleptics during neonatal development produce long term changes in the subsequent development of central DA systems in the offspring. The results of animal studies indicate that subtle changes in offspring development can be detected using a variety of behavioural and biochemical measures. There is a lack of information, however, regarding the effects of neuroleptic administration specifically during the prenatal period of development when the interrelationships between presynaptic terminals and postsynaptic target sites are first being established. The main goal of this thesis was to investigate this question in greater detail, as is outlined in the "Hypothesis and Rationale" presented next.

HYPOTHESIS AND RATIONALE

The hypothesis tested by this study was that the administration of haloperidol during the third week of prenatal development in the rat alters the normal ontogeny and function of central dopamine (DA) neurons. The question asked was, whether prenatal haloperidol administration during initial synaptogenesis interferes with the normal development of pre-synaptic DA neurons and their postsynaptic target sites. If so, does this lead to long-term morphological, biochemical and behavioural changes in brain DA function of the offspring?

To test these hypotheses, both presynaptic and postsynaptic aspects of DA neural development were investigated. The effects of haloperidol on the development of presynaptic DA neurons was assessed by histofluorescence analysis (de la Torre and Surgeon, 1976) of normal DA innervation in terminal areas of the brain. These included the striatum of the nigrostriatal DA system, and the nucleus accumbens and olfactory tubercle of the mesolimbic system. The pattern of DA innervation to these areas was compared in offspring whose mothers received either haloperidol or vehicle treatment during the third week of pregnancy.

To test the effects of haloperidol on the development of post-synaptic components of the DA system, two parameters were investigated: (1) the activity of the DA-sensitive adenylate cyclase (Mishra et al. 1974), and (2) the number and affinity of postsynaptic DA receptors as labelled by the high-affinity ligand ^3H -spiroperidol (Seeman et al., 1975; 1976). These parameters were compared in offspring whose

mothers received haloperidol or vehicle alone (control) during the third trimester. The offspring used in the adenylate cyclase series of experiments were littermates of those used in the histofluorescence studies, so that the results might be correlated. DA-sensitive adenylate cyclase activity and ^3H -spiroperidol binding was measured in the offspring striatum since this is the best characterized DA terminal area of the brain, as well as the most accessible.

To determine whether changes in the morphological and biochemical development of DA systems might produce significant changes in their functional integrity, two functions that are DA-mediated were also assessed in the offspring. These included (1) locomotor and exploratory behaviour as assessed by observation of spontaneous open field behaviour (Janssen et al., 1965; Steof et al., 1978) and (2) hormonal regulation as indicated by pituitary prolactin secretion measured both in vivo and in vitro. The behaviours are indicative of nigrostriatal and mesolimbic function (Guyton, 1972; Pijnenburg et al., 1975), while prolactin regulation is mediated by the tuberoinfundibular DA system (Meites, 1977).

Again, littermates of the haloperidol and control offspring of the morphological and biochemical studies were used in these experiments.

Most of the measures, notably DA-sensitive adenylate cyclase, ^3H -spiroperidol receptor binding, open field behaviour and prolactin regulation have been used in studies of the adult DA system following chronic neuroleptic treatment (Introduction, Part E). Almost nothing is known about prenatal neuroleptic effects on these aspects of DA function in the developing animal. Hence, in addition to contributing new information, the results of the present study can be compared to those in the

literature on the adult. This provides an ideal opportunity to determine whether response mechanisms of the developing DA system are the same as that of the adult, and if not, then how they differ.

Another important question addressed by this thesis work is whether alterations produced by haloperidol blockade during early synaptogenesis may be long-term, perhaps even permanent. In other words, does prenatal haloperidol treatment produce alterations in any of the parameters measured that can be detected much later in offspring development, that is, when they are reaching adulthood? To answer this question, all experimental determinations included offspring that were 25 days old since this is the age when the morphological and biochemical parameters of brain DA neurons analyzed are reaching the adult stage. See Figure 8 for a schematic overview. In addition, the histofluorescent pattern of target area innervation and the ontogeny of the DA-sensitive adenylate cyclase activity was evaluated prior to this stage to determine whether temporary changes might be occurring before postnatal day 25. Behaviour and adenylate cyclase activity were also measured on postnatal day 60 to determine whether alterations in DA function might still be apparent later in adulthood.

The Neuroleptic Haloperidol

Haloperidol, a member of the butyrophenone class of neuroleptics (Figure 7) was chosen as the test drug in this study for a number of important reasons. It is one of the most potent neuroleptics available. A comparison of the various classes of neuroleptics versus their clinical

efficacy indicate that haloperidol and spiroperidol, both butyrophenones, exert their antipsychotic effects at doses 10 to 100-fold lower than other neuroleptics, for example the phenothiazines (Seeman et al., 1978). Hence one can use very low doses of haloperidol and avoid the many nonspecific side-effects associated with the use of high doses.

Haloperidol is a very potent antagonist of brain DA receptors (Iversen 1975; Seeman et al., 1978). It shows a high affinity for binding to postsynaptic DA receptors (Seeman et al., 1975; Burt et al., 1975). The affinity constant of haloperidol for binding to the ^3H -spiroperidol receptor in the striatum is much higher than most other neuroleptics. In contrast, there is virtually no haloperidol binding in non-DA containing brain areas such as the cerebellum (Snyder et al., 1976). Furthermore, destruction of the striatum with kainic acid abolishes ^3H -spiroperidol binding sites while destruction of preterminal nigrostriatal axons is without effect (Nagy et al., 1978). This indicates that high affinity haloperidol binding is associated with DA receptors at postsynaptic sites.

Studies on the regional distribution of tritiated haloperidol demonstrate a preferential accumulation of haloperidol in DA-rich terminal areas of the brain, including striatum and pituitary (Janssen and Allewijn, 1969). There is some indication, however, that haloperidol may bind to the receptors of other monoamines, for example serotonin, but the binding affinity of haloperidol for these receptors is much lower than that for the DA receptor (Creese and Snyder, 1978). Also, haloperidol is known to enhance Dopa synthesis in monoamine neurons, but its thresh-

hold dose is much lower in DA-rich compared to norepinephrine-rich areas of the rat brain (Carlsson et al., 1976). Hence, haloperidol shows a preferential affinity for DA-rich brain regions and interactions with other monoamine systems are likely to occur only at high dose levels.

Haloperidol effects on the adult DA system are already well characterized as was described in the Introduction (Part E). In summary, haloperidol is a competitive inhibitor of postsynaptic DA-sensitive adenylate cyclase in the striatum (Iversen, 1975), mesolimbic nucleus accumbens (Rosenfeld et al., 1979), and the pituitary. Acute treatment with high doses of haloperidol produce catalepsy in the rat, a result of blocking DA-regulated motor movement (Baez et al., 1979). Haloperidol also stimulates prolactin secretion, an effect that is mediated by blocking pituitary DA receptors that are normally under the tonic inhibitory influence of the tuberoinfundibular DA system (Meites, 1977).

Besides characterization of the acute effects of haloperidol on brain DA systems, the effects of chronic haloperidol treatment has also been well studied in the adult rat. The results of these studies, as described in the Introduction (Part E), indicate that long-term administration of haloperidol produces DA receptor supersensitivity. This supersensitivity is expressed in a number of ways including an increase in number of DA receptors, in DA-sensitive adenylate cyclase activity, and in locomotor behaviour (Muller and Seeman, 1978). To determine whether haloperidol produces the same effects on developing DA systems, these same parameters were investigated in offspring after haloperidol treatment during the third trimester.

Almost nothing is known about the effects of haloperidol on pre-natal development of DA systems. Haloperidol does penetrate the placental barrier and reach the fetal brain, owing to its highly lipophilic nature (Mirkin, 1973). It also passes via the mother's milk while nursing (Ibid). When given during the early postnatal period for example to nursing rabbits, it can produce retardation of motor development in the offspring (Lundborg, 1972). In addition, several human case studies also report extrapyramidal reactions, that may be DA-mediated, after maternal ingestion of haloperidol during pregnancy (Goldberg and Di Mascio, 1978).

These data, along with the available data on the adult system indicate that haloperidol is a potent antagonist of postsynaptic DA receptors and can serve as a useful tool in blocking trophic interactions between presynaptic and postsynaptic neurons during prenatal development.

The Rat as an Experimental Animal

The Wistar rat was chosen as the experimental animal in this study for a number of important reasons. The major DA pathways in the brain of the rat have already been mapped out using a variety of histofluorescence techniques (Dahlstrom and Fuxe, 1964a, 1964b). This provides a basis for comparison of the results in the present study using the glyoxylate fluorescence technique (de la Torre and Surgeon, 1976). Also since the DA terminal areas in the brains of neonatal rats and their reaction to prenatal haloperidol treatment have not yet been investigated using this technique, the results of the present investigation serve to extend current knowledge in this area.

The rat has been the animal of choice for many of the parameters measured in this investigation, including striatal DA-sensitive adeny- late cyclase activity and DA receptor binding studies. These have been well characterized in the adult rat (Kebabian and Calne, 1979; Titeler et al., 1978) and very recently, their ontogeny has also been investigated (Coyle and Campochiaro, 1976; Pardo et al., 1977). This serves not only as a basic for comparison of the results obtained in the present study, but also in comparing and contrasting haloperidol effects on both the adult and developing rat. Since the experimental techniques using rat brain tissues are already established, the use of rats facilitates the incorporation of these procedures.

The rat also has a repertoire of distinct DA-mediated behaviours that can be readily measured. For example, spontaneous locomotor activity can be assessed by observation in an open field chamber and by recording the number of squares crossed per fixed interval of time (Janssen et al., 1965). Exploratory and emotional behaviour can also be assessed by monitoring rearing, grooming and bolus deposits in the same situation (Stoof et al., 1978). A significant difference in the behaviours of rats receiving prenatal haloperidol administration compared to controls would provide an indication that the drug treatment interfered with the normal development of brain DA systems, hence altered the expression of normal DA-mediated behaviours.

The regulation of prolactin secretion by tuberoinfundibular DA neurons has also been fairly well studied in the rat. The procedure for obtaining serum prolactin determinations from trunk blood is relatively simple and unstressful to the rat which is important since prolactin is

a highly stress-responsive hormone (Seggie et al., 1976). The remaining brains and pituitaries can be simultaneously used for receptor binding assays and incubation studies, respectively, so that correlations among these measures can be determined. Also, a sensitive radioimmunoassay for rat prolactin is now commercially available (Results, Part 5).

Another important factor is that the development of the rat, starting from conception, is quite rapid so that the effects of prenatal haloperidol treatment on subsequent development from birth to adulthood (60 days) can be determined in a relatively short time span. To facilitate such studies, it is possible to order timed-pregnant Wistar rats. This has at least two important advantages. First, any number of experimental and control dams can be treated simultaneously and on the precise days of gestation desired, for example, day 15 to 21. Secondly, both experimental and control offspring can be fostered simultaneously right after birth, then monitored together throughout postnatal development. This is important in view of inter-animal variations, possible seasonal variations and probably a great number of unknown variables as well (Spyker, 1975).

Finally, a rough analogy can be drawn between the stages of development in the rat and that of the human. For example, the third week of gestation in the rat corresponds to the second trimester of pregnancy in the human (Joffe, 1969). Rats, therefore, are born prematurely in terms of the analagous stage of development in the human. This has at least two important implications. The first is that one can administer a drug, such as haloperidol, during a discrete period in the rat's gestation that corresponds to the desired stage of human develop-

ment. Tentative generalizations can therefore be made about the observed experimental effects and the corresponding stage of vulnerability in the human. The second implication is that the drug must be administered during the rat's prenatal stage of development to reproduce as closely as possible its potential effects during human pregnancy. Many studies in which neuroleptics were administered during the early postnatal period in the rat have been taken to indicate the potential toxicity of these drugs if administered during pregnancy. These postnatal studies, however, fail to reproduce precisely the direct effects of neuroleptics on maternal-fetal interactions that take place only during pregnancy.

Hence the Wistar rat was chosen as the experimental animal in the present study because the morphological, biochemical and functional development of DA systems in its brain are relatively amenable for investigation. Since these have been well characterized in the adult, it will also be possible to compare the effects of haloperidol on the developing animal and the adult.

OUTLINE OF EXPERIMENTAL DESIGN

The experiments were based on the design that is outlined schematically in Figure 8. The development of the offspring was assessed after maternal treatment with either haloperidol or vehicle (control), using a combination of tests that were done at various ages from postnatal day 2 to 60 days. Details of the methods used are described in Methods and Results, parts A to E. In this section, the sequence of procedures used is briefly described to provide an overall perspective on the different series of experiments carried out. Details of the treatment procedure, that was constant for all experiments, are discussed here.

Haloperidol was administered once daily to timed-pregnant rats during the third trimester from gestational day 15 to 21 (Figure 8). Treatment was terminated at birth and the offspring of both haloperidol-treated and vehicle-treated controls were fostered within 12 hours after birth to lactating dams who had received no prior experimental handling. The effects of prenatal haloperidol treatment on offspring development was assessed using measures of both presynaptic and postsynaptic DA systems. Wherever possible, littermates of each experimental group were used for each of the different tests and at the different ages tested.

The morphological development of presynaptic DA neurons was assessed at postnatal day 15 and day 25 using the glyoxylate histofluorescence technique of de la Torre and Surgeon, (1976) to determine whether temporary or permanent changes were induced by the prenatal haloperidol

OUTLINE OF EXPERIMENTAL DESIGN

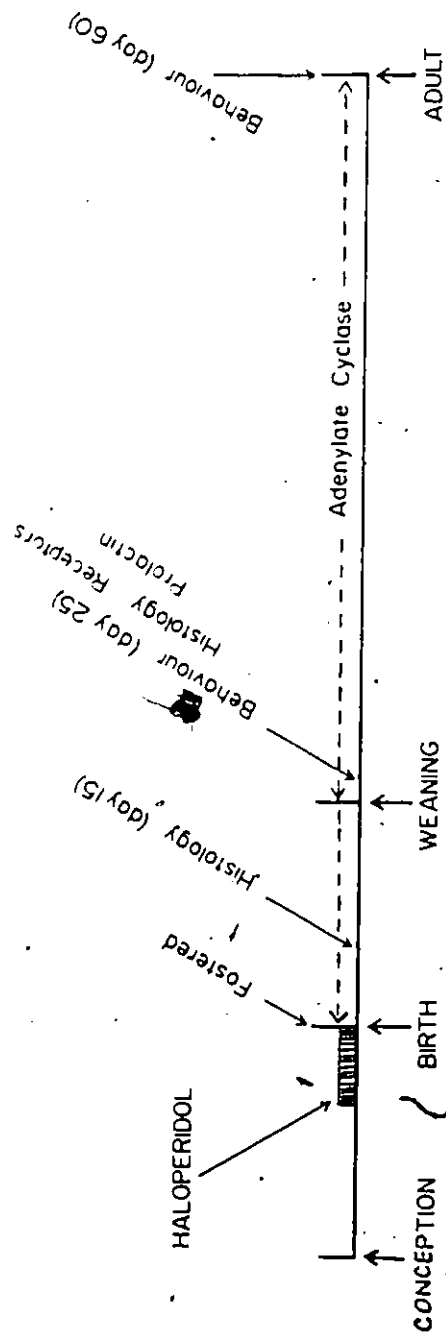


Figure 8: Haloperidol, or vehicle (control) was injected once daily in maternal rats during the last week of gestation when fetal DA neurons first form synapses. At birth, all offspring were fostered by untreated lactating dams. Adenylate cyclase activity was measured from birth to day 60 to establish the normal pattern of ontogeny as well as to determine treatment effects. The histological developments of DA neurons was initially assessed at postnatal day 15 when complete neural tracts could first be visualized. They were assessed again at postnatal day 25 when these neurons reach maturity. At the same time, postnatal DA receptors, prolactin secretion and offspring behaviour was analyzed to compare the effects of prenatal treatment on postnatal development and on brain DA function. The behaviour of the offspring was analyzed also on day 60 (adult) to determine the relative permanence of altered DA function. For further details see Methods and Results, Parts A to E.

treatment (Methods and Results, Part A). The ontogeny of postsynaptic DA-sensitive adenylate cyclase activity in the striatum of offspring was determined at intermittent periods from postnatal day 2 to day 60 to establish whether prenatal treatment produced changes in normal pattern of ontogeny (Part B). Postnatal day 25 served as a focal point. This included not only striatal DA-sensitive adenylate cyclase activity and histofluorescence analysis, but also DA receptor binding using ^3H -Spiroperidol as ligand (Part C) and DA regulation of prolactin secretion both in vivo and in vitro (Part E). To determine whether haloperidol produced any permanent changes in brain DA function, the behaviour of haloperidol and control pups was recorded on postnatal days 22-25 as well as on day 60 (part D). In addition, the gross morphological development and weights of offspring were monitored at weekly intervals until weaning on postnatal day 21 (Part D).

Animals and Maintenance

Timed-pregnant Wistar rats were obtained from Biobreeding Labs, Montreal. Two groups of rats were always obtained at the same time. One group that was 13 days pregnant on arrival were used as drug treated mothers. They were allowed to accommodate to their new housing environment for 2 days prior to the start of haloperidol treatment. The second group that was approximately 18 days pregnant on arrival were used as foster mothers for the offspring of the treated mothers. They were allowed to give birth and tend to their own litters until they were replaced by offspring of the treated mothers.

All rats were housed individually in a quiet, environmentally controlled room within animal quarters. Food and water were freely

available. Unnecessary handling or disturbance of mothers and their litters was strictly avoided. Experimental procedures that were carried out over a number of days, such as injections, were always performed at the same time each day.

Drugs and Chemicals

All chemicals used in this study were of analytical grade and were obtained from Sigma Chemical Company, St. Louis, Mo., Fisher Scientific Company, New Jersey, N.Y., and J.T. Baker Chemical Company, Phillipsburg, N.J., unless otherwise indicated. The drugs dopamine and reserpine were obtained from Sigma, and fluphenazine from Squibb and Sons Ltd., Montreal, Quebec. Haloperidol was a gift from McNeil Laboratories Ltd., Stouffville, Ontario.

Drug Preparation

Haloperidol was first dissolved in a few drops of lactic acid (13 molar) then brought up to volume in deionized water. Fresh preparations were made on alternate days and stored in a dark, tightly capped vial at 4°C between injections. The solution was allowed to reach room temperature before injection into the rats. The control solution contained vehicle alone and was prepared in exactly the same way except that the drug was omitted.

Treatment Procedure

Injections of haloperidol and vehicle were begun on day 15 of gestation and terminated on the day of delivery, one week later. Each morning, between 1000 and 1100 hours, each rat was weighed then given an

injection subcutaneously (s.c.) in the nape of the neck and returned to its home cage. Their daily food and water intake was also monitored throughout the treatment period.

Initially, there were 5 pairs of experimental groups, based on the dose of haloperidol administered. Each group consisted of at least 6 litters. Controls, also consisting of at least 6 litters each, were treated simultaneously with each of the haloperidol groups treated. The groups consisted of the following:

1. Very low dose group that received 0.26 mg/kg s.c. of haloperidol and corresponding vehicle controls;
2. Low dose group that received 0.6 mg/kg s.c. of haloperidol and controls;
3. Mid dose group that received 1.2 mg/kg s.c. of haloperidol and controls;
4. High dose group that received 2.5 mg/kg s.c. of haloperidol and controls;
5. Very high dose group that received 3.2 mg/kg s.c. of haloperidol and controls.

The very high dose group was abandoned after the first series of experiments due to the toxic side effects produced by haloperidol at this dose. Maternal rats receiving this dose were catatonic for many hours after this injection. Their food and water intake dropped drastically accompanied by a lack in normal weight gain as pregnancy progressed. There was close to 50% mortality in their litters at birth or shortly thereafter. The subsequent weight gain and morphological development of the surviving offspring was also significantly retarded compared to the controls. The

very low dose group was also not continued so that the number of animals handled at any one time could be reduced. In subsequent experiments, only the low, mid and high-dose groups of animals were run.

Fostering Procedure

Within 12 hours after birth, the number of pups in each litter was recorded, including live-births and still-births. The pups of both haloperidol and control mothers were then transferred to the home cages of foster mothers who had received no prior drug treatment nor experimental handling. The number of pups in the original litters of the foster mothers was also recorded at their delivery earlier. They were removed and discarded immediately prior to transfer of the experimental offspring. Each experimental litter that was fostered contained 8 pups, including both males and females. Wherever possible, entire litters from original mothers were transferred together to the nests of the foster mothers. Foster mothers that had delivered less than 8 of their own pups were not used for the transfer. The pups were weighed at weekly intervals and briefly examined for physical abnormalities, then immediately returned to the nest (Methods and Results, Part D). Any abnormalities or additional mortalities over the next few weeks were recorded. At 21 days of age the pups were weaned. Males and females were separated and housed in groups of 6 per cage. After day 25, the remaining offspring were housed in groups of 3 per cage to avoid overcrowding.

Summary

As outlined in Figure 8, this study incorporated five different experimental measures to compare and evaluate the development of offspring

whose mothers were treated with haloperidol during the third trimester. The morphological, biochemical (DA-sensitive adenylate cyclase and DA receptors) and functional (prolactin regulation and behaviour) development of brain DA systems of the offspring was evaluated using rats that were littermates within the same experimental groups wherever possible.

Correlations were made based on the data obtained from all experiments done on postnatal day 25.

Details of the methods and results obtained are described individually in the next section of this thesis (Parts A to E) although in reality the experiments were run simultaneously and/or consecutively. The final section of this thesis consists of a summary of the conclusions that can be drawn from the culminated results of the study, as well as a discussion of their overall implications with regard to plasticity in development.

METHODS AND RESULTS

A EFFECTS OF PRENATAL HALOPERIDOL ON PRESYNAPTIC DOPAMINE NEURAL DEVELOPMENT

Introduction

Dopamine (DA) neurons become differentiated by prenatal day 15 in the rat (Coyle, 1974; Loizou, 1971; 1972). Neural projections from DA nuclei in the brainstem then grow towards terminal areas in the forebrain where they first begin to form synapses (Loizou, 1972; Kellogg and Wennerstrom, 1974). Since complete neuronal pathways are not yet established at this time, early neuroblasts cannot be carrying out the same function of innervation as they do when matured. Thus, during the third trimester of the rat, DA appears to subserve a function that is unique to the initial stages of synaptogenesis.

Preliminary studies indicate that DA plays an important role in the interactions between presynaptic and postsynaptic elements of the developing system. For example, Tennyson et al. (1979) recently found that treatment of rabbits with reserpine, that depletes DA storage and release, during this critical stage of prenatal development led to a reduction in number and maturity of presynaptic DA terminals. It also led to degenerative changes in postsynaptic target cells in late fetal life. Hence, DA seems to be involved in at least two important functions: first, in directing the outgrowth of its axons towards appropriate target sites and secondly, in establishing appropriate connections with postsynaptic sites.

In view of the apparent importance of DA availability during the third trimester, it was hypothesized that blockade of postsynaptic DA sites at this time would interfere with the normal interactions between pre- and postsynaptic sites and lead to abnormalities in presynaptic development. Haloperidol is a potent antagonist of postsynaptic DA binding sites (Iversen, 1975; Seeman et al., 1978). Administration of haloperidol during the third trimester, therefore, is likely to impede interactions with presynaptic terminals and lead to abnormalities in their development. This may lead to abnormalities in their innervation of terminal sites in the brain. This might subsequently lead to abnormalities in postsynaptic development, which is the subject of the following sections of this thesis (Methods and Results, Parts C and D). The question of whether haloperidol alters the normal pattern of presynaptic DA innervation of terminal sites is considered first in this series of experiments.

DA is synthesized and released by developing terminals even prior to birth (Coyle and Henry, 1973; Loizou et al., 1972). These terminals can be visualized using histofluorescence techniques (Coyle, 1974; Loizou, 1971; 1972). Application of histofluorescence techniques has enabled visualization of the nigrostriatal tract as early as prenatal day 17 in the rat (Olson et al., 1972). These techniques have also been used to determine whether there is an increase or decrease of DA in terminal regions of the brain by comparing the intensity of fluorescence emitted in the presence or absence of any given treatment (Loizou, 1972; Olson et al., 1972). The most sensitive of these techniques, the glyoxylate fluorescence technique (de la Torre and Surgeon, 1976) is used in the present

study to determine whether haloperidol treatment during the third trimester in the rat leads to abnormal presynaptic DA neural innervation of terminal areas of the brain, specifically the striatum, nucleus accumbens and olfactory tubercle. A photometric technique was devised by the author to measure the intensity of fluorescence emitted from each of the areas examined. See Figure 10A for a schematic of brain areas analyzed.

Methods

Treatment Procedures: Haloperidol or vehicle were administered to Wistar rats during the third trimester as was described previously (Outline of Experimental Design).

Animals: In preliminary experiments, offspring at postnatal ages day 2, 7, 11, 15, 20, 25 and 30 were used in an attempt to reconstruct and visualize the nigrostriatal tract using the glyoxylate fluorescence method. At least 2 rat pup brains were used at each of these ages. In subsequent experiments, pups at postnatal day 15 and day 25 of age were chosen to determine the effects of prenatal haloperidol treatment on the development of DA innervation to terminal areas of the brain, including striatum, nucleus accumbens and olfactory tubercle. At least 6 pairs of haloperidol and control brains were prepared for each of the three haloperidol doses tested (0.6 mg/kg, 1.2 mg/kg and 2.5 mg/kg) both at postnatal day 15 and day 25.

Tissue Preparation: The rat pups were decapitated and their brains carefully excised to avoid distortions, then placed on foil squares on dry ice till frozen. The brains used in the preliminary experiments were hemi-sectioned longitudinally along the midline when partially hardened,

then replaced on dry ice to complete freezing.

In order to cut longitudinal parasagittal sections, the brains were mounted individually with temporal lobes facing down on the cryostat chuck and stabilized with Tissue-Tek II embedding medium (Miles Laboratories Inc., Illinois). They were then placed into the cryostat (AO Cryo-Cut Microtome, American Optical Corporation, Buffalo, New York) and allowed to equilibrate at a temperature of -20°C before sectioning was begun. After approximately 20 minutes, serial sections were taken in a medial to lateral direction, relative to the half brain mounted. The sections were cut at 30 micron (μ) thickness and picked up individually on warm microscope slides, ready for processing.

When frontal brain sections were taken, a control plus haloperidol brain were mounted together on the same cryostat chuck. They were stabilized on the chuck with Tissue-Tek II embedding medium, and allowed to equilibrate at -20°C in the cryostat holding chamber. The anterior fore-brains were trimmed off, using a razor, then by thick sectioning with the cryostat blade until the point where the right and left halves of the corpus callosum became opposed in the frontal plane. The approximate coordinate used for 15 day old experimental offspring was anterior 6.2 mm using the 10-day old rat as standard, and for 25 day old experimental offspring was anterior 7.5 mm, using the 21-day old rat as standard according to the rat stereotaxic atlas of Sherwood and Timiras (1970).

Serial frontal sections, 30μ thick, were made in a rostral to caudal direction up to the approximate coordinates of anterior 5.9 mm (10-day standard) and 7.0 mm (21-day standard) according to Sherwood and Timiras (1970). Consecutive sections, 90μ apart were picked up on warm microscope

slides. Since 2 brains were mounted together on the same chuck, each slide held 2 brain sections, one haloperidol and one control at approximately the same coordinates. A set of 10 slides at a time were then processed with the glyoxylic acid reaction (de la Torre and Surgeon, 1976). The procedure was repeated for each set of 10 slides till all brains, both haloperidol-control and control-control pairs, were processed.

Glyoxylic Acid Reaction: The brain sections were processed according to the SPG method of de la Torre and Surgeon (1976; personal communication) with the addition of a few modifications (Appendix 1) included in the following account. The contents of the glyoxylic acid (SPG) solution, included 200 mM sucrose, 236 mM phosphate (monobasic) and 1% glyoxylic acid (Sigma Chemical Company). The reagents were dissolved in 100 mls of deionized water, titrated to a pH of 7.4 with 1 Normal (N) sodium hydroxide and brought up to a final volume of 150 mls. In summary, the procedure consisted of dipping each slide into the SPG solution for 3 seconds then draining and drying it under a stream of cool air. In this way, 10 slides could be accumulated within 20 minutes and placed together in an oven at $80^{\circ} \pm 1^{\circ}\text{C}$ to initiate the glyoxylic acid reaction. A container of dessicant (Drierite, CaSO_4) was kept in the oven to maintain low humidity. The slides were incubated in the oven for exactly 5 minutes then removed and placed for 90 seconds on a hot plate at 80°C to reduce autofluorescence of air bubbles. A drop of warm mineral oil (USP Thin) was placed on each slide before coverslipping. The slides were then ready for analysis under the fluorescence microscope.

Analysis of Brain Sections: The brain sections were viewed with a Zeiss Universal microscope using a HBO 200 W/4 mercury lamp as light source. To the microscope was attached an Epi-fluorescence condenser III

RS. A UG-1 exciter filter and a 47 barrier filter were inserted to select for the fluorescence emission band including 360-470 nm wavelengths. The intensity of fluorescence emitted from each brain area examined was determined using a photomultiplier system that was devised by the author and attached to the microscope (see below and Appendix 2). Intensity readings were recorded for each terminal brain area within the corresponding haloperidol-control pairs, as well as control-control pairs on each microscope slide processed (Figure 10A). Each slide, therefore, provided paired readings for the striatum, nucleus accumbens, olfactory tubercle as well as background readings from the cortex of each. No readings were taken of the longitudinal, parasagittal series of brain sections since the nigro-striatal tract was too narrow to fill the viewing field of the microscope at low magnification. Hence the intensity of fluorescence was too low to activate the photomultiplier sensor system.

Photomultiplier System: To make comparisons of the amount of fluorescence emitted by DA terminal areas in the brains of haloperidol and control offspring, it was necessary to devise a means by which the intensity of fluorescence could be quantitated. The author, therefore, devised a photometric system that was designed for attachment to the Universal fluorescence microscope used to view the brain sections (technical assistance: C. Ikeson and M. Holmes). This consisted of a cadmium sulfide photocell (trademark: Archer R No. 270-116) that was mounted in the barrel of a 500 cc syringe and stabilized with a rubber ring. This component was inserted into the phototube of the microscope. The leads from the photocell were connected with insulated wires to a Ballantine 3026A digital

FLUORESCENCE MICROSCOPE-PHOTOMULTIPLIER SYSTEM

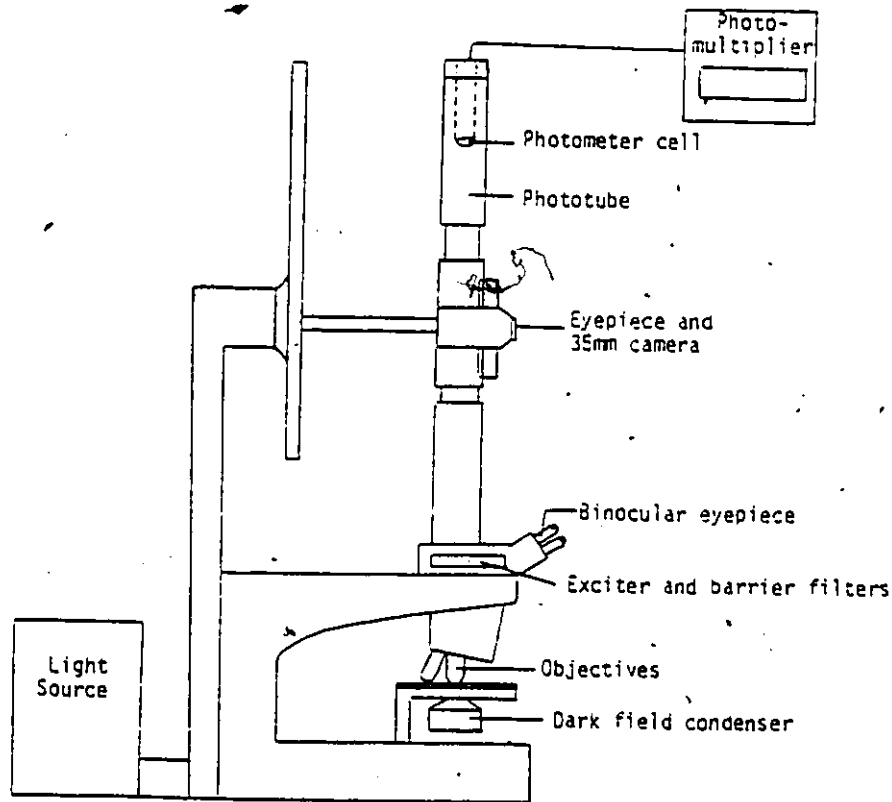


Figure 9: Schematic of the photomultiplier system, devised by the author, that was attached to the fluorescence microscope. The photometer cell was inserted into the phototube of the fluorescence microscope. The photocell was connected to a Ballantine photomultiplier recorder with digital display. The fluorescence microscope was equipped with a built-in camera system. The brain sections were viewed through the binocular eyepiece. The image could be projected up to the camera system for final focusing and photography. The image could be projected further up into the phototube where the photometer cell was exposed to the fluorescence light. The signal was detected by the photomultiplier recorder that provided a digital readout for the relative intensity of light emitted by the brain section under view. See text for further details.

LOCALIZATION OF PHOTOMETER READINGS

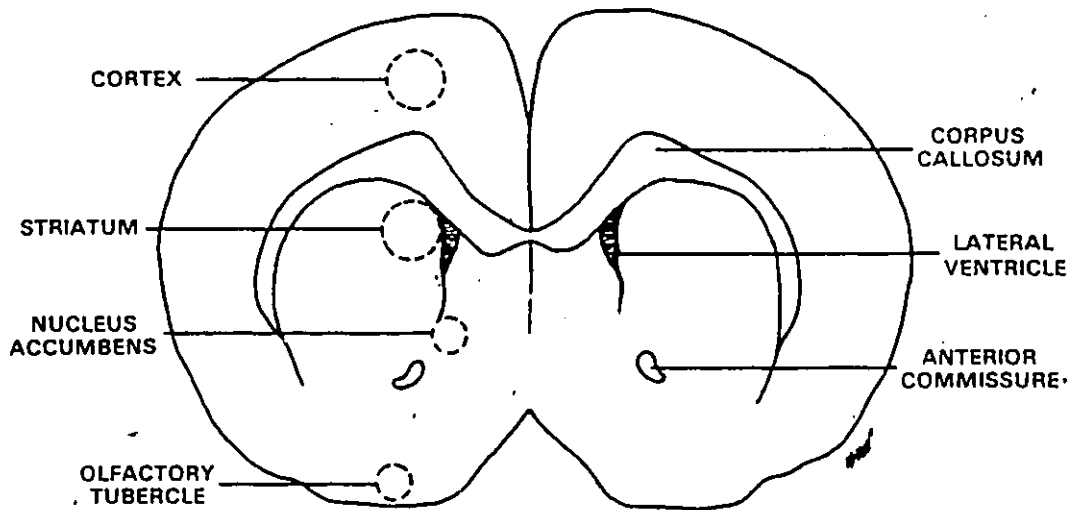


Figure 10A: Schematic of a frontal section through a rat brain. The dotted circles indicate the approximate areas within the cortex, striatum, nucleus accumbens and olfactory tubercle that were analyzed using the fluorescence microscope photomultiplier system. Photometric readings of these same areas within pairs of haloperidol and control brains were compared and photographed.

multimeter (Figure 9). As the intensity of light emitted by each brain section being viewed under the microscope differed, it produced a corresponding change in the resistance of the photocell. This change in resistance was detected by the multimeter component and indicated by a change in numerical readout. Hence differences in the fluorescence emitted from terminal areas in various brain sections were recorded as numerical differences. This provided an objective means for comparing fluorescence intensity, that reflects DA content (de la Torre and Surgeon, 1976) in terminal areas of the brains of offspring that received prenatal haloperidol or vehicle treatment.

Photomicrography: The brain sections were photographed using Ektachrome ASA 400 film and a Zeiss C35 camera-back that was mounted onto the phototube of the microscope. The most appropriate exposure time for each of the brain terminal areas examined was determined prior to the start of this study by systematically experimenting with different film speeds and exposure times to obtain the best photographic results.

Results

Attempts to Determine the Rate of Nigrostriatal Fibre Outgrowth:

Initially, sagittal sections of the offspring brains were used to assess the development of the nigrostriatal tract and determine whether prenatal haloperidol treatment altered the rate of outgrowth of these DA fibres. It was hypothesized that if haloperidol blocked interactions between pre-synaptic terminals and their target sites in the striatum, this would produce a decrease in numbers of DA fibres and in their rate of growth into the striatum. Hence, an attempt was made to reconstruct the nigrostriatal tracts of the control and haloperidol using photomontages and comparing them at regular intervals during postnatal development.

Figure 10B is an example of a photomontage of the nigrostriatal tract of a control offspring aged 15 days.

A number of problems arose that led me to abandon this technique. The first was the excessive time required to accurately reconstruct the nigrostriatal tract at different postnatal ages. During this period of development, particularly the first 2 postnatal weeks, offspring brains are still developing rapidly and expanding, hence the relationship of brain structures were continually changing. The technique of sectioning the brains and reconstructing the nigrostriatal tract, therefore, had to be continually modified. This became increasingly difficult since as the brains matured, the nigrostriatal tract no longer followed a relatively linear pattern of outgrowth, but deviated in a medial to lateral and a ventral to dorsal direction. Hence, a planar section through the brain at different levels would include only a small part of the curving nigrostriatal tract at any one time.

Other problems were also encountered with this procedure. Nigrostriatal DA fibres of the neonatal brain are very fine and grow together in dense bundles, thereby making it difficult to accurately estimate their number at the light microscope level. Another problem is that their growth is not uniform. Some fibres had already reached the striatum at birth while the rest gradually terminated there during the first few postnatal weeks. As a result, there was no uniform front of fibre projections that could be assessed in terms of rate of outgrowth.

Attempts were made to measure the intensity of fluorescence and relate this to density of fibres in the nigrostriatal tract. This proved difficult since the viewing field was not filled by the width of the tract

NIGROSTRIATAL TRACT OF 15-DAY OLD RAT

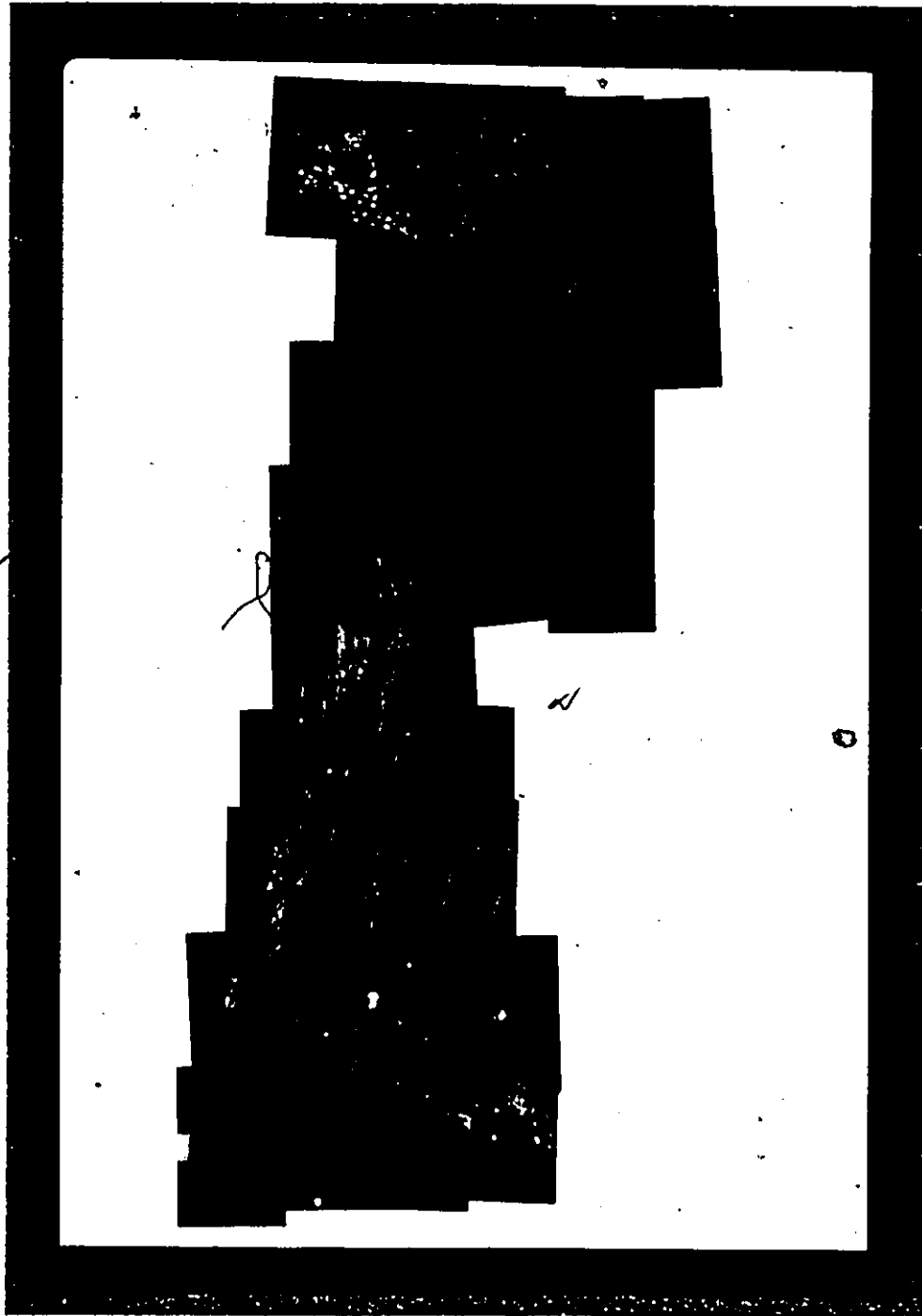


Figure 10B: Photomontage reconstructing the nigrostriatal tract of a 15-day old rat. Brain was sectioned longitudinally in an oblique parasagittal plane. Serial photographs (40x objective) were then taken of a brain section including the substantia nigra (cluster of fluorescent cells at right) and dopamine fibres (fluorescent striations) projecting towards the striatum (left area).

unless very high magnifications were used. Increasing the magnification reduced the portion of the tract that was being sampled at any one time. Hence, accuracy in sampling the same section of the nigrostriatal tract in both haloperidol and control offspring brains was hindered. This was further complicated by the deviant course of nigrostriatal fibre outgrowth with maturation.

Long-term Decrease in Striatal Fluorescence after Prenatal Haloperidol: The striata of offspring whose mothers received haloperidol injections during the third trimester were compared to vehicle-controls at postnatal day 15 and day 25. The intensity of DA-induced fluorescence in the striata of haloperidol offspring was reduced compared to controls at both postnatal ages. The degree of fluorescence depletion was particularly marked at postnatal day 15, but still apparent at postnatal day 25. Photomicrographs of striata from haloperidol (2.5 mg/kg) and control offspring aged 15 days are shown at low magnification in Figure 11 (A and B).

On postnatal day 15 (Figure 11A) one could see the characteristic islands of bright green fluorescence in the striatum surrounded by areas of diffuse light green fluorescence described by others using different fluorescence techniques (Loizou, 1971; 1972; Coyle, 1974). These represent clusters of DA terminals entering the striatum surrounded by finer, more dispersed DA fibres. Also seen were dark, rounded areas devoid of fluorescence that represent the non-catecholamine containing cross-tracts of the internal capsule which traverse the striatum at different levels. Superior to the striatum is another band of non-fluorescing tracts that course through the corpus callosum which forms the upper boundary of the striatum.

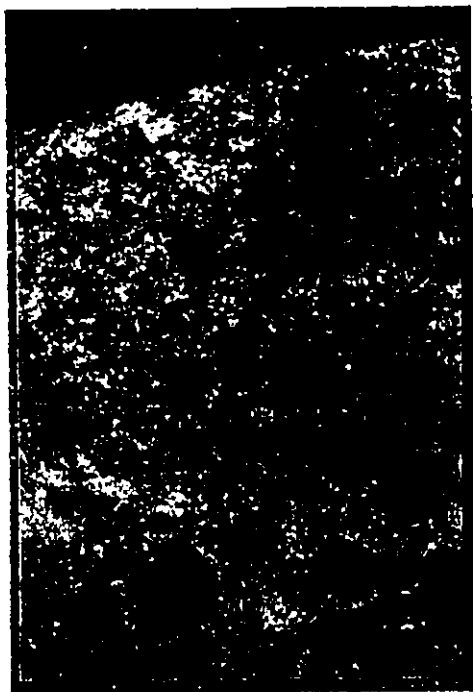


Figure 11A
Striatum at 15 days,
Low power view (10 x objective)

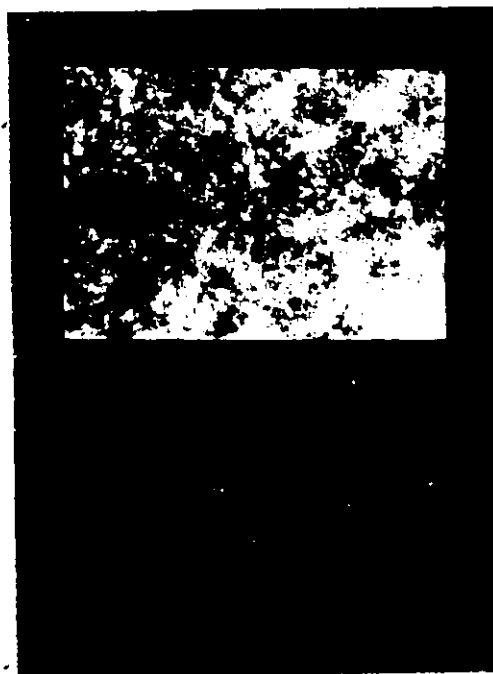


Figure 11B
Striatum at 15 days (40 x obj.)
Control (top) vs haloperidol (bottom)

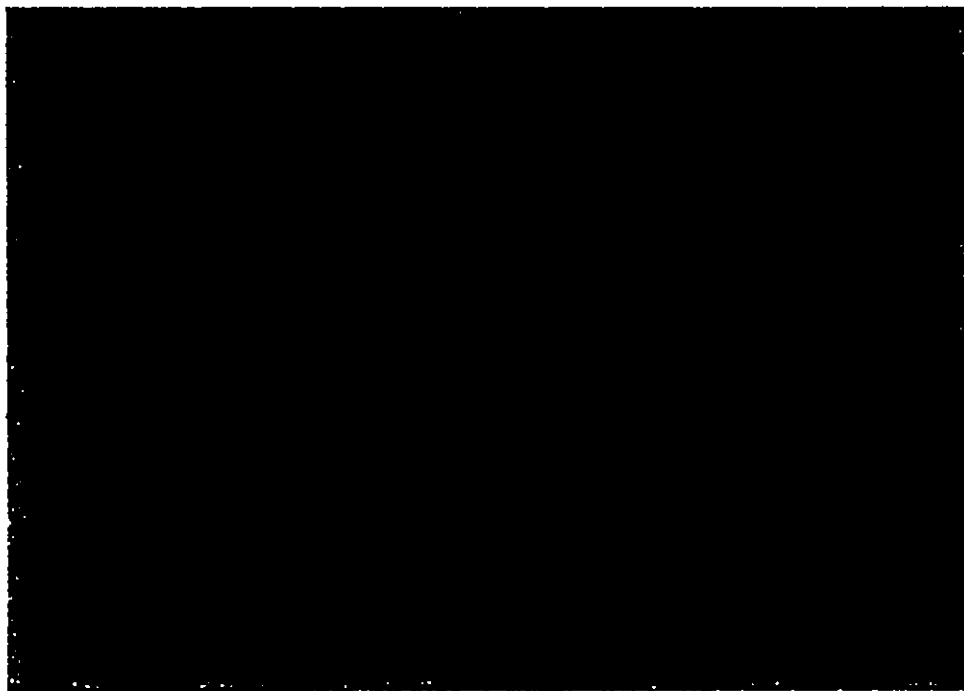


Figure 11C and D
Striatum at 25 days (40 x obj.)
Control brain (left) and haloperidol brain (right)
from sections prepared on the same slide.

The striatum of the 25 day old control rat offspring is shown in Figure 11C, (40 x objective). One feature that distinguished the striatum at this later stage of development was the relative increase in intensity of the diffuse fluorescence, making the islands of fluorescence less apparent. This increase in general fluorescence intensity has also been observed by others and is attributed to the increase in endogenous DA content in the striatum at this age (Fuxe et al., 1974; Olson et al., 1972).

In comparing the haloperidol striatum with that of controls at postnatal day 15 (Figure 11A and B) one could see a marked decrease in intensity of DA-induced fluorescence. The areas that normally emitted a diffuse light green fluorescence were depleted to near background levels. The more punctate islands of fluorescence were also reduced in haloperidol striata compared to controls.

This decrease in fluorescence within the striata of haloperidol offspring was still evident at postnatal day 25 (Figure 11C and D). Again, there was a general decrease in the diffuse type of fluorescence as well as a reduction in the punctate pattern compared to controls. The relative degree of decrease in striatal fluorescence within haloperidol brains at this age, however, was less than that observed at day 15.

A number of control comparisons were also made at postnatal day 15 and day 25 to determine the extent of variation in fluorescence due to daily experimental fluctuations and inter-animal differences. In the first case, the cerebral cortex of control and haloperidol offspring (that normally emits low background fluorescence) were compared. In the second case, pairs of brain sections from control offspring of the same

age were compared. There were no major differences in intensity of fluorescence within these comparisons (Table 1). Cortical fluorescence did not vary significantly between groups nor did emissions from other brain areas within groups.

Estimation of Decreased Fluorescence in Striata of Haloperidol

Offspring: The intensity of fluorescence emitted by striatal sections of haloperidol and control brains at postnatal day 15 and day 25 was measured using the photomultiplier system. Table 1 presents a summary of the difference in readings obtained in a series of experiments in which mothers of offspring were treated with 1.2 mg/kg or 2.5 mg/kg haloperidol or vehicle during the third trimester.

The data indicate that there was a decrease in intensity of fluorescence emitted by the striata of haloperidol offspring, both at postnatal day 15 and day 25. This was true for both doses of haloperidol tested. Generally speaking, there did not seem to be a consistent dose-response relationship between the amount of haloperidol administered prenatally and the intensity of fluorescence emitted by the striatum: There was, however, a drop in the degree of difference between haloperidol and controls by postnatal day 25 compared to day 15. For example, in the 1.2 mg/kg dose series of experiments, the postnatal day 15 difference was -55 whereas by day 25 it was -30 (Table 1).

To determine the extent of variation due to inter-animal differences, pairs of control-control brain sections were also compared. The results indicate that in the majority of cases, there was little or no difference in readings obtained from the striata of controls. Differences in readings of less than 10 were considered insignificant. Occasionally,

Table 1

DIFFERENCES IN FLUORESCENCE INTENSITY IN BRAIN AREAS
AFTER PRENATAL HALOPERIDOL ADMINISTRATION

Haloperidol (1.2 mg/kg)	POSTNATAL DAY 15		POSTNATAL DAY 25	
	<u>Con-Con</u>	<u>Hal-Con</u>	<u>Con-Con</u>	<u>Hal-Con</u>
Cortex	0	-15	0	0
Striatum	-10	-55	+15	-30
N. Accumbens	0	+35	0	+80
Olfactory Tub.	0	+45	0	+75
Sub. Nigra	--	-150	--	-65

Haloperidol (2.5 mg/kg)	POSTNATAL DAY 15		POSTNATAL DAY 25	
	<u>Con-Con</u>	<u>Hal-Con</u>	<u>Con-Con</u>	<u>Hal-Con</u>
Cortex	0	0	0	0
Striatum	0	-40	0	-25
N. Accumbens	-15	+45	0	+75
Olfactory Tub.	0	+45	0	+55
Sub. Nigra	--	-80	--	-60

The values represent differences in photometric readings obtained in comparisons of brain areas from control pairs (Con-Con) and haloperidol-control pairs (Hal-Con) of offspring brain sections at postnatal day 15 and day 25 after the administration of haloperidol at doses of 1.2 mg/kg and 2.5 mg/kg during the third trimester. Each value is derived from triplicate readings of 3 brains sections from at least 6 offspring at each dose of haloperidol tested. The data were analyzed using the Nonparametric Runs Test and determined significant at $p < .05$.

there were differences in readings of greater than 10, for example, in the 1.2 mg/kg dose series of experiments at postnatal day 25 (Table 1). These differences, however, were not consistent and always less than those observed in haloperidol-control comparisons. There were no consistent differences in the control-control comparisons of striatal fluorescence in the 2.5 mg/kg dose series of experiments.

Estimation of Decreased Fluorescence Intensity in Substantia Nigra of Haloperidol Offspring: Frontal sections of the brainstems that remained after forebrain analyses, were similarly processed and analyzed to determine the extent of fluorescence depletion. Photometric readings of the substantia nigra of control and haloperidol offspring were then compared. The results indicate that there was a large decrease in fluorescence intensity within the substantia nigra of haloperidol offspring compared to controls (Table 1). The decrease was particularly marked at postnatal day 15. This was true for both the 1.2 mg/kg and 2.5 mg/kg dose series of experiments.

It was not possible to determine whether the observed decrease in fluorescence of the substantia nigra was due to a decrease in number of cell bodies or a decrease in intensity within the same number of cell bodies. These results indicate, however, that the decrease in DA-induced fluorescence after prenatal haloperidol involved not only DA terminals in the striatum, but also cell bodies in the substantia nigra. There was also a positive correlation between substantia nigra and striatal data in that the decrease in fluorescence intensity was most marked at postnatal day 15 and became less apparent by postnatal day 25 (Table 1).

Long-Term Increase in Mesolimbic Fluorescence after Prenatal

Haloperidol: Two mesolimbic terminal areas, the nucleus accumbens and olfactory tubercle, could be seen in the same brain sections used to analyze DA-induced fluorescence in the striatum. The fluorescence in these areas therefore was also compared in haloperidol and control offspring at postnatal day 15 and day 25. In contrast to that observed in the striatum, there was an increase in the intensity of fluorescence emitted from both the nucleus accumbens and olfactory tubercle of haloperidol offspring compared to controls. The degree of increase appeared to be greatest by postnatal day 25.

Photomicrographs of nucleus accumbens and olfactory tubercle from haloperidol (2.5 mg/kg) and control offspring at postnatal day 25 are shown in Figure 12. In comparing either of these 2 mesolimbic areas with the striatum of controls (Figure 11C), one can see a number of distinguishing features. The mesolimbic areas contain a relatively coarse, punctate fluorescence rather than the fine pattern observed in the striatum. There is less of the diffuse light green fluorescence distribution and there are no distinct islands of fluorescence. The pattern of fluorescence in the nucleus accumbens compared to the olfactory tubercle is quite similar suggesting that there is a similarity in their pattern of innervation. These characteristic features have also been observed by others using different fluorescence techniques (Fuxe et al., 1974; Olson et al., 1972).

In comparing the intensity of fluorescence of mesolimbic structures in haloperidol sections with that of controls, one could see a marked increase not only in intensity of fluorescence generally, but also in number of punctate terminals (Figure 12A and B; C and D). A number of

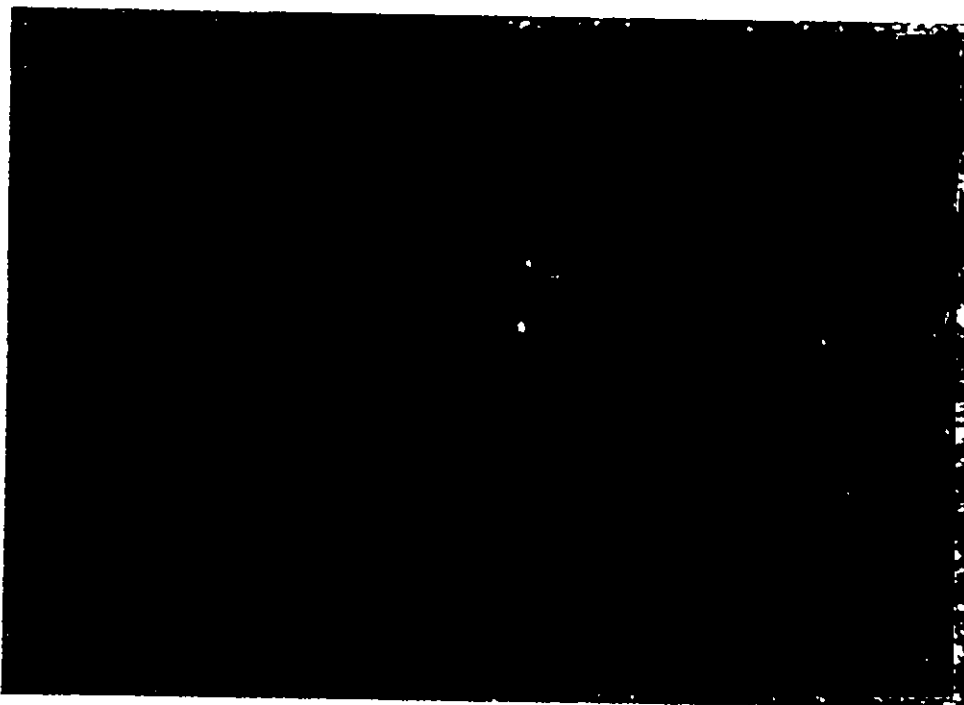


Figure 12 A and B
Nucleus accumbens at 25 days (40 x obj.)
Control (left) and haloperidol (right) from sections
prepared on the same slide.

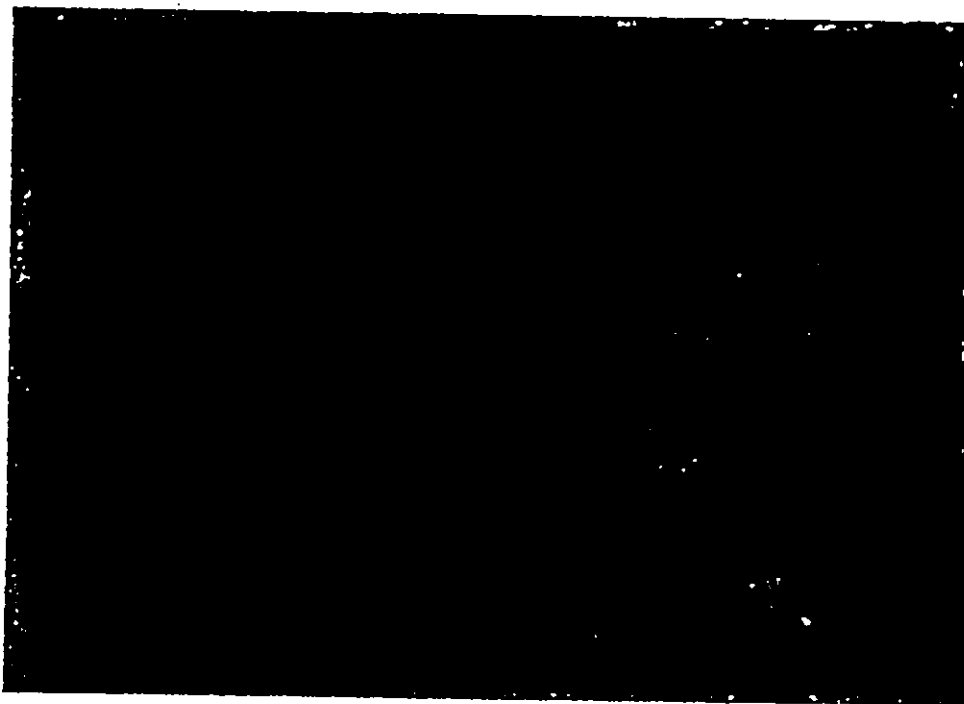


Figure 12 C and D
Olfactory tubercle at 25 days (40 x obj.)
Control (left) and haloperidol (right) from sections prepared
on the same slide.

bright green patches of fluorescence seem to appear within these areas of haloperidol brains. A similar pattern of changes was observed at postnatal day 15, although the relative difference in intensity between haloperidol and control sections was less than that at the later stage of development.

Estimation of Increased Mesolimbic Fluorescence of Haloperidol

Offspring: The differences in readings obtained in mesolimbic terminal areas of haloperidol and control brains are summarized in Table 1. Both the nucleus accumbens and the olfactory tubercle of haloperidol offspring show an increase in the intensity of fluorescence emitted at postnatal day 15 and day 25. The largest increases were apparent at postnatal day 25. For example, the nucleus accumbens of day 25 haloperidol offspring in the 1.2 mg/kg dose series showed an increase of +35 compared to controls. At prenatal day 15, the difference was +80. The relative increases in fluorescence intensity were similar in both 1.2 mg/kg and 2.5 mg/kg dose series of experiments with no apparent dose-response relationship.

To test for inter-animal variations, the nucleus accumbens and olfactory tubercle of control-control pairs of brain sections were also analyzed. As seen in Table 1, there were no consistent differences in fluorescence intensity emitted by these areas in vehicle controls at either postnatal day 15 or day 25. Occasionally, small variations were noted but these differences were usually less than those observed in haloperidol-control comparisons. When differences did appear, they were usually related to artifactual cracks and damage to the tissue resulting from processing that could be seen when viewed later at lower magnification.

Discussion

The results of this study indicate that the administration of haloperidol during the last week of prenatal development in the rat interferes with the normal development of presynaptic DA neurons and produces long-term changes in the normal pattern of innervation of terminal areas in the brain. These changes were measured as differences in the intensity of DA-induced fluorescence emitted from brain sections processed by the glyoxylate method of de la Torre and Surgeon (1976) that was modified by the author for use with neonatal brain tissue. The intensity of fluorescence emitted by brain sections was measured using a photomultiplier system devised by the author and cross-checked with the Leitz Photovolt 520M Photometer (Appendix 2). The brains of haloperidol and control offspring were processed using frontal sections so that three DA terminal areas: the striatum, nucleus accumbens, and olfactory tubercle could be analyzed within the same sections. The brains were double-mounted so that haloperidol-control or control-control pairs of brain sections could be processed and analyzed simultaneously on the same slides for the most direct comparisons. The brain sections were analyzed on postnatal day 15 and day 25 to determine the relative permanence of the observed changes.

A major finding in this study was that the nigrostriatal and mesolimbic-DA systems responded differently to haloperidol administration during the third trimester. The striatum of haloperidol offspring showed a decrease in the intensity of fluorescence whereas the nucleus accumbens and olfactory tubercle within the same brain sections showed an increase compared to controls. In comparing the normal pattern of glyoxylate induced fluorescence in these areas, one could see that there were

differences in the types and distribution of DA terminals. For example, nucleus accumbens and olfactory tubercle contained a coarse punctate pattern of fluorescence whereas the striatum contained a very fine, diffuse pattern of fluorescence. This description is similar to that reported by others using different histofluorescence techniques (Fuxe et al., 1974; Olson et al., 1972). The observation that the intensity of fluorescence and its pattern of distribution change in haloperidol offspring compared to controls provides the first morphological evidence that the development of central DA neurons is altered by prenatal haloperidol treatment and that the direction of change varies in different DA systems. The fact that these observations are still apparent by postnatal day 25 when DA systems are reaching maturity indicates that these changes are of a persistent, perhaps permanent nature.

There are a number of possible mechanisms that could account for the observed differences in response of nigrostriatal and mesolimbic systems to prenatal haloperidol administration. The results of studies in adult rats provide pharmacological (Creese et al., 1975; Seeman et al., 1978; Titeler et al., 1978; Thal et al., 1978), biochemical (Scatton et al., 1975; Julou et al., 1977; Asper et al., 1973; Bowers et al., 1974) and behavioural (Ungerstedt and Ljungberg, 1977; Kelly et al., 1975) evidence that the reactivity and function of these two DA systems differs (Introduction, Part B). For example, the mesolimbic and nigrostriatal systems respond differently to acute and chronic treatment with neuroleptics, including haloperidol, during adulthood (Scatton et al., 1975; 1976; Julou et al., 1977). In particular, long-term treatment produces a tolerance effect on rate of DA synthesis in the striatum but not in the

mesolimbic system (Asper et al., 1973; Bowers et al., 1974).

Since developing DA neurons share many properties in common with the adult system (Coyle and Henry, 1973) consistent differences in metabolic response of these two systems to prenatal haloperidol treatment may account for the opposite reactions observed in the present experiments. That is, haloperidol may induce a more rapid rate of DA synthesis within mesolimbic neurons that would appear as increased fluorescence intensity in terminal areas, whereas the tolerance effect might produce the opposite response in the nigrostriatal system. Hence, the observed differences in fluorescence intensity may be due to haloperidol-induced alterations in preterminal DA content that has long-term consequence on their subsequent development.

The consequences of haloperidol effects on DA availability may be different depending on the particular neural systems involved. In the case of the nigrostriatal system, there was a decrease in both the diffuse light green fluorescence and also in the amount of fine punctate fluorescence (Figure 11). This suggests not only a decrease in general DA content but perhaps also a deficit in number of presynaptic terminals reaching the striatum. A similar drop in fluorescence within offspring striata was observed by Tennyson et al. (1979) who administered the DA-depleting drug, reserpine, to rabbits during a comparative period of prenatal development. With the help of electron microscopic analysis, they reported many degenerative changes within the striatum including a decrease in presynaptic terminal growth. Taken together, these observations support the hypothesis that reduced availability of DA during prenatal development leads to a deficit in DA innervation of the striatum in

offspring. Since, in the present study, this deficit was somewhat less apparent by postnatal day 25, it may be initially due to a retardation in rate of nigrostriatal outgrowth. This may account for the larger decrease in striatal fluorescence at postnatal day 15 and a partial recovery by the later stage (Table 1).

In contrast to the nigrostriatal system, the mesolimbic terminal areas of haloperidol offspring showed an increase in clusters of punctate fluorescence. The possibility that this may be due to increased pre-terminal DA content and the fact that the two DA systems show a difference in pattern of innervation has already been discussed. An alternate hypothesis to account for these observations is that prenatal haloperidol antagonism leads to a compensatory increase in number of fibres innervating mesolimbic sites. Prenatal haloperidol antagonism might produce a selective blockade of feedback from postsynaptic target sites that may lead to a compensatory increase in presynaptic terminal growth. Johnston et al., (1979) have recently reported an increase in the density of nor-epinephrine innervation of rat cortex after selective destruction of target cells in the cortex during fetal development. An earlier study by Sachs and Jonsson (1975) showed a similar hyperplasia of noradrenergic fibres after prenatal treatment with a catecholamine-specific toxin, 6-hydroxydopamine. They found that attenuation of the development of noradrenergic neurons in one part of the brain lead to a compensatory hyperplasia of fibres to the other target regions.

The findings of the latter study may account not only for the increase in mesolimbic fluorescence observed in the present study, but also for the simultaneous decrease in nigrostriatal fluorescence. The

intensity of fluorescence was decreased not only in the striatum, but also in the cell body area of the substantia nigra (Table 1). There is evidence, on anatomical grounds, for a feedback pathway between this area and mesolimbic efferents (Nauta et al., 1978). In addition, there is anatomical proximity between mesolimbic and nigrostriatal feedback loops that offers the potential for interactions between these DA systems (Nauta et al., 1978). In view of the likelihood that the function of these two systems is closely integrated, it is not surprising that deficits in the development of the nigrostriatal system may lead to compensatory changes in the growth of the mesolimbic system, particularly if the growth of the two systems is not completely synchronous. This might also account for there being a progressive increase in mesolimbic fluorescence that is most apparent by postnatal day 25 (Table 1).

The foregoing hypotheses to account for changes in fluorescence emitted by nigrostriatal and mesolimbic systems are still conjectural and further microscopic and neurochemical studies must be done to test their validity. The results of the present study do, however, indicate that prenatal treatment with haloperidol interferes with the normal development of presynaptic DA neurons and that the effects of this treatment on nigrostriatal development differs from that of the mesolimbic DA system. Furthermore, in both cases, the duration of effects appear to be long term, perhaps even permanent.

B - ONTOGENY OF STRIATAL DOPAMINE-SENSITIVE ADENYLATE CYCLASE FOLLOWING PRENATAL HALOPERIDOL

Introduction

The results of previous experiments (Part A) provide morphological evidence that presynaptic dopamine (DA) neural development is altered by haloperidol treatment during the third trimester. The observed decrease in DA-induced fluorescence indicates that there may be a decrease in DA content in the striatum of the offspring during synaptogenesis. The experiments described in this section were designed to determine whether this decrease of DA in the striatum would affect the development of the postsynaptic enzyme, DA-sensitive adenylyate cyclase, that is thought to be coupled to DA receptors in the striatum (Kebabian et al., 1972; Greengard, 1976). At the time this work began, nothing was known about the normal ontogeny of the DA-sensitive adenylyate cyclase in the striatum of the rat. A number of prior studies in the literature had described the ontogeny of adenylyate cyclases in different parts of the brain, either in the whole brain (Schmidt et al., 1970; Kohrman, 1973) or in large subdivisions of the brain (Weiss, 1971; Von Hungen et al., 1973; Perkins and Moore, 1973). The results of these studies indicated there to be a difference in the developmental pattern of adenylyate cyclases in various parts of the brain. The first objective of this study, therefore, was to establish the normal developmental pattern of the adenylyate cyclase that is specifically sensitive to DA within the rat striatum.

Striatal DA-sensitive adenylyate cyclase activity has been studied

rath~~er~~ extensively in the adult rat. Many studies report that chronic treatment with neuroleptics, including haloperidol, produce an increase in DA-sensitive adenylate cyclase activity (Gnegy et al., 1977; Burkard, and Bartolini, 1974; Iwatsubo and Clouet, 1975) although this is not unanimous (Rotrosen et al., 1975; Heal et al., 1976; Hyttel, 1978). Since this enzyme is thought to be coupled to the postsynaptic DA receptor (Mishra et al., 1974; Iversen, 1975; Greengard, 1976) these data have been taken to indicate that chronic haloperidol treatment produces postsynaptic DA receptor supersensitivity (Gnegy et al., 1977; Burkard and Bartholini, 1974; Iwatsubo and Clouet, 1975). Nothing, however, is known about the effects of prenatal haloperidol administration on offspring DA-sensitive adenylate cyclase activity in the striatum. The second objective of this study, therefore, was to determine whether the administration of haloperidol during the third trimester alters the normal development of postsynaptic DA-sensitive adenylate cyclase.

Methods

Treatment Procedures: Haloperidol or vehicle were administered to Wistar rats during the third trimester, and the offspring fostered at birth, as described in the Experimental Design section of this thesis.

Animals: To establish the normal ontogeny of DA-sensitive adenylate cyclase in the striatum of the rat, the activity of this enzyme was measured in striatal homogenates of rat pups starting at postnatal day 2 until 60 days. Since other brain adenylate cyclases show a rapid rate of development during the earlier stages of development (Van Hungen et al., 1973) striatal DA-sensitive adenylate cyclase was measured at frequent

intervals, that is 3-day intervals up to postnatal day 15, and 5-day intervals up to postnatal day 25. To determine whether any further changes in activity occurred during the later stages of development, DA-sensitive adenylate cyclase was measured at longer intervals, for example, 10 days apart, up to the postnatal day 60.

Once the normal pattern of striatal DA-sensitive adenylate cyclase ontogeny was established, the enzyme activity in the striata of haloperidol and control offspring were compared at 5 and 7 day intervals during postnatal development. To control for inter-experimental variations, enzyme activity in the striata of both haloperidol and control offspring of any given age were analyzed within the same assay. To avoid possible inter-litter variations, haloperidol and control offspring drawn simultaneously from a number of litters, usually 6 vehicle and 6 haloperidol, at each stage of development analyzed. Consequently, the littermates of these offspring that remained were used for subsequent assays as they became progressively older, thereby providing internal consistency in sampling across all ages analyzed. In addition, some of these littermates were used for the other morphological, biochemical and behavioural measures included in this thesis study so that correlations between these measures could be assessed (Methods and Results, Parts A, C and D).

Dopamine-Sensitive Adenylate Cyclase Assay: The activity of striatal DA-sensitive adenylate cyclase was determined using the method of Mishra and Makman (1974). Rat pups of the appropriate ages were removed from their litters and immediately decapitated. Their striata were dissected out within 60 seconds. Striatal tissue of pups less than a week old were pooled within groups. The tissue was quickly weighed then

immersed in ice cold 2mM Tris-maleate buffer (pH 7.4) plus 0.8mM EGTA (ethylene bis (oxyethylenitrilo)-tetraacetic acid) and homogenized at a dilution of 1:75 (weight:volume). All samples were assayed in duplicate. Striatal homogenate (50 μ l) was added to the reaction mixture containing 80mM tris-maleate, 10mM theophylline and 2mM magnesium sulfate. The activity of DA-sensitive adenylate cyclase was measured in the presence of 10 μ M DA and 10 μ M fluphenazine in a final volume of 200 μ l. The reaction was initiated by the addition of 1mM ATP. Samples were incubated at 30°C for 2.5 minutes. The reaction was terminated by placing samples in a water bath at 100°C for 2.5 minutes. Samples were centrifuged at low speed (900 g) in a Sorvall GLC-1 centrifuge for 10 minutes. Supernates were removed and placed on ice or stored at -40°C.

Assay of Adenosine 3'5'-cyclic Monophosphate (cyclic AMP): The product of the adenylate cyclase reaction, cyclic AMP, was measured using the competitive binding assay of Brown et al., (1971). Other assay systems were also tested in preliminary experiments, including the cyclic nucleotide chromatographic system of Krishna and Birnbaumer (1970) and the commercially prepared cyclic AMP Assay Kit (TRK 432 - Amersham/ Searle). The results obtained with all three systems were the same. Hence, the method of Brown et al. (1971) was chosen on the basis of its comparatively lower cost per sample and the relative simplicity of the procedures involved.

The assay included the addition of 1 picomole (pmole) in 100 μ l of tritiated cyclic AMP (specific activity: 21 Ci/mole, New England Nuclear) to all assay tubes that were arranged in duplicate. Standard curves were constructed using dilutions of cyclic AMP in the range from

0 to 6.0 pmoles. Tissue samples (25 μ l) were added in place of cyclic AMP standards and brought up to a volume of 50 μ l with deionized water. The reaction was initiated by the addition of 50 μ l of binding protein prepared from rat brain tissue and purified according to the method of Dr. R. Mishra, Department of Pharmacology, McMaster Health Science Centre (personal communication, Appendix 3). The samples were incubated in a double ice bath for 90 minutes in the cold room. The reaction was terminated by the addition of 1 ml of 0.2 M potassium phosphate buffer (pH 6.0). Cyclic AMP bound to binding protein was separated from the unbound by filtration through cellulose filters (Millipore 0.45 μ m HA) using a filtering manifold (Millipore 03025) at a negative pressure of 20 mm Hg. The filters were dried for 10 minutes under a heat lamp source then immersed in 5 mls of Scintillation cocktail composed of 5g PPO (2,5-diphenyloxazole), 0.1g POPOP (1,4-Bis(Z-(5-Phenyloxazolyl) Benzene) per litre of Toluene. The samples were equilibrated in the cold room then counted in a Beckman LS-230 scintillation counter.

Protein Determinations: The amount of protein in striatal homogenates was measured using the Lowry Protein Assay (Lowry et al., 1951). Standard curves were constructed using dilutions of bovine serum albumin (BSA). Optical densities of the samples were determined using a Spectronic 700 Colorimeter (Bausch and Lomb).

Data Analysis: The data was analyzed by analysis of variance (ANOVA) that compared treatment (haloperidol vs vehicle) by age of offspring (described above) by drug dose (0.26 vs 0.6 vs 1.2 mg/kg of haloperidol) effects on each of the 3 parameters of DA-sensitive adenylate cyclase activity measured : basal, DA-stimulated and fluphenazine inhibited activity.

Results

Normal Ontogeny of Striatal Dopamine-Sensitive Adenylate Cyclase:

DA-sensitive adenylate cyclase activity in the striata of normal rats was measured starting postnatal day 2 to 60 days after birth. The normal pattern of ontogeny is shown in Figure 14. Adenylate cyclase activity was expressed as pmoles of cyclic AMP produced per mg of protein per minute of incubation. Basal activity was measured in the presence of the substrate, 1mM ATP. DA-stimulated activity was determined by the addition of 10 μ M dopamine. Since there are a variety of catecholamine-sensitive adenylate cyclases in the brain, it was necessary to determine whether that being measured in this preparation was the specific DA-sensitive adenylate cyclase. To test this, 10 μ M fluphenazine, a specific DA antagonist was also added to the reaction mixture at the different ages. As shown in Figure 1, the addition of fluphenazine completely inhibited DA-stimulated adenylate cyclase activity. This activity approximated basal levels indicating that the enzyme being measured was the specific DA-sensitive enzyme.

In the absence of substrate ATP, levels of endogenous cyclic AMP in striatal homogenates were very low, less than 50 pmoles/mg of protein throughout development (Figure 14). Basal adenylate cyclase activity was also low during the first postnatal week, ranging from 50 to 75 pmoles of cyclic AMP produced per mg protein. This level of activity gradually increased with age till it reached approximately 200 pmoles/mg/minute by postnatal day 25. Basal activity then remained at this level until postnatal day 60, the oldest age tested.

Striatal adenylate cyclase activity increased in response to 10 μ M

ONTOGENY OF STRIATAL DA ADENYLATE CYCLASE

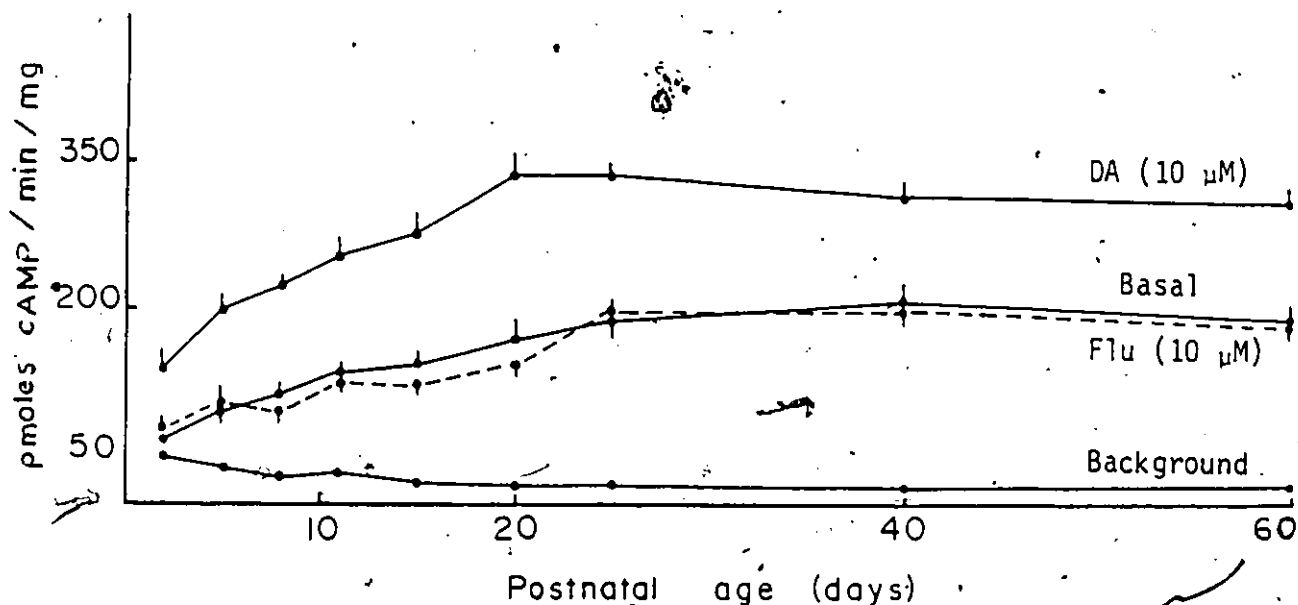


Figure 14: The normal ontogeny of dopamine-sensitive adenylylate cyclase activity in the striatum of rats is shown above. Striatal homogenates were prepared from normal rats aged 2 days to 60 days postnatally. Four parameters were measured in quadruplicate within each assay: (1) background activity (endogenous cyclic AMP in the absence of exogenous ATP); (2) basal activity (addition of 1 mM ATP); (3) DA-stimulated activity (1 mM ATP + 10 μM DA); and (4) fluphenazine-inhibited activity (1 mM ATP + 10 μM DA + 10 μM Flu). Each point represents the mean ± sem of at least 8 separate assays. Striatal tissue was pooled on postnatal day 2 to provide sufficient homogenate for assay.

DA as early as postnatal day 2 (Figure 14). This sensitivity appeared to be greatest during the first two postnatal weeks at which time adenylylate cyclase activity increased 2.5 fold with the addition of $10\mu\text{M}$ DA. During the subsequent week of development as basal activities continued to rise, DA produced only a 2 fold increase in striatal adenylylate cyclase activity. Then when basal activity reached a peak after the third postnatal week, the level of sensitivity to DA dropped slightly and remained relatively stable up to 60 days. During this stage of development, $10\mu\text{M}$ DA produced a 1.5 fold increase in striatal adenylylate cyclase activity over basal levels.

Ontogeny of Striatal Adenylylate Cyclase after Prenatal Haloperidol:

Once the normal ontogeny of striatal cyclase was established, the effect of prenatal haloperidol administration on this development was evaluated. DA-sensitive adenylylate cyclase activity was evaluated in striata of offspring whose mothers received either 0.26, 0.6 or 1.2 mg/kg of haloperidol daily during the third trimester. Cyclase activity was sampled at key stages of development determined in the preliminary series of experiments, including postnatal days 2, 15, 25, 40 and day 60. These data are summarized in Figure 15.

In all three series of experiments, basal (1mM ATP), DA ($10\mu\text{M}$) stimulated and fluphenazine ($10\mu\text{M}$) inhibited adenylylate cyclase activity was measured in the striata of haloperidol and control offspring. Since fluphenazine always reduced cyclase activity to basal levels, only DA-stimulated and basal activities have been included in Figure 2. Also since background levels of cyclic AMP were negligible, as in the first series of experiments, they have not been included in these graphs.

ONTOGENY OF DA-SENSITIVE ADENYLATE CYCLASE

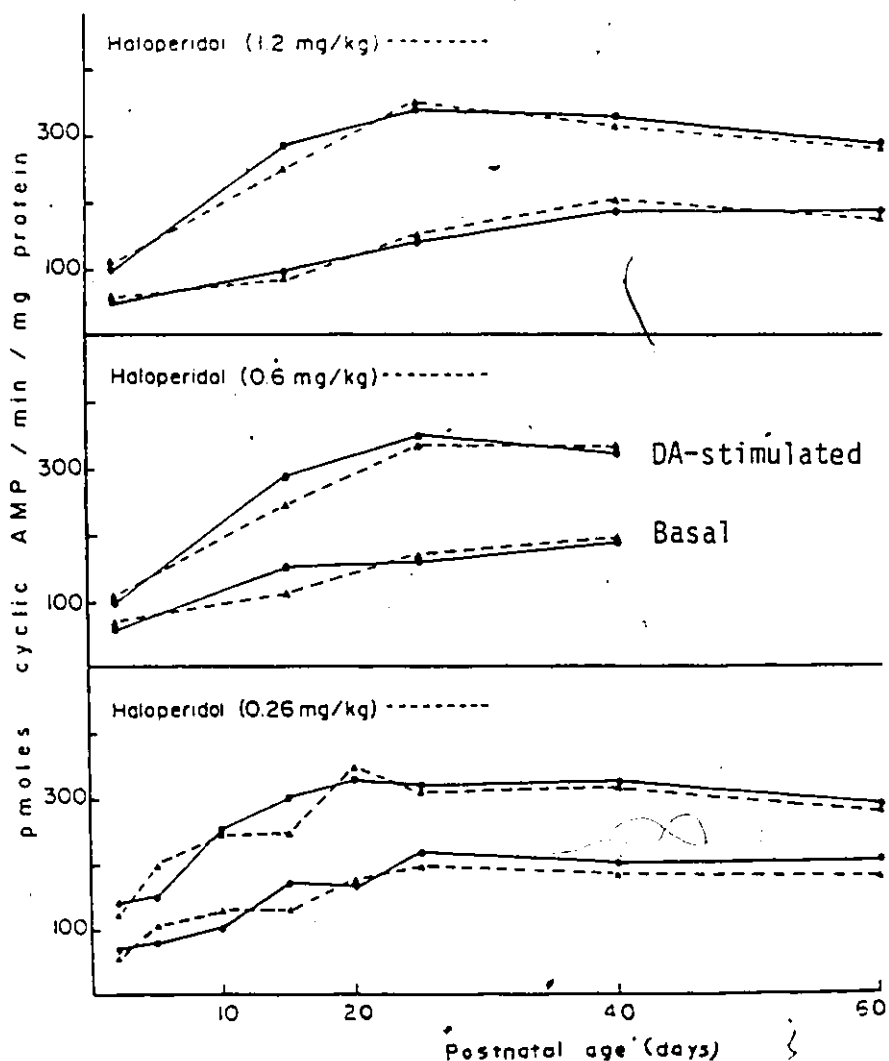


Figure 15: DA-sensitive adenylate cyclase activity in striata of offspring after maternal treatment with haloperidol (0.26, 0.6 or 1.2 mg/kg) or vehicle (—) is summarized above. Basal (1 mM ATP) and DA-stimulated (10 μ M DA) activity from postnatal day 2 to 60 is shown. Each point represents the mean of at least 8 separate assays. There was no significant difference in the activity of DA-sensitive adenylate cyclase activity of haloperidol compared to control offspring at any age.

DA-sensitive adenylate cyclase activity was compared in striatal homogenates of haloperidol and control offspring that were always measured in the same assay at each respective stage in development. As can be seen in Figure 15, there was no significant difference in either basal or DA-stimulated adenylate cyclase activity at any of the ages tested. Despite increasing the dose of haloperidol administered during the third trimester from 0.26 mg/kg to 1.2 mg/kg, there was no significant change in the ontogeny of striatal cyclase in haloperidol offspring striata compared to controls.

The results of statistical analysis (ANOVA) indicated that there was a significant ($p < .01$) age effect on basal, DA-stimulated and fluphenazine-inhibited adenylate cyclase activity. That is, there was a significant increase in all three with age up to postnatal day 25. Thereafter, no further significant changes occurred in these measures. There was no significant difference in background levels of endogenous cyclic AMP levels at any age tested nor after any dose of haloperidol administered.

There was also no significant dose-effect on any of these measures. There was no change in the normal ontogenic pattern of basal, DA-stimulated or fluphenazine-inhibited DA-sensitive adenylate cyclase activity in the striata of offspring at any age following maternal administration of haloperidol at any of the doses tested.

Discussion

DA-sensitive adenylate cyclase in the striatum of the neonate shows a low basal activity during the first few days after birth (Figure

14). The specific activity of the enzyme then increases progressively until reaching adult activity by 3-4 weeks after birth. DA produces a significant stimulation of cyclic AMP production as early as postnatal day 2. The absolute amount of cyclic AMP formed is only about 40% of that measured in the adult, although the stimulation by $10\mu\text{M}$ DA over basal activity is higher in the neonate as compared to the adult stage. This is because the DA-sensitive activity increases 2.5 fold from postnatal day 2 to adulthood, whereas the increase in basal activity is 4-fold. These data were confirmed by the results of Coyle and Campochiaro (1976) who reported their study on the ontogeny of DA-cholinergic interactions in the striatum just as these experiments were completed. Their experimental procedures differed somewhat in that they used cell free homogenates of striatal tissue, different assay systems and $50\mu\text{M}$ DA to stimulate cyclase activity. Despite these differences, they found a similar pattern of basal and DA-sensitive adenylate cyclase ontogeny in rat striatum. In addition, they found that the developmental rise in the activity of DA-sensitive cyclase parallels but clearly precedes the developmental increase in three presynaptic markers for DA terminals: tyrosine hydroxylase, endogenous DA and synaptosomal uptake of ^3H -DA. Taken together, these data appear to indicate that the development of postsynaptic DA-sensitive cyclase precedes the ingrowth of DA terminals.

The results of these thesis experiments also agree with those recently reported by Enjalbert et al. (1978a) who compared the ontogeny of DA-sensitive and serotonin-sensitive cyclase in different areas of rat and guinea-pig brains. They found a similar pattern of ontogeny of DA-sensitive cyclase in rat striatum as observed in this study, although their

procedures also differed to some extent. For example, they used different assay systems and a higher dose of DA, 100 μ M, to stimulate cyclase activity. Nevertheless, they found similar increases in basal relative to DA-sensitive cyclase activity from birth to adulthood. Also in agreement with the results of this study, they found that striatal DA-sensitive adenylate cyclase was very sensitive to inhibition by 10 μ M fluphenazine, an effect that is apparent as early as postnatal day 2. Fluphenazine used at this concentration had no effect on basal adenylate cyclase activity (Enjalbert et al., 1978b). On the basis of these data, it appears that although there are some similarities between serotonin and DA-sensitive adenylate cyclases, they show important differences in terms of their distribution, pattern of ontogeny and sensitivities to DA antagonists. Both cyclases, in turn have properties that are very different from other catecholamine cyclases in the brain (Enjalbert et al., 1978a; 1978b).

Also of interest to this study are the findings of Pardo et al. (1977) who recently investigated the ontogeny of DA receptor binding in the rat striatum using 3H-haloperidol as the ligand. In contrast to the developmental pattern of DA-sensitive adenylate cyclase observed in this study, they found that 3H-haloperidol binding is very low at birth and remains low during the first postnatal week. DA-sensitive cyclase activity during this period shows significant increases in specific activity. (Figure 14). Between the third and fourth weeks, 3H-haloperidol binding is reported to double, while only a minimal increase in cyclase activity is observed at this time. These differences in developmental pattern leave unresolved the relationship between postsynaptic DA-sensitive cyclase and the 3H-haloperidol labeled receptor. Pardo et al.

(1977) concluded that the earlier development of the DA-sensitive adenylylate cyclase supports the hypothesis that the postsynaptic DA receptor labeled by 3H-haloperidol and the cyclase may be separate entities. They also proposed that these receptors are associated with postsynaptic cholinergic neurons that show a postnatal pattern of development that closely parallels the ontogeny of 3H-haloperidol binding sites (Pardo et al., 1977).

The results of the second phase of experiments, investigating the effects of prenatal haloperidol treatment on the development of DA-sensitive adenylylate cyclase in striata of offspring, give further support to the hypothesis that this enzyme and the receptors that bind haloperidol may be separate entities. There was no change in the ontogeny of cyclase activity at any of the ages tested from postnatal day 2 to adulthood. The ontogeny of striatal cyclase was the same in control and haloperidol offspring whose mothers received haloperidol in doses of 0.26, 0.6 and 1.2 mg/kg (Figure 15). These doses of haloperidol have previously been found to alter other aspects of DA neural development. For example, Lundborg (1972) reported that the administration of haloperidol at the dose equivalent of 0.2 mg/kg to nursing rabbits for one week led to a retardation of motor development in the offspring. This effect was attributed to the passage of haloperidol via the mother's milk to block DA receptors in the brains of offspring during synaptogenesis. The results of the histofluorescence study described previously indicate that haloperidol at 1.2 mg/kg interferes with presynaptic terminal development and produces a long term decrease in striatal DA content (Methods and Results, Part A; Plach et al., 1980). Hence, the lack of effect on striatal DA-sensitive cyclase

indicates that haloperidol may be acting at some site that is independent of this enzyme.

The results of studies using adult animals indicate that chronic haloperidol treatment produces changes in striatal receptors that bind butyrophenones like haloperidol and spiroperidol (Muller and Seeman, 1978) despite an inconsistent effect on striatal cyclase (Gnegy et al., 1977; Heal et al., 1976; Hyttel, 1978). If the DA-sensitive adenylyate cyclase and the postsynaptic haloperidol receptor are separate entities, then the outstanding question is whether one might expect to find receptor changes in the absence of cyclase changes in offspring striata after haloperidol treatment during early development. This is one of the questions investigated by the author in a subsequent series of experiments (Methods and Results, Part C).

C EFFECTS OF PRENATAL HALOPERIDOL ON POSTSYNAPTIC STRIATAL DOPAMINE RECEPTORS

Introduction

The third trimester of the rat is the critical period of initial synaptogenesis of brain dopamine (DA) systems (Loizou, 1972; Kellogg and Wennerstrom, 1974). It is likely, therefore, to be the period when central DA receptors are just beginning to develop. The question raised by this study is whether the availability of DA to interact with postsynaptic target sites is critical to the normal development of postsynaptic receptors. It is hypothesized that blockade of postsynaptic DA sites during the third trimester will interfere with the normal development of DA receptors.

Haloperidol is a potent antagonist of postsynaptic DA receptors (Iversen, 1975; Seeman et al., 1975). Muller and Seeman (1978) have recently reviewed the evidence that chronic treatment with haloperidol and similar neuroleptics in adult rats produces an increase in postsynaptic DA receptors in the striatum. This increase may be a compensatory mechanism by which the DA system increases postsynaptic responsiveness to compensate for a reduction in DA availability (Dismukes and Daly, 1976; Muller and Seeman, 1978). Nothing is known about the effects of haloperidol treatment during the discrete period of initial synaptogenesis. It is known that a reduction in the availability of DA at this time leads to long term defects in both presynaptic and postsynaptic components of the striatal DA system (Tennyson et al., 1979). In addition, the results of the histo-

fluorescence study described previously in this thesis indicate that haloperidol treatment during the third trimester produces a deficit in DA terminal development in the striatum of offspring, an effect that persists as long as postnatal day 25 (Methods and Results, Part A). The purpose of the present study is to determine whether this deficit in the development of striatal innervation leads to a concurrent alteration in postsynaptic striatal DA receptors at postnatal day 25 and whether the effects produced by haloperidol are similar to those observed in the adult.

Methods

Treatment Procedures: Haloperidol or vehicle were administered to Wistar rats during the third trimester as was described in the Experimental Design of this thesis. The offspring were used for the binding studies when they reached 25 days of age (Figure 8). In the series of experiments on adult animals, male Wistar rats (approximately 300 grams) received daily injections with increasing doses of haloperidol for 3 weeks as described by Friend et al. (1978). The protocol involved daily injection with 0.6 mg/kg of haloperidol for the first 4 days, then 1.2 mg/kg and 2.5 mg/kg for the next 2 sets of 4 days followed by 5.0 mg/kg for 8 days. Controls received injections of the vehicle that was used to dissolve each dose of haloperidol. A 6 day washout period was allowed before the animals were decapitated and the binding experiments were done.

Tissue Preparation: The tissue was prepared according to the method of Seeman et al. (1975, 1976, personal communication). Immediately after decapitation, the striatal tissue was dissected out on ice, then placed in ice cold TEAN buffer containing 14mM Tris, 5mM Na₂EDTA, 1.1mM ascorbate,

12.5 μ M nialamide and titrated to pH 7.4 with HCl. The stock solution of TEAN was stored at 10X concentration at -70 $^{\circ}$ C. Portions were thawed and diluted as required before each assay.

For homogenization and assay, striatal tissue was pooled within groups. The tissue was homogenized in a dilution of 10X volume using a Sorvall rotary homogenizer and teflon pestle rotated at 500 rpm using 20 up and down strokes. Equal volumes of buffer were used to rinse out the homogenizing vessel, then the homogenate was centrifuged in a Sorvall RC2B centrifuge at 30,000 g for 20 minutes at 4 $^{\circ}$ C. The supernate was discarded and the pellet resuspended in ice cold TEAN at 25 3 mg/ml using a Brinkmann polytron (Rexdale, Ontario) for 20 seconds. The striatal tissue from haloperidol and control brains were always prepared at the same time, using the same materials and buffers.

Dopamine Receptor Binding Assay: 3 H-spiroperidol (specific activity 25.64 Ci/mmol) obtained from New England Nuclear (Boston, Mass.) was used as the ligand. The procedures followed were those of Seeman et al. (1975, 1976, personal communication). In summary, the incubation mixture consisted of 0.2 ml of (+) or (-) butaclamol (1 μ M) plus 0.2 ml of 3 H-spiroperidol at each of the following concentrations: 0.05, 0.15, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 nM. All samples were done in triplicate. The incubation was initiated by the addition of 0.2 ml of resuspended striatal homogenate (final dilution of 25 mg wet weight/ml) in a staggered fashion and vortexing (DeLuxe Mixer, Scientific Products, Illinois) after each addition. The incubation was done at room temperature for 30 minutes, vortexing every 5 minutes. Separation of membrane-bound 3 H-spiroperidol was accomplished by pipetting 0.5 mls of sample with a Pipetman (Gilson,

France) onto a glass fibre filter (Whatman GF 1B, 24 mm diameter) supported by a stainless steel grid and washing twice with 5 mls each of TEAN buffer. Care was taken to ensure that the filtration was standardized under a constant negative pressure of -25mm HG over a period of 10 seconds. The filters were placed in Nalgene baggies (Sybron/Nalge, New York) and immersed in 3 mls of Canamix Scintillant (Canatech Inc., Dorval, P.Q.) then allowed to equilibrate for 10 hours in the cold room before counting in a Beckman LS230 scintillation counter.

Protein Determinations: The protein concentration in striatal homogenates was determined using the standard Lowry Protein Assay (Lowry et al., 1951). Standard curves were constructed using dilutions of bovine serum albumin (BSA, Sigma). The optical densities were determined using a Spectronic 700 colorimeter (Bausch and Lomb).

Data Analysis: Scatchard analysis (Scatchard, 1949) was used to determine the number of binding sites and the affinity of the binding in each experiment. Specific binding was defined as the amount of ^3H -spiroperidol bound in the presence of (-) butaclamol minus that bound in the presence of (+) butaclamol. The data for each experiment was graphed on a Scatchard plot in which the amount of ligand Bound was plotted against the amount Bound/Free for each of the 8 concentrations of ^3H -spiroperidol. The amount bound at each concentration of ^3H -spiroperidol was determined as specific binding (dpm) converted to concentration in femtomoles per milligram (mg) of protein. The amount Free at each concentration of ^3H -spiroperidol was determined as the total number of counts (dpm) converted to concentration (nM) of free ligand.

Linear regression analysis was used to determine the number of

binding sites and their affinity for each binding experiment. The values calculated included X and Y intercepts, slopes of the regression lines and correlation coefficients. The number of receptor binding sites (Bmax) was indicated by the X intercept and the binding affinity (the dissociation constant, KD) was determined by the negative reciprocal of the slope of the regression line (Scatchard, 1949). For statistical analysis, the results of all binding assays within each series of experiments were compared using a multiple linear regression analysis and F tests (Kleinbaum and Kupper, 1978).

Results

Increased Striatal Binding in Adult Rats After Chronic Haloperidol

Treatment: The purpose for testing the effects of chronic haloperidol treatment on striatal receptor binding in the adult rat was two-fold. First, it was necessary to determine what effects this treatment would have specifically on striatal ³H-spiroperidol binding, since this was the ligand to be used in subsequent experiments. Secondly, the results of these experiments would provide a standard for comparison of the effects of haloperidol treatment during prenatal development on offspring striatal receptor binding.

³H-spiroperidol binding in the striata of haloperidol-treated and control (vehicle-treated) adult rats was assayed within the same experiment on three separate occasions using separate pools of tissue. The data from both haloperidol and control striata are presented on the same graph in Figure 16. Scatchard analysis of these data indicated that the administration of haloperidol for 3 weeks produced an increase in the

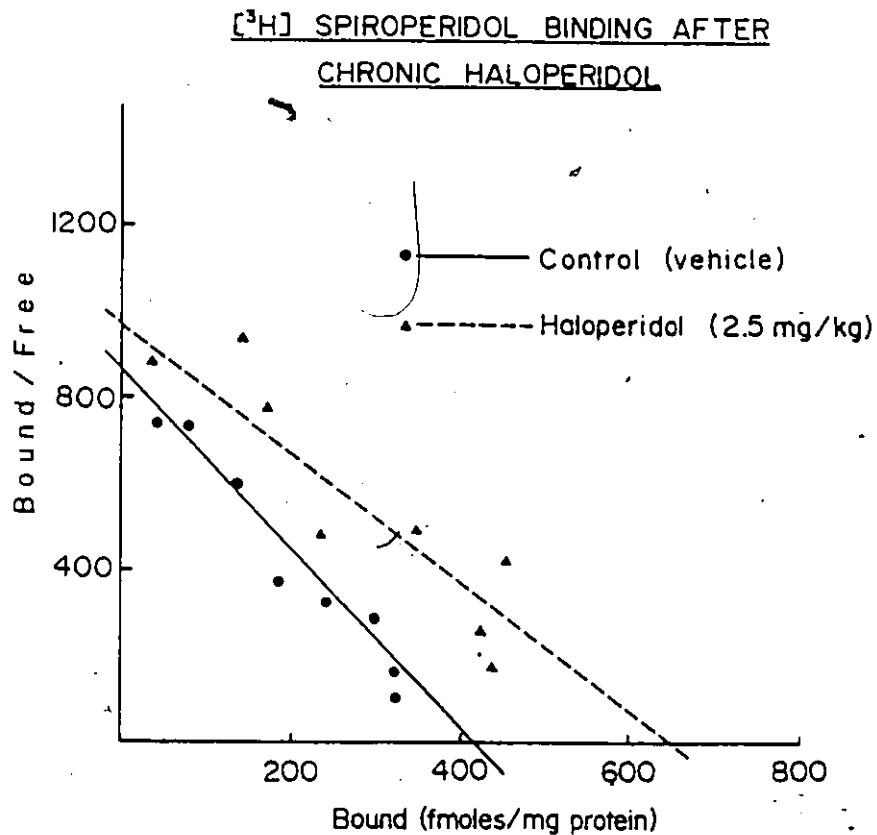


Figure 16: Scatchard plots of ³H-spiroperidol binding in the striata of adult male rats after 3 weeks treatment with haloperidol or vehicle. B_{max} values for haloperidol and control striata are 682 and 420 fmoles/mg protein respective. K_D values are .68 nM and .48 nM respectively. The correlation coefficients of the points on these regression lines are .95 and .97 for haloperidol and control data. See text for further details.

number of ^3H -spiroperidol binding sites within the adult striatum.

As seen in Figure 16. The number of binding sites (B_{max}) in haloperidol striata was 682 fmoles/mg protein while that in controls was 420 fmoles/mg protein. This represented a 38% increase in ^3H -spiroperidol binding sites within the striatum of haloperidol animals. The values for the second and third experiments of this series were 650 and 587 for haloperidols and 393 and 362 for controls, respectively (graphs not shown). The increase in binding sites in haloperidol compared to controls was 40% and 38% respectively. Statistical comparisons of the X intercepts within these experiments indicated that the B_{max} values of haloperidol and control were significantly different ($p < .05$) in all cases. These data indicated that the increase in number of binding sites was highly reproducible and that inter-experimental variation was relatively small.

The binding affinity (KD) of ^3H -spiroperidol sites was .48nM in control striata and .68nM in haloperidol striata. In the second and third experiments of this series, the values were .66 and .65nM for controls and .65 and .52nM for haloperidol striata respectively. Multiple regression analysis indicated that there was no statistically significant difference in the binding affinities within haloperidol and control striatum.

The correlation coefficients of the data presented in Figure 16 were .97 and .95 for control and haloperidol data, respectively. They ranged from .95 to .98 for the data obtained in all three binding assays done in this series of experiments. This indicated that there was a relatively high correlation between the slopes of the regression lines as estimated by linear regression analysis, and the individual sets of points

they represented.

Decreased Striatal Binding in Day 25 Offspring After Prenatal

Haloperidol: ³H-spiroperidol binding in the striata of offspring whose mothers received haloperidol injections during the third trimester was analyzed on postnatal day 25. Three series of experiments were done in which either 0.6, 1.2 or 2.5 mg/kg of haloperidol was administered. Within each of these series of experiments, at least 3 separate binding assays were done, including both haloperidol and control striatal tissue within the same assay. The procedures followed were the same as those used in the adult binding experiments.

The results of striatal ³H-spiroperidol binding in day 25 offspring whose mothers received 1.2 mg/kg are shown in Figure 17. Scatchard plots of both haloperidol and control data are presented on the same graph. The values for Bmax were 569 fmoles/mg in controls and 422 fmoles/mg in haloperidol striata. The KD values were .57nM and .47nM, respectively. Regression analysis of the data indicated a correlation coefficient of .92 and .96 respectively. A comparison of the slopes of these regression lines and their X intercepts using t-test analysis indicated that there was a significant decrease (P <.001) in the Bmax of haloperidol striata while there was no significant difference in the binding affinities for ³H-spiroperidol.

The results obtained in the other 2 binding assays within this series of experiments, as well as those within the 0.6 mg/kg and 2.5 mg/kg series of experiments all show a similar pattern. These data are summarized in Table 2. A comparison of Bmax values indicated that there was a 25-30% decrease in number of ³H-spiroperidol binding sites in haloperidol

[³H] SPIROPERIDOL BINDING IN STRIATA
AFTER PRENATAL HALOPERIDOL

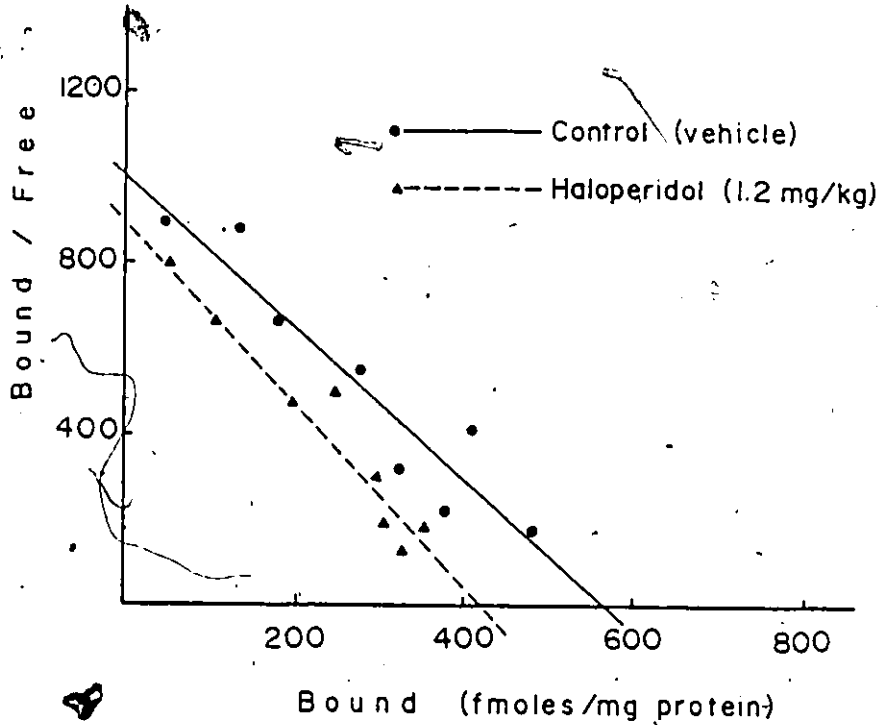


Figure 17: Scatchard plots of ³H-spiroperidol binding in the striata of 25 day old offspring after maternal treatment with haloperidol or vehicle during the third trimester. Bmax values for haloperidol and control striata are 422 and 569 fmoles/mg protein, respectively. KD values are .47 nM and .57 nM. The correlation coefficients of the points on these regression lines are .96 and .92 for haloperidol and control data. See text for further details.

Table 2

SUMMARY OF ³H-SPIROPERIDOL BINDING IN STRIATA OF DAY 25 OFFSPRING
AFTER HALOPERIDOL ADMINISTRATION DURING THE THIRD TRIMESTER

Haloperidol Series	Group	\bar{x} Bmax (Range) fmoles/mg protein	\bar{x} KD (Range) nanomolar	r	Range
0.6 mg/kg n=2	Con	520 (491 - 549)	0.54 (0.53 - 0.54)	0.92	0.95
	Hal	498 (417 - 578)	0.44 (0.33 - 0.55)	0.92	0.97
1.2 mg/kg n=3	Con	578 (563 - 604)*	0.54 (0.49 - 0.57)	0.92	0.98
	Hal	437 (422 - 459)*	0.46 (0.40 - 0.47)	0.95	0.96
2.5 mg/kg n=3	Con	573 (553 - 593)*	0.55 (0.49 - 0.59)	0.92	0.94
	Hal	399 (382 - 417)*	0.39 (0.33 - 0.48)	0.93	0.98

Properties of ³H-Spiroperidol binding in striata of day 25 offspring whose mothers were treated during the third trimester with haloperidol at doses of 0.6, 1.2 and 2.5 mg/kg were determined using Scatchard analysis. Above are summarized the means of Bmax (number of binding sites) and KD (binding affinity) values as well as their range and correlation coefficients (r) from individual binding assays (n =) at each dose of haloperidol tested. The asterisk (*) indicates that there is a significant difference (p < .01) in the data obtained from the striata of haloperidol (Hal) and control (Con) offspring that were assayed concurrently within the same binding experiments.

offspring striata compared to controls assayed within the same experiments. In the case of the 1.2 mg/kg and 2.5 mg/kg series of experiments, this decrease was statistically significant at $p < .01$. In the case of the 0.6 mg/kg series of experiments, the lowest dose of haloperidol tested, there was more variation in B_{max} values than observed at the higher doses tested, hence, although the same trend was apparent, the difference did not reach statistical significance.

There appeared to be a slight increase in the affinity of 3H -spiroperidol binding in haloperidol offspring striata compared to controls. The results of the multiple regression analysis, however, indicated that the slopes of the regression lines for haloperidol and control data were not significantly different.

Increased Striatal Binding in Maternal Rats after Haloperidol

Treatment during the Third Trimester: In several experiments, the 3H -spiroperidol binding in the striata of maternal rats after haloperidol treatment during the third trimester was also analyzed. The purpose of these experiments was two-fold. First, it was important to determine whether this treatment was sufficient to produce changes in striatal receptor binding in maternal rats. The results would serve as a direct comparison for the effects of this treatment on their offspring. Secondly, the effects of maternal haloperidol treatment during the final week of pregnancy would be compared with those obtained in the preliminary experiments, after chronic treatment of adult males. This was of interest since most studies reported in the literature use higher doses of haloperidol and longer periods of treatment than were used in the present experiments (Muller and Seeman, 1978). It was necessary, therefore, to

determine the potency of haloperidol at the doses and under the same conditions used in these experiments.

The effects of haloperidol treatment during the third trimester on ³H-spiroperidol binding in maternal striata are presented in Figure 18. These experiments were done after a 6-day washout period to provide time for the elimination of endogenous haloperidol from the maternal system. Scatchard plots of both control and haloperidol data are shown in the same graph. The dose of haloperidol, 1.2 mg/kg, used in this experiment was the same as that used in the offspring experiments whose data is shown in Figure 17. In fact, these offspring were drawn from litters that were delivered by these maternal rats.

Maternal treatment with haloperidol led to an increase in the number of ³H-spiroperidol binding sites (Figure 18). The Bmax value for control striata was 357.1 fmoles/mg protein while that of haloperidol was 645 fmoles/mg protein. This represented a 44% increase in number of binding sites in haloperidol striata compared to controls. The KD values were .43nM and .69nM respectively and the correlation coefficients were .95 and .96 respectively. Similar binding data was obtained from other maternal striata after the administration of different doses of haloperidol. For example, after treatment with haloperidol at 2.5 mg/kg, control striata showed a Bmax of 362 fmoles/mg while that of haloperidol was 578 fmoles/mg. The respective KD values were .52nM and .49nM and correlation coefficients were .94 and .99.

Multiple regression analysis of all maternal binding data combined indicated that there was a significant increase ($p < .001$) in the number of binding sites in haloperidol striata compared to controls. This

[³H] SPIROPERIDOL BINDING IN MATERNAL STRIATA
AFTER HALOPERIDOL TREATMENT

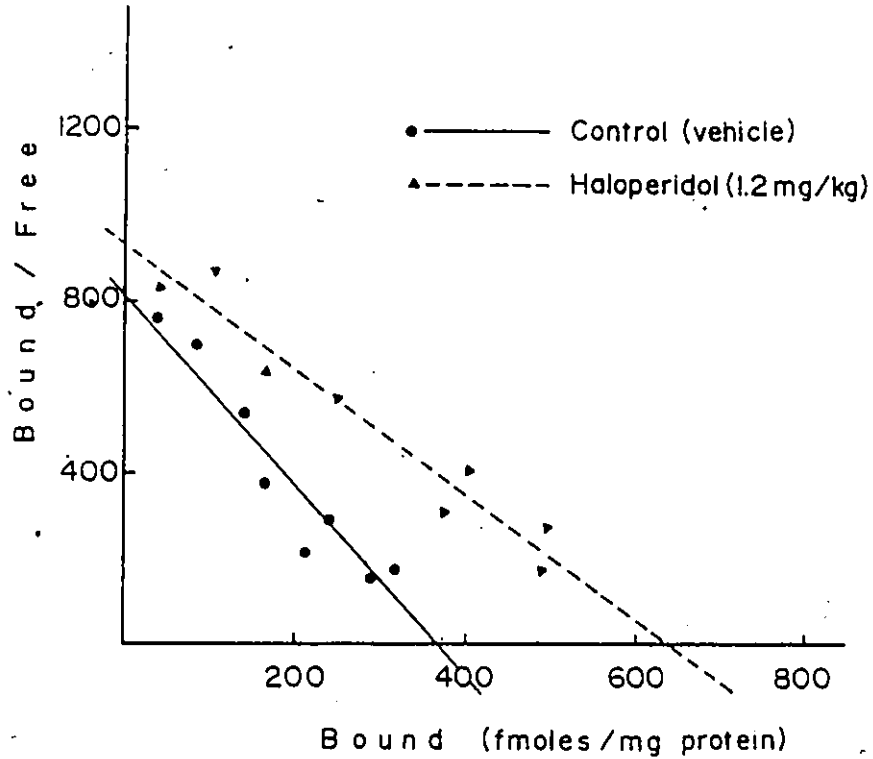


Figure 18: Scatchard plots of ³H-spiroperidol binding in the striata of those maternal rats whose offspring data is shown in the previous graph (Figure 17). Bmax values for haloperidol and control striata are 645 and 357 fmoles/mg protein, respectively. KD values are .69 nM and .43 nM, respectively. The correlation coefficients of the points on these regression lines are .96 and .95 for haloperidol and control data. See text for further details.

is similar to the results obtained in the adult males following chronic haloperidol treatment. Unlike the adult male study, however, maternal data showed more variation in KD values when comparing the slopes of the regression lines for haloperidols and controls. Statistical analysis of all experiments combined indicated that there was a significant difference ($p < .025$) in the binding affinities of striatal haloperidol sites compared to controls.

Discussion

Scatchard analysis of ^3H -spiroperidol binding in the striata of adult rats indicated that the daily administration of haloperidol for 3 weeks produced a significant increase in the number of binding sites in this DA-terminal area of the brain. This finding is in agreement with most studies in the literature as recently reviewed by Muller and Seeman (1978) in which a variety of different neuroleptics were administered and binding properties determined using different labelled ligands. The choice of ^3H -spiroperidol in this study is based on the fact that it belongs to the butyrophenone class of neuroleptics, as does haloperidol, and is thought to bind specifically to the same postsynaptic DA receptors (Leysen et al., 1978). In addition, at the time this study was undertaken, ^3H -spiroperidol was the ligand with the highest specific activity, higher than that of ^3H -haloperidol. Hence it provided the best measure of specific binding to postsynaptic receptors in the striatum.

The results of binding studies in maternal rats indicate that, like the previous study, striatal ^3H -spiroperidol binding increases significantly after the administration of haloperidol during the third tri-

mester. Although the duration of haloperidol treatment of maternal rats was only one week, this was sufficient to alter the number of postsynaptic receptors to the same degree as observed after 3 weeks treatment of the adult male rats. This rapid increase in number of binding sites was also observed by Burt et al. (1977) who studied ^3H -haloperidol binding in rat striata after one week of treatment with low doses of haloperidol. These data attest to the potency of haloperidol in altering the properties of postsynaptic receptors in the rat striatum.

The effects of haloperidol administration during the third trimester on ^3H -spiroperidol binding in the striata of offspring were in direct contrast to that observed in both adult studies. The results of binding experiments in 25-day old offspring indicate that the prenatal administration of haloperidol produced a significant decrease in the number of striatal receptors. These data are in agreement with those recently reported by Rösengarten and Friedhoff (1979). In that study, one dose of haloperidol (2.5 mg/kg) was administered to rats during the whole period of gestation beginning day 4 or 5 after conception. ^3H -spiroperidol binding in caudate homogenates of the offspring assessed by a technique different from that used in this study, also showed a similar decrease, an effect that was apparent as long as 60 days after birth. In addition, they reported the results of 2 Scatchard plots based on data from 14-day old pups that indicated a 34% and 47% decrease in binding sites in haloperidol striata compared to controls. This decrease is somewhat greater than that found in this study, although the reported affinities of the receptors were approximately the same.

The results of this study extend these findings by demonstrating

that the administration of haloperidol, even at lower doses and for a shorter duration during gestation, also produce a significant decrease in striatal receptor binding in postnatal day 25 offspring, as measured by Scatchard analyses. That the relative decrease is somewhat less may be accounted for not only by the shorter duration of treatment, but perhaps also to some age-related compensation for altered postsynaptic receptor number. For example, Rosengarten and Friedhoff (1979) demonstrated smaller decreases in ^3H -spiroperidol binding at postnatal day 35 compared to day 14 in offspring receiving the same prenatal treatment. Hence, given time, there may be partial recovery from the reduced number of striatal receptors induced by prenatal haloperidol administration.

The decrease in ^3H -spiroperidol binding sites in striata of offspring contrasts with the increase found after haloperidol treatment of adult rats, both adult males and maternal rats. These contrasting results emphasize the difference between the immature compared to the mature DA system in reacting to haloperidol antagonism. DA synapses are not fully established in the prenatal rat, hence the presence of haloperidol at this time likely interferes with important interactions between the developing pre- and postsynaptic components of this system. This ultimately may lead to a deficit in the development of postsynaptic DA receptors, an effect that may be permanent. In contrast, the postsynaptic DA receptor system in the adult rat is established and any increase in the number of the receptors following chronic blockade with haloperidol is reversible after withdrawal of the drug (Muller and Seeman, 1978). Hence, the effects of chronic haloperidol treatment on DA receptors in the adult striatum cannot be generalized to other stages of development, particularly not the

prenatal stage of development.

In an attempt to understand the mechanisms that might account for the reduction in DA receptor number, it is of value to consider the concurrent changes observed in the striata of these offspring using the other morphological and biochemical measure employed in this thesis study. The initial observations, using histofluorescence techniques, that the development of presynaptic DA neurons into the striatum of haloperidol offspring was deficient suggests that this reduced availability of DA during the latter part of prenatal development was at least in part responsible for the deficit in normal receptor development (Methods and Results, Part A). In support of this interpretation are the findings of Tennyson et al. (1979) who attempted to evaluate the role of DA as an important agent in directing the normal development of the nigrostriatal system. They found that the administration of reserpine that depleted DA availability during the same prenatal period of initial synaptogenesis, led to degenerative changes in the striatum of rabbits. They demonstrated using light- and electron-microscopic techniques that the striata of the fetuses whose mothers had received reserpine contained fewer and less mature presynaptic terminals and a variety of degenerative profiles in cell bodies and in processes that appeared to be dendritic within the striatum (Tennyson et al., 1978, 1979). These presumably could be the postsynaptic sites where DA receptor development is taking place. Hence alterations in their morphology might lead to a reduction in number of DA receptors in the striatum. It would be of great interest to determine using electron-microscopic techniques whether prenatal haloperidol produced similar types of degenerative changes.

That prenatal haloperidol treatment does not lead to complete postsynaptic degeneration of target sites is indicated by the finding that the ontogeny of DA-sensitive adenylate cyclase was not appreciably changed in striata of haloperidol offspring compared to controls (Plach et al., 1979; Methods and Results, Part B). The concurrent reduction in number of postsynaptic DA receptors, however, indicates that specific postsynaptic changes did occur and that the observed DA receptor changes took place independent of the DA-sensitive adenylate cyclase system. These observations support the hypothesis that these two postsynaptic components of the DA system are not subject to the same presynaptic influences (Iversen, 1975) and may not be coupled in a direct one-to-one relationship (Pardo et al., 1977). These data also provide further evidence for the existence of more than one type of DA receptor (Seeman et al. 1978; Keabian and Calne, 1979; Hyttel, 1978). That is, the ³H-spiroperidol labelled receptor that was affected by prenatal haloperidol blockade in these experiments appears to be independent of that which may be coupled to the DA-sensitive adenylate cyclase system.

D - EFFECTS OF PRENATAL HALOPERIDOL ON OFFSPRING BEHAVIOUR

Introduction

There is increasing evidence that the prenatal administration of centrally-acting drugs produces significant changes in the behaviour of the offspring despite an absence of overt morphological teratogenesis (Coyle et al., 1976; Spyker, 1975; Kolata, 1978; Vorhees et al., 1979). DA neurons in the brain play an important role in the regulation of motor movement (Guyton, 1972; Denny-Brown and Yanagisawa, 1976). Lundborg (1972) found that the administration of the potent DA antagonist, haloperidol, to nursing rabbits for a period of one week lead to a retardation in the motor development of the offspring. It was suggested that this effect might be due to the passage of haloperidol to the brains of the offspring where it blocked DA receptors during the time when dopaminergic synapses are undergoing maturation. This provided the first evidence that the administration of haloperidol during development can alter motor behaviour of offspring.

Very little is known about the effects of DA antagonists on motor behaviour of offspring when administered during the prenatal period of development. Clark et al., (1970) found that maternal administration of chlorpromazine from day 12 to 15 of gestation produced significant changes in the locomotor activity of the offspring tested in an open field chamber. These offspring showed greater spontaneous motor activity compared to controls on postnatal day 18. The morphological data described previously in this thesis indicate that the administration of haloperidol

from day 15 to 21 of gestation produced long term deficit in the development of both presynaptic DA terminals (Methods and Results, Part A) as well as postsynaptic DA receptors (Part C). Since DA neurons play such an important role in the regulation of motor behaviour, it was of interest to determine whether this deficit in pre- and postsynaptic DA neural development would lead to long-term changes in the spontaneous behaviour of offspring.

Methods

Treatment Procedures: Haloperidol or vehicle was administered to Wistar rats from day 15 to 21 of gestation and the offspring were fostered at birth as described in the Experimental Design section of this thesis. The offspring were weaned on postnatal day 21 and recaged in groups of six according to sex and prenatal treatment. In an initial series of experiments, offspring behaviour was assessed in the open field chamber on 4 consecutive days from day 22 to 25 and again from day 60 to 63. In subsequent experiments, locomotor behaviour was assessed only on first exposure to the open field chamber on postnatal day 25 and postnatal day 60. The offspring were tested at these ages to evaluate whether the prenatal haloperidol treatment produced temporary (day 25) or long-term (day 60) changes in behaviour. The animals used in this behavioural study were littermates of those used in the morphological and biochemical studies described previously (Methods and Results, Parts A and B).

Assessment of Maternal Pregnancy: Maternal weight, as well as food and water intake was monitored daily during the treatment period. The rate of weight gain and dietary intake of rats receiving injections

with haloperidol were then compared with those receiving vehicle alone to determine whether drug injections produced any notable effects on the normal course of pregnancy. In addition, the litter size and number of still-births delivered were also compared to determine whether the doses of haloperidol used were detrimental to the viability of the offspring.

Assessment of Offspring Physical Development: Both haloperidol and control offspring were weighed at weekly intervals to compare rate of weight gain. At the same time, the physical development of the pups was briefly assessed to determine whether the prenatal drug treatment produced any gross deficits in postnatal development. The parameters compared included ability to raise the head, pivot, lift the body off a flat surface, and age of eye opening. This assessment was made while the pups were placed on the scale for weighing and did not involve any extra handling. Each pup was returned to the nest within 30 seconds of removal.

Behavioural Testing: All behavioural testing was done between 1200-1500 hours in a quiet, isolated room with uniform lighting. The offspring were tested individually within an open field chamber (25 x 25 x 50 inches) with floor marked off in 5 x 5 square inches (Figure 19). Each animal was observed for a 2 minute period during which the number of squares crossed, the frequencies of rearing and grooming, as well as the number of boluses deposited were recorded (Figure 20). Both male and female offspring from haloperidol and control groups were tested in randomized order. The walls and floor of the chamber were wiped with dilute acetic acid after each run to mask any olfactory cues that might be left by the previous rat. The same procedure was used at both post-

OPEN FIELD CHAMBER

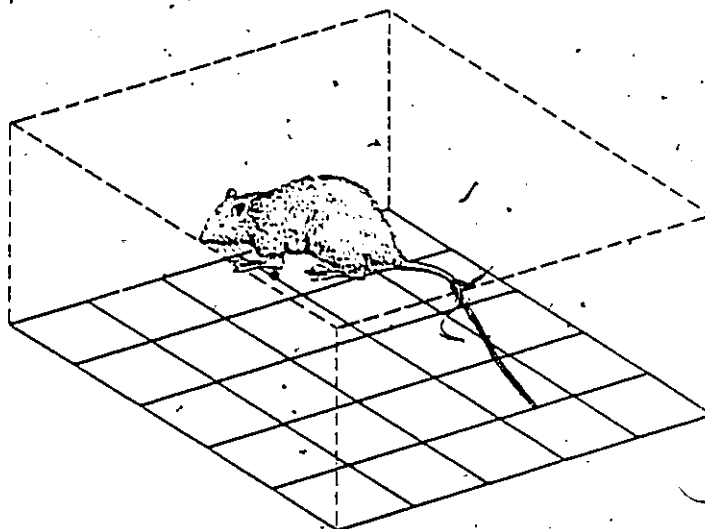


Figure 19: The dimensions of the open field chamber were 25 x 25 x 50 inches with floor marked off in 5 x 5 inch squares. Each rat was placed in the corner square and observed for a 2-minute period. The walls and floor were wiped with dilute acetic acid after each run to mask any olfactory cues that might be left by the previous rat. Both 25 and 60 day old rats were tested in this chamber.

OPEN FIELD BEHAVIOUR

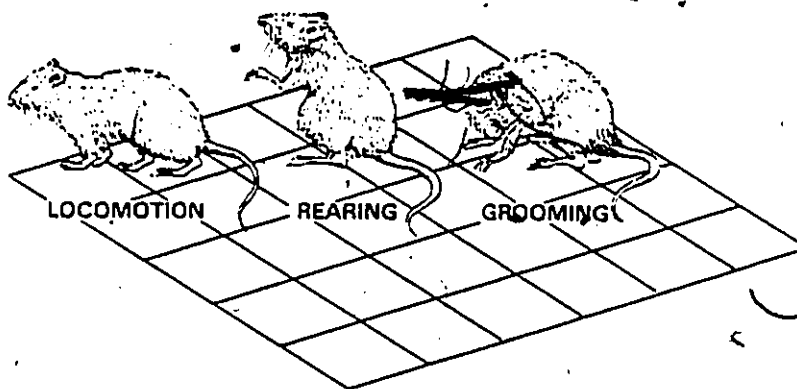


Figure 20: Four aspects of spontaneous behaviour were recorded during a 2-minute observation period: (1) Locomotion, scored as the number of squares crossed; (2) Rearing, scored as the number of times that the forepaws were raised off the floor; (3) frequency of Grooming; and (4) the number of Boluses desposited (not shown). Both male and female offspring from haloperidol and control groups were tested individually, in randomized order.

natal day 25 and day 60.

Statistical Analysis: The data was analyzed using a 3-way analysis of variance (ANOVA) that compared treatment (haloperidol versus vehicle-control) by sex (male versus female offspring) by dose (0.26 versus 1.2 versus 2.5 mg/kg of haloperidol) effects on each of the four behavioural measures scored: number of squares crossed, frequency of rearing, frequency of grooming and number of boluses deposited. Data from postnatal day 25 and day 60 were analyzed separately since these included different sets of offspring.

Results

Progress of Maternal Pregnancy: There was no significant difference in the rate of weight gain nor in the food and water intake of maternal rats receiving daily injections with haloperidol compared to vehicle during the third trimester.* Figure 21 summarizes the weights of maternal rats receiving either vehicle (control) or haloperidol 1.2 mg/kg and 2.5 mg/kg from gestation days 15 to 21. The rats were also weighed on gestation day 14, prior to the start of treatment, to establish a baseline and to ensure that the mean starting weights in all 3 groups was approximately the same.

Similarly, there was no significant difference between haloperidol

* Footnote: In an initial pilot study, one group of maternal rats received 3.2 mg/kg of haloperidol, the highest dose tested. Unlike the lower doses tested, this dose of haloperidol produced a marked sedating effect on maternal rats. Their food and water intake was severely reduced and their litters less viable, showing a 50% mortality rate by the end of the first postnatal week, even when fostered by normal dams. This dose was omitted in subsequent experiments.

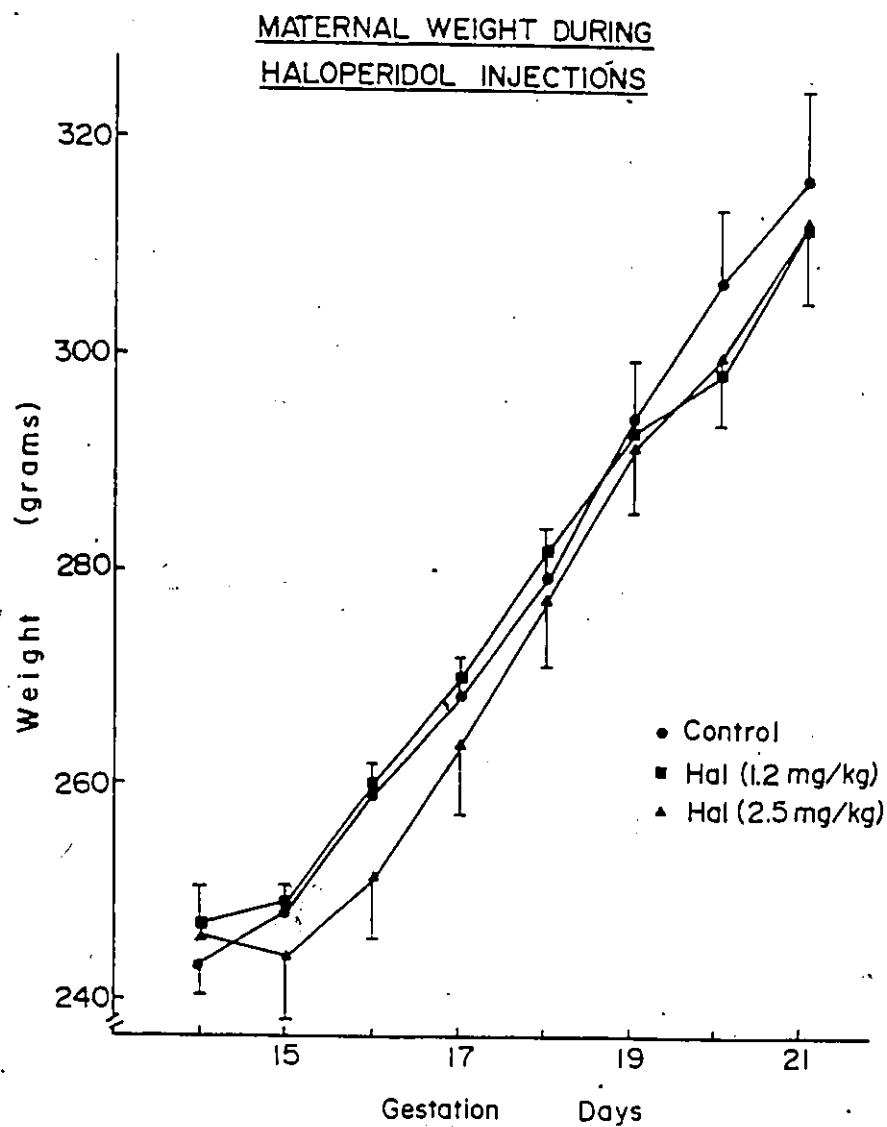


Figure 21: The mean weights (+ sem) of maternal rats receiving either vehicle (control) or haloperidol (1.2 mg/kg or 2.5 mg/kg) from gestation days 15 to 21 are summarized above. There were 8 rats in each group. The day 14 values are also included to show the mean weights prior to the start of treatment.

treated and control dams in terms of delivery date, litter sizes and litter viability. The offspring were delivered on day 21 and day 22 in litter sizes that ranged from 8-15 pups, with a mean of 10 pups each. The incidence of stillbirths was 1 per 15 live births on average with larger litters usually containing the greatest number of stillbirths.

Comparison of Offspring physical Development: There was no significant difference in the physical development of offspring whose mothers received haloperidol compared to controls. The weekly weight gain of pups whose mothers received 1.2 and 2.5 mg/kg of haloperidol or vehicle alone are summarized in Figure 22. The weights of male and female pups together are shown. The age of eye-opening, ability to raise the head, pivot and to lift the body off a flat surface was similar in all groups of offspring.

Open Field Behaviour: The results of statistical analysis (ANOVA) indicate that at both postnatal day 25 and day 60, haloperidol offspring showed greater spontaneous locomotor activity than controls. That is, there was a significant treatment effect ($p < 0.001$). See Figure 23 for the test results of postnatal day 25. Similar data were obtained at postnatal day 60 (Figure 24).

There was also a significant sex effect ($p < 0.01$) on open field behaviour on postnatal day 25 and day 60. This effect was greatest on day 60 (Figure 24). Female offspring from both haloperidol and control groups crossed a greater number of squares and reared more often than males of the same litters. This difference between male and female behaviour was greatest in offspring whose mothers received haloperidol treatment during pregnancy.

WEEKLY PUP WEIGHTS AFTER PRENATAL
HALOPERIDOL (1.2 and 2.5 mg/kg)

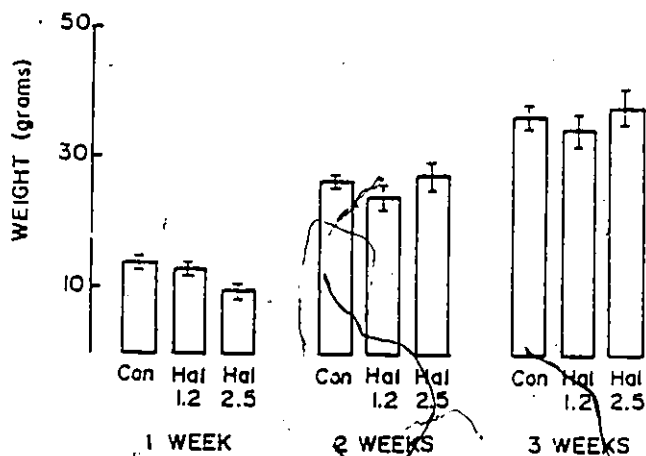


Figure 22: The weights (mean \pm sem) of offspring of maternal rats, treated with 1.2 or 2.5, mg/kg of haloperidol (Hal) or with vehicle (Con) during the third week of gestation are summarized. Both male and female rats (n = 25 to 30 per group) were weighed at weekly intervals.

OPEN FIELD BEHAVIOUR - POSTNATAL DAY 25

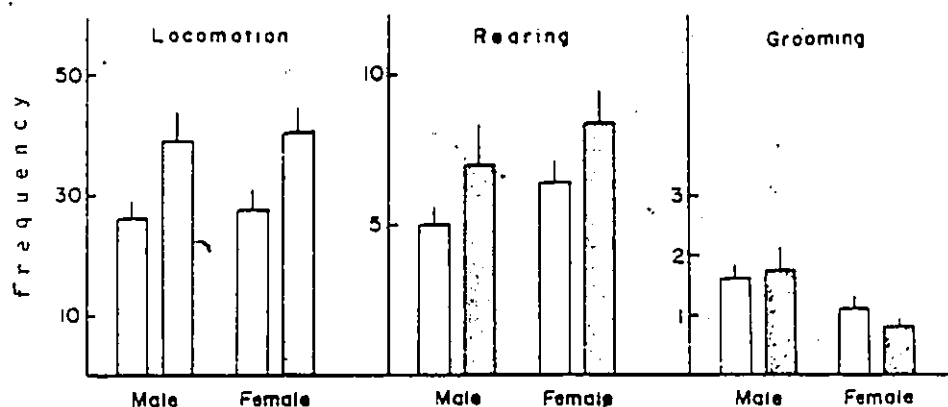


Figure 23: The open field behaviour (locomotion, rearing and grooming) of male and female, 25 day old offspring of maternal rats treated with 2.5 mg/kg of haloperidol (dark bars) or vehicle (light bars) during the third trimester. Each bar represents the mean \pm sem score of 18-20 rats. See text for details.

OPEN FIELD BEHAVIOUR - POSTNATAL DAY 60

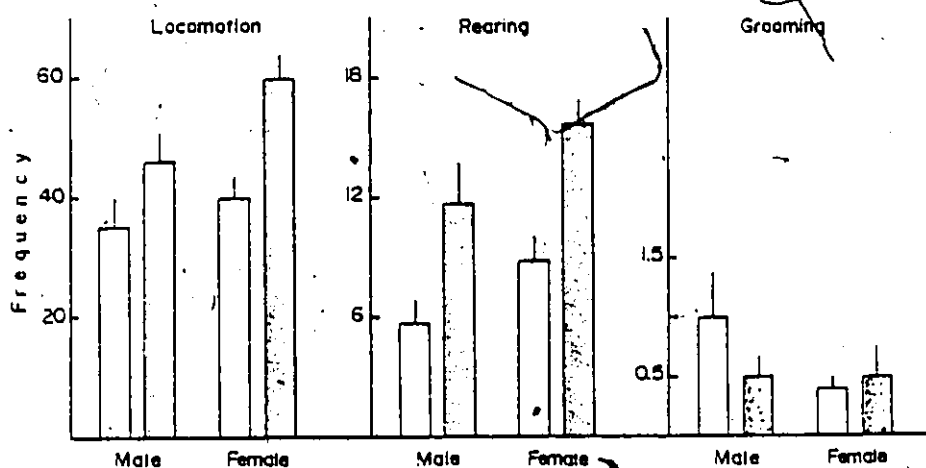


Figure 24: The open field behaviour of 60 day old male and female offspring of maternal rats treated with 2.5 mg/kg haloperidol (dark bars) or vehicle (light bars) during the third trimester. Each bar represents the mean \pm sem of 10-15 rats. See text for details.

In the case of grooming behaviour, the difference between haloperidol and control offspring was less striking, although there was still a significant sex by treatment effect ($p < 0.05$). Females generally groomed less frequently than males (Figures 23 and 24). Haloperidol offspring usually groomed less frequently than controls of the same age although this was variable.

There was no significant difference in the number of boluses deposited regardless of treatment or sex. This was true for both post-natal day 25 and day 60 results (not shown). There was a high frequency of zero scores for this measure.

With regard to dose-response relationships, there was an increase in number of squares traversed and in frequency of rearing with increase in dose of haloperidol administered during the prenatal period. However, due to the intergroup variations, these values did not reach statistical significance. There was no significant dose-response effect on grooming behaviour nor number of boluses deposited (not shown).

In initial experiments where offspring behaviour was tested on four consecutive days (day 22 to day 25 and day 60 to day 63), significant differences between haloperidol and control behaviour were observed only on first exposure to the open field chamber. These differences were consistent with those described above. On repeated exposure to the same environment, these differences between haloperidol and control offspring behaviour were no longer evident. Figure 25 (A and B) shows the behaviour (number of squares crossed and frequency of rearing) of female offspring (controls and haloperidol of the 1.2 mg/kg series) on first to fourth exposure to the open field chamber after weaning. On test day 1,

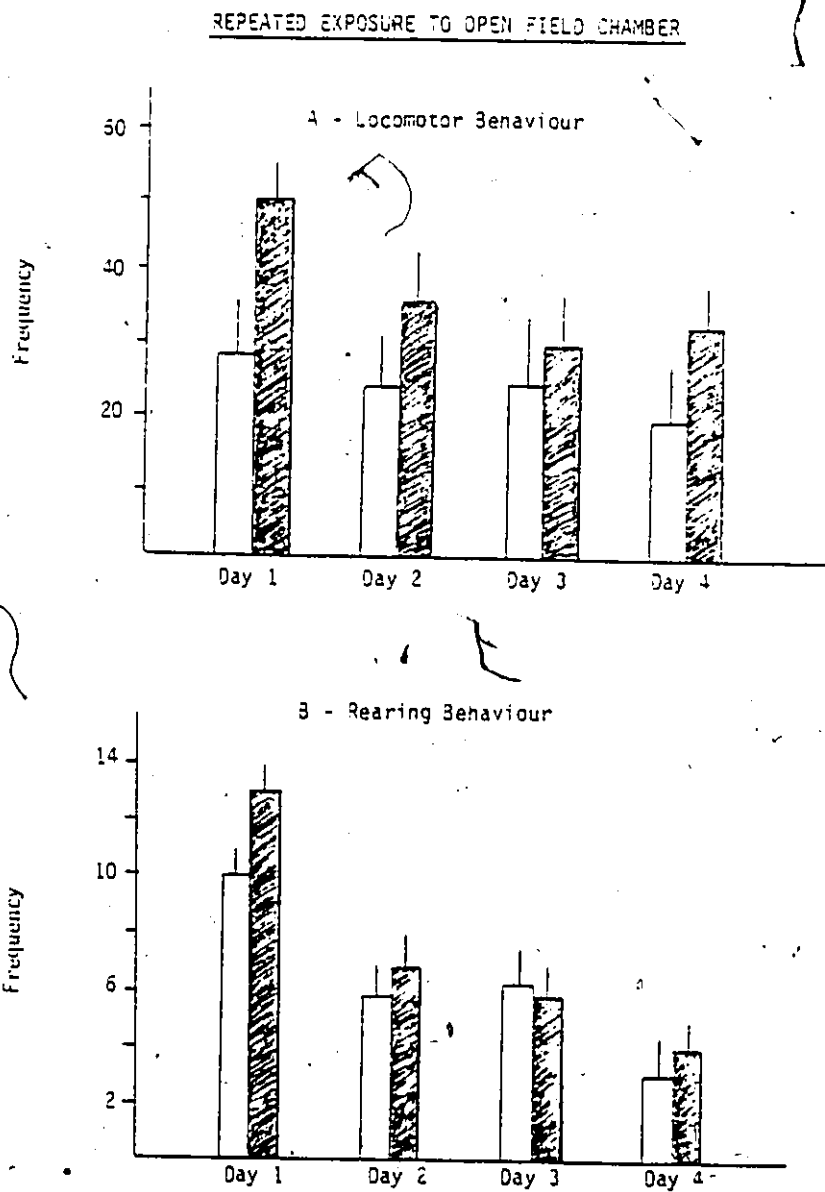


Figure 25: These graphs summarize the open field activity, A - Locomotor Behaviour, and B - Rearing Behaviour, of female offspring (haloperidol - dark bars, and controls - light bars) during 2 minute test periods on four consecutive days after weaning at 21 days of age. Each bar represents the mean \pm sem of 15 rats per group. See text for further details.

haloperidol offspring crossed a greater number of squares ($p < .05$) and reared more frequently than controls ($p < .01$). There was no significant difference between haloperidol and controls on subsequent test days when these measures showed a general decline in frequency.

Discussion

The results of this study indicate that prenatal administration of haloperidol during the third trimester produces long-term behavioural hyperactivity in the offspring. Haloperidol offspring showed locomotor hyperactivity as indicated by crossing a greater number of squares and rearing more frequently than controls during a 2-minute observation period in the open field chamber. This finding is in agreement with those of O'Donnell and Seiden (1980) who found a similar locomotor hyperactivity of offspring tested in stabilimeter cages following chronic haloperidol administration to maternal rats for the duration of pregnancy. Similarly, Clark et al., (1970) reported that offspring of rats treated prenatally with the neuroleptic chlorpromazine also showed locomotor hyperactivity when tested in an open field chamber.

The results of the experiments presented here extend those of previous studies in a number of important ways. For example, in previous studies, haloperidol was administered chronically for the duration of pregnancy (O'Donnell and Seiden, 1980; Rosengarten and Friedhoff, 1979). In this study, maternal treatment with haloperidol was acute. That is, haloperidol was administered only during the third trimester, the critical period of initial synaptogenesis of central DA neurons (Loizou, 1972; Coyle and Henry, 1973). In addition, offspring were fostered by untreated

maternal rats immediately after birth to avoid confounding results with possible postnatal effects of haloperidol (Joffe, 1969). Thus, the locomotor hyperactivity of offspring observed in this study is most likely attributable to adverse effects of haloperidol on DA synaptogenesis and underlines the potential toxicity of this neuroleptic during prenatal development.

The potential toxicity of haloperidol requires that not only offspring development but also maternal progress during pregnancy should be closely monitored, especially during the treatment period. Most studies of prenatal drug effects have omitted to report this, although it is essential in demonstrating that the observed effects on offspring behaviour are not merely due to nonspecific side-effects of drug-treatment (Coyle et al., 1976; Joffe, 1969). For example, drug-induced nutritional deficits during pregnancy are known to produce abnormalities in offspring behaviour (Smart and Dobbing, 1971; Altman and McCrady, 1972). In this study, a whole range of doses of haloperidol was tested, including very low doses (0.20 mg/kg) that had no observable effect on maternal or offspring behaviour and very high doses (3.2 mg/kg) that were detrimental to maternal rats. Treatment with this high dose, for example, lead to reduced nutritional intake and weight gain as well as decreased viability of their offspring. The data presented here was obtained from those experiments in which both maternal and offspring progress was closely monitored and found not to differ from controls. Hence the observed effects of prenatal haloperidol reported here are not likely due to nonspecific side-effects of the treatment.

This study extends the findings of previous studies in that

offspring behaviour was evaluated at two distinct periods of postnatal development, 25 and 60 days after birth. The observed hyperactivity of haloperidol offspring at both these ages indicate that the prenatal effects of haloperidol produce long term, not merely temporary, changes in behaviour. Other studies investigating the neonatal effects of haloperidol have evaluated offspring behaviour during the early postnatal period, in particular. For example, Lundborg (1972) reported that haloperidol administration to nursing rabbits produced a temporary retardation of offspring motor development during the first two weeks of life. O'Donnell and Seiden (1980) found that chronic prenatal haloperidol treatment with doses in the same range used in this study, produced locomotor hyperactivity in offspring tested during the first few weeks after birth. The results of this study indicate that the haloperidol-mediated hyperactivity is not just a temporary change but extends into the adult life of the offspring. Hence, haloperidol may be a behavioural teratogen without producing overt morphological abnormalities (Coyle et al., 1976; Vorhees et al., 1979; Spyker, 1975; Mello, 1975; Kolata, 1978).

Another important variable generally overlooked in perinatal drug studies is the sex difference in rat behaviour (Fonseca, et al., 1976) so that the behavioural responses of males and females are not investigated separately and are often erroneously considered as the same. Masur (1972) however, provides evidence for important sex differences in the "emotionality" and behaviour of rats using an open field situation. The open field chamber offers the advantage over other techniques in that it provides a non-invasive means for evaluating spontaneous behaviour and allows for the assessment of not only motor behaviour but also emotional

responsiveness. Both these aspects of behaviour were analyzed in male and female offspring following prenatal treatment with haloperidol or vehicle in this study.

There was a significant sex difference in locomotor activity (number of squares crossed and rearing) as well as grooming behaviour. Female offspring were generally more active and groomed less frequently than the males. This was the case for littermates within control groups, but was particularly evident in comparisons of male and female offspring from haloperidol groups at 60 days of age. Fonesca et al. (1976) also found sex differences in the open field behaviour of rats following treatment with a variety of psychotropic drugs during the neonatal stage of development. One interpretation of these findings is that perhaps psychotropic drugs, in this case haloperidol, can influence the maturation of brain mechanisms that control hormonal function, hence differentially affect male and female offspring. In support of this hypothesis are the findings of the hormonal study described next in this thesis (Part E) that indicate a differential effect of haloperidol on prolactin secretion in male and female (Plach et al., 1980b). Hence, male and female offspring appear to be differentially sensitive to prenatal treatment with haloperidol.

Another significant finding regarding the open field behaviour of offspring was that the activity of haloperidol offspring, both males and females, differed from controls on first exposure to the chamber but did not differ after repeated presentations to the same situation. Initial exposure to a novel situation might be expected to elicit an emotional reaction, whereas repeated presentations to a familiar environment may

not. Hence the observed hyperactivity during first exposure to the open field chamber may be interpreted as an emotional reaction to a novel situation (Joffe, 1969; Masur, 1972). Indeed, both haloperidol and control rats were more active on the first day compared to subsequent test days. However, in comparing the two treatment groups, haloperidol offspring were significantly more hyperactive than controls, suggesting an excessive emotional response to a novel situation. Hence prenatal treatment with haloperidol may produce a subtle increase in the emotional behaviour of offspring in situations of stress.

In terms of underlying mechanisms, the finding that haloperidol offspring display altered behavioural responses to a novel environment is similar to that observed by Stoof et al. (1978) following lesioning of central DA neurons in rat pups. They found that depletion of brain DA by intraventricular 6-hydroxydopamine injections lead to a prolonged phase of restless locomotion (increased locomotion and rearing, decreased grooming). Similarly, Shaywitz et al. (1976) also found that rat pups with lesioned DA systems were more active than control littermates when exposed to a novel environment. The previous morphological and biochemical data already described in this thesis indicate that maternal haloperidol administration produced a deficit in the development of presynaptic DA terminals (Part A) and a decrease in postsynaptic DA receptor development (Part C) in the striatum of offspring (Plach et al., 1979; 1980). This may be analogous to the aforementioned neonatal lesion studies that also produced a depletion of brain DA.

The finding of hyperactivity in animals with reduced DA function may at first appear paradoxical since in adult rats, hyperactivity is

associated with an increase in postsynaptic DA receptors (Muller and Seeman, 1978). However, evidence is now accumulating to indicate that the effects of drugs on the developing animals are not necessarily the same as that of the adult due to the immaturity of the brain and other organs as well as inability to metabolize drugs effectively (Spyker, 1975; Coyle et al., 1976). Shaywitz et al. (1976) have proposed that this apparent paradoxical response to brain DA depletion in developing animals may be explained by considering DA as modulator of excitatory noradrenergic neural activity. In normal rats, activity is constrained by adequate concentrations of brain DA (Ungerstedt, 1974; Ungerstedt and Tomas, 1977; Stoof et al., 1978). Depletion of brain DA removes this constraint and allows activity to occur unchecked (Shaywitz et al., 1976). Thus hyperactivity may be due to an imbalance between an excitatory noradrenergic system and inhibitory DA system.

The aforementioned hypothesis has recently been questioned (Stoof et al., 1978). An alternate interpretation of these data emphasizes the emotional aspect of the behavioural response to a novel situation. Stoof et al. (1978) suggest that the hyperactivity indicates an inability to habituate to a novel environment. Habituation can be considered a simple form of learning that can be tested using an active avoidance paradigm. In support of this hypothesis, Ahlenius et al. (1977) found that neonatal penfluridol (a DA antagonist) treatment produced both a deficit in the acquisition of an active avoidance response and an increase in locomotor activity prepuberally.

Consistent with the interpretation of the observed hyperactivity as an increased emotional response of haloperidol offspring is the

previous finding described in this thesis (Part A) of an increase in DA-induced fluorescence in the mesolimbic areas of the brains of these offspring (Plach et al., 1980). The mesolimbic system has been indicated in the regulation of emotionality (Stevens, 1979) as well as motor activity (Kelly et al., 1975; Pijnenburg et al., 1975). Hence increased DA in the mesolimbic system may be responsible for the hyperactivity reflecting increased emotional reactivity of offspring after prenatal haloperidol treatment.

Nevertheless, it should be pointed out that from an anatomical point of view, the striatum is strategically positioned to mediate information transfer from the cerebral cortex, thalamus and substantia nigra (Kemp and Powell, 1971; Mehler and Nauta, 1974; Fibiger et al., 1972; Hattori et al., 1973). It has been suggested that this position involves an integrating function in sensory and motor activity (Ungerstedt, 1974). Hence the nigrostriatal system may be most influential in mediating the observed behavioural effects of haloperidol. The situation is further complicated, however, by the existence of connections between the nigrostriatal and mesolimbic systems (Lindvall and Bjorklund, 1974). This connection likely allows for an integration in the activity of these two DA systems so that both may play an important role in mediating the observed behavioural effects.

Despite these complexities in interpreting the basis for the observed behavioural results in this study, it is clear that treatment with haloperidol during initial synaptogenesis of DA systems produces changes in the locomotor activity and emotional responses of offspring and that these behavioural effects occur at relatively low

doses of haloperidol that do not produce overt morphological abnormalities. The implication of these findings is that haloperidol may be a behavioural teratogen (Coyle et al., 1976; Spyker, 1975) and caution should be advised in the use of this drug clinically, particularly in the young and during pregnancy.

E - EFFECTS OF PRENATAL HALOPERIDOL ON PROLACTIN SECRETION

Introduction

Dopamine (DA) secreted by the tuberoinfundibular system is thought to be the main inhibitory factor involved in the control of prolactin secretion (MacLeod and Lehmeyer, 1974; MacLeod, 1976). It is released from nerve endings in the median eminence and transported to the pituitary prolactin-secreting cells by the hypothalamic-hypophyseal portal system (Fuxe, 1965; Ben-Jonathan et al., 1977). DA receptors have been found in the anterior pituitary (Creese et al., 1977; Brown et al., 1976). The presence of DA receptors here and their absence in the median eminence supports the postulate that DA and dopaminergic drugs exert their effects on prolactin secretion at the level of the anterior pituitary (Brown et al., 1976; 1979).

DA antagonists, such as haloperidol, produce an increase in pituitary prolactin secretion by a disinhibitory mechanism, thereby raising serum prolactin levels both in humans (Sachar, 1978) and in rats (Dickerman et al., 1972). Increased prolactin secretion following DA antagonism is observed as early as postnatal day 3 in the rat (Ojeda and McCann, 1974). This indicates that DA receptors are already functional and that DA regulation of prolactin secretion is already established by this age. It is known from studies in adult rats that reducing the availability of DA in the pituitary, by synthesis inhibition (Annunziato and Moore, 1977) or by medial-basal hypothalamic lesions (Cheung and Weiner, 1976; 1978) increases the sensitivity of pituitary DA receptors.

Chronic treatment with neuroleptics such as haloperidol may also increase the sensitivity of these receptors (Lal et al., 1977), although this has not been resolved (Ravitz and Moore, 1977). Since the previous results of this thesis (Part C; Plach et al., 1979) and other studies (Rosengarten and Friedhoff, 1979) indicate that haloperidol treatment during the third trimester alters the normal development of postsynaptic DA receptors within the brain, it was of interest to determine whether this treatment also alters DA receptor development in the pituitary. The absence of nerve terminals in the anterior pituitary allows one to study a population of DA receptors that is wholly postsynaptic and to investigate receptor sensitivity using a relatively accessible and precise parameter under dopaminergic control, that is, prolactin secretion (Labrie et al., 1979). It was postulated that if haloperidol administration during the third trimester alters DA receptor development in the pituitary, then this would lead to long-term changes in pituitary sensitivity to DA and DA antagonists as indicated by altered prolactin secretion.

Methods

Treatment Procedures: Haloperidol or vehicle were administered to Wistar rats during the third trimester and the offspring were fostered at birth, as described in the Experimental Design section of this thesis. The offspring were weaned on postnatal day 21 and recaged within groups, separating males from females. On postnatal day 25, the offspring were assigned to one of two series of experiments: (1) to test the sensitivity of the pituitary to an acute challenge with haloperidol in vivo, or (2) to test pituitary sensitivity to direct inhibition by DA in vitro.

1. In vivo Experiments In this series of experiments, half of the haloperidol and half of the control offspring received an acute injection of haloperidol (0.3 mg/kg s.c.). The other half of each group received a control injection of vehicle. The order of the injections was randomized. Both male and female offspring were included. Exactly 60 minutes after injection each rat was decapitated and trunk blood was collected. The samples were undisturbed for 1 hour at room temperature to allow clotting to take place. They were then centrifuged in a Beckman J6 centrifuge at 1000 g at -4°C for 30 minutes. Supernatant serum was carefully drawn off and transferred to fresh tubes that were capped tightly and stored at -70°C until assayed.

2. In vitro Experiments On postnatal day 25, haloperidol and control offspring were decapitated and their pituitaries were excised. The anterior pituitaries were separated from the posterior portions and then bisected. The hemipituitaries (anterior halves) were placed individually into separate tubes containing Medium 199 (Gibco, Toronto, Ontario). The hemipituitaries were preincubated in 3 ml of aerated (5% CO_2 and 95% O_2) Medium 199 for 1 hour at 37°C . They were then transferred in randomized order into tubes containing fresh medium plus various doses of DA ranging from 10^{-9}M to 10^{-5}M , or no DA to establish baseline. The hemipituitaries were incubated under aerated conditions (as above) in a shaking water bath at 37°C for 3 hours. Then 400 μl of medium from each sample was drawn off and diluted 1:5 in BSA buffer (pH 7.6) and stored at -20°C till assayed for prolactin content. In two replications of this study, samples were drawn at 90 minutes, as well as 3 hours after incubation, and assayed for comparison.

Rat Prolactin Assay: Serum and incubation media were assayed by the laboratory of Dr. G.M. Brown, Department of Neurosciences, McMaster University, using a radioimmunoassay for rat prolactin with materials provided by the National Pituitary Agency and funded by the National Institute of Arthritis Metabolism and Digestive Disease. All samples within a given experiment were measured in duplicate in the same assay to minimize intra-experimental variation. The coefficients of variation for interassay and intrassay determinations ranged from 6.8% to 11.3% at the time that these experiments were done.

Data Analysis: The results obtained in each series of experiments were analyzed using analysis of variance and covariance including repeated measures and Students t-test.

Pilot Study: Before it was possible to determine whether prenatal treatment with haloperidol produced long-term alterations in pituitary sensitivity of offspring, it was necessary to establish the normal level of sensitivity in control rats at 25 days of age. Haloperidol was chosen as the challenge to test the same population of pituitary receptors that would likely be affected by the prenatal treatment. Since nothing was known about the effects of a haloperidol challenge on serum prolactin levels in normal 25 day old rats, it was necessary to establish a normal dose-response curve. This would also facilitate the choice of an appropriate dose of haloperidol to be used as the challenge in the subsequent prenatal experiments.

Normal 25 day old Wistar rats were given a single injection of haloperidol at one of the following doses: 0.02, 0.1, 0.3 and 0.5 mg/kg (s.c.), or vehicle alone. The injections were administered in a random-

ized sequence to 10 rats per dose of haloperidol tested (N = 50). An additional set of 10 rats received no injections and were decapitated immediately at the start of the experiment to control for possible extraneous effects of experimental handling, since prolactin is known to be a stress-responsive hormone (Seggie and Brown, 1975; 1976). The remaining groups of rats were decapitated exactly 60 minutes after their injections. Trunk blood was collected for serum prolactin determinations. Both male and female rats were used in this study.

Results

Normal Serum Prolactin Response to a Haloperidol Challenge on Postnatal Day 25: A normal dose-response curve of serum prolactin levels of 25-day old rats injected with various doses of haloperidol is presented in Figure 26. There was no difference in serum prolactin levels of rats that were killed immediately and those injected with vehicle and killed 60 minutes later, indicating that the experimental procedures did not produce sufficient stress to alter baseline prolactin secretion one hour after injections. There was no change in serum prolactin levels after injection with 0.02 mg/kg of haloperidol, the values being similar to baseline levels. Injection with greater doses of haloperidol, however, produced significant increases ($p < .01$) in serum prolactin levels. The maximum serum prolactin response in this study was obtained after injection with 0.5 mg/kg of haloperidol.

A sex difference in serum prolactin response was observed following haloperidol injections at mid range doses (Figure 26). Serum prolactin levels of females were significantly greater ($p < .01$) than that of males

SERUM PROLACTIN RESPONSE TO A
HALOPERIDOL CHALLENGE

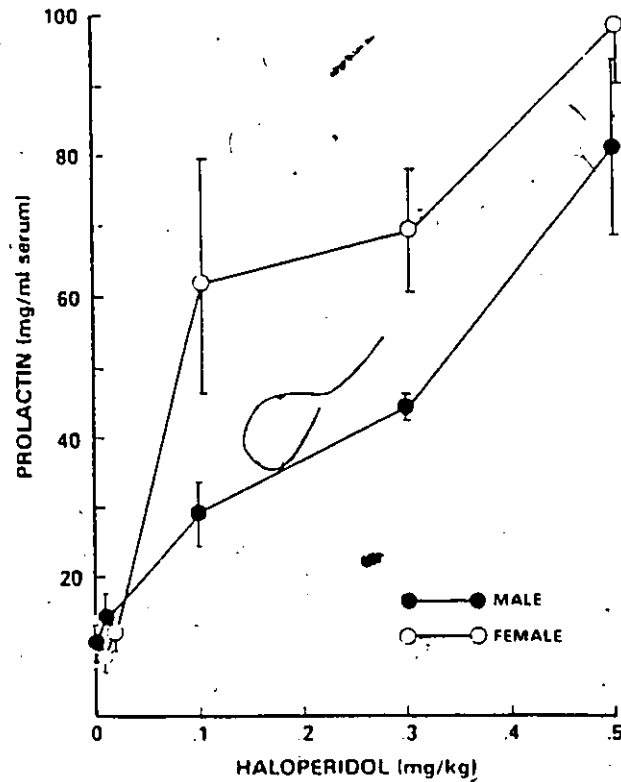


Figure 26: Normal 25 day old rats received a single injection with haloperidol (0.02, 0.1, 0.3 or 0.5 mg/kg s.c.) or vehicle alone and serum prolactin levels (mean \pm sem) determined 1 hour postinjection. Both male and female rats (n = 8 each per dose) were tested.

following injections with 0.1 and 0.3 mg/kg of haloperidol. No differences were observed after injections with 0.02 or 0.5 mg/kg of haloperidol. This indicated that at postnatal day 25, female rats were more sensitive to haloperidol than males.

Based on this normal dose-response curve of serum prolactin response of 25 day old rats to haloperidol over a range of doses, one dose of haloperidol was chosen to be used as the challenge dose to test the responsivity of offspring from the prenatal experiments. Since it was not known whether maternal treatment with haloperidol might produce an increase or a decrease in pituitary sensitivity of their offspring, the midrange dose of 0.3 mg/kg of haloperidol was chosen as the challenge dose (Figure 26). This allowed for the detection of changes in serum prolactin secretion in either direction.

Altered Serum Prolactin Response Following Prenatal Haloperidol:

Serum prolactin response of offspring whose mothers received treatment with either haloperidol (2.5 mg/kg) or vehicle during the third trimester were compared 1 hour following a challenge with 0.3 mg/kg of haloperidol on postnatal day 25 (Figure 27). Half the offspring in each group received a control challenge of vehicle only. Both male and female offspring were used in these studies. These experiments were replicated on 2 separate occasions.

There was no significant difference in serum prolactin levels of control or haloperidol offspring following the challenge with vehicle (Figure 27). Serum prolactin values were 10 to 15 ng/ml, in agreement with those observed in the pilot dose-response study (Figure 26). Also as found earlier, serum prolactin levels of female rats (58 ng/ml \pm

SERUM PROLACTIN RESPONSE FOLLOWING PRENATAL HALOPERIDOL

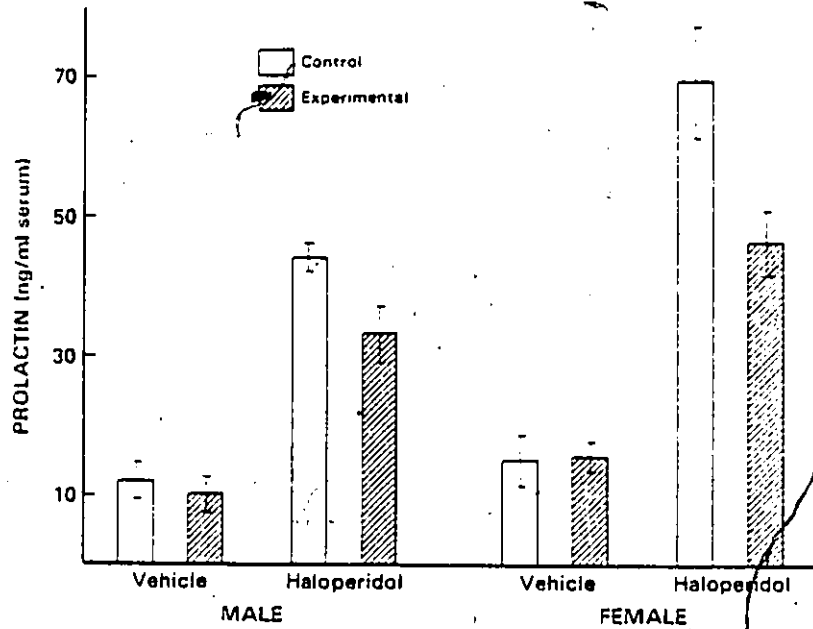


Figure 27: On postnatal day 25, half the offspring of maternal rats treated with 2.5 mg/kg of haloperidol (experimental) or vehicle (control) received a test injection of haloperidol (0.3 mg/kg s.c.). The other half received vehicle alone. Serum prolactin levels (mean \pm sem) of both males and females (n = 8 each per group) were determined 1 hour postinjection.

6, N = 24) were significantly greater than males (40 ng/ml \pm 3; N = 24) in response to the haloperidol challenge (p < .02). In addition, serum prolactin values of control offspring as a group (male + female/2; 56 ng/ml \pm 5; N = 24) were greater (p < .01) than that of offspring whose mothers were treated with haloperidol (40 ng/ml +4; N = 24).

Since offspring of maternal rats treated with haloperidol showed altered serum prolactin levels to a subsequent haloperidol challenge on postnatal day 25, it appeared that prenatal exposure to haloperidol produced a long-term change in the sensitivity of their pituitary receptors to DA inhibition in vivo.

Pituitary Sensitivity to Dopamine In Vitro Following Prenatal

Haloperidol : To determine whether the observed difference in prolactin response to haloperidol challenge on postnatal day 25 represented an increase or decrease in DA receptor sensitivity in pituitaries of haloperidol offspring, it was necessary to measure pituitary sensitivity more directly. Hence, the direct effects of DA on anterior pituitary prolactin secretion was measured using an organ culture system (Methods #2, In vitro Experiments) . The dose-response curve of pituitary prolactin secretion of haloperidol and control offspring in response to a range of DA concentrations were then compared. These experiments were replicated on 3 separate occasions, twice using male offspring and once including female offspring.

The effects of DA (10^{-9} to 10^{-5} M) on pituitary prolactin secretion (% of control) after a 3 hour incubation in vitro are summarized in Figure 28. The mean prolactin values (\pm Sem) of controls are shown in the top graph while that of haloperidol groups are shown in the bottom

DOSE-RESPONSE CURVES OF PROLACTIN SECRETION IN VITRO

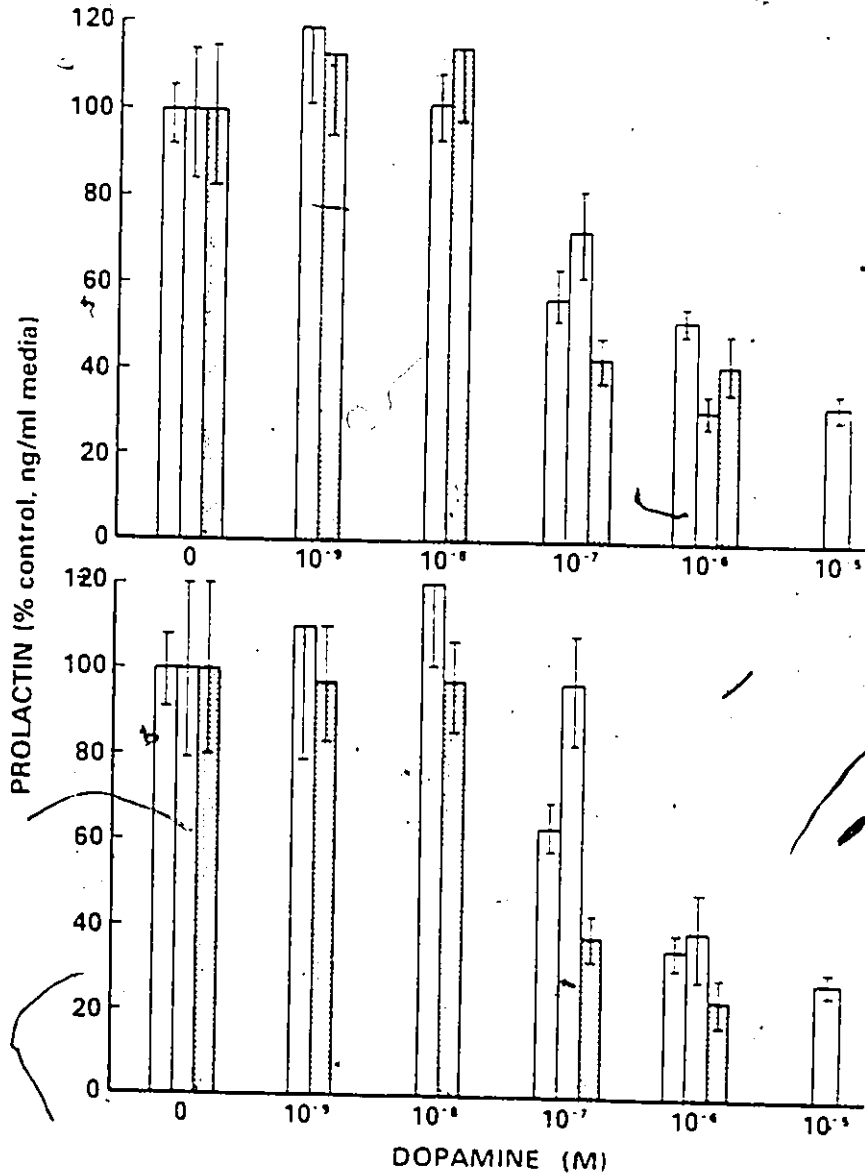


Figure 28: Hemipituitaries of control (top graph) and haloperidol offspring (bottom graph) were incubated with dopamine (0, 10⁻⁹ to 10⁻⁵ M) and the amount of prolactin secreted (mean ± sem) after 3 hours incubation determined. The results of replicate experiments using anterior pituitaries of male (light bars) and female (dark bars) offspring are summarized above as prolactin secretion (% of control). See text for further details.

graph. There was no significant difference in pituitary sensitivity to DA inhibition in haloperidol compared to control offspring. While the dose-response curves in initial experiments showed a significant shift to the left ($p < .001$) the subsequent 2 replications did not bear out this relationship.

There was, however, a significant difference between DA effects on pituitaries from female offspring (shaded bars) compared to males (unshaded bars, Figure 28). This sex difference in pituitary sensitivity to DA inhibition in vitro is indicated more clearly in Figure 29. As was found in the previous in vivo studies, females showed a greater ($p < .01$) prolactin response to DA inhibition than did males of the same age. For example, female pituitary prolactin secretion was almost totally suppressed by 10^{-7} M DA while that of males was not maximally inhibited until 10^{-6} M DA. Hence, the threshold dose of DA required to inhibit pituitary prolactin secretion is approximately 10-fold greater in males compared to females, indicating the greater sensitivity of the female pituitaries.

Discussion

The results of this study indicate that maternal treatment with haloperidol during the third trimester altered the normal prolactin response of offspring to DA inhibition in vivo. When challenged with haloperidol (0.3 mg/kg) on postnatal day 25, the serum prolactin response of these offspring was significantly less than that of controls. This indicates that prenatal treatment with haloperidol can produce a long-term change in the sensitivity of offspring pituitaries.

SEX DIFFERENCE IN SENSITIVITY TO
DOPAMINE IN VITRO

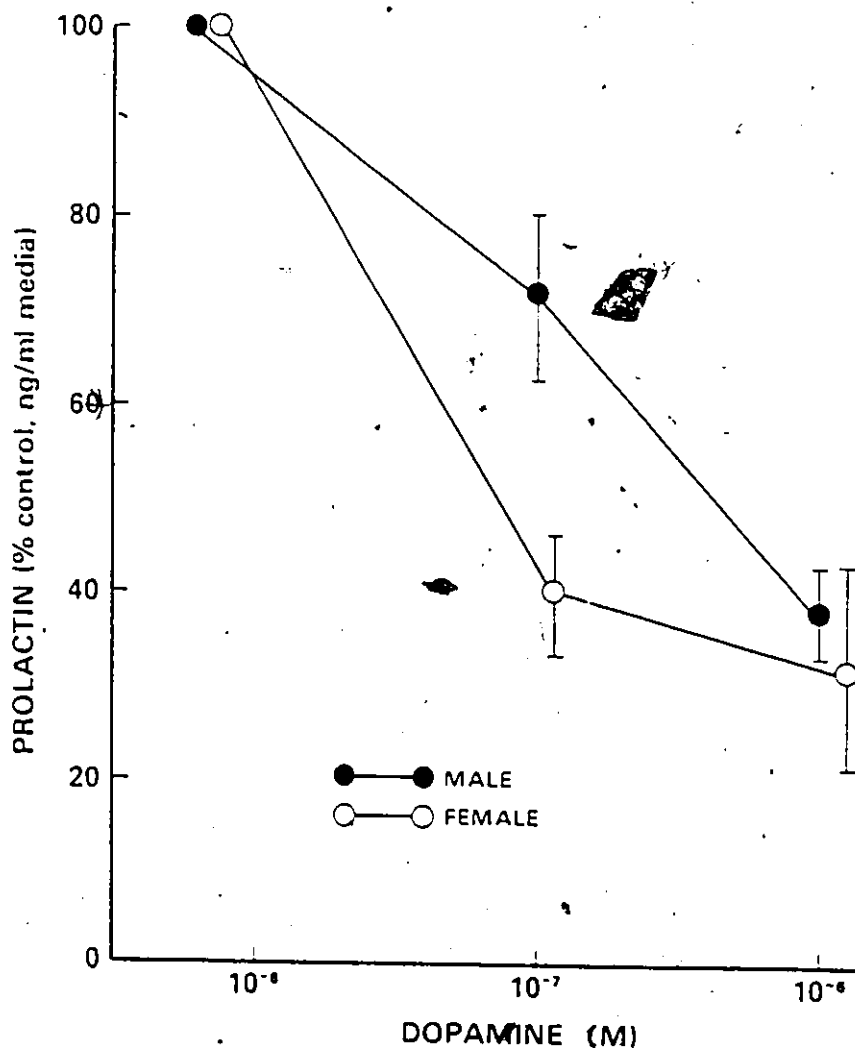


Figure 29: This figure is a summary of the data shown in figure 28 and compares prolactin secretion of pituitaries from male and female haloperidol offspring after 3 hours incubation with dopamine (1.0^{-8} to 10^{-6} M). The data are expressed as percent of control. Pituitaries from 25 day old female rats showed significantly greater sensitivity ($p < 0.1$) to DA inhibition in vitro than that of males. See text for further details.

No difference was found between haloperidol and control offspring in terms of the sensitivity of their pituitaries to direct DA stimulation in vitro. The dose-response curves of haloperidol and control anterior pituitary prolactin secretion in the presence of DA within the range of concentrations including 0 to 10^{-5} μ M during 90 minute and 3 hour incubation periods were not significantly different. These data suggest, therefore, that factors other than pituitary DA receptor sensitivity may be responsible for the observed alterations in prolactin release in vivo. What these other factors might be could not be determined by the results of these experiments.

A consistent sex difference in prolactin response to DA manipulation was observed both in the in vivo and in vitro experiments. In the initial dose-response study, a single injection of haloperidol at a dose of 0.1 mg/kg or greater produced a significant increase in serum prolactin levels of normal 25 day old rats. Females, however, showed a comparatively greater increase in serum prolactin than males at 0.1 and 0.3 mg/kg doses of haloperidol. Hence female rats at 25 days of age, are more sensitive to the effects of haloperidol than males of the same age. These findings extend those of Dohler and Wuttke (1974) who found that serum prolactin levels of female rats more closely approximated that of adulthood than that of males between day 21 and day 25 after birth. The results of this thesis study provide the first indication that there is also a sex difference in pituitary sensitivity to DA inhibition by haloperidol as early as postnatal day 25.

Prenatal treatment with haloperidol did not alter this differential response when tested either in vivo or in vitro. When challenged

with haloperidol, in vivo, the decrease in serum prolactin levels of females was more marked than that of males. Furthermore, pituitaries from female offspring were more sensitive to direct DA-inhibition in vitro than males. In fact, a 10-fold greater concentration of DA was required for maximal inhibition of pituitary prolactin secretion of males compared to females. These data support the more general conclusion that DA receptors in the pituitary of female rats are more sensitive than that of males in the regulation of prolactin secretion by postnatal day 25.

In summary, these data indicate that treatment with haloperidol during the third trimester produces long-term changes in the sensitivity of offspring pituitaries as indicated by reduced serum prolactin response at postnatal day 25. The mechanism for this altered sensitivity could not be determined from these series of experiments. It is clear, however, that there is a sex difference in pituitary sensitivity to DA inhibition in that female offspring always showed greater reactivity in prolactin response when tested both in vivo and in vitro.

CONCLUSION

To conclude this thesis, the significant findings of this study are highlighted along with a brief discussion of the data in terms of their contributing new information about the role of dopamine (DA) in prenatal development and the effects of haloperidol on the subsequent development and function of brain DA systems.

Trophic Interactions during DA Synaptogenesis in the Striatum

The results of the morphological and pharmacological experiments (Methods and Results, Parts A and C) indicate that the administration of haloperidol, a potent DA antagonist, during the prenatal period of synaptogenesis produces two significant effects:

1. a deficit in the development of presynaptic DA terminals within the striatum of rat offspring;
2. a deficit in the development of postsynaptic DA receptors within their striatum.

These findings support the hypothesis that there exist important trophic interactions between developing DA neurons and their target cells during initial synaptogenesis (third trimester in the rat) that are critical to the normal ontogeny of brain DA systems.

It has previously been postulated that monoamines, including DA, norepinephrine and serotonin, have important trophic effects during morphogenesis of the brain prior to their function as transmitters (McMahon, 1974; Vernadakis and Gibson, 1974; Lanier et al., 1976). This includes an important influence on cell division, motility and differentiation (Lauder and Krebs, 1978). Tennyson et al. (1979) also recently

provided evidence that depletion of nigrostriatal DA leads to a deficit in DA innervation of the striatum and degenerative changes in the striatal cells of rabbit fetuses after maternal administration of reserpine during the period of neural differentiation and early synaptogenesis of this system. The results of this thesis study provide the first evidence that the administration of a postsynaptic DA antagonist during the third trimester in the rat, a period when differentiation is already complete but synaptogenesis is just beginning (Loizou, 1972; Coyle, 1974), produces long term deficits in the development of both presynaptic DA terminals and postsynaptic DA receptors (Methods and Results, Parts A and C). Furthermore, these deficits in DA neural development of offspring are evident as long as 4 weeks after birth when DA systems in the rat should be reaching maturity (Loizou, 1972; Plach et al., 1979; 1980a,b).

As to why postsynaptic DA blockade during synaptogenesis leads to a deficit in DA terminal development and in number of postsynaptic DA receptors, one can only speculate since the sequence of events associated with synaptic formation has not yet been adequately determined (Lund, 1978). Some insight, however, may be gained from in vitro studies of synaptogenesis such as that of the cholinergic neuromuscular junction. Alpha-bungarotoxin, a derivative of snake venom, has often been used to bind irreversibly with postsynaptic receptors, so that with the addition of appropriate labels, the receptors can be visualized and followed during development. Devreotes and Farnbrough (1976) found that receptor molecules may be produced within the muscle cell and transferred to the surface. These receptors move about the membrane within high density

patches until innervated by neural axons, in which case they migrate and remain solely within the region of innervation (Anderson and Cohen, 1977; Anderson et al., 1977). It is interesting to note that neurons make initial contact with muscles even though the receptors are blocked by alpha-bungarotoxin (Cohen, 1972). From these data, it appears that receptors are present early and become organized by nerve terminals but do not necessarily direct their initial contact with the target cell (Lund, 1978).

The sequence of events in the next stage of synaptogenesis is also poorly understood. The results of studies on the developing superior cervical ganglion in vitro indicate that once initial contact is made between the growing nerve terminal and target cell, the maintenance of this connection as well as the appropriate biosynthetic activities of both depend on trophic interactions between the two (Hendry, 1976). Trophic factors may include retrograde transport of nerve growth factor from the target cell that influences catecholamine synthesis in the presynaptic terminal (Black et al., 1971) and presynaptic impulse transmission that releases transmitter to act at postsynaptic sites, although there is very little information available at this time (Lund, 1978). What appears to be important, however, is that these interactions take place during a critical period after initial contact is made, otherwise the synaptic connections and normal activity of their pre- and post-synaptic components is not maintained (Lund, 1978; Hendry, 1976).

If a similar sequence of events occurs during synaptogenesis within the brain, then the effects observed in this study following prenatal haloperidol treatment may be the result of the following processes.

It may be that, despite a relatively normal outgrowth of DA terminals towards striatal targets, initial contact with postsynaptic sites was lost due to persistent blockade of DA receptors by haloperidol. DA terminals are known to be functionally active prior to birth since prenatal treatment with reserpine depletes preterminal DA, an effect that is dependent on impulse transmission (Tennyson et al., 1979; Anden, 1967). Any release of DA, probably in small amounts due to the immaturity of the terminals at this time, did not have adequate access to postsynaptic sites because of antagonism by haloperidol. This deficit in access of DA to postsynaptic sites may have failed to signal appropriate retrograde trophic influences required for the maintenance of presynaptic terminals. This lack of feedback may have led to a decline in presynaptic striatal terminals, either by reducing the number of terminals surviving or by inadequate stimulation of presynaptic biosynthetic activities. Either of these would reduce endogenous levels of DA, as was observed in the fluorescence microscopy analyses (Methods and Results, Part A). Deficits in presynaptic terminals might further reduce the availability of DA to stimulate receptor production by postsynaptic cells leading therefore to the deficit in number of postsynaptic receptors in striata of offspring observed in this study.

It must be emphasized that the foregoing interpretation is merely hypothetical at this time. It is based on the assumption that synaptogenesis in the brain involves similar trophic interactions as observed in the periphery, including synaptogenesis of the neuromuscular junction (Anderson et al., 1977) and sympathetic ganglia (Hendry, 1976). It also assumes that the properties of haloperidol action in the developing :

nervous system is similar to that of the adult so that the observed effects are not due mainly to cytotoxic effects of the drug. To verify these assumptions, it is necessary to incorporate more specialized techniques including electron microscopy, neural cell cultures and microassay systems, which would be the next stage in these analyses.

Effects of Haloperidol on the Adult vs Developing DA System

The results of the receptor binding studies (Part C) indicate that developing DA neurons react differently to postsynaptic antagonism by haloperidol than those of the adult. It has previously been reported that the chronic administration of haloperidol to adult rats produces a significant increase in postsynaptic DA receptors (Muller and Seeman, 1978). The results of this study confirm and further extend that observation. Both adult males treated with haloperidol for 3 weeks as well as adult females treated with haloperidol during the third week of pregnancy, showed a significant increase in striatal DA receptor number when measured in binding experiments using ^3H -spiroperidol as ligand (Part C; Plach et al., 1979). In contrast, the offspring of maternal rats receiving haloperidol treatment showed a significant decrease in number of striatal ^3H -spiroperidol binding sites when analyzed on postnatal day 25. The latter finding has since been confirmed by Rosengarten and Friedhoff (1979), although they administered haloperidol at higher doses and for the entire period of gestation.

The contrast in observed effects of haloperidol on the adult versus prenatal DA system emphasizes the different role that DA may play at these two stages of development. The role of DA as a trophic agent

in the development of appropriate connections between a presynaptic neuron and the target cell has already been discussed. The effect of haloperidol during this prenatal period appears to be disruption of the dynamic interactions between pre- and post-synaptic neurons leading to a deficit in the appropriate development of postsynaptic receptors. By the adult stage, DA neurons have already established connections with target cells, so that in this case DA serves as a transmitter signalling activation of presynaptic neurons by binding with postsynaptic receptors. The response to prolonged blockade with haloperidol at this stage was to increase the number of postsynaptic receptors. The essential difference between the developing and mature neural system therefore is that the developing neuron adjusts the production of its receptors according to the relative degree of input from the presynaptic terminal, the limits of which have not yet been set, while in the adult stage, the limits have already been set and the subsequent adjustments are designed to remain within normal limits of function (Lund, 1978; Dismukes and Daly, 1976; Kerr, 1975).

Development of DA Receptors and DA-sensitive Adenylate Cyclase

The next important observation made in this study is that despite the deficit in postsynaptic DA receptors, there was no significant change in the DA-sensitive adenylate cyclase (Methods and Results, Part B) that was previously thought to be directly associated with these receptors (Kebabian et al., 1972). This indicates two things: first, that DA receptors can vary independent of changes in the activity of this enzyme. This supports the hypothesis that there may be more than one type of DA receptor in the brain, and that not all are coupled to DA-sensitive

adenylate cyclase (Kebabian and Calne, 1979; Titeler et al., 1978). The receptors analyzed in this study, labelled by the ligand ^3H -spiroperidol, belong to the class of receptors referred to as the D-2 receptors (Kebabian and Calne, 1979). The postulate that D-2 receptors are not coupled to DA-sensitive adenylyate cyclase is confirmed by the results of this study since prenatal haloperidol treatment produced a deficit in ^3H -spiroperidol binding sites without effecting DA-sensitive adenylyate cyclase (Methods and Results, Part B).

Secondly, these results indicate that the development of D-2 receptors may be dependent on the normal development of presynaptic DA terminals while the development of the DA-sensitive adenylyate cyclase is not. This suggests that these two entities are regulated by different influences during synaptogenesis. From the studies of Pardo et al. (1977) and Coyle and Campochiaro (1976) it is apparent that the ontogeny of striatal DA receptors and DA-sensitive adenylyate cyclase follow a different pattern. Besides this difference in time-course, it appears that there is also a difference in the nature of trophic factors influencing these two postsynaptic entities (Iversen, 1975). Hence, antagonism of postsynaptic sites by haloperidol that blocked the access of DA and perhaps other presynaptic trophic influences from interacting with these sites interfered with the normal development of the D-2 receptor but not the DA-sensitive adenylyate cyclase.

Finally, the results of these experiments indicate that a given drug (in this case haloperidol) can have differential effects on the development of different receptor populations within the striatum. The implication is that in future studies, continued consideration must be

given to the existence of multiple classes of receptors and their differential response to a given treatment. Otherwise, erroneous conclusions may be drawn from the observations made of only one set of receptors. A case in point within this thesis is that if only DA-sensitive adenylate cyclase was measured, the conclusion may have been that prenatal haloperidol treatment has no effect on the normal development of postsynaptic striatal DA receptors. It would be of interest next to determine whether this differential responsivity during development is a general feature of all brain transmitter systems with multiple classes of receptors.

Increased Locomotor and Emotional Behaviour of Offspring

The question of whether deficits in pre- and post-synaptic development in the brains of offspring would effect their behaviour was tested using an open field chamber. This procedure has the advantage over other behavioural techniques in that it allows noninvasive observation of the spontaneous activity of the offspring including the individual components of their behaviour (Stoof et al., 1978). The results of these analyses indicated that 25 day old-offspring whose mothers were treated with haloperidol showed a significant increase in restless behaviour (increased number of squares crossed and frequency of rearing with a decrease in grooming behaviour) compared to controls (Methods and Results, Part D; Plach et al., 1980a). In accord with these findings O'Donnell and Seiden (1980) have also reported that haloperidol offspring showed locomotor hyperactivity up to postnatal day 28 when placed in a stabilimeter. The mothers of these offspring, however, had received haloperidol

in very large doses, in addition to those used in this study, and were treated for the duration of pregnancy (Ibid).

This thesis served to extend these observations, not only by differentiating what aspects of behaviour were increased, but also by demonstrating that this hyperactivity was still apparent 60 days after birth, which is considered adulthood in the rat (Joffe, 1969). In addition, since there is a sex difference in rat behaviour (Fonseca et al., 1976) both male and female behaviour was recorded and analyzed separately, a consideration that is often overlooked in developmental studies of behaviour (Coyle et al., 1976). The results of these experiments indicated that the spontaneous behaviours of female rats, particularly locomotion and rearing, was greater than male littermates and that this sex difference was most apparent in haloperidol littermates at 60 days of age. This suggests that perhaps female offspring were more sensitive to prenatal treatment with haloperidol. With regard to both sexes, it is clear that this treatment produced a long term, perhaps permanent change in behaviour despite the fact that relatively low doses of drug were administered and the period of treatment was only one week.

A particularly interesting feature of this behaviour was that the hyperactivity was most apparent on first exposure to the open field chamber and declined on subsequent exposures to the same situation. Since first exposure to the novel situation may represent a stress it is proposed that the observed hyperactivity may be an emotional reaction (Joffe, 1969). Hence the significantly greater hyperactivity of haloperidol offspring compared to controls of the same age and sex may indicate an increased emotional reactivity due to the prenatal treatment.

with haloperidol. If this can be generalized to the human, it has serious implications for the use of drugs like haloperidol during pregnancy. In particular, they may lead to subtle changes in the behaviour of the offspring, perhaps involving emotional reactivity under stress, despite their not producing observable changes in the physical development of these offspring. Clearly, there is a need for further work in the area of behavioural teratogenesis (Coyle et al., 1976; Spyker, 1975).

The question that remains unresolved is "what is the basis of the observed changes in behaviour?". The results of this thesis provide a number of correlations that provide a focus for further study. For example, there is a positive correlation between the increase in locomotor and emotional behaviour of haloperidol offspring and an increase in DA-induced fluorescence in the mesolimbic areas of their brains. Since the mesolimbic system may be involved in the regulation of emotional (Stevens, 1979) and locomotor behaviour (Kelly et al., 1975; Pijnenburg et al., 1975) it may be that enhanced activity within this system is responsible for mediating the behavioural hyperactivity. Confirmation of this hypothesis requires further analysis of electrophysiological activity within this system compared to others, for example, the nigrostriatal system that is also involved in the control of motor behaviour (Guyton, 1972), to determine whether the prenatal administration of haloperidol effected the level of activity in these neurons. In addition, it would be of interest to do a quantitative analysis of both DA and norepinephrine in these specific brain areas of haloperidol and control offspring since both have been implicated in the regulation of locomotor activity (Snyder, 1973).

Alternatively, there is a negative correlation between the observed increase in locomotor behaviour and decrease in DA-induced fluorescence in the striatum. DA is known to exert an inhibitory influence on the activity of some cells within the striatum (Guyton, 1972). Hence, a decrease in DA would release these cells from a tonic suppression and perhaps mediate an increase in locomotor activity. Unfortunately, not enough is known yet about the precise mechanisms by which DA neurons regulate locomotor and perhaps emotional behaviour, so that these questions remain unresolved at this time.

Differential Effects of Haloperidol on the Development of Mesolimbic vs Nigrostriatal DA Systems

The data from the fluorescence microscopy analysis described in the Methods and Results, Part A indicate that the prenatal administration of haloperidol produces different effects on the development of the mesolimbic compared to nigrostriatal DA systems. While DA-induced fluorescence in the striatum of haloperidol offspring was decreased compared to controls on postnatal days 15 and 25, it was increased in nucleus accumbens and olfactory tubercle regions of the same brain sections. The reason for this difference is at this time unclear, although there are a number of important contributory factors that should be considered.

The nigrostriatal and mesolimbic DA systems differ in a number of important ways as is indicated by the results of pharmacological, biochemical and behavioural studies that have already been described in the Introduction (Parts B and C) of this thesis. Of particular interest, with regard to the results of this thesis is the evidence that the ontogeny of

these two DA systems, as well as their sensitivity to haloperidol may be different. For example, Olson et al. (1972) found that DA terminals that appear as "dotted fluorescence" in the mesolimbic areas develop earlier than the "diffuse fluorescence" type found mainly in the striatum. The rate of DA turnover in the former is comparatively slower than that of the latter (Olson et al., 1977). Ohman et al. (1977) found that chronic administration of haloperidol produced a greater increase in the rate of DA synthesis in the striatum compared to mesolimbic neurons. Furthermore, Engel and Lundborg (1976) have found that treatment of nursing rats with the neuroleptic penfluridol lead to a reduced turnover in mesolimbic DA neurons of offspring but not that of the striatal system.

It is not surprising in this study, therefore, that DA fluorescence may be increased in the mesolimbic system while it is decreased in the striatum. For example, the developing axons of the two systems may have a different growth rate and target distance so that haloperidol treatment for a discrete period may have a differential effect on them. Couple to that the observed reduction in turnover rate within mesolimbic neurons induced by haloperidol, that could lead to an accumulation of DA within this system (Engel and Lundborg, 1976). In addition, Engel and Lundborg (1976) report that impulse flow in mesolimbic neurons is apparently decreased following neonatal penfluridol treatment. If haloperidol produces similar effects, then the increased fluorescence observed in this study may be due to an accumulation of endogenous DA subsequent to reduced impulse flow and transmitter release from these neurons.

Determination of whether or not these hypotheses are accurate depends on further investigations using microscopic, biochemical and

electrophysiological techniques. Nevertheless, the results of this study indicate that further attention should be directed at comparing the differential effects of various treatments on different DA systems in the brain. Otherwise, one runs the risk of drawing inaccurate conclusions from data limited to a single pathway that may not be applicable to the others.

Abnormal DA Regulation of Prolactin Secretion

The tuberoinfundibular (TIF) DA system differs from the other brain DA systems in that its postsynaptic targets are prolactin secreting cells in the pituitary. There is a distinct separation between presynaptic DA terminals in the hypothalamus and postsynaptic receptor sites in the pituitary. This provided an opportunity to study the effects of prenatal antagonism with haloperidol on the development of a population of DA receptors that is wholly postsynaptic (Brown et al., 1976; 1979). An added advantage was that the development of these receptors could be evaluated using a relatively accessible and precise parameter under DA control in vivo, that is, prolactin secretion (Labrie et al., 1979).

DA neurons in the TIF system exert a tonic inhibitory influence on pituitary prolactin secretion (MacLeod, 1976). To test whether prenatal haloperidol treatment altered this control, 25 day old offspring were challenged with a low dose of haloperidol that was demonstrated in prior experiments to increase serum prolactin levels in normal rats at this age (Methods and Results, Part E). A comparison of serum prolactin response one hour after the challenge injection with haloperidol indicated that the serum prolactin levels of offspring from haloperidol treated mothers did not rise to the same degree as those whose mothers received vehicle

alone (Plach et al., 1980b). This suggested that in accord with the results in the striatum, DA receptors on prolactin secreting cells in the pituitary did not develop normally due to prenatal antagonism with haloperidol, hence the sensitivity of these cells to DA was abnormal:

To test this hypothesis, anterior pituitary cells from haloperidol and control offspring were stimulated directly by DA in vitro and dose-response curves of prolactin secretion were compared (Part E). The dose-response curves in several series of experiments, however, did not appear to be significantly different. It does not seem likely, therefore, that the observed abnormality in serum prolactin response can be entirely accounted for by an abnormality in pituitary DA receptors.

A number of alternate explanations might be proposed. For example, in view of the changes in development of presynaptic DA terminals in the nigrostriatal and mesolimbic systems, it may be that prenatal haloperidol treatment also effected TIF DA terminal development. If this lead to an increase in number of terminals or an excess in DA content, then the challenge dose of haloperidol used in the first series of experiments may have been insufficient to compete with endogenous DA levels, hence the rise in serum prolactin would be less than expected, which is what was observed (Part E). To test this hypothesis, it would be necessary to carry out microscopic analysis and precise quantitation of DA content in TIF neurons. Initial attempts were made in this respect, but the results of these experiments were not available at the time this thesis was compiled.

Another point of particular interest was the consistent sex difference in prolactin secretion that was also observed in the behav-

avioural studies already described. Female rats, at 25 days of age, showed a greater increase in prolactin secretion in response to a haloperidol challenge (in vivo) as well as to direct stimulation by DA (in vitro) than did males that were their littermates. Dohler and Wuttke (1974) have found that serum prolactin levels of female rats are closer to adult levels by postnatal day 25 than are the males. The results of this study confirm and extend this observation by indicating that there is also a significant sex difference in response to haloperidol treatment at this age.

On the basis of the behavioural and prolactin results of this thesis, it appears that female rats are more sensitive to both stimulation and inhibition of DA receptors, whether these receptors are involved in the regulation of locomotor behaviour by the brain or regulation of prolactin secretion by the pituitary. Prenatal treatment with haloperidol, however, does not appear to change this difference in sensitivity. The implication of these observations is that further systematic study should be made of the sex differences in behavioural and hormonal responses to haloperidol since it may be of considerable importance in the clinical use of neuroleptics by women of child-bearing age, as described in the following.

Clinical Implications

The results of this thesis indicate that treatment of maternal rats with the neuroleptic, haloperidol during the third trimester produces abnormalities in the development of brain DA neurons and post-synaptic receptors that may lead to long term abnormalities of behaviour and hormonal regulation in the offspring. Haloperidol and other neuro-

leptics are used not only in the treatment of psychoses but also as an adjunct in labour to reduce emotional tension, control nausea or to potentiate analgesia (Goldberg and DiMascio, 1978). Neuroleptics can cross the placental barrier and detectable concentrations have been reported in fetal circulation (Mirkin, 1973). There have been reports of neurological deficits in newborns, especially following treatment with neuroleptics during the last trimester (Goldberg and DiMascio, 1978). Behavioural abnormalities, including extrapyramidal reactions, hypotonicity and lethargy have also been reported (Hill et al., 1966; Tamer et al., 1969; Levy and Wisniewski, 1974; Hammond and Roselars, 1970).

A complicating factor in most neonatal studies is the concurrent use of a number of different drugs so that it is not clear which drug, or combination of drugs, may be detrimental. One factor that stands out, however, is that very few reports attribute teratogenic effects to neonatal treatment with neuroleptics, aside from some evidence that chlorpromazine can produce retinopathies in the newborn (Goldberg and DiMascio, 1978). Here, the results of this thesis may shed some light.

In preliminary experiments, it was demonstrated that only high doses of haloperidol (for example, 3.2 mg/kg in the rat) produced complications in maternal pregnancy and reduced the viability of the offspring (Methods and Results, Part D). Those that survived past the trauma of birth were much smaller in size and within the first week, 50 percent had died. At lower doses, however, no overt physical abnormalities were observed. Instead, there appeared to be long term subtle changes in behaviour and hormonal regulation of the offspring (Parts D and E). Offspring of maternal rats treated with haloperidol were hyper-

active, especially when placed in a novel environment, which suggests an increase in emotionality (Joffe, 1969). In addition, these offspring did not show a normal prolactin response to a later challenge injection with haloperidol, that normally causes serum levels of this hormone to increase.

Besides demonstrating that prenatal treatment with haloperidol produced abnormal development of brain DA systems, these data demonstrate that the effects produced are subtle, perhaps only expressed when challenged by a mild stress. Furthermore, the behavioural and hormonal changes were still apparent long after birth when the offspring were reaching maturity. For example, haloperidol offspring were still hyperactive at 60 days after birth, considered adult in the rat. Hence, haloperidol may be a behavioural teratogen (Coyle et al., 1976; Spyer, 1975). In other words, the administration of this drug during pregnancy may lead to subtle and long term changes in behaviour in the absence of overt physical abnormalities in the offspring.

The implications of these findings are that offspring of mothers ingesting neuroleptics may develop abnormal brain DA neurons and thereby are predisposed to long term problems, particularly in stressful situations, that are a consequence of abnormal function within this neural system. For example, the child or young adult may be predisposed to affective reactions, psychoses, or perhaps even schizophrenia (Snyder et al., 1974; Stevens, 1979). Of course, this remains theoretical at this point and much further study is required to determine whether the results of this thesis are generalizable to other mammals and primates, especially man. Nevertheless, it is clear that studying the effects of prenatal administration of neuroleptics is of value not only in providing a

better understanding of the development of brain DA neurons but also in determining the potential clinical toxicity of these drugs in terms of subtle, long term consequences on behaviour and hormonal regulation.

APPENDIX 1

MODIFICATION OF SPG METHOD FOR PROCESSING OF NEONATAL BRAIN TISSUE

Initially the neonatal brain tissue was processed precisely according to the method described by de la Torre and Surgeon (1976; personal communication). There were a number of problems, however, that required modification of the technique. The first problem was the excessive cracking and tearing of the tissue during sectioning in the cryostat. This was a particularly difficult problem since 2 brains were being sectioned at the same time (side by side) and valuable samples were being lost this way. Part of the problem appeared to arise from the neonatal brain tissue being too brittle at the recommended temperature of minus 30°C. There also seemed to be an inconsistency in the density of the tissues as they were being sectioned that lead to variations in the thickness of sections when picked up with the microscope slides. These problems were eliminated first, by sectioning in the cryostat at the slightly higher temperature of minus 20°C, which prevented the tissue from becoming too brittle. Secondly, the tissues were placed in the cryostat holding-chamber for at least 15 to 20 minutes before sectioning, which allowed the tissues to equilibrate to the temperature and conditions of the cryostat. This not only provided consistency in tissue section thickness but also facilitated a more rapid processing of the neonatal brains.

Another problem that required improvement was the nonspecific background fluorescence found in neonatal brain sections, particularly

during the first 2 postnatal weeks when it was most apparent. Background fluorescence after this stage was less of a problem since levels of endogenous DA-induced fluorescence became relatively greater afterwards. According to the procedures of another glyoxylate fluorescence technique, described by Lindvall and Bjorklund (1974), it is necessary that the tissues be processed under very dry conditions during the reaction. This principle therefore was incorporated in these experiments by placing a dessicant, Drierite, (CaSO_4), into the oven as it was heating up to 80°C and leaving it there while the slides were incubating. This maintained a low humidity environment during the incubation period and reduced the nonspecific background fluorescence in neonatal brain sections.

A difficulty that arose in the preparation of slides for viewing and storage was the presence of bubbles in the medium between the slide and the coverslip. The medium that was recommended for coverslipping was mineral oil, without specification of grade. The author found that the USP thin grade mineral oil was best for minimizing the presence of bubbles. In addition, gradually warming up the mineral oil to about 40°C before applying a drop to each slide reduced the amount required before coverslipping, hence the coverslips remained stationary during storage. This also prevented damage of tissue sections due to shearing pressures.

APPENDIX 2

PHOTOMULTIPLIER SYSTEM

STANDARDIZATION OF PHOTOMETRIC READINGS

A problem that arose in the readings obtained with the photomultiplier system was the occurrence of a gradual shift in photocell resistance over time with exposure to a light source. A graph showing this drift in readings over a period of 75 minutes is shown in Figure 13. Records of photometric readings on exposure to fluorescence light were taken on several different occasions, each time showing the same linear pattern of drift. Since this drift could present a bias in readings taken from haloperidol and control brain sections, depending on which was recorded first, the procedure for taking readings was standardized to avoid potential bias.

The standardized procedure was as follows. The fluorescence microscope and photomultiplier system was turned on and allowed to warm up for 15 minutes before readings began. Then multiple readings were taken of each brain section examined. For example, when comparing the striata of a haloperidol brain and its corresponding control on the same slide, alternate 30 second readings were taken of each striatum interspersed with 30 second delays of darkness between each reading. Readings were taken over a period of 1 hour followed by a period (20 minutes) of darkness to allow the system to return to its initial starting point again.

The triplicate readings obtained from each section were graphed

BACKGROUND DRIFT IN UNSTANDARDIZED PHOTOMETRIC READINGS

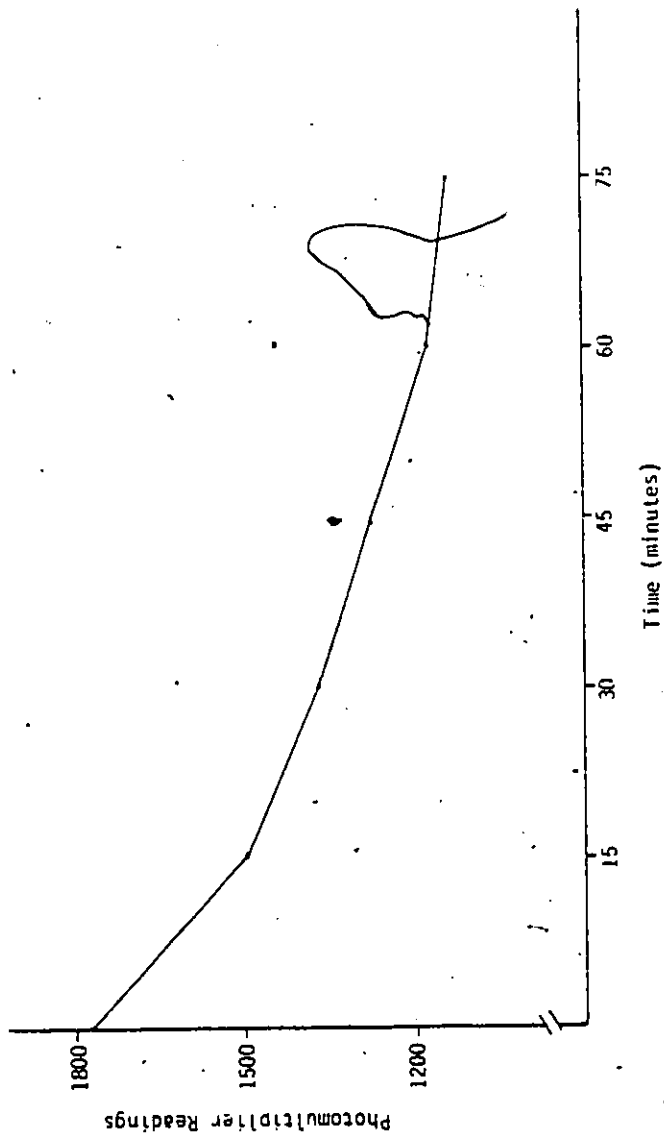


Figure 13: To test the stability of photocell resistance over time, photomultiplier readings were recorded during a 75 minute exposure of the system to continuous fluorescence light. The recordings showed a consistent pattern of initial drop in resistance during the first 15 minutes of exposure, followed by a slower, almost linear drift during the next 60 minutes. -See text for details on standardization readings.

over time and the mean of the slope recorded. The difference between these means was then compared to determine the difference in intensity of fluorescence emitted from the haloperidol and control striatum (Table 1, Methods and Results, Part A). The same procedure was repeated for each terminal area examined including the nucleus accumbens, olfactory tubercle, cortex, as well as striatum. At least 3 slides for every pair of brain sections examined was analyzed this way. This includes all haloperidol-control pairs at the different doses of haloperidol given prenatally, as well as control-control pairs.

TESTING THE SENSITIVITY OF THE PHOTOMULTIPLIER SYSTEM VERSUS SUBJECTIVE DISCRIMINATION

Before the photomultiplier system was used to analyze the fluorescence intensity emitted by experimental brain sections, a test of its sensitivity was first carried out using a double-blind procedure. A set of 20 photographic slides were presented to 6 people within the Department of Neurosciences (McMaster University). These slides were organized into pairs which ranged in fluorescence intensity from being very different to being essentially the same. The observers were asked to discern which one of each pair of slides was brighter or whether they were the same. Another individual, not involved in this test, took photometric readings of the brain sections from which these slides were prepared. The results of these two tests were then compared.

The photomultiplier readings for this set of slides ranged from 0-10 in the "no different" category, 15-30 in the "slightly different", 40-60 in the "moderately different" and greater than 80 in the "very

different" categories. In general, each observer had difficulty in distinguishing fluorescence intensity in 4 slides out of 20, these being either "no different" or only "slightly different". There was some variation in which of the 10 slides that fell into these two categories presented the most difficulty to the observers. The remaining 8 slides, that were "moderately different" in fluorescence intensity, and 2 slides that were "very different" presented no problems in discrimination.

The results of this test indicate that the photomultiplier system is at least as sensitive, if not slightly more sensitive to differences in intensity of fluorescence that can be detected by direct visual comparison of two slides presented simultaneously. The advantage of the photomultiplier system, however, is two-fold. First, it provides objective readings of the intensity of fluorescence emitted by brain sections. Secondly, this system does not require that 2 slides be presented simultaneously for comparison, as is necessary in subjective assessments especially where the differences between slides are small. This is particularly important since only one section at a time can be viewed under the fluorescence microscope when comparing brain sections of haloperidol and control offspring.

VERIFICATION OF THE PHOTOMULTIPLIER SYSTEM

An additional test to verify the sensitivity of the photomultiplier system was carried out when a commercial photometer, the Photovolt 520M produced by the Leitz Company of Canada, was purchased by Dr. G.M. Brown, Department of Neurosciences, McMaster University. The Leitz system was designed for the same function as that devised by the author,

that is, to quantitate intensity of fluorescence emitted by tissues observed with a fluorescence microscope. The Leitz photometer system, however, arrived in the Department just after the author's experiments were completed.

A series of slides that had been previously prepared were analyzed using both the photomultiplier system devised by the author, and the Photovolt 520M Leitz system. The author took readings of these slides using the photomultiplier system while a technician in Dr. Brown's laboratory took readings of the same slides on the Leitz system. These readings were then compared.

In all cases, the values obtained by the two systems showed close agreement. Large differences in readings were obtained from sections that emitted very different intensities of fluorescence and vice versa. The Leitz Photovolt 520M, however, showed a higher sensitivity at the low ranges of fluorescence intensity and gave less fluctuation in readings owing to the addition of a stabilizer to the Leitz power source so that fewer repeated readings were necessary. Nevertheless, the results obtained by the two systems in comparing relative differences in fluorescence intensity were the same.

APPENDIX 3

PROTOCOL FOR PREPARATION OF BRAIN BINDING PROTEIN

1. Decapitate rats and immediately remove brains to beaker with 1% NaCl, on ice.
2. Homogenize tissue in 3 volumes of ice-cold 4mM EDTA, titrated to pH 7.0 with 0.1 N NaOH, using waring blender at low speed for 2 minutes.
3. Centrifuge at 15000 rpm, 4°C for 30 minutes.
4. Decant supernate into a beaker on ice and titrate to pH 4.8 with 1.0 N HAC (glacial acidic). Continue stirring for 10 minutes.
5. Centrifuge at 15,000 rpm, 4°C for 30 minutes.
6. Decant supernate into a beaker on ice and titrate to pH 6.5 with 1.0 M k PO₄ buffer (pH 7.2). Measure final volume.
7. Gradually add ammonium sulfate crystals (Sigma, special grade) at 3.25 g/10 ml of supernate. Continue stirring for 30 minutes, on ice.
8. Centrifuge at 11,000 rpm, 4°C for 20 minutes.
9. Decant and discard supernate.
10. Dissolve pellet in 5 mM K PO₄ buffer plus 2 mM EDTA (pH 7.0). Use 0.24 ml of buffer/gram of starting tissue.
11. Pour into dialysis tubing* and dialyze in 20 volumes of same buffer for 16 hours at 4°C, replacing fresh buffer 2 times during this period.
12. Centrifuge remaining content of dialysis tubing at 15,000 rpm, 4°C for 30 minutes.
13. Decant supernate without disturbing pellet and divide into approxi-

mately 200 μ l portions for storage in plastic tubes.

14. Cap tubes tightly and store frozen (-70°C).

* Dialysis tubing ($\frac{1}{8}$ inch diameter) should be presoaked overnight in 0.2 M EDTA at 4°C , then rinsed with deionized water prior to filling and dialysis.

This protocol was drawn up in consultation with Dr. R. Mishra, Department of Pharmacology, McMaster University, whose laboratory was employing the same procedure.

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