

THE ACTION OF PSYCHOACTIVE DRUGS ON THE RELEASE OF
NEUROTRANSMITTERS IN THE BRAINS OF CONDITIONED ANIMALS

by

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ABSTRACT

Specific animal behavioural models are employed to evaluate neuroleptics which might be useful clinically. These drugs are known to influence neurotransmission in the central nervous system. The conditioned avoidance behavioural model was chosen as a suitable system in which to study the effects of psychoactive drugs on neurotransmitters in the brain. This study was designed to make comparisons of neurotransmitter release patterns in brain superfusates from animals subjected to different behavioural strategies. These included naive rats, rats whose behaviour had been modified by avoidance-escape training schedules, and rats whose conditioning had been extinguished by drug treatment. The drugs chosen for this study were trazodone (100 mg/kg) and haloperidol (0.1 mg/kg); both extinguished the conditioned response leaving the escape response intact.

A modified brain slicer was used to obtain tissue from two regions of the brain established in reports of stereotaxic lesioning experiments as being implicated in conditioned avoidance behaviour. Superfused slices were electrically stimulated in a specifically designed chamber with microgold electrodes. Noradrenaline, dopamine and dihydroxyphenylacetic acid in superfusate fractions were measured using high performance liquid chromatography with electrochemical detection.

Experiments on slices preloaded with tritiated dopamine established the conditions for subsequent studies of the release of endogenous neurotransmitters. After serial stimulation release of the radiolabel was found to be Ca^{2+} dependent, a criterion for neurotransmission. Trazodone caused a significant

increase in ^3H -dopamine released from the slices by electrical stimulation. Preliminary studies with haloperidol showed a decrease in ^3H -dopamine release.

Studies on electrically stimulated release of noradrenaline and dopamine from corticolimbic and corticostriatal showed:

1. In all brain slices - a significant increase over basal (non-stimulated) release.

2. In brain slices from conditioned rats compared with brain slices from naive rats - a significant decrease.

3. In brain slices from rats with conditioning extinguished
(a) by trazodone - a significant increase over conditioned rat brain release figures, the naive pattern was re-established.

(b) by haloperidol - a significant reduction in dopamine released from one of the regions of the brain in comparison with the conditioned mode.

This study indicates that conditioning may be correlated with changes in dopamine and noradrenaline release patterns in specific areas of rat brain. These modifications appear to be reversible by trazodone but not by haloperidol, even though both drugs extinguished the learned response. In the light of these results possible differences in the mechanisms of psychoactive drug action are discussed.

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ABBREVIATIONS

mg/min	milligram per minute
μ M	micro molar
mg/kg	milligram per kilogram
nm	nanometer
mm	millimetre
mV	millivolt
Hz	Hertz
mM	milli molar
cms	centimeters
i.v.	intravenous
ng/g	nanogram per gram
pA	pico ampere
Fig	figure
C.N.S.	central nervous system
B.C.	Before Christ
L.S.D.	lysergic acid diethylamide
DA	dopamine
NA	noradrenaline
5-HT	5-hydroxytryptamine
D β H	dopamine- β -hydroxylase
PNMT	phenylethanol-N-methyltransferase
L-DOPA	L-3-4-dihydroxyphenylalanine
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
COMT	catechol-o-methyl-transferase
Ach	acetylcholine
NMJ	neuromuscular junction
ATP	adenosine triphosphate
Ca ²⁺	calcium ions
TEA	tetraethylammonium ions
K	potassium
Na	sodium
GABA	gamma-aminobutyric acid
AgCl	silver chloride
ms	millisecond
V	Volts
ADP	adenosine diphosphate
AIB	aminoisobutyrate
g	grams
mg	milligram
MHPG	3-methoxy-4-hydroxyphenylglycol
i.p.	intra peritoneal
5HIAA	5-hydroxyindolacetic acid
HVA	homovanillic acid
DOPAC	dihydroxyphenylacetic acid
CR	conditioned response
UCR	unconditioned response
UCS	unconditioned stimulus
CS	conditioning stimulus
mA	milli ampere
CAR	conditioned avoidance response
UER	unconditioned escape response
EF	escape failure
NSM	non-signal movement
S.e.m.	standard error of the mean
Tc ^{99m}	technitium

O ₂	oxygen
CO ₂	carbon dioxide
EGTA	ethyleneglycoltetracetic acid
BAL	2,3-dimercaptopropanol
HPLC	high performance liquid chromatography
Ag	silver
SOS	sodium octylsulphate
DHBA	dihydroxybenzylamine
2 DG	2 deoxyglucose

SECTION 1: GENERAL INTRODUCTION

The introduction of chlorpromazine into psychiatric therapy was a most important advance, and resulted in the development of a whole new era of psychotropic drugs which in turn have shed light on aspects of the neurophysiology and pathophysiology of the central nervous system.

The psychoactive properties of these drugs were tested using specific animal behaviour models such as the conditioned avoidance behavioural system. These drugs were subsequently used in clinical trials after which they were prescribed therapeutically.

The advances in the understanding of the mode of action of these drugs depended upon the discovery of neurotransmitters, neuronal pathways and the establishment of the synapse and neurotransmission. This led to the identification of vesicles and pre and post synaptic membranes separated by a synaptic cleft. On the basis of the presence of synaptic vesicles came the proposition of the 'quantal' aspect of transmitter release and the mode of chemical transmission. From studies of synaptic transmission evolved the ideas of modulation of release and the subsequent development of these ideas by means of drugs. Initial studies of release mechanisms and the influence of drugs on this release were done using tissue preloaded with radiolabelled amine and subsequently released using electrical or chemical stimuli. From these studies arose the "receptor blockade" and "coupling blockade" theories of antipsychotic drug action. With the development of sensitive techniques for the estimation of catecholamines, endogenous transmitter release studies became possible.

The aim of this project was to study how conditioning may be correlated with changes in dopamine and noradrenaline release patterns in specific areas of rat brain and how haloperidol and trazodone may influence this release.

Historical account of psychoactive drugs

Agents used in the treatment of psychiatric disorders are often described as psychoactive. Psychoactive agents which alter central nervous system (CNS) function include both psychotoxic and psychotropic substances. Psychotoxic substances are natural products and herbal extracts used mainly for pleasure purposes whereas the psychotropic drugs are used for psychiatric conditions. Products such as opioids, alcohol, cannabis and tobacco formed part of ancient folklore in many parts of the world, especially with respect to religious practices. These substances had limited therapeutic use until recent times. Over 1500 compounds have been described primarily as psychoactive (Usdin, 1978).

The use of psychoactive drugs by many different cultures goes back into ancient times. For example, soma, which is a hallucinogenic fungus, has been traced back to the times of the earliest Aryan invaders of India, and is repeatedly referred to in the Vedas which are religious Hindu writings composed between the 15th and 5th centuries B.C. Aldous Huxley (1932), in his book *Brave New World*, gives the name soma to his fictional 'social' drug of the future. Cannabis can be traced back at least 8000 years, and coca leaves were found in the early mummy bundles in Peru. Commenting on *The Odyssey*, presumed to be written by Homer in 900 B.C., both Schmeideberg (1918) and Lewin (1920) believe that the drink Helen gave to Telemachus, Peisistratus and Menelaus in Sparta, contained opium. The drink

was given in order to alleviate a sad and sorrowful atmosphere, engendered during discussions about Odysseus, who had failed to return from the Trojan war. Homer describes it as follows:

"Then Helen, daughter of Zeus, took other counsel.

Straightaway she cast into the wine of which they were drinking a drug to quit all pain and strife, and bring forgetfulness of every ill. Who so should drink this down, when it is mingled in the bowl, would not in the course of that day let a tear fall down over his cheeks, no, not though his mother and father should lie there dead, or though before his face men should slay with the sword his brother or dear son, and his own eyes behold it." Homer, The Odyssey IV, 220-223.

Translated by A. T. Murray.

Mandrake, an alkaloid of the Solanaceae plant family, is a psychoactive agent which in large doses caused mental aberrations, but in small doses was used to combat depressive states with suicidal tendencies and forms of motor hyperexcitability. For these reasons Mandrake was thought to be a "drug for insanity". Homer also describes another drug which Lewin (1920) believes to be the mandrake root (mandragora). The gods are said to have sent Hermes (Argeiphontes) with the magic drug to protect Odysseus against Circe. Odysseus describes the event as

"... So saying, Argeiphontes gave me the herb, drawing it from the ground, and showing me its nature. At the root it was black, but its flower was like milk. Moly the gods call it, and it is hard for mortalmen to dig; but with the gods all things are possible." Homer, The Odyssey X, 302-307. Translated by A. T. Murray.

The use of psychoactive drugs evolved along two pathways. The first was in the use of the drug to modify normal behaviour and to produce altered states of feeling for religious, ceremonial or recreational purposes. The second was to alleviate mental illness. In 1845, Moreau de Tours suggested that hashish intoxication provided a model psychosis useful in the study of insanity. It was thirty years later that Freud suggested the potential use of cocaine in pharmacotherapy. Soon after Freud's study with cocaine, Kraepelin founded the first laboratory of clinical psychopharmacology in Dorpat, where he evaluated psychological effects of drugs in man. Sen & Bose (1931) published the first report on the use of *Rauwolfia serpentina* in the treatment of insanity. This was followed by the use of insulin shock in 1933, leptazol-induced convulsions in 1934, and electroconvulsive therapy in 1937 for the treatment of both depression and schizophrenia. In 1938 Hoffman synthesised lysergic acid diethylamide (LSD) and discovered its psychoactivity in 1943 when he happened to inhale some.

Historical precedence with respect to the antipsychotics should be given to the use of the *Rauwolfia* plant in the treatment of insanity (Sen & Bose, 1931). The active principle, reserpine, was extracted from *Rauwolfia serpentina*, and first used in a clinical trial by Kline (1958). Pure reserpine was later synthesised by R. B. Woodward in the United States. Reserpine, in its ability to deplete monoamines from vesicular storage sites in neurons, exerts antipsychotic effects which are weak and often associated with severe side effects which include excessive salivation, diarrhoea and sedation.

In 1893, August Heinrich Bernthsen synthesised phenothiazine, or thiodiphenylamine, by heating diphenylamine with sulphur, an event that was destined to revolutionise

psychiatry. About the same time in 1896, Emil Kraepelin as cited by Caldwell (1978) defined the psychiatric syndrome which he called 'dementia praecox' and which is now called schizophrenia. Phenothiazine was the progenitor of a group of drugs that have since been demonstrated to be of value in the treatment of schizophrenia, especially chlorpromazine.

Prior to the synthesis of chlorpromazine, promethazine, (an antihistamine) was introduced into surgery in 1949, by Laborit as a centrally acting autonomic drug to mitigate against shock. In 1950, Paul Charpentier synthesised chlorpromazine, 57 years after Bernthsen first synthesised phenothiazine. Paul Charpentier sent samples of chlorpromazine to the pharmacologist Simone Courvoisier. Courvoisier, Fournel, Ducrot, Kolsky & Koetschet, (1953) published a paper on the drug listing an amazing number of actions, including gangliolytic, adrenolytic, antifibrillatory, antioedematous, antipyretic, antishock, anticonvulsant, antiemetic and the inhibition of conditioned reflexes. Laborit, Huguenard & Alluaume (1952) as cited by Caldwell (1978), noted that chlorpromazine by itself did not cause a loss of consciousness but produced only a tendency to sleep and a marked lack of interest in what was going on. These central effects became known as "ataractic" meaning untroubled or neuroleptic, a term introduced in the early 1950's by Delay & Deniker, neuron, meaning nerve, and lepsis, meaning seizure. Laborit, Hamon, Paraine and Velluz, at a meeting of the Société Médico-Psychologique in Paris in 1952, described the beneficial effects of chlorpromazine in a manic patient (Swazey, 1978). This was followed by a report by Delay & Deniker, (1952) as cited by Baldessarini (1980) on the treatment of mental illness by chlorpromazine.

In the years that followed the discovery of chlorpromazine,

many structural analogues similar to chlorpromazine were synthesised. They were first tested using animal behaviour model systems, and subsequently in clinical trials after which they were prescribed therapeutically. Particularly important in this regard is the work of Janssen working in Belgium in the 1950's with a group of pethidine analogues in the hope of developing an improved analgesic agent. He found that propiophenones had analgesic effects, but the addition of one methylene group produced a butyrophenone with quite unexpected neuroleptic and analgesic effects in animals. Modification of the butyrophenone structure led to the development of a neuroleptic with potency similar to the piperazine phenothiazines or thioxanthenes. In 1958 the first of these products, haloperidol, became available in clinical psychiatry (Janssen & Van Bever, 1978).

Animal models for screening drugs

Since the advent of neuroleptics, several attempts have been made in the field of psychopharmacology, to find a specific animal model of human psychosis, which would function both as a reliable predictor of neuroleptic activity and as a tool to further our understanding of the mode of action of psychoactive drugs. Needless to say such a behavioural model has not been found. Psychoses can manifest themselves by disturbances in interpersonal relationships, internal conflict, anxiety and depression often revealed only through verbalization. Such essential characteristics of human mental disease cannot be reproduced in animals. However, there exist several animal tests which are used to screen for potential neuroleptic activity. Once the properties of the drug have been established in animal tests, new structurally analogous compounds with similar actions can be synthesised. Such animal tests include inhibition of

conditioned avoidance responses, inhibition of exploratory behaviour, inhibition of electroencephalogram (EEG) arousal, antagonism of brain dopamine, neuroleptic-induced catalepsy, ptosis and sedation (Janssen & Van Bever, 1978). Although the study of the action of drugs on animal behaviour has not yielded much enlightenment with respect to the mode of action of drugs in abnormal human behaviour, such studies have led to screening procedures for selection of drugs in the treatment of mental illness.

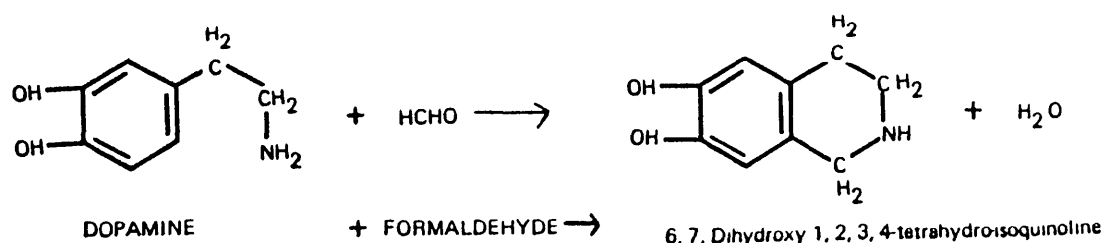
The behavioural effects of psychoactive drugs are studied using techniques based on conditioned reactions. These techniques, although originally derived from Pavlov's classical experiments, do not conform to the original with regard to stimuli presented and responses elicited. The screening procedure most commonly used for the evaluation of neuroleptic drugs has been the conditioned avoidance behavioural model (p81 of this thesis).

Neurons, neurotransmitters and their pathways

Advances in our understanding of the mode of action of such drugs was dependent upon insight into how the brain worked. The discovery of neurotransmitters, the mapping of the neurons containing them, followed by an appreciation of the importance of the synapse, laid the foundation for progress in our understanding of neuroleptic drug action. The discovery of these neurotransmitters was due to the development of very sensitive and specific histochemical fluorescence techniques used for the demonstration of the existence of dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in the CNS. This technique was introduced into neurochemistry by Falck (1962). The method is based on the principle that amines can be transformed into

intensely fluorescent isoquinoline derivatives by condensation with formaldehyde gas.

The dopamine reaction would be:-



Using the Falck and Hillarp technique (Falck, Hillarp, Thieme & Torp, 1962), Dahlström & Fuxe (1964) found that nerve cell bodies in the lower brainstem, contained low concentrations of catecholamines and 5-HT. The cell cytoplasm fluoresced weakly, principally in the perinuclear zone. The nerve cell bodies gave rise to thin unmyelinated fibres which divided into fine terminal branches on reaching the area of the brain to be innervated. The fluorescence in terminals was concentrated in small enlargements of the terminal branches called 'varicosities'; these also occur on the axon itself. From evidence provided by microscopic studies, amines were shown to be present in granules or vesicles inside the varicosities (p28). Dahlström & Fuxe (1964) described the distribution of catecholamine neuron groups and classified them primarily on the basis of topographical and morphological evidence, into twelve groups, designated A1-12. These cell groups occur in the medulla oblongata, pons, mesencephalon, caudal thalamus and mediobasal hypothalamus. Later two neuron groups A13 and A14 were found. A13 in the zona incerta and the caudal hypothalamus (Fuxe, Hökfelt & Ungerstedt, 1969), and A14 in the periventricular gray

matter of the anterior hypothalamus and preoptic regions (Björklund & Nobin, 1973).

Using the Falck & Hillarp technique alone, it was not possible to localise low levels of catecholamines in the axons. In order to trace catecholamines in axons, mechanical (Anden, Dahlström, Fuxe, Larsson, Olson & Ungerstedt, 1966) and chemical lesioning studies using 6-hydroxydopamine (Ungerstedt, 1971) had to be applied. Results from these studies, together with the Falck & Hillarp technique, provided coarse pictures of chemical pathways in the intact animal.

With the introduction of the immunohistochemical techniques of Geffen, Livett & Rush (1969) it became possible to differentiate between dopamine, noradrenaline, and 5HT at the cellular level, and to trace their pathways along their entire length in the intact animal. The immunohistochemical technique based on the ability of antibody molecules to bind specific substances (antigens) involved the use of antiserum against the catecholamine biosynthetic enzyme dopamine- β -hydroxylase (D β H) and the adrenergic vesicle protein chromogranin. Thus, neurons which contain only tyrosine hydroxylase and aromatic L-amino acid decarboxylase but not D β H and phenylethanol^{amine}-N-methyltransferase (PNMT) probably represent DA neurons. If, in addition, the neurons contain D β H but not PNMT the neurons probably contain NA as the transmitter. If the neurons contain all the four enzymes in the catecholamine synthesis the neuron probably contains adrenaline. This technique allowed a more precise neuroanatomical identification of monoaminergic cell bodies and their fibre tracts than the conventional histochemical techniques.

A relatively new technique for morphological identification and mapping of transmitter specific neuronal systems has been the

autoradiographic technique (Hokfelt & Ljungdahl, 1971). It is based on the finding that one of the biochemically specialised properties of a catecholaminergic neuron is its ability to selectively take up the transmitter substance by means of a specific high-affinity transport mechanism. This technique has been reviewed by Iversen (1978).

The data on the noradrenergic and dopaminergic neuronal pathways described below have been based on use of the immunohistochemical technique (Geffen et al, 1969; Hokfelt, Fuxe & Goldstein, 1975) autoradiographic tracing studies (Hokfelt & Ljungdahl, 1971) and information derived using the formaldehyde and glyoxylic acid fluorescence techniques (Falck et al, 1962; Lindvall & Björklund, 1974) in combination with lesioning studies (Anden et al, 1966).

Figure 1 shows the ascending forebrain dopamine neuronal systems. Neurons which synthesise dopamine are found in the mesencephalon, the diencephalon and the telencephalon. The mesencephalic dopaminergic cells have been described as forming three cell groups, A8, A9 and A10. The cells of the A8 group are located in the mesencephalic reticular formation. This group merges ventromedially with the A9 group, found in the zona compacta part of the substantia nigra. The A10 group is limited ventrally by the interpeduncular nucleus. Dopaminergic cell groups A11, A12, A13 and A14 have been recognised in the diencephalon (Björklund & Nobin, 1973). Cells of the A11 group are located in the caudal hypothalamic periventricular region, dorsal to the infundibular nucleus, which contains most of the cells of the A12 group. Group A13 is located in the zona incerta, whereas the A14 group constitute a rostral continuation of the A12 group. The dopaminergic cells in the outer zone of the olfactory bulb are designated A15.

Sagittal representation of the principal dopamine (DA) pathways in the rat brain

(Modified from Ungerstedt, 1971)

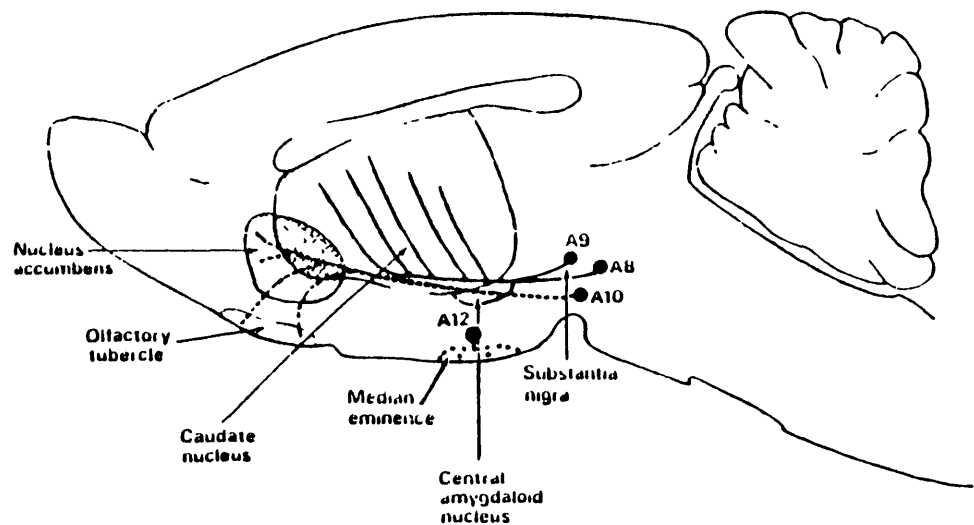


FIG 1: Shows the nigrostriatal pathways (-) from the substantia nigra (cell bodies A8 and A10). The mesolimbic pathways (----) originate from the A10 cell body. The "short" tubero-infundibular DA neurons (....) have their cell bodies in the arcuate nucleus (A12) in the hypothalamus. (Taken from Livett, 1973)

FIG. Sagittal representation of the rat brain, showing the principal ascending and descending NA pathways

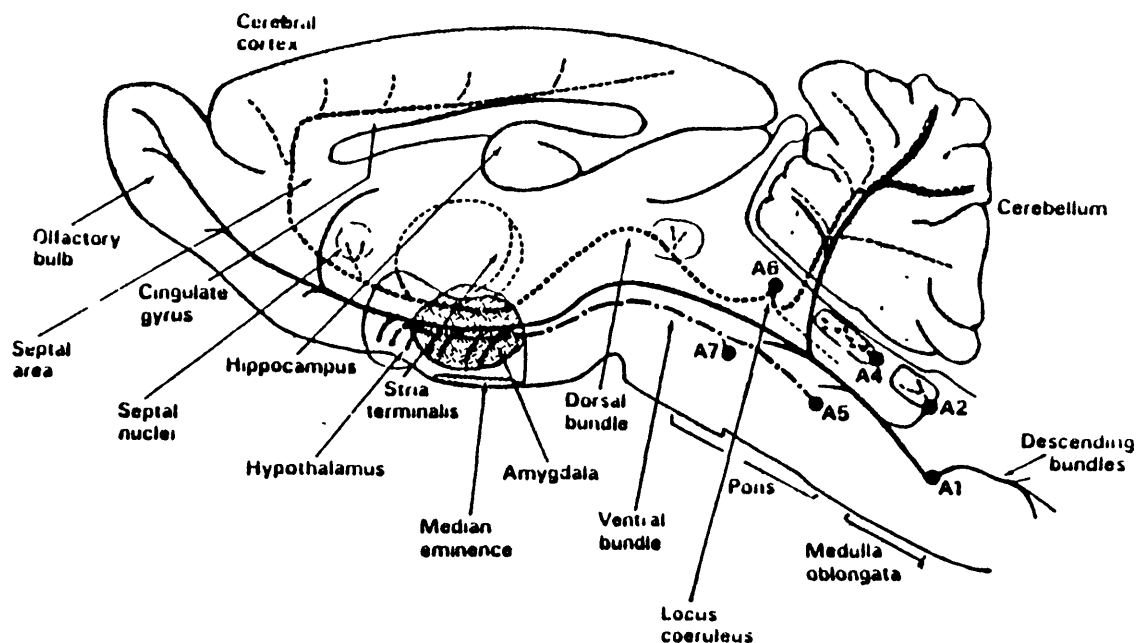


FIG 2: Shown are the cell bodies in the locus coeruleus (A6) giving rise to pathways (---) innervating the cortical regions. The dorsal bundle which arises from A6 also innervates areas of the amygdala and anterior hypothalamus, short descending pathway innervates the lower brain-stem. (-) are the pathways arising from the A1 cell group. (-.-.-) are pathways from A5 and A7 cell groups. Pathways arising from A4 cell group send axons towards the A6 group. (Photograph taken from Livett, 1973.)

The A8 and A9 groups give rise to a large pathway that ascends in the lateral hypothalamic area from where its fibres diverge dorsally and laterally. These fibres traverse the internal capsule and fan out in the caudate nucleus and the putamen where they form a dense network of terminals. This is the nigrostriatal dopaminergic system. Some of the fibres of this system also enter the amygdaloid nucleus. The second system, the mesolimbic dopaminergic system originates mainly from the A10 cell groups. Their fibres ascend medial to the nigrostriatal system in the medial forebrain bundle and are distributed to the following telencephalic structures; nucleus of the stria terminalis, the olfactory tubercle, the nucleus accumbens, the lateral septal nucleus and parts of the frontal, cingulate and entorhinal cortex.

The tubero-infundibular dopaminergic system is the third dopaminergic system which arises from cells of the A12 group. The axons of these cells pass to the rostroventral part of the infundibulum and innervate the external layer of the median eminence.

The cells of groups A11, A13 and A14 give rise to short, intradiencephalic projections which have been designated the incerto-hypothalamic dopaminergic system.

The noradrenergic pathways have been mapped by fluorescence histochemistry and by biochemical analysis after selective lesions in the brain (Dahlström & Fuxe, 1964; Ungerstedt, 1971).

Figure 2 shows both descending and ascending NA pathways. There are two descending pathways, the first descending NA fibres originate in the most caudal group of the cell bodies, A1, and run down in the anterior and lateral funiculi to innervate the gray matter of the spinal cord, the other descending pathway originates in the A6 locus coeruleus neuron bodies, and

innervates the lower brain stem nuclei.

The ascending NA pathways comprise a dorsal and a ventral bundle of axons. The dorsal bundle originates in the lower locus coeruleus A6 and innervates the cortex, hippocampus and the cerebellum. The ventral bundle originates in the cell groups A1, A2, A5 and A7 in the pons and medulla oblongata and gives rise to pathways innervating the lower brainstem, hypothalamus and limbic system. Another pathway arises in the A4 cell group and sends axons towards the A6 group. These axons probably merge with the other ascending axons to provide terminals to the medial brain stem and cerebellum. The elucidation of the monoamines and their pathways, was followed by intensive studies on the roles played by these amines in CNS functions. Considerable evidence has been accumulated identifying these monoamines as neurotransmitters in the nervous system (Carlsson, 1967; Bloom & Giarman, 1968). The identification and mapping of these neurotransmitters led to questions concerning their involvement in the process of neurotransmission.

Historical concept of chemical transmission

The concept of chemical neurotransmission as 'cited by Von Euler (1981) dates back to 1877 when Du Bois-Reymond expressed the idea that nerve effect on muscle was due either to a chemical secretion or else had to be electric. He even suggested that lactic acid and ammonia might function as neurotransmitters.

In 1904 Elliott proposed that "adrenaline might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery." This proposal was made as a result of his studies on the innervation of the bladder and urethra in the cat. He found a parallelism between the effect of adrenaline and

stimulation of the sympathetic nerve, this led him to suggest the idea of a release of adrenaline in the periphery as a result of nerve impulses.

It was not until the classic experiments of Loewi in 1921 on frog hearts that the concept of chemical transmission was established. The experiments of Loewi (1921) demonstrated the release of Vagusstoff (which was later shown to be acetylcholine [ACH]) or Acceleransstoff (later shown to be adrenaline) on stimulation of frog cardiac nerves either slowed or accelerated, respectively, when administered to a second isolated frog heart. Loewi's fundamental experiment consisted of isolating hearts of two frogs, the first with its nerves and the second without. The hearts were then attached to Straub cannulae filled with Ringer solution. The vagus nerve of the first heart was then electrically stimulated and the Ringer solution then transferred to the second heart. This in turn slowed the beat of the second heart as if its vagus had been stimulated (due to Vagusstoff). An objection raised to Loewi's experiment was that in his experiments both the vagus and the sympathetic nerves were stimulated, yet the result was a slowing of the heart. The reason for this is related to seasonal changes in the sensitivity of the amphibian heart. Stimulation of the vago-sympathetic nerve tract leads to cardiac acceleration only in the spring, and Loewi made use of this phenomenon to demonstrate in the spring of 1921 that the fluid which bathed the heart contained an 'Acceleransstoff'. It is important to note that the frog is an exception in that it releases adrenaline from sympathetic nerves whereas most other species release noradrenaline. It was difficult for Loewi's opponents to believe his simple reasoning that, if on stimulating the nerve the response of the organ was inhibitory, the substance liberated was inhibitory, while, if the

effect was excitatory the substance was excitatory. The inhibitory substance Vagusstoff was later shown to be acetylcholine and Acceleransstoff was identified as adrenaline. This experiment became the foundation of the theory of chemical transmission in the nervous system.

A prerequisite for the action of a chemical neurotransmitter is the existence of "receptors" on the reacting cells, which are specific for the released transmitter, with the ability to transfer the chemical signal to the target cell. The development of this concept was due to Langley's work on nicotine (Langley, 1906) From his investigations on the effect of curare on nicotinic stimulation of skeletal muscle he postulated a specific recognition site - the "receptive substance", the occupation of which, can initiate or inhibit responses, by responding to different types of molecules, either of an excitatory or inhibitory nature.

In order to discuss transmitter action at the molecular level the morphology of the synapse will be described together with a definition of synaptic transmission. A detailed analysis of the synapse has been done by Papas & Purpura (1972). The word synapse was originally used to mean "the surface of separation between neurons", (Sherrington, 1906). It is now clear that there are two modes of synaptic transmission; chemical and electrotonic. It is well established that transmission at most synapses in the CNS of vertebrates is chemically mediated. Electrotonic synapses exhibit two characteristics which distinguish them morphologically from chemical synapses. First, the synaptic cleft of such synapses, if present, is very small. Secondly, the area of close apposition exhibits a symmetric appearance. In the mammalian CNS there are only a few regions at which electrotonic synapses have been demonstrated. One of these

regions is the mesencephalic trigeminal nucleus (Hinrichsen & Larramendi, 1970). In this thesis only chemical transmission will be discussed.

A distinct synaptic cleft is present at most chemically transmitting synapses when viewed under the electron microscope. This separates the pre and post synaptic membranes and provides morphological evidence for the physiological distinctiveness of pre and post synaptic elements (Eccles, 1964). The presynaptic element contains a number of structures, these include glycogen particles, mitochondria and tubular elements of endoplasmic reticulum. The most outstanding feature of the presynaptic terminals is that they contain synaptic vesicles. These vesicles are clustered close to the presynaptic membrane. The presynaptic component may be an axon terminal which synapses with a dendrite, cell body or an axon, thus forming axo-dendritic, axo-somatic or axo-axonal synapses. The axon need not necessarily be the presynaptic component, and it is possible to have dendro-dendritic, somato-dendritic and somato-somatic synapses.

The postsynaptic membrane "senses" the presence of a chemical neurotransmitter by means of receptors that, somehow, modulate its permeability to inorganic ions. The first post synaptic specialisation to be found was described by Palade (1954) and Palay (1956) as "localised thickening" of the postsynaptic membrane. Palay (1958) illustrates that such thickenings lie directly opposite the presynaptic dense material in CNS synapses and considered them to be postsynaptic components of the 'synaptic complex'. Deeper cytoplasmic structures are associated with the postsynaptic dense material in certain synapses. Taxi (1961), working on frog sympathetic nerve synapses, found a second dense stratum separated from the post synaptic densities by wispy filamentous material.

In different synapses the dense fuzz under the postsynaptic membrane varies in thickness. Gray (1959), using osmium tetroxide fixation and block staining in an ethanolic solution of phosphotungstic acid, noted that in the cerebral cortex the dense fuzz (which can be stained selectively without staining the postsynaptic membrane), under postsynaptic regions is thicker at postsynaptic regions of dendritic spines than it is at postsynaptic sites on neuronal cell bodies. He proposed that in the cerebral cortex two types of synaptic complexes could be distinguished - Type I - characterised by thick postsynaptic dense material that covers a relatively large area and by a relatively broad synaptic cleft containing a prominent intermediate dense line, and a type II complex characterised by a thinner postsynaptic dense material covering a relatively small area and by a narrower synaptic cleft without a prominent dense line. The synaptic complexes on dendritic spines are type I and those on neuronal cell bodies or mainstream dendrites as type II. However, in many regions of the CNS, synapses have since been found which do not fit all the criteria for type I or type II (Gray & Guillery, 1966).

Vesicles

The presence of vesicles in the presynaptic terminal and the dimensions of the cleft separating the pre and post synaptic membranes at the junctional region were of most immediate interest with regard to chemical transmission. Sjöstrand (1953) described granules in the synaptic knobs of the guinea-pig retina. Palade (1954), Palay (1954; 1956) and Fernandez-Moran (1957) noted the presence of vesicles in the mammalian CNS. Robertson (1956) described vesicles at the neuromuscular junction. The term 'synaptic vesicles' was coined by De Robertis

& Bennett (1954; 1955) studying frog sympathetic ganglia and earthworm nerve cord neuropil. The implication of synaptic vesicles in the transport of a chemical transmitter such as acetylcholine to the presynaptic membrane was made by De Robertis & Bennett (1955) and by Del Castillo & Katz (1955) and Palay (1956).

Synaptic vesicles in the CNS of vertebrates can be subdivided into agranular and dense-cored varieties (granular). Agranular synaptic vesicles are spherical, about 40-50 nm in diameter with a clear central zone and are the most commonly encountered vesicles in the nerve terminals studied in the CNS. Dense core vesicles are generally divided into small (40-60 nm diameter) and large (80-100 nm diameter) and are found mainly in the peripheral nervous system (Wolfe, Potter, Richardson & Axelrod, 1962; Hökfelt, 1968; Tranzer, Thoenen, Snipes & Richards, 1969).

The dense core vesicles encountered in the CNS were of the order of 60-150 nm in diameter (Soltelo, 1971).

Noradrenaline is stored in large dense cored vesicles (large vesicles) and small dense cored vesicles (small vesicles). Large dense cored vesicles were originally isolated from bovine splenic nerve by Von Euler & Hillarp (1956).

Synthesis and Metabolism of Catecholamines in the Brain

The catecholamines are synthesised from the amino acid L-tyrosine. Once inside the neuron L-tyrosine is hydroxylated to L-3-4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, (an enzyme requiring molecular oxygen, ferrous ions and a tetrahydropteridine cofactor). This is the rate-limiting step in the synthesis of catecholamines. L-DOPA is decarboxylated by DOPA decarboxylase to dopamine. Noradrenergic terminals contain dopamine β hydroxylase which converts dopamine into

noradrenaline. Various metabolites arise from each of the catecholamines and these are formed in reactions mediated principally by two enzymes; monoamine oxidase (MAO) and catechol-O-methyl-transferase (COMT). Figure 3 shows both synthesis and metabolism of NA and DA. Once synthesised, the catecholamines are stored in vesicles at nerve endings.

Dahlström and Haggendal (1966), in estimating the NA content of cell bodies of one pair of cervical ganglia of the cat, and the NA content of the area (neck and head tissue) innervated by the ganglia, found the distribution of NA between the cell bodies and the terminals was in the ratio 1:300. The estimation of NA content in the small and large vesicles were based on fluorometric techniques.

Fillenz (1972) and Fillenz & Pollard (1976), working on different sympathetic nerve terminals, provided evidence to suggest that the small vesicles are not a homogenous population. Their studies were carried out using dichromate and permanganate oxidation and the quantitative analysis of the core size of the small vesicles in various sympathetic nerve terminals. In sympathetic nerve terminals of different organs (these included the vas deferens, spleen and heart ventricle of the rat) the authors found that the mean core volume and the core size distribution of vesicles differed. Fillenz, Howe & West (1976) also showed that the NA content of the vesicles in the nerve terminals of the rat heart, following inhibition of monoamine oxidase and administration of noradrenaline, was related to the core size of the small vesicles. This study showed that the small vesicles were heterogeneous with respect to noradrenaline content. In adrenergic neurons, large dense cored vesicles were present in both the body of the axon and in the terminals, the small dense cored vesicles were found mainly in the terminals

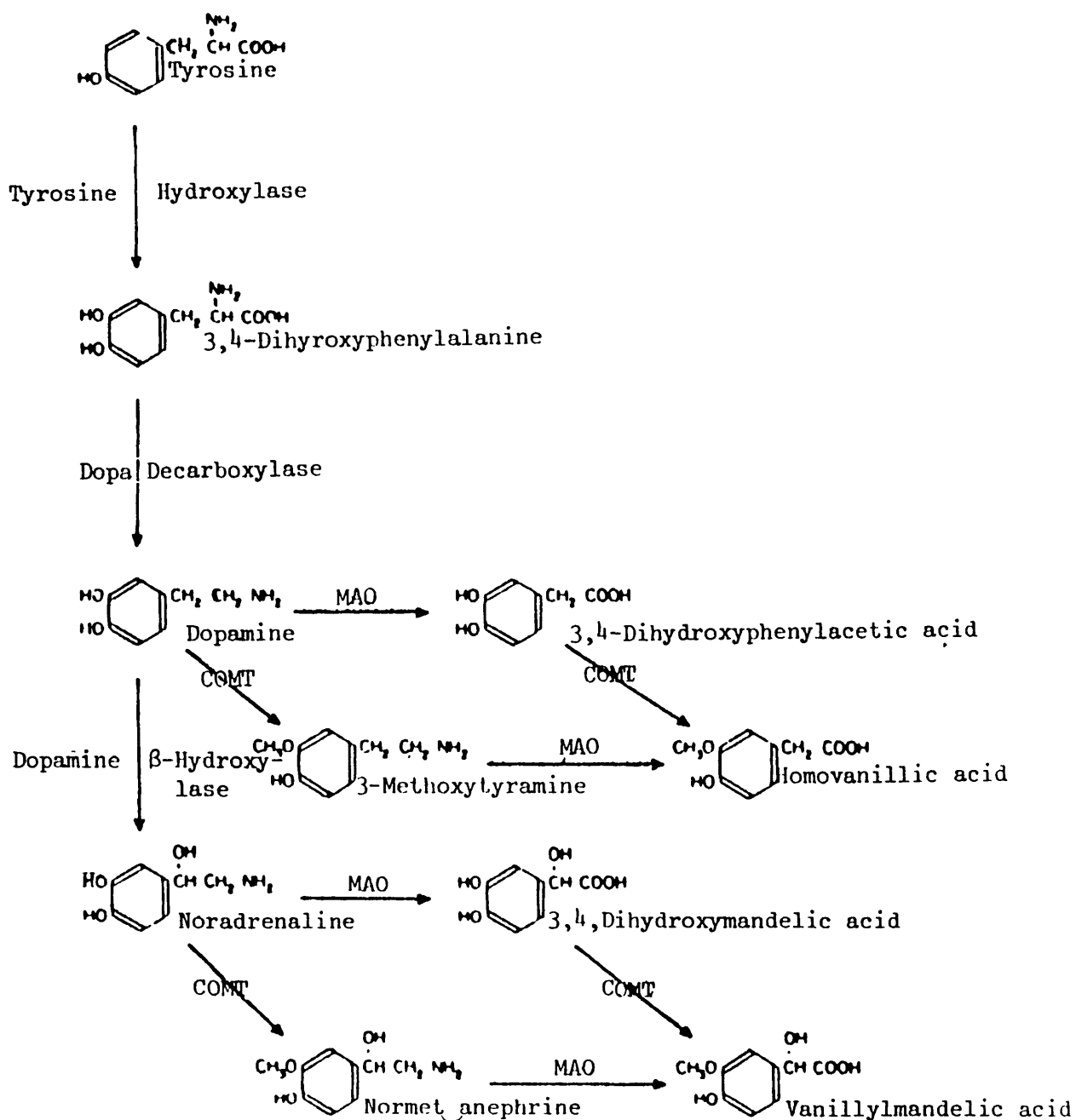


FIG 3: Simplified representation of main pathways in the formation and degradation of dopamine and noradrenaline. MAO = Monoamine oxidase. COMT = Catechol-O-methyltransferase. (Modified from Carlsson 1967)

(Tranzer, 1973). The exact function of the small and large vesicles is not very clear. Nelson & Mollinoff (1976) showed that large vesicles function as auxiliary storage sites. Fillenz and Howe (1971) showed that large and small vesicles participate in NA release.

About the same time as Grillo & Palay (1962) reported heterogeneity of NA vesicles, Trendelenburg (1961) investigated the sympathomimetic action of tyramine. He found that electrical stimulation and tyramine treatment evoked very similar responses in cat nictating membrane. Pretreatment of the animal with reserpine equally reduced the responses evoked by nerve stimulation or tyramine treatment. He decided that nerve stimulation and tyramine treatment were operating on the same NA pool. He posed the question as to why a reduction of the response to tyramine could only be observed after pronounced depletion of the stores by reserpine, and postulated two pools of NA, one mobilised by reserpine (B) and one much less affected (A).

Subsequent studies of Crout, Muskins & Trendelenberg (1962) on the effect of tyramine on guinea-pig atria supported Trendelenberg's work. They found that it required over 90% depletion by reserpine of the NA stores of guinea-pig atria before the sympathomimetic action of tyramine on atria could be significantly reduced. They deduced there were two stores of NA, one of which was important for the action of tyramine. Furthermore NA stores could be refilled by adding NA to the atria preparations and the action of tyramine could be restored, even when the observed content of NA following refilling was 2.2% of normal.

Kopin, Breese, Krauss & Weise (1968) lent further support to this hypothesis with results obtained from experiments on cat

spleen perfusate with ^{14}C tyrosine (NA precursor). On rapid stimulation of the splenic nerve the specific activity of the ^{14}C -NA released into the perfusate was found to be higher than that found in the spleen tissue, suggesting that newly synthesised ^{14}C -NA was released from the "available" pool. When spleens were treated with ^3H -NA, after stimulation, the specific activity of the released ^3H -NA was initially similar to that in the spleen, suggesting equilibration with all pools of NA. After continuous nerve stimulation however, the specific activity of the perfusates fell to one third of that found in the spleen, suggesting that the immediately available stores had been depleted.

By the 1970s the concept of a small readily releasable NA pool and a large reserve pool was well established. Thierry, Blanc & Glowinski (1971) investigated the possibility that the small NA pool may be composed of newly synthesised NA. To test this hypothesis they carried out a series of experiments. Using the intracisternal route they injected rats with ^3H NA and ^3H tyrosine, and 5 hours later killed the animals and analysed the brain stems. Both substances resulted in the same biphasic disappearance of the label from the tissue. Phase I occurred after 15-30 minutes and phase II after 180-260 minutes. From these data they postulated a short life compartment (A) and a longer life compartment (B), thus evidence for compartmentation in the brain emerged. In further experiments a stress of short duration (15 minutes) induced by electric shocks to the feet was found to increase the utilisation of ^3H -NA newly taken up or newly synthesised from ^3H -tyrosine, having no effect on the ^3H -NA stored for a longer period. However, in prolonged application of stress (180 minutes) ^3H -NA utilisation was increased from both compartments.

As yet no synthesis of ideas had emerged relating vesicular heterogeneity with two (or more) storage pools of noradrenaline. Fillenz & Howe (1971) were interested in transmitter release from vesicular stores. They studied hearts of normal and phenoxybenzamine treated rats. Phenoxybenzamine was used because it increases reflex stimulation of sympathetic nerve activity (Dontas & Nickerson, 1957). A reduction in NA stored in the vesicle would therefore be taken as a measure of transmitter release. These workers isolated two groups of vesicles from normal and treated rat heart muscle preparations, using sucrose density gradient centrifugation techniques. They found that both groups of vesicles from phenoxybenzamine treated rats were NA depleted, but that the smallest vesicles showed greater depletion. Thus evidence was provided that peripherally at least, the small vesicle noradrenergic pool contained the most easily mobilised neurotransmitter.

Evidence for the two compartment theory has been amassed for NA in the peripheral system. Such compartments of transmitters have also been established for CNS transmitters such as ACH, DA and 5HT. The compartmentation of DA in striatal nerve terminals is a controversial issue (Costa, Carenzi, Cheney, Guidotti, Racagni & Zivkovic, 1974; Glowinski, 1974). Costa et al (1974) assumed a single open compartment for the synthesis of DA from intravenously administered radioactive tyrosine and calculating the turnover rates for DA. The term 'turnover' is employed to describe an estimate or an index of the rate at which the catecholamines in the brain are being utilized. The time course of the specific activity of DOPA was the same as that of tyrosine in striatal tissue but from this evidence it is not possible to conclude as to whether or not synthesis of DA occurs in one specific compartment.

The main argument of Glowinski (1974) for the possible existence of two striatal DA pools is the observation of a biphasic decline of the amine following intraperitoneal injection of the DA-synthesis inhibitor, α -methyl-p-tyrosine.

Korf, Grasdijk & Westerink (1976), using electrical stimulation of the nigrostriatal dopaminergic pathway of the rat also found a biphasic decline of striatal DA. In probenecid-treated animals, DA levels remained unchanged during stimulation. However, after treatment with α -methyl-p-tyrosine, DA levels in the striatum declined biphasically. The first rapid phase was concerned with a pool of DA of about 20%, and this might represent a releasable pool. This biphasic decline was also observed by Javoy & Glowinski (1971). They injected rats with α -methyl p tyrosine and found the disappearance of DA in the striatum occurred in two phases, and suggested that this may correspond to amine utilization in both the 'functional' and a main storage compartment in dopaminergic terminals. Korf, Roth & Aghajanian (1973) provided evidence for the two compartment storage of NA in the brain. They found that after administration of α -methyl-p-tyrosine there was a biphasic decline of NA in the cerebral cortex during stimulation of the locus coeruleus. They explained these results by assuming that NA in the nerve terminals was stored in two different compartments, one that is readily releasable and one that is not.

Another explanation of the biphasic decline of NA in the cerebral cortex after stimulation of the locus coeruleus could be a transynaptic regulation of the transmitter release. This was explained by postulating that after initial release of NA a rapidly acting post synaptic feedback mechanism may be initiated.

Mechanisms of transmitter release

Many workers have studied mechanisms of neurotransmitter release. A generally accepted theory for the release of monoamines is the process of exocytosis. This process involves the attachment and fusion of vesicular membranes to the cell membrane, followed by an opening of the outer membrane and emptying of the soluble contents of the vesicle into the extracellular synaptic space. This has been established from evidence provided by experiments carried out mostly at the neuromuscular junction.

Current hypotheses concerning the release of catecholamines were developed after observations made by Von Euler and Hillarp (1956) that catecholamines are stored in granular vesicles in the adrenal medulla, and in noradrenergic nerves such as the bovine splenic nerve.

De Robertis & Vaz Ferveira (1957), from their studies on the adrenal medulla using electron microscopy techniques, suggested that release may occur via exocytosis. Coupland (1965) provided electron microscopic evidence for exocytosis. Smith & Winkler (1972) postulated several mechanisms whereby release of a transmitter can occur. These mechanisms are divided into two modes of transmitter release and are:

1. release occurring via the cytosol.
2. release occurring directly from vesicles.

Release via the cytosol is highly unlikely and evidence against such release has been provided below. The most accepted hypothesis of release is that of exocytosis or the vesicular hypothesis. Most of the evidence for the vesicular hypothesis has been provided using the neuromuscular junction, and such

release mechanisms have not been fully established in the CNS.

Release from chromaffin cells of the adrenal medulla was thought to occur by exocytosis, because besides catecholamines, high molecular weight proteins (chromogranins and dopamine β hydroxylase, the constituents of the chromaffin granules), were released into the cleft. However, tyrosine hydroxylase and lactate dehydrogenase were not released as they are not constituents of the chromaffin granule but are present in the cytosol (Smith & Winkler, 1972).

Mechanism of NA release via the cytosol

Large amounts of NA have been shown to be stored free in the cytosol. In experiments on sympathetically innervated tissue, treatment of an animal with reserpine caused the depletion of NA from the vesicle. Treatment with monoamine oxidase inhibitor prevented the breakdown of NA and thus, an increase in cytosol NA was observed. Smith & Winkler (1972) have suggested possible mechanisms of release via the cytosol, where release can occur by the destruction of the storage complex, or by the inhibition of the active uptake of the amine into vesicles. Other mechanisms may involve loss of NA from the vesicle into the cytosol, and across the cell membrane spontaneously. Spontaneous release from the cytosol differs from quantal release from the nerve terminals, for example, spontaneous efflux is slower than release evoked by nerve stimulation. Lindmar & Loffelholz (1972) found that spontaneous release of NA in the nerve terminals of the heart is temperature dependent whereas release induced by electrical stimulation is not. In animals treated with reserpine, but not MAO inhibitor, the NA lost from the vesicles into the cytosol is destroyed by the MAO of the mitochondria and leaves the terminals as metabolites, whereas after electrical

stimulation NA released is found as NA (Kopin, 1972). It is accepted that release via the cytosol is possible but occurs only through administration of drugs or spontaneously, NA released by electrical stimulation does not seem to go via the cytosol, otherwise it would be 'released' as NA metabolites.

Mechanism of release via the vesicles

The first understanding of the mechanisms underlying the secretion of chemical neurotransmitters came from the studies of Bernard Katz and colleagues. Discovery of the spontaneously occurring miniature end-plate potentials (Fatt & Katz, 1952) at the frog neuromuscular junction led to the formulation of the quantum hypothesis by del Castillo & Katz (1954b). Intracellular recordings were made from the end-plate regions of single muscle fibres of isolated frog nerve-muscle preparation and the end-plate potential produced by motor nerve stimulation was measured. The experiments established that miniature end-plate potentials (mepps) observed to occur spontaneously at the neuromuscular junction were due to the release of multimolecular amounts or quanta of acetylcholine (ACH). These quanta were shown to originate from the presynaptic endings and to be equivalent in size to the basic unit of transmitter release, therefore the end plate potential (epp) was made up of several mepps. Del Castillo & Katz (1954b) explained the multiple relationship of epp and mepp by assuming that the transmitter release is derived from many small storage packages or vesicles, rather than one large reservoir, thus one quantum of transmitter represented the contents of one vesicle.

Heuser, Reese, Dennis, Jan, Y., Jan, L. & Evans (1979), using freeze fracture techniques followed by electron microscopy, found a close correlation between the detectable exocytotic site

and quantal release measured electrophysiologically, hence providing strong evidence that transmitter release involves exocytosis. They used a machine that was able to freeze tissues by contact with a super-cold metal block, which also had a timing circuit able to trigger stimulation of the frog neuromuscular junction in the last few milliseconds before being frozen. Nerve terminals were frozen during transmitter release and synaptic vesicles were seen in the act of exocytosis. They used 4-aminopyridine to increase the number of transmitter quanta discharged with each nerve impulse and found that the number of exocytotic vesicles caught by quick-freezing increased commensurately, indicating that one vesicle undergoes exocytosis for each quantum that is discharged.

The presence of soluble vesicular proteins in the perfusates of stimulated, sympathetically innervated organs would suggest release by exocytosis. Using enzyme assay methods, Gewirtz & Kopin (1970) demonstrated Ca^{2+} dependent release of D β H on stimulation of the cat splenic nerve. Geffen & Livett (1971), using immunological methods, showed the release of both D β H and chromogranin from the spleens of several species. Tyrosine hydroxylase and dopa decarboxylase found in the cytoplasm are not released upon stimulation of the splenic nerve (Smith, De Potter, Moerman, De Schaepdryver, 1970). This evidence strongly indicates that release occurs via exocytosis. Smith et al (1970) also showed that dopa decarboxylase in spite of being a smaller molecule than either D β H and chromagranin, was not released, suggesting that release did not occur via the cytoplasm or that the entire intracellular contents leaked out non-specifically.

Lucy (1970) suggested that the suspected fusion between vesicular and plasma membrane during exocytosis may be due to the presence of lysolecithin (palmitoylglycerophosphorylcholine).

This led to the hypothesis that the rearrangement of the bimolecular leaflet form of phospholipids in membranes to a micellar form, by lysolecithin, enables the membranes to fuse.

The mechanism involved in the creation of an opening of the cell membranes or in the thinning out of the membrane (fenestration) to expel the vesicular content is still not clear. Kanno & Loewenstein (1966) showed that D β H and chromogranin which are small proteins can cross tight junctions between cells, suggesting a micropore formation during fenestration to allow the passage of larger vesicles.

Evidence for different pools of ACH does not comply with the vesicle hypothesis

ACH exists in central neurons in three forms, free ACH, labile-bound ACH and stable-bound ACH (Whittaker, 1959; Whittaker, Michaelson & Kirkland, 1964; Marchbanks, 1968). Free ACH exists in the cytoplasm of the neuron, and may be located in the perikaryon or axon. Stable-bound ACH survives hypotonic treatment and is present in synaptic vesicles, whereas the labile-bound form fails to survive this treatment and is destroyed in the absence of a choline esterase inhibitor. Labile bound ACH occurs predominantly in the cytoplasm of the terminal.

Chakrin & Whittaker (1969) and Whittaker (1970) found that the three kinds of ACH differed in specific radioactivity following injection of radioactive choline in vivo, suggesting that they could not have arisen from a single homogeneous pool. Their studies suggest three distinct pools of ACH.

The vesicular hypothesis of transmitter release does not consider the other pools of ACH. If there is some dynamic interaction between the different pools, the vesicular pool need not necessarily take part in the final stage in the release of

the transmitter. The concept of three pools of ACH has arisen from studies using CNS material. Originally evidence for the vesicle hypothesis of transmitter release was based on studies carried out on the neuromuscular junction (NMJ). As the neuromuscular junction is difficult to fractionate, the distribution of ACH between different pools is uncertain. It is important to question whether the transmission process is the same in CNS synapses as in neuromuscular junctions. Quantal neurotransmission has not been categorically demonstrated for the mammalian central synapse.

Fate of vesicles after release

It is rational to assume that vesicle membrane material cannot keep adding to the cell membrane. An approach for obtaining direct evidence for vesicle recycling in the near future is likely to be the application of immunochemical methods. A precondition for the success of immunochemical studies is the existence of fractions of pure antigens (synaptic vesicles and (or) synaptic plasma membranes or components derived from them) either for raising antibodies or for subsequent testing of the specificity of antisera obtained. Whittaker & Stadler (1980) obtained initial success using synaptic vesicular antigens derived from the electric organ vesicles of Torpedo, which bound specifically to nerve terminals in the brains of a number of vertebrates. This technique can be developed further to elucidate the fate of vesicles after exocytosis of the intravesicular material.

Zimmerman, Stadler & Whittaker (1981) suggested a model for recycling of synaptic vesicles at the cholinergic nerve terminals. The authors stimulated the cholinergic nerve terminal of the Torpedo electric organ at a low frequency and observed

that at the onset of stimulation the synaptic vesicles became heterogeneous. Using fine structural, biochemical and physical methods it has been shown that whilst most vesicles are in a resting state, some would have already released their vesicular content and been recycled. Vesicles are then preloaded with newly synthesised ACH and adenosine triphosphate (ATP), and the contents are released in preference to those of the resting vesicles. Continued stimulation would cause the resting vesicles to be activated and recycled. Zimmerman, et al (1981) suggest that vesicles containing newly synthesised ACH lie close to the cell membrane and as a result of stimulus-induced influx of Ca^{2+} ions, vesicles release the ACH and are then recycled. Experiments of Zimmermann et al (1981) on the Torpedo electric organs suggest that increasing the stimulus frequency or duration at first causes a depletion of the transmitter whilst vesicle recycling continues. Eventually, endocytosis of membranes (i.e. recycling of vesicles) does not follow exocytosis of Ach and there is a depletion of vesicles.

It has been suggested by Smith et al (1970) that the membranes of the large noradrenergic vesicles are also retrieved after exocytosis of the neurotransmitter and contribute to the formation of small noradrenergic vesicles. If this is so, then the small NA vesicles should contain D β H and Bisby, Fillenz & Smith (1973) have found this to be the case in the small vesicles in the nerve terminals of the rat vas deferens. Tranzer (1973) suggested that there are small NA vesicles which do not contain D β H and these may be derived from cisternae of the smooth endoplasmic reticulum. Using peroxidase-chase techniques Heuser & Reese (1973) also proposed that synaptic vesicles are incorporated into the cell membrane and recycled by an endocytotic process.

Ceccarelli, Hurlbut & Munro (1972) on the basis of peroxidase uptake studies backed up the idea that newly synthesised transmitter was preferentially released, that vesicles empty by temporary fusion with the presynaptic terminal, and are retrieved and remain in the vicinity of the terminal for refilling and re-use. Van Herreveld & Trubatch (1975), using rapid freezing techniques with electronmicroscopy after KCl stimulation of frog brain, provided evidence supporting the experiments of Ceccarelli et al (1972). The authors observed that in the resting state the vesicles are round and close to the cell membrane; on stimulation with potassium chloride and the resulting Ca^{2+} influx, the vesicles become elongated and fuse with the presynaptic terminal membrane and squeeze out their contents into the synaptic gap. After release of the contents the vesicles are refilled with cytoplasmic fluid and move away from the release site, (see Figure 4 below). These observations were not seen in unstimulated brain or those that had not been bathed in Ca^{2+} depleted media.

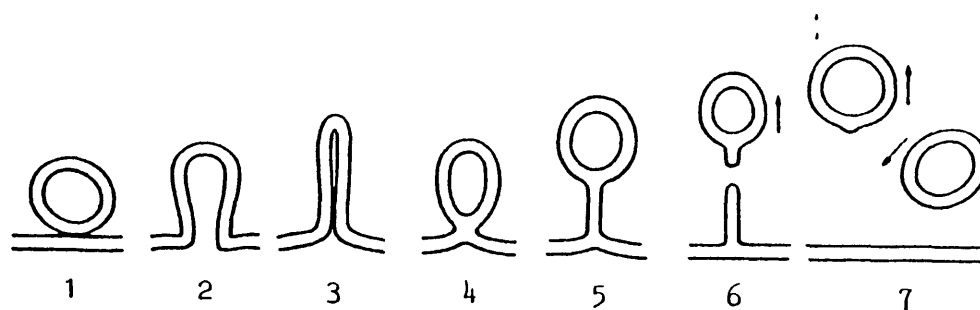


FIG 4: A possible sequence of transmitter release and vesicle recycling. (Taken from Traubach & Van Harreveld, 1981.)

Role of Ca^{2+} in the release process

Long before Elliot's (1904) enunciation of the concept of chemical neurotransmission Locke (1894) as cited by Harvey & MacIntosh (1940) recognised that calcium ions were required for neuromuscular transmission. Locke found that a muscle would not respond to nerve stimulation after 1-2 hours in a calcium deficient medium, although it would still contract upon direct stimulation.

The classical study of Harvey & MacIntosh (1940) subsequently showed that, in sympathetic ganglia, Ca^{2+} ions are required for acetylcholine (ACH) release. The authors used the eserinated isolated perfused cervical sympathetic ganglion of the cat and assayed the output of ACH. Their experiment showed that in the absence of calcium there was no release of acetylcholine from preganglionic nerve endings, either during electrical stimulation of the sympathetic trunk or after application of potassium.

It was established by del Castillo & Katz (1954a) and del Castillo & Stark (1952), working on the frog neuromuscular junction, that when the concentration of calcium was low, or the magnesium concentration high, the end plate potential was reduced to a minimal amplitude and became identical in size and shape to the spontaneously occurring miniature end-plate potentials.

The discovery of quantal release of ACH was made at the same time as the discovery of vesicles at synaptic nerve terminals (De Robertis & Bennett, 1954; 1955) (see p38 of this thesis). Kuffler (1944) showed that at the frog neuromuscular junction if the tissue was deprived of Ca^{2+} there was a decrease in end-plate potential recorded extracellularly, whilst the nerves could still conduct impulses and the sensitivity of the end-plate to applied ACH was not diminished. Kuffler concluded that the major effect

of calcium deprivation was due to a decrease in transmitter release. del Castillo & Stark (1952) confirmed this result at the frog neuromuscular junction using intracellular recording techniques.

Katz & Miledi (1965) and Katz & Miledi (1967b), working on the myoneural junctions of the frog's sartorius muscle provided direct evidence that the action of calcium is on the release process and not mediated by alterations in impulse conduction through the distal nerve terminals. They showed that when Ca^{2+} was present at the time of depolarisation ACH release could be triggered by focal current pulses without nerve impulses. This led to the enunciation of the "Calcium Hypothesis". The hypothesis was based on the postulation that depolarisation causes a selective increase in the permeability of the presynaptic terminal plasma membrane to Ca^{2+} , so that Ca^{2+} then enters the terminal and moves down a steep electrochemical gradient. The rise in the intracellular Ca^{2+} then causes the fusion of the synaptic vesicle membrane with the plasma membrane by an unknown mechanism and the vesicular contents are extruded into the synaptic cleft probably via exocytosis (see p39 of this thesis).

The calcium hypothesis has been verified by experiments on the squid giant synapse. Katz & Miledi (1967b); Katz & Miledi (1969); Kusano, Livengood & Werman (1967) found that depolarisation of tetrodotoxin-blocked terminals sufficed to trigger an increase in Ca^{2+} conductance and release of ACH. It appeared that as tetrodotoxin blocked the sodium channels, sodium influx was not essential for transmitter release. The size of the depolarisation-induced inward current and the amount of transmitter released depended upon the concentration of Ca^{2+} in the bathing media. Katz & Miledi (1969) carried out these experiments on the giant synapse in the stellate ganglion of the

squid. They found that the abolition of nerve impulses by tetrodotoxin does not prevent the release of ACH from nerve endings in response to a locally induced action potential. The local response was elicited from the terminal parts of the presynaptic axon after intracellular injection of tetraethyl ammonium ions (TEA). These studies provided further evidence to show that depolarisation leads to transmitter release via inward movement of calcium ions, and that the opening up of 'calcium gates' at specific sites of release is not prevented by tetrodotoxin.

Llinas & Nicholson (1975) provided further support for the calcium hypothesis. Their studies were carried out using the calcium sensitive photoprotein Aequorin to demonstrate directly the association between the rise in intracellular calcium and transmitter release. Their studies were carried out on the squid, where Aequorin was injected into the squid presynaptic terminals, and the depolarisation induced increase in intracellular calcium was measured by the increased light output. These experiments proved that in order to trigger transmitter release a rise in intracellular calcium was necessary.

Douglas & Rubin (1961), working on secretion from the adrenal medulla, also showed that Ca^{2+} was required for transmitter secretion. They coined the term stimulus-secretion 'coupling' where depolarisation leads to Ca^{2+} uptake and then secretion of transmitter (or hormone in this case).

The mammalian central synapses, due to their complexity and small size, prove difficult as material for the stimulus secretion coupling studies that have been described for the neuromuscular junction. The synaptosomal preparation (Gray & Whittaker, 1962) has been used by Blaustein (1975) to study neurotransmitter

release, using high potassium ion concentrations as a stimulus. The synaptosomes take up Ca^{2+} and release neurotransmitter substances in a Ca^{2+} -dependent manner. Rapid filtration techniques (Kreuger, Mordecai & Blaustein, 1980) have also been employed to study the properties of calcium channels by measuring $^{45}\text{Ca}^{2+}$ uptake in rat brain synaptosome preparations (Nachshen & Blaustein, 1980). Baldessarini & Kopin (1966), using brain slices, showed that electric field stimulation of the slices triggered the Ca^{2+} -dependent release of ^3H -noradrenaline. Release of preloaded ^3H -dopamine in brain slices by electrical stimulation has also shown to be Ca^{2+} ion dependent (Farnebo & Hamerger^b, 1971).

The mechanism for Ca^{2+} entry studied on the squid synapse suggested that the process was manifested as an external Ca^{2+} dependent net inward current (Katz & Miledi, 1969) which is associated with a rise in intracellular Ca^{2+} (Llinas & Nicholson, 1975). Depolarisation triggers the opening of Ca^{2+} selective channels which are not affected by tetrodotoxin (Katz & Miledi, 1969).

Calcium channel

Calcium channels are molecular structures that selectively allow Ca^{2+} ions to cross the membrane in response to a depolarisation of the membrane. Upon depolarisation the Ca^{2+} channel opens and allows Ca^{2+} to move into the cell, this movement is a passive process driven by the Ca^{2+} electrochemical gradient. Intracellular $[\text{Ca}^{2+}]_i$ is usually less than one millionth of the extracellular calcium $[\text{Ca}^{2+}]_o$. There is considerable variation in the properties of Ca^{2+} channels found in different membranes, and therefore the Ca^{2+} channels may also differ in molecular structure (Hagiwara &

Byerly, 1983).

Ca^{2+} channels have been found in almost all excitable tissues. As yet no substance exists which specifically blocks the calcium channel as tetrodotoxin does the Na^+ channel. Ca^{2+} currents are blocked by polyvalent metal ions e.g. Cd^{2+} , Mn^{2+} and also Mg^{2+} . The other Ca^{2+} blocker is the drug verapamil.

Studies by De Lorenzo (1982) showed that the Ca^{2+} receptor protein in the brain tissue called calmodulin may be involved in neurosecretion. Calmodulin is thought to regulate the effects of Ca^{2+} at the synapse and is present on synaptic vesicles. Using preparations of synaptic vesicles (De Lorenzo & Freedman, 1977, 1978) the responsiveness of these vesicles was found to depend on both the binding of endogenous calmodulin to the vesicle membrane and the stabilisation of the enzyme systems involved in mediating the effects of Ca^{2+} on vesicle function (De Lorenzo, 1980).

De Lorenzo, Freedman, Yohe & Mauer (1979) developed a technique to remove endogenous vesicle-bound calmodulin from the vesicle membrane without significantly altering the neurotransmitter content of the vesicles. This preparation was then used to study the effect of calmodulin on Ca^{2+} stimulated vesicle neurotransmitter release. De Lorenzo et al (1979) showed that effects of Ca^{2+} on vesicle neurotransmission were dependent on calmodulin and inhibited by trifluoperazine, a calmodulin inhibitor. Ca^{2+} entry into synaptosomes induced using the Ca^{2+} ionophore A23187 was also inhibited with trifluoperazine. It appeared that trifluoperazine was acting by directly inhibiting the Ca^{2+} -stimulated system within the nerve terminal. It appears from these studies that calmodulin plays a role in neurosecretion.

Study of synaptic modulation

The study of synaptic modulation of transmitter release in the CNS presents with difficulties due to the complexity of neuronal organisation. Central catecholamine containing varicosities are within the reach of many other neurotransmitter substances. Neurotransmitters may be released from nerve endings that impinge upon the varicosities forming axo-axonic synapses or may be released from dendrites (Groves, Wilson, Young & Rebec, 1975). A drug, in influencing transmitter release, instead of acting directly on catecholaminergic varicosities, may first act on neighbouring terminals or dendrites, or on the cell bodies from which they project, or on glial cells. Neurotransmitter release and the subsequent extrapolation of synaptic modulation of the release by means of drugs, can be studied using various techniques. These techniques in the CNS include the use of:

1. brain slices
2. synaptosomal preparations
3. in vivo experiments on small brain areas using push-pull cannula techniques.

The best method for studying neurotransmitter release is to determine the overflow of the transmitter and its metabolites from the tissue into the superfusion media. In this manner the effect of drugs on transmitter release can be studied.

Brain slices

Most investigations on central presynaptic mechanisms have been carried out using brain slices. Numerous references can be found to studies on mammalian brain slices (Kerkut & Wheal, 1981; McIlwain, 1975; Elliott, 1969). In using brain slices which contain axon terminals and not cell bodies, the possibility of the drug affecting the firing rate of the neurons can be

eliminated. For example, striatal slices can be used to study dopamine release without interference from the cell bodies in the pars compacta of the substantia nigra. The advantage of slice preparations is that they permit the selection of specific areas of the brain. The other advantage is that the superfusion of thin slices allows one to study the effects of drugs at the organised cellular level by monitoring drug-induced release or blockade of release of substances into the superfusate. McIlwain and his group carried out the most detailed study on brain slice metabolism and they developed a technique of slicing whereby a slice remained viable for several hours (McIlwain, 1961; McIlwain & Rodnight, 1962; McIlwain & Batchelard, 1976). Since the 1920's many biochemical studies have been carried out on the brain. Otto Warburg introduced the tissue slice technique as a method for studying, by simple and easily controlled in vitro methods, the metabolism of tissues of which the structure had not been extensively disrupted. The disadvantage of the slice preparation is that the tissue is not in a normal state because it is separated from its normal inputs. Wholly integrated synaptic events are lost as well as tonic excitatory and inhibitory influences. This means that although one can study the local event taking place in a specified anatomical site it would be difficult to directly relate the situation to an in vivo condition. In the preparation and manipulation of brain tissue slices, damage to the superficial layers of cells is inevitable. Cellular processes are severed and cells may undergo degeneration, additionally it is difficult to maintain intact circuitry because cell input-output orientation is not contained within the lamella of the slice.

Swelling of slices which indicates tissue damage has been extensively studied. Swelling was defined by Varon and McIlwain

(1961) as occurring in three phases. First a thin film of Kreb's adheres to the slices, then a large swelling appears during preparation of slices at a rate of $2.4 \mu\text{l}/100 \text{ mg}/\text{min}$, a process which is not reversible in spite of subsequent incubation in oxygenated physiological medium. This maybe associated with intracellular changes as there is no change in inulin space. Finally, slow swelling occurs on incubation at a rate of $0.1 \mu\text{l}/100 \text{ mg}/\text{min}$ and probably associated with extracellular swelling since inulin space is increased on incubation. This contributes to a 28% increase in slice weight per hour. Using swollen slices, and slices in which swelling has been prevented by dextran, Banay-Schwartz, Gergely & Lajtha (1974) showed that swelling does not impair the ability to transport amino acids.

Garfinkel, London, Dzubow & Nicklas (1975) provided information to show that brain slices were as viable as the intact brain by the use of a computer model of intermediary metabolism. Data obtained from in vivo studies showed that metabolism is divided between two compartments. In order to match the data obtained under in vivo conditions they had to have an additional compartment which corresponded to brain tissue damaged during preparation. However the organisation of metabolism was largely unchanged and there was an overall similarity between in vivo metabolism and metabolism in slices.

In most studies of presynaptic regulation, the neurotransmitter stores in brain slices have been labelled by means of radioactive dopamine or noradrenaline; in the case of dopamine release studies, slices have also been incubated with radioactive tyrosine. It is possible that in the study of preloaded catecholamines, the label may be taken up into cells other than those containing the endogenous transmitter, and therefore could be released upon stimulation from inappropriate

cells.

Cohen (1974) carried out a systematic electron microscopic study of swelling in relation to the thickness of mouse brain slice. Slices greater than 0.3 mm consisted of an outer layer of 'damaged' tissue and an intact core. Slices less than 0.17 mm consisted entirely of damaged tissue. In both cases the damaged tissue was more susceptible to swelling and leakage. Studies of McIlwain and Bachelard (1976) and McIlwain (1975) summarise the ability of brain slices to maintain normal metabolic processes in vitro. They note that even without exogenously added metabolic substrate, oxygen is absorbed and carbon dioxide evolved. In general, respiration rates are about half of those occurring in vivo and this is independent of slice size. Physiological studies also show that brain slices are durable and viable in vitro. Intracellular recordings originally done by Yamamoto & McIlwain (1966), demonstrate that slices can actively maintain membrane potentials of about 60 mV, normal synaptic phenomena such as postsynaptic potentials following stimulation occur and electrical activity spreads to an appreciable portion of the slice.

To what extent the release of preloaded labelled transmitter is akin to endogenous transmitter release is not clear. Glowinski (1975) describes nerve endings as containing at least two pools of catecholamines with different dynamic properties. A newly synthesised or recently taken up transmitter first enters a "functional" pool from which major release occurs. With time, the possibility of the transmitter molecule being released declines and it is stored in a "reserve" pool, which contributes to release when an increased rate of neuronal firing (stimulation) occurs. As the preincubation time with the labelled transmitter for slices is about thirty-forty minutes and

thirty minutes for superfusion or 'washout', the overflow of labelled transmitter will predominantly originate from the reserve pool. How the modulation of transmitter release from the functional and reserve pools may or may not differ is not clear. Study of endogenous transmitter release may clarify these problems.

The basal release of labelled noradrenaline is not affected by tetrodotoxin (Baldessarini, 1975) nor by omission of Ca^{2+} (Taube, Starke & Borowski, 1977). Electrically stimulated evoked release is completely abolished by tetrodotoxin or lack of calcium.

The basal release of ^3H -dopamine is markedly reduced during superfusion with tetrodotoxin or in a Ca^{2+} free media (Dismukes & Mulder, 1977). The electrically evoked release of ^3H -DA is completely calcium ion dependent and blocked by tetrodotoxin (Farnebo, 1971; Dismukes & Mulder, 1977). It appears that basal release of NA and DA have different characteristics.

Release using synaptosomes

The technique for preparing synaptosomes in order to study transmitter release has been reviewed by Bradford (1975). Release of endogenous and preloaded transmitter can be measured, using electrical stimulation, high potassium and veratridine as stimulating agents. It is suggested that synaptosomes could offer the possibility of studying drug effects purely on the presynaptic terminals, however, some synaptosomes have been shown by Jones (1975) to be still attached to postsynaptic membranes and site of drug action may involve either or both.

In vivo experiments to study transmitter release

The release of transmitter in small brain areas has been studied in vivo using push-pull cannula techniques (Bartholini,

Stadler, Gadea Ciria & Lloyd, 1976; Nieoullon, Cheramy & Glowinski, 1977).

The results of the effect of drugs on the release of transmitter in vivo are more difficult to interpret than the results obtained using slices or synaptosomal preparations. Nieoullon et al (1977), estimated the evoked release of the transmitter from the nerve endings by electrically stimulating catecholaminergic cell bodies in vivo. The effect of drugs on this release could be explained as being presynaptic, however the external stimulus is superimposed upon total endogenous activity and many factors could be contributing to the modulation of the release.

Different types of negative modulation of transmitter release

In studies of synaptic transmission, modulation of transmitter release takes a prominent position. Three types of negative modulation of transmitter release can occur. These include presynaptic, transynaptic and nonsynaptic modulation. See Figures a-c below.

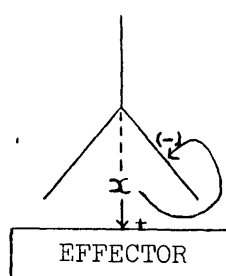


FIG a: Presynaptic autoinhibition

This type of modulation is characterised by autoinhibition where transmitter released from a varicosity inhibits further release from the same varicosity.

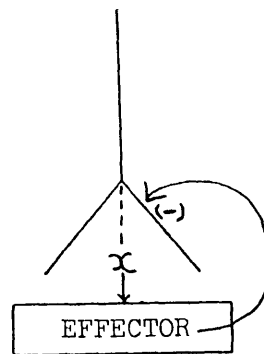


FIG b: Transsynaptic modulation

A trans-synaptic modulator is a substance formed by and released from a postsynaptic cell which has the capacity to inhibit the release of the transmitter from the presynaptic nerve endings.

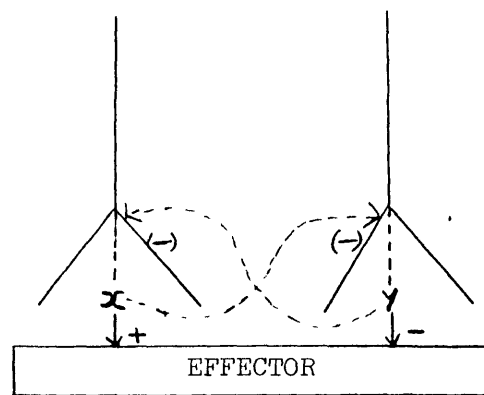


FIG c: Non-synaptic modulation

In non-synaptic modulation, one set of neurons decreases the influence of another set. The inhibitory agent or the modulator is released from axon terminals at a distance from effector cells and diffuses over some micrometres to exert its inhibitory effect

on a neighbouring neuron.

Modulation of NA release via presynaptic α -adrenoceptors

It was reported in 1957 by Brown & Gillespie that phenoxybenzamine enhanced the overflow of noradrenaline released by electrical stimulation of the postganglionic sympathetic fibres to the spleen. Initially this effect was thought to be due to the block of α -adrenoceptors on the effector organ. When it was found that phenoxybenzamine inhibits neuronal and extra neuronal re-uptake of NA (Hertting, 1965; Iversen, 1965; Eisenfeld, Axelrod & Krakoff, 1967) it was suggested that the drug effect was due to the inhibition of re-uptake.

Cocaine, known to inhibit neuronal uptake of NA without blocking α -adrenoreceptors, was tested on the perfused spleen preparation and failed to increase the stimulation evoked release of NA even at concentrations at which maximal inhibition of neuronal uptake of NA is obtained (Blakeley, Brown & Ferry, 1963). These results refuted the view that the increase in stimulation-evoked transmitter overflow in the presence of phenoxybenzamine was due to the inhibition of neuronal uptake of NA.

Having excluded inhibition of enzymatic degradation and inhibition of re-uptake as explanations for the effect of phenoxybenzamine, Häggendal (1969) and Hedqvist (1969) proposed that the drug might increase release per impulse either by a direct action on nerve terminals or by the existence of a trans-synaptic regulation for the release of NA whereby transmitter output would be controlled by the activity of the effector cell. However, the trans-synaptic feedback hypothesis was ruled out when it was found that α -adrenolytic drugs also increase the overflow of NA from guinea-pig atria (McCulloch,

Rand & Story, 1972), rabbit heart (Starke, Montel & Schumann, 1971) and cat heart (Farah & Langer, 1974) preparations where the adrenoceptors that mediate the response of the effector organ are of the β -type.

In 1971 Kirpekar & Puig, working on the perfused cat spleen, proposed that -

"NA released by nerve stimulation acts on presynaptic sites to inhibit its own release by a negative feedback mechanism. Adrenoceptor blocking agents enhance the NA overflow from spleen because they remove this autoinhibition by blocking the presynaptic α sites."

Therefore according to this proposal, presynaptic adrenoceptors are involved in the regulation of NA release through a negative feedback mechanism mediated by the transmitter itself (Enero, Langer, Rothlin & Stefano, 1972; Langer, 1977; Starke, 1977).

Once NA release elicited by nerve stimulation reaches a threshold concentration in the synaptic cleft, it activates presynaptic α adrenoceptors triggering a negative feedback mechanism that inhibits further release of the neurotransmitter (Langer, 1973). In support of this hypothesis it was shown that α adrenoceptor agonists inhibit NA release elicited during nerve stimulation (Kirpekar, Furchgott, Wakade & Prat, 1973; Starke, Endo, Taube & Borowski, 1975; Langer, Dubocovich & Celuch, 1975) while an increase in the stimulation-evoked release of NA is obtained in the presence of α adrenoceptor blocking agents (Enero et al, 1972; Starke, Montel & Schumann, 1971). The effects are observed irrespective of the α or β nature of the post-synaptic adrenoceptor.

The regulation of neurotransmitter release by presynaptic adrenoceptor is calcium ion dependent (Stjarne, 1973; Starke &

Montel, 1974) and the mechanism is operational at low and intermediate frequencies (Dubocovich & Langer, 1976).

The term presynaptic autoreceptor is used for receptors that are probably located on axon terminals and involved in the regulation of the Ca^{2+} dependent release of a neurotransmitter by the transmitter itself. How presynaptic autoreceptors modulate release is not clear, a possible clue is the involvement of calcium, as only Ca^{2+} -dependent models of release are modulated. In the rabbit heart, oxymetazoline decreases, whereas phentolamine increases the Ca^{2+} dependent release of NA elicited by electrical nerve stimulation, or by high K^+ , but not the Ca^{2+} independent basal efflux, nor the Ca^{2+} -independent release evoked by tyramine (Starke & Montel, 1974). Autoreceptor activation seem to make less Ca^{2+} available for transmitter release. The decrease of stimulus-evoked release of a transmitter may be due firstly to a block in the conduction of action potential to the axon terminals, hence fewer terminals open their gates for Ca^{2+} ions. Secondly it is possible that even though the axon terminals are invaded by the action potential, they allow less Ca^{2+} entry and electrosecretory coupling in each individual terminal is depressed. In the presence of high K^+ , when a drug reduces the transmitter release, the first possibility can be excluded because high K^+ depolarises all axons simultaneously and action potentials which are sensitive to tetrodotoxin do not contribute to release.

Göthert, Pohl & Wehking (1979) found that release of NA could be produced by re-introduction of Ca^{2+} after exposure of the tissue to Ca^{2+} free media, provided the tissue is kept depolarised with high K^+ . Presynaptic receptor antagonists increase, and agonists decrease this Ca^{2+} dependent release of NA, presumably because Ca^{2+} ions enter through

potential-sensitive channels. This experiment supports the idea that autoreceptors are somehow linked to voltage controlled Ca^{2+} -channels.

The presynaptic negative feedback mechanism for NA requires a threshold concentration of the transmitter released by nerve impulses into the synaptic gap. When reserpine is used to deplete the endogenous NA stores the effectiveness of phenoxybenzamine in enhancing the ^3H -NA release is reduced (Enero & Langer, 1973).

Rand, Story, Allen, Glover & McCulloch (1973) found that in guinea-pig atria on electrical stimulation, the overflow of NA per pulse decreases with successive pulses and this effect is reversed by phenoxybenzamine. Starke (1977) suggested this may be due to greater blockade of autoreceptors by the antagonist. In support of this theory Baumann & Koella, (1980) found that in cortical slices of the rat there was, over a frequency range of 0.2-1.6hz, a decrease in NA released per pulse with increasing frequency. The agonist clonidine totally blocked release per pulse with increasing frequency as the effects of NA released and the agonist were additive. The presynaptic autoregulatory theory rests on two separate findings. These are that phenoxybenzamine, an α -receptor antagonist, increases the stimulated release of labelled noradrenaline whereas exogenous noradrenaline inhibits this release. It is suggested that a single unit modulates whether it is occupied by an agonist or an antagonist. Results obtained by Kalsner (1979) do not support such an unitary hypothesis of presynaptic modulation. In his experiments on the guinea-pig vas deferens, he found that upon single pulse stimulation, when no modulation of ^3H -NA release can occur, phenoxybenzamine enhanced the release of preloaded ^3H -NA.

Rand, Story, Allen, Glover & McCulloch (1973), using the

guinea-pig atria, found that phenoxybenzamine did not increase the efflux of tritium after single pulse administration. Whether the observations from these two experiments vary due to different organs or to different experimental procedures is not clear.

Kalsner (1980) later suggested that exogenous NA and phenoxybenzamine worked by different mechanisms. Using a test system whereby he increased the frequencies of stimulation of the vas deferens at two different pulse rates (10 and 50 pulses) he found that as the frequency increased phenoxybenzamine had a diminishing effect on ^3H -NA release irrespective of pulse rate. The depression of ^3H -NA release by exogenous NA was independent of frequency, but pulse rate dependent (60% depression at 10 pulses and 30% at 50 pulses). On the basis of these differences Kalsner suggested that both substances work by different mechanisms or at different sites.

However, there is further evidence in the literature that presynaptic modulation of NA release is frequency dependent. Brown & Gillespie (1957) found that at a frequency of 10 pulses/sec the α blocking agent dibenamine caused an increase in NA release, but at 30 pulses/sec release was not enhanced. Kirpekar & Cervoni (1963), using phenoxybenzamine, confirmed that at 30 pulses/sec NA release was not enhanced, whereas at lower frequencies it was. α -receptor agonists are more effective in reducing responses to sympathetic nerve stimulation at low frequencies than at high frequencies. Armstrong & Boura (1973) found that the increased heart rate of pithed rats caused by sympathetic nerve stimulation was depressed by clonidine at frequencies of 0.25-1 Hz but not significantly changed at frequencies of 2-8 Hz. Starke, Montel, Gayk & Merker (1974) found that in superfused strips of rabbit pulmonary artery the

inhibition caused by clonidine was greater at a frequency of 2 Hz than at a high stimulation frequency of 8 Hz. From these studies and the controversial results of Kalsner it is not clear to what extent modulation of release is frequency dependent.

Calcium and presynaptic events

As previously discussed the flux of Ca^{2+} ions into the intracellular presynaptic terminal is an essential element in neuronal secretion of a transmitter (p44).

Stimulation of the frog neuromuscular junction by a single impulse or a brief train causes subsequent impulses to release increased amounts of transmitter (del Castillo & Katz, 1954b). This phenomenon is referred to as facilitation. Katz & Miledi (1965) proposed facilitation to be due to each nerve impulse leaving some calcium bound to an active internal site; this bound calcium would be added to by the calcium bound during subsequent impulses thereby increasing transmitter release.

Facilitation is supposed to occur up to 2 Hz frequency of stimulation and modulation between 2 and 4 Hz; beyond this no modulation of transmitter release is thought to occur, due to high intracellular Ca^{2+} ions causing desensitisation of presynaptic receptors (Starke, 1977).

The outcome of Kalsner's studies using single pulse stimulation, was to question the unitary hypothesis of presynaptic modulation. He further questioned the role of bound calcium in presynaptic modulation. Kalsner (1981) carried out a series of experiments on guinea-pig atria (preloaded with ^3H -NA) in which he varied both the Ca^{2+} concentrations in the Krebs' solution, and the frequencies of stimulation. He expected to find that using reduced calcium Krebs', and across the whole range of frequencies (1 Hz-15 Hz) of stimulation there would be

increased inhibition of tritium release in the presence of exogenous NA as there would be insufficient inflow of Ca^{2+} to switch off the regulator. At high frequency of stimulation, further inhibition of tritium overflow by exogenous NA should have been observed. However, he found inhibition to be constant throughout the range of frequencies employed.

With standard calcium Krebs' Kalsner expected reduced inhibition across the range of frequencies as now sufficient Ca^{2+} was available for desensitisation. He found greater inhibition of tritium release by exogenous NA at low frequency stimulation, but at high frequency the inhibition declined as expected. With high calcium Krebs' modulation would not be expected at any frequency if the calcium desensitisation story was true. Kalsner found similar patterns to the standard Ca^{2+} Krebs' pattern. It may be concluded that the role of calcium in modulation mechanisms is not clear. It should be noted that if Ca^{2+} is an essential element in neuronal secretion of a transmitter, then experiments of Kalsner in reduced Ca^{2+} Krebs' need to be questioned. It is quite possible that his observed results may be due to a lack of threshold levels of Ca^{2+} .

The exact sites and ionic and biochemical mechanisms involved in the modulation of presynaptic NA release are not completely understood. This is partly due to the anatomical complexity of nerve terminals. At present it is not known whether each varicosity is invaded by a nerve impulse and that transmitters are secreted from each varicosity.

A great deal of work still needs to be done in order to understand the role of presynaptic regulation in such highly branched and varicose terminals. The electrophysiology of a single "bouton-en-passage terminal" and distribution of

presynaptic sites within it and its relationship to a neighbouring terminal may help understand presynaptic regulation.

Evidence for presynaptic α adrenoceptors in the CNS

Farnebo & Hamberger (1971); Starke, Endo, Taube & Borowski (1975); Taube, Starke & Borowski (1977) have provided evidence to show that noradrenaline agonists reduce the release of ^3H -monoamines from brain slices upon electrical stimulation. Dismukes & Mulder (1976) provided evidence that oxymetazoline, an α receptor agonist reduces, and phentolamine, an α receptor antagonist increases depolarisation-induced release of ^3H -NA from superfused neocortical slices of the rat in a dose-dependent manner (using potassium and electrical stimulation).

Presynaptic dopamine receptors

The mechanisms involved in the regulation of DA release are controversial. Farnebo & Hamberger in 1971 reported that rat striatal slices preloaded with ^3H -DA, when electrically stimulated in the presence of the dopamine receptor agonist apomorphine reduced the evoked release of tritium, whereas the antagonists chlorpromazine (10^{-6}M) and pimozide (10^{-6}M) increased it. On the basis of this evidence they proposed the concept that DA release in the CNS is modulated by dopamine receptors, probably located on the dopaminergic nerve terminal. A year later it was demonstrated by Carlsson, Kehr, Lindqvist, Magnusson & Atack (1972) and Kehr, Carlsson, Lindqvist, Magnusson & Atack (1972), that the dopamine released controls the rate of dopamine synthesis by activation of presynaptic dopamine autoreceptors located in nerve terminals of central dopaminergic neurons. Several groups of workers found that they could not reproduce results obtained by Farnebo & Hamberger (1971). Dismukes &

Mulder (1977), using striatal slices preloaded with ^3H -DA found that apomorphine did not have any affect on the evoked release of tritium. Seeman & Lee (1975), Dismukes & Mulder (1977) and Iversen, Roganovski & Miller, (1976) found that several neuroleptics caused a decrease in the stimulus evoked release of tritium. Seeman & Lee (1975) and Dismukes & Mulder (1977) used brain slices and stimulated the release of ^3H -DA by electrical methods, whereas Iversen et al (1976) used striatal synaptosomes stimulated by protoveratrine. Seeman & Lee (1975) and Dismukes & Mulder (1977) replicated the experimental procedure of Farnebo & Hamberger (1971) using striatal slices and electrical stimulation. In spite of adhering as closely as possible to a single experimental procedure the results produced by these two groups of workers were not quite consistent. Dismukes & Mulder (1977) found that the haloperidol concentrations which inhibited the stimulation evoked release of tritium by 50% (IC_{50}) were different from those obtained by Seeman & Lee (1975). Dismukes & Mulder (1977) found the IC_{50} for haloperidol to be $2 \times 10^{-6}\text{M}$. Seeman & Lee (1975) found IC_{50} for haloperidol to be 10^{-7}M . IC_{50} for haloperidol obtained by Iversen & al (1976), using a synaptosomal preparation, was found to be 2×10^{-6} , similar to the result obtained by Dismukes & Mulder (1977).

Westfall, Besson, Giorguleff & Glowinski (1976), using the method for continuously superfusing striatal slices with ^3H -tyrosine and causing a potassium chloride (50mM) evoked release of ^3H -DA, found similar inhibitor effects of neuroleptics to those of Farnebo & Hamberger (1971). In the presence of fluphenazine (10^{-7}M) and benztropine (10^{-6}M) the potassium evoked release of ^3H DA was increased. When both drugs were administered together their effects were additive. The

effect of benztropine may be due to inhibition of re-uptake of dopamine. However, fluphenazine is a weak uptake inhibitor and its effect may be facilitatory.

The contradictory results obtained by different groups of workers are difficult to explain. Differences in dissection and preparation of brain slices and different experimental procedures may be additive in producing incompatible results. Dismukes & Mulder (1977) suggested that the contradictory results may result if pre-synaptic receptors modulate release, as these receptors may be located away from nerve endings and therefore the cutting of slices becomes a critical factor.

Pharmacological characteristics of presynaptic dopamine autoreceptors

Westfall, & al (1976) suggested that the synthesis and release of dopamine do not follow the same course, therefore whether the receptors modulating the release of dopamine and those controlling the synthesis of the transmitter are the same remains an open question. Furthermore it is not known whether the cell body dopamine autoreceptors that modulate the firing of the dopamine neurons and those receptors that modulate synthesis and release in the nerve terminal are the same. Inhibition of the dopaminergic cell activity has been demonstrated after iontophoretic application of dopamine, apomorphine and bromocriptine. The inhibition of the dopaminergic firing obtained by iontophoretic application of dopamine agonists was blocked by antagonists of the phenothiazine series (Aghajanian & Bunney, 1977; Bannon, Grace, Bunney & Roth, 1980). The results suggest the possible existence of DA receptors, not only in the terminals, but also at the soma-dendritic regions of the dopaminergic neurons. The dopamine autoreceptors in the

nigrostriatal dopaminergic neuron are not associated with the dopamine sensitive adenylate-cyclase (Kebabian & Saavedra, 1976; Krueger, Forn, Walters, Roth & Greengard, 1976). This evidence would favour the view that these presynaptic autoreceptors correspond to the D₂ subtype of dopamine receptors as classified by Kebabian & Calne (1979).

Influence of the frequency of nerve stimulation on the presynaptic autoregulation of dopamine release

Presynaptic autoregulation of DA release has also been found to be frequency dependent. Kamal, Arbilla & Langer, in unpublished observations, found that in slices of rabbit caudate nucleus preloaded with ³H-DA the inhibition of ³H-DA release by 0.1 μM apomorphine was more pronounced at a frequency of 3 Hz than at 10 Hz and the facilitation of ³H-DA release by haloperidol (0.01 μM) was only observed at 3 Hz.

These results suggest that modulation of DA release through presynaptic autoreceptors is less effective at high frequencies of stimulation. However, a threshold concentration of DA at the synaptic gap is necessary to trigger the disinhibitory effect of DA receptor antagonists. To support this suggestion, Arbilla, Langer and Lehmann (1981) found that, in the cat caudate slices the DA receptor antagonist sulpiride was less effective in facilitating the release of dopamine elicited at 1 Hz than at 3 Hz.

The absence of presynaptic regulation of DA release at high frequencies of stimulation is akin to the regulation of NA release through presynaptic α-adrenoceptors. Brown & Gillespie (1957); Kirkpekar & Cervoni (1963) have shown reduced effectiveness of α-adrenoceptor blocking agents in enhancing NA release at high frequencies of stimulation. This view on the

frequency dependence of release has been opposed by Kalsner (see p60 of this thesis).

Effect of chronic treatment with neuroleptics on the sensitivity of the presynaptic autoreceptors modulating DA release

Presynaptic dopamine autoreceptors modulating release have been shown to undergo changes in sensitivity after chronic treatment with neuroleptics known to block postsynaptic receptors. Arbilla, Nowak & Langer (1982) found that after chronic treatment of rabbits with haloperidol for four weeks, the inhibition of release of preloaded ^3H -DA from slices of caudate nucleus in the presence of apomorphine was markedly increased. The results suggest the development of presynaptic receptor supersensitivity to the inhibitory effect of apomorphine on the release of ^3H -DA. Conversely, chronic haloperidol treatment was found to result in subsensitivity of the dopamine autoreceptors to the facilitatory affect of S-Sulpiride on transmitter release (unpublished results by Nowak, Arbilla & Langer). Chronic neuroleptic treatment also causes changes in the sensitivity of the postsynaptic DA receptors (Muller & Seeman, 1978).

It is possible that supersensitivity of presynaptic DA receptors to agonists developed during chronic haloperidol treatment may enhance the presynaptic autoregulation of dopaminergic transmission. In the treatment of psychoses this effect would contribute towards a reduction in dopaminergic activity. It is not clear how presynaptic DA receptor supersensitivity may relate to postsynaptic DA receptor sensitivity.

Dendritic release of dopamine

The first data suggesting dendritic release of DA was reported by Geffen, Jessell, Cuello & Iversen in 1976. Figure 5 illustrates the dopaminergic nigrostriatal pathway. The pars compacta of the substantia nigra contains mainly cell bodies whereas the pars reticulata is rich in dopamine-containing dendrites. The terminal dendrites of the DA neurons are varicose in nature and contain clusters of vesicles (Hajdu, Hassler & Bak, 1973). Geffen & al (1976) showed that the release of preloaded ^3H -DA from slices of the substantia nigra using high potassium, was calcium ion dependant. The release was more pronounced in slices prepared from the pars reticulata than those from the pars compacta.

In vivo dendritic release of DA has been examined using cannula techniques (Nieoullon, Chéramy & Glowinski, 1977). This method involved the insertion of a push-pull cannula into the cat's substantia nigra and continuous superfusion with artificial cerebrospinal fluid containing ^3H tyrosine (the precursor of DA), the release of the synthesised ^3H -DA is then measured in the superfusing media. The ^3H -DA release induced by potassium chloride was found to be Ca^{2+} dependent. The spontaneous release of ^3H -DA from dendrites was not reduced by tetrodotoxin, which is in contrast to the release of ^3H -DA from the caudate nucleus which is markedly reduced by tetrodotoxin. The effect on dendritic release indicates that fast sodium channels are not involved in the spontaneous release of the ^3H -labelled transmitter from dendrites.

Neuronal pathways which project to the substantia nigra are shown to influence directly or indirectly the release of DA from dendrites. Bunney, Aghajanian & Roth (1973) showed the existence of DA receptors on dendrites in the rat substantia

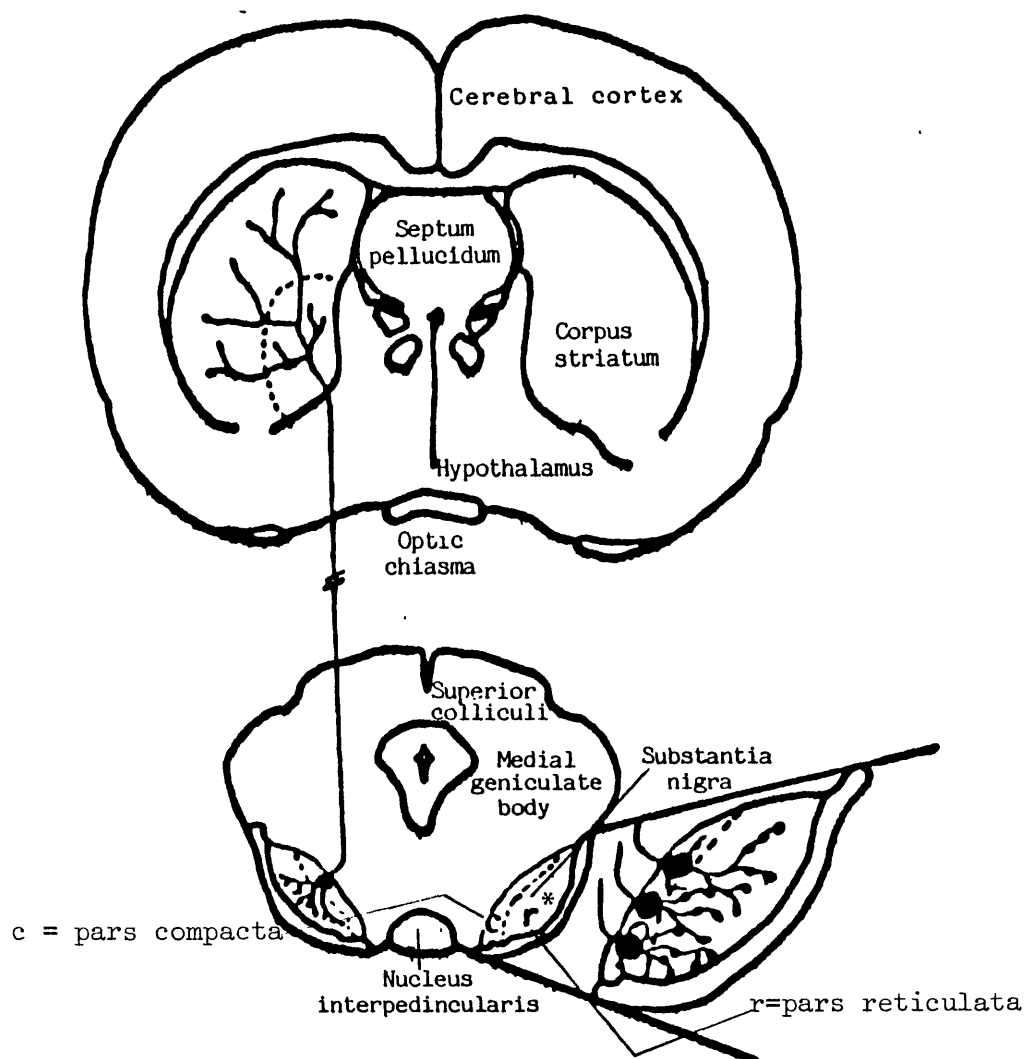


FIG 5: Diagram showing dopaminergic nigro striatal pathway. Cell bodies of dopaminergic neurons are confined to the pars compacta with a segregation of their axonal and dendritic processes as shown magnified *. The dendrites of the neurons radiate ventromedially into the pars reticulata whereas the axons project to the corpus striatum (rostromedially). Some dendrites may be seen in the pars compacta. (Modified from Geffen, Jessel, Cuello & Iversen (1976))

nigra. Systemic application of apomorphine reduced the firing rate of DA cells and this effect could be prevented using neuroleptics (Aghajanian & Bunney, 1977).

Groves, Wilson, Young & Rebec (1975) observed that the application of haloperidol into the pars compacta, produced an increase in neuronal firing rates of the dopaminergic neurons there. They explained this increase in neuronal firing rate as resulting from the blockade of an intrinsic dopaminergic inhibitory mechanism in the substantia nigra. In addition, they noted that the application of amphetamine to the pars compacta would cause an inhibition of firing of dopaminergic neurons, the dopaminergic neurons being inhibited by DA released from their own dendrites in response to amphetamine administration.

DA released from dendrites has been shown to regulate presynaptic release of gamma aminobutyric acid (GABA) from terminals of the striato-nigral GABA neurons, the DA receptors associated with DA sensitive adenylate cyclase are involved (Van der Heyden, Venema & Korf, 1980) and are located on the GABA terminals in the nigral region.

DA exerts an excitatory effect on the nigrothalamic neurons which contribute to a presynaptic neuronal loop by which dendritic DA in one substantia nigra influences the activity of the contralateral nigrostriatal dopaminergic neurons (Cheramy, Levieil & Glowinski, 1981).

It is still not clear if DA released from dendrites acts at a distance from its release sites. Important questions concerning dendritic release have still not been answered. These include the storage of the transmitter, and events contributing to the release procedure. In the pars reticulata, dendro-dendritic synapses (Wilson, Groves & Fifkova, 1977) and

dendro-axonic junctions (Hattori, McGeer & McGeer, 1979) have been observed, therefore a clear morphological basis needs to be established in the connections between dopaminergic dendrites and nigral neurons and the afferent fibres.

The nigrostriatal dopaminergic neurons in their function as processors of information within the basal ganglia contribute not only by filtering information delivered to the striatum by afferent pathways such as the cortico and thalamostriatal neurons, but also by processing information within the substantia nigra.

Neurotransmitter release studies in vitro

Werman (1966), in his article on the criteria for identification of a neurotransmitter, stressed the importance of the demonstration of calcium ion dependent release of the transmitter. This criterion involves three interrelated concepts, the physiological stimulation of the presynaptic neuron, the release of the transmitter and the dependency of this release on calcium ions.

McIlwain's extensive studies on brain slices (1963), showing that they would remain viable for several hours when maintained in oxygenated physiological saline, led to their extensive use for the study of neurotransmitter release.

These procedures involved the preloading of brain slices with ^3H -transmitter such as ^3H -noradrenaline and "wash off" of excess label by a continuous superfusion system and the subsequent collection of the released tritium in the superfusate fractions. The release of ^3H -noradrenaline and ^3H -dopamine from brain slices was studied by Baldessarini & Kopin (1967) and Farnebo & Hamberger (1971) and found to be calcium ion dependent.

There are several means of inducing transmitter release, the

two methods described below include: 1. electrical stimulation.
2. potassium-induced stimulation.

Electrical stimulation

Electrical stimulation has been the most favoured method of releasing transmitters, probably due to it being more akin to normal nerve activity and also because it releases only suspected transmitter substances, Baldessarini & Kopin (1967) and not non-transmitter substances, (Hopkin & Neal, 1971; Hammerstad & Cutler, 1972).

A possible drawback, namely the raised temperature in the electrode chamber caused by electrical stimulation was shown by Baldessarini & Kopin (1966) not to affect the release of ^3H -noradrenaline. They demonstrated that when the temperature of the bath was raised from 37° to 40°C there were insignificant differences in the amounts of ^3H -noradrenaline released from brain slices.

The metals generally used as electrode material are gold, platinum and silver. These metals have been extensively studied by McIlwain (1963) in metabolic experiments. Electrode material needs to be carefully selected when it is to function in physiological saline in which metabolic changes in superfused slices are to be measured. Silver has been used for recording potentials in electrophysiological experiments; under these circumstances deleterious effects of silver have not been seen. However, if pulses of alternating current of 100/sec, 5V and 0.3ms are applied to silver electrodes in physiological saline, they undergo 'chloriding' which is a formation of silver chloride (AgCl) on their surface.

In neurochemical studies, platinum is not a very desirable metal for use, because of its function as a catalyst in aqueous

solution at room temperature. Platinum is particularly active as a catalyst when electrically stimulated, stimulating pulses or currents normally used in electrophysiological experiments are able to catalyse oxidation of glucose in oxygenated bicarbonate saline. Gold has been found to be a suitable material for neurochemical studies, as no deleterious effects have been seen on application of sinewave alternating currents to gold electrodes in oxygenated glucose saline. McIlwain (1975) did not observe any interaction between the gold electrodes and bicarbonate saline on examination of oxygen uptake, substrate utilisation and acid formation, after a period of electrical stimulation.

The metabolic effect of electrical stimulation of brain tissues has been described by McIlwain (1975) as:

1. Electrical stimulation momentarily alters the electrical charge at polarised membranes of the component cells in the tissue.
2. These membranes respond with transitory increase in permeability to Na^+ allowing greater Na^+ entry. This accentuates the depolarisation caused by the initial permeability change and subsequently causes increased permeability to K^+ which leaves the cells and thus the membrane regains its negative charge.
3. Slowly, with the utilisation of ATP, Na^+ movements are reversed.
4. The by-products of ATP utilisation, ADP and inorganic phosphates then accelerate the oxidative and glycolytic processes of phosphorylation which result in the resynthesis of ATP.

Potassium-induced depolarisation

High extracellular concentrations of potassium are frequently used as a substitute for electrical stimulation and as a convenient means of releasing known or putative neurotransmitters (Hillman & McIlwain, 1961). Mann, Tennenbaum & Quastel (1939) first used high extracellular concentrations of potassium for releasing endogenous acetylcholine from brain slices. The effects of high K^+ in inducing release of transmitters is complex, and besides depolarising membranes, high K^+ has shown to cause an increase of the influx of sodium and calcium ions and cause brain tissue to swell (Arnfred & Hertz, 1971). The degree of tissue swelling induced by electrical stimulation is much lower than that due to high concentrations of potassium (Keeseey, Wallgreen & McIlwain, 1965). At high potassium concentration the specificity to release transmitter-like substances is lost (Mulder & Snyder, 1974). Vargas & Orrego (1976) carried out studies on superfused neocortical slices to show if release using high potassium was a specific means for inducing transmitter release from central neurons. They used high potassium to release ^3H -NA - a known transmitter and ^{14}C aminoisobutyrate (AIB) a known non-transmitter. Their results showed that both ^3H -NA and ^{14}C -AIB are released at 50mM K^+ concentration although the release of ^{14}C -AIB is much smaller than that of ^3H -NA. Thus, the use of high K^+ has more limited specificity than electrical stimulation (Orrego, Jänkelevich, Ceruti & Ferrera, 1974).

Vargas & Orrego (1976) found the K^+ stimulated release of ^3H -NA to be Ca^{2+} ion dependent. They interpreted this effect as being due to high K^+ causing an increased calcium influx leading to increased concentration of Ca^{2+} ions in the cytoplasm of nerve ending (Ishida & Yoneda, 1974), thus triggering transmitter

secretion. The release of ^{14}C -AIB was not Ca^{2+} dependent, and the effect difficult to interpret. The origins of the released ^{14}C -AIB was not clear, it may have been released from neurons or glial cells. High potassium can release cytoplasmic pools of NA GABA (Vargas, de Lorenzo & Orrego, 1977) and acetylcholine (Collier & Katz, 1971).

Introduction to drugs used

Drugs which influence human CNS functions and animal behaviour appear to act by interfering with central monoamine neurotransmission. These drugs can therefore be used as tools for elucidating the roles of monoamines in mediating different kinds of behaviour (Dews & Morse, 1961; Cook & Kelleher, 1963; Herz, 1960).

Drugs used in this investigation were haloperidol and trazodone. Haloperidol was chosen because it is a typical antipsychotic drug widely used in clinical practice. Trazodone was chosen because it is an unusual drug in that although used as an antidepressant, it extinguishes the conditioned avoidance response in the rat, in a manner similar to an antipsychotic.

Haloperidol

Haloperidol, a butyrophenone, was first synthesised in 1956 by Janssen and his colleagues in Belgium (Janssen, van der Westeringh, Jageneau, Domoen, Hermans, van Daele, Schelleken, van der Eycken & Niemegeers, 1959).

After initial animal studies the drug was first given to patients at the psychiatric clinic of Liege University by Divry, Bobou, Collard, Pinchard & Nols (1958). The results of this pilot trial found the drug to be highly effective as a psychomotor sedative within 5-15 minutes of intravenous injection

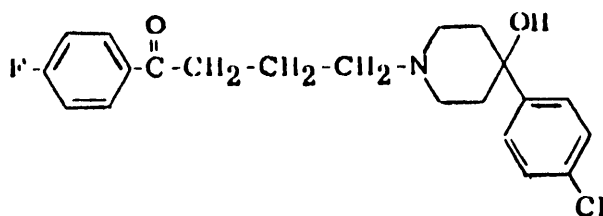
at doses of 3-15 mg and the effect lasted for 3-5 hours.

Haloperidol became the drug of choice in the emergency treatment of psychomotor agitation.

After this initial pilot trial on haloperidol several trials were organised in western Europe and the first meeting on haloperidol was organised in Beerse in 1959. Within two years of this symposium the drug came into use in psychiatric clinics in most western European countries.

Pharmacology of haloperidol

The chemical structure of haloperidol is shown below.



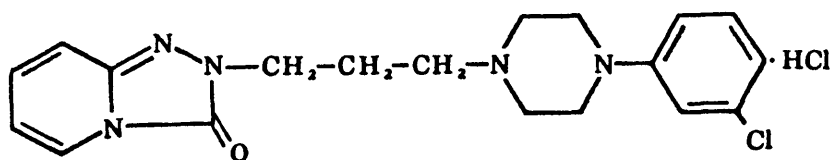
Haloperidol: 4'-fluoro-4-[4-hydroxy-4-(4-chlorophenyl)piperidino]butyrophenone

Haloperidol is a typical potent neuroleptic drug or major tranquilliser. The literature on the pharmacology of haloperidol is extensive, and its pharmacology will only be described briefly. The most common effect of neuroleptics appears to be the inhibition of dopaminergic neurotransmission, by blockade of postsynaptic DA receptors. Long term use of neuroleptics often causes tardive dyskinesia and this has been suggested as being due to supersensitivity of the postsynaptic DA receptors (Yarbrough, 1975). This supersensitivity may be as a result of prolonged disuse of the receptors. Besides blocking postsynaptic DA receptors, neuroleptics have been shown to block presynaptic DA receptors (p64 of this thesis). Haloperidol has powerful surface tension reducing properties, at 10^{-7}M concentration, it

will form a monolayer on water or water-lipid interphases in a manner similar to detergents (Seeman & Bialy, 1963). There exists for neuroleptics, a correlation between neuroleptic potency (that is the dose injected into an animal for obtaining a given neuroleptic effect) and surface tension lowering activity. This effect suggests that the mechanism of action of haloperidol involves the formation of monolayers on biological structures. Haloperidol is also known to have antiemetic action (Janssen, Niemegeers & Schellekens, 1965). Radioactive haloperidol (0.1 mg) given orally to Sprague-Dawley rats (200 g) was found to be maximally absorbed within 3 to 5 hours and mainly excreted via the urine and faeces within 72 hours. Haloperidol is metabolised by an N-dealkylation reaction, the resultant fragments can be conjugated with glucuronic acid and it is believed that all the metabolites of haloperidol are inactive (Forsman & Uhman, 1977). Haloperidol selectively inhibits conditioned avoidance behaviour whilst leaving the unconditioned escape response intact.

Trazodone

Trazodone, chemically 2-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-1,2,4-triazolo[4,3-a] pyridin-3(2H) one, is a new antidepressant with a molecular weight of 408.33. Its chemical structure is shown below.



Trazodone hydrochloride 2-(3-[m-chlorophenyl]
1-piperazinyl] propyl)-1,2,4-triazolo[4,3-a]pyridin-3(2H) one

Silvestrini (1967) revealed that trazodone definitely had CNS activity. It is currently marketed as an antidepressant in Great Britain under the name Molipaxin. Trazodone has a unique spectrum of pharmacological activity since it does not resemble known drugs belonging to any of the three major psychotropic classes, namely antidepressants, antipsychotics or anxiolytics.

Animal studies show that although it is used as an antidepressant in man it does not possess most of the effects regarded as being typical of antidepressants. Unlike tricyclic antidepressants, it does not have antireserpine activity, does not potentiate methylamphetamine-induced anorexia or potentiate the effects of catecholamines on L-dopa. (Domino, 1974; Garattini, 1974; Koe, 1976; Longo & Scotti de Carolis, 1974). It is not a monoamine oxidase inhibitor or an anticholinergic agent (Pecknold, Ritzolu & Ban, 1976). Trazodone possesses neuroleptic properties in that it reduces spontaneous motor activity and exploratory behaviour and blocks responses to painful stimuli and conditioned avoidance responses at doses which do not affect the unconditioned response (Angelucci & Bolle, 1974; Gatti, 1974; Silvestrini, Cioli, Burberi & Catanese, 1968). But unlike neuroleptics, it has only weak hypothermic activity, does not inhibit apomorphine or amphetamine-induced stereotyped behaviour or possess cataleptogenic activity, even in higher doses (Domino, 1974; Longo & Scotti de Carolis, 1974; Silvestrini et al, 1968). Trazodone occupies cortical α_2 receptors and enhances noradrenaline efflux from slices of rat occipital cortex (Clements-Jewery, Robson & Chidley, 1980). Garattini (1974) produced data to show that trazodone decreases the levels of brain noradrenaline in rats. The reduction of

noradrenaline in the rat brain is not accompanied by any change in the major metabolite of noradrenaline that is 3-methoxy-4-hydroxyphenylglycol (MHPG) sulphate (Schauberg, Schildkraut, Breese & Kopin, 1968). In rats given probenecid (to inhibit transport of the metabolite) there is an increase in MHPG-sulphate in the trazodone treated rats. Garattini (1974), in studying noradrenaline uptake mechanisms by injecting ^3H -noradrenaline intraventricularly before and after administration of trazodone 50 mg/kg i.p., found accumulation of noradrenaline to be confined to the brain stem. Like desipramine, trazodone inhibits the accumulation of ^3H NA in the whole brain by causing an increase of normetanephrine, without any change in the deaminated acid metabolites.

The effect of trazodone on brain levels of 5-hydroxyindolacetic acid (5HIAA), 5-hydroxytryptamine (5HT), noradrenaline and dopamine were studied by Angelucci & Bolle (1974). They found that at low doses trazodone reduces 5-HIAA, whilst at higher doses it decreases 5HIAA and increases 5HT.

Trazodone (Baran, Maj, Rogoz & Skuza, 1979) inhibits the head-twitch response to 5-HTP or 5-methoxytryptamine, tryptamine-induced convulsions and the hypothermia produced by various 5-HT agonists. This potent central 5-HT antagonist activity has also been observed for other antidepressants e.g. mianserin (Maj, Sowinska, Baran, Gancarozyk & Rawlów, 1978). Compounds showing potent 5-HT antagonist activity are those used therapeutically in the treatment of anxiety-agitation, but whether the clinical use of trazodone is related to its 5-HT antagonist action has not been elucidated.

Stefanini, Fadoa, Porceddu & Gessa (1976) investigated whether trazodone shares with neuroleptics and narcotic analgesics (Laverty & Sharman, 1965) the property of stimulating

dopamine synthesis. Their results showed that trazodone increased homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) levels in the rat brain. At 25 mg/kg there was a 96% increase in HVA and a 56% increase in DOPAC. At dose levels of 25-100 mg/kg trazodone does not influence dopamine concentrations. It can be concluded from the authors' results that trazodone shares with neuroleptics the capacity of increasing brain dopamine metabolites. The theory proposed by Nyback & Sedvall (1968), for the neuroleptics may provide an explanation of the increase in dopamine synthesis. A block of dopamine receptors by trazodone could, by a compensatory feedback response, increase the synthesis of dopamine. As already mentioned, trazodone does not cause catalepsy, in a manner similar to clozapine.

Stefanini et al (1976) suggested that the dissociation of the increased dopamine turnover from the cataleptogenic effect may lie in the fact that trazodone induced blockade of the dopamine receptors from nerve terminals is overcome by increased liberation of dopamine from nerve terminals. A similar theory was proposed by Bartholini, Haefele, Jalfre, Keller & Pletcher (1972) for clozapine.

Sulpiride also stimulates dopamine turnover without causing catalepsy (Tagliamonte, De Montis, Olanas, Vargiu, Corsini & Gessa, 1975). A theory similar to Tagliamonte et al (1975) can be proposed for trazodone. It is possible that trazodone stimulation of dopamine synthesis is independent of the blockade of postsynaptic dopamine receptors. As Carlsson (1975) suggested, it is possible that these substances block the postulated pre-synaptic receptors at the dopamine nerve terminals thereby preventing the inhibitory control of its own synthesis.

From the data available this far it is not clear how

trazodone acts. It is still not known whether its effects on 5HT or on the catecholaminergic systems are related to its clinical use as an antidepressant.

It is difficult to correlate any one of the biochemical changes observed in the brain with the pharmacological effects of trazodone. If trazodone inhibits 5HT uptake (Yatmatsu, Kaneko, Yamanishi, Kitahara & Ohtake, 1974; Cassano, Castrogiovanni & Conti, 1974) in the brain, this may explain the total increase in sleeping time observed in animals after trazodone treatment.

Trazodone, when given orally to man, is rapidly and almost completely absorbed (Jauch, Kopitar & Prox, 1976). In non-starving volunteers 50 mg given orally produced peak serum levels within 120 minutes (Allorital, 1975 unpublished report). Plasma halflife of labelled trazodone was found to be between 1 hour initially to 13 hours in non starving volunteers (Jauch et al, 1976). The drug is metabolised and excreted via the urine and faeces and its metabolites have no clinical importance.

SECTION 2: BEHAVIOURAL METHODS

2.1. Introduction

The study of the psychotropic properties of new drugs has met with the problem of developing suitable evaluative methods in animals and the additional problem is the relevance of these findings to man. Methods used in studying the behavioural effects of psychotropic drugs are based on conditioned reactions; these methods, although derived in principle from a classic Pavlovian experiment, show little conformity to the original with regard to stimuli presented or responses elicited. The method employed to characterise the major tranquilising drugs has been the conditioned avoidance behavioural system.

History of conditioning

The term conditioning refers to two procedures, known as classical conditioning and instrumental conditioning. The terms classical and instrumental conditioning were first introduced into the literature by Hilgard & Marquis (1940). The history of classical conditioning is dominated by the early work of Pavlov (1849-1936), and instrumental conditioning procedures stem from the work of Thorndike & Bekhterev. It was whilst studying digestive glands that Pavlov came across the phenomenon of conditioning. Pavlov observed as early as 1880, that sham feeding, in which food passed through an oesophageal fistula and not to the stomach, still produced gastric secretion. This led him to suggest that the stimulation provided by the taste of food and the act of swallowing influenced the stomach, causing 'psychic secretion'. This type of experiment needed surgical skills and was tedious, so Pavlov started work involving salivary secretion which was easier to measure.

The first experiment on salivary secretion by Pavlov was crude, it involved showing a dog a piece of bread, and evidence for conditioning was the salivation caused by sight of food prior to eating. These studies were later followed by the use of a conditioning stimulus such as a bell to cause salivation. Pavlov placed food in a dog's mouth and observed that salivation occurred. (The food is termed the unconditioned stimulus UCS, and salivation the unconditioned reflex or response UCR.) Then Pavlov rang a bell just prior to food presentation, and after a few trials the dog salivated at the sound of the bell. (The bell is termed the conditioning stimulus CS - the dog's response the conditioned response CR.) An association between the food and the bell was established so that the response originally made to food was then made to the bell. Pavlov's studies were first published in 1899 as a thesis entitled "Observations upon salivary secretions" (Pavlov, 1927). Pavlov's experimental work was limited to the salivary response of dogs but the terms he used in dealing with his data are those currently used in discussion of all types of conditioning: conditioned and unconditioned stimuli, reinforcement, extinction, discrimination. In 1906, Pavlov, at Charing Cross Hospital, gave the Huxley lecture entitled "The Scientific Investigation of the Psychical Faculties or Processes in the Higher Animals".

In a typical instrumental conditioning experiment a rat is placed in a sound proof compartment with a bar, which when pressed produces food. With practice, the response of pressing the bar became part of the rat's behaviour as long as he was hungry and received food for pressing the lever. Bekhterev's contribution to conditioning procedures was through his systematic study of association-reflexes. In his experiments with dogs, he studied the withdrawal of a paw and respiratory

responses evoked by electric shock stimulation. The training procedures derived from the work of Bekhterev (1932) and Thorndike (1911) differ from Pavlovian procedures in that the subject's behaviour was instrumental to the production of reward or avoidance of punishment. Hence the term instrumental conditioning. A distinction is made between classical and instrumental conditioning in that classical conditioning is basically a process of association by contiguity whereas instrumental conditioning operates on the environment. Table 1 shows the terms used for classical and instrumental conditioning by several different workers.

To distinguish between a variety of instrumental conditioning procedures, Thorndike (1911) used the terms annoyers and satisfiers or negative and positive reinforcers. By the use of positive reinforcement a reward training procedure can be designed where the animal gets a reward (food) after producing a correct response. Negative reinforcers lead to avoidance training procedures where an animal learns to avoid an electric shock by responding to a warning signal, for example, the conditioned avoidance behavioural paradigm. A distinction can be made by considering whether reinforcement is contingent upon the occurrence of the conditioned response. In classical conditioning the unconditioned response occurs whether or not a conditioned response occurs. By contrast in instrumental conditioning the conditioned response must occur if reward is to be obtained or punishment avoided. The distinction between classical and instrumental conditioning procedures is based on the consequence of the conditioned response. Skinner (1938) observed that under natural conditions an animal makes two kinds of behavioural responses, ELICITED and EMITTED, and suggested an hypothesis that these two forms of behaviour are subject

Author(s)	Term for classical conditioning	Term for instrumental conditioning
Thorndike (1911)	Associative shifting	Trial-and-error learning
Miller & Konorski (1928); Konorski & Miller (1937a, 1937b)	Type I	Type II
Skinner (1937)	Type S, or Respondent	Type R, or Operant
Schlosberg (1937)	Conditioning	Success learning
Hilgard & Marquis (1940)	Classical conditioning	Instrumental conditioning
Mowrer (1947)	Conditioning	Problem solving

TABLE 1: Terms used by different authors for classical and instrumental conditioning. (Taken from Hilgard & Marquis, 1940.)

respectively to respondent or type S (classical) and operant or Type R (instrumental) conditioning.

Elicited responses are those that can be induced reliably by a specific stimulus and under normal conditions only by that stimulus or one very similar to it. Such responses are reflexive, for example, withdrawal of a limb to a painful stimulus or salivation to food. Elicited responses are precisely determined by the properties of the eliciting stimulus (its frequency of presentation, duration and intensity) and are objectively quantified by their latency of onset, and the amplitude and intensity of eliciting stimulus required to induce them. Emitted responses by contrast, are not induced by any identifiable stimulus. If a rat is placed in a small chamber with a lever on the wall, the rat emits a whole variety of responses, none of which is obviously elicited by stimulus in the environment. The lever is connected so that when it is pressed a food reward drops into the cage. At first the rat manipulates the lever by chance but the food reward reinforces the emitted response.

Both elicited and emitted responses may be modified by conditioning, and it is generally accepted that elicited responses are most easily conditioned classically and emitted responses operantly.

It was suggested by Miller & Konorski (1928), and by Skinner (1935) that instrumental conditioning be considered as being distinct from classical conditioning. The differences in their viewpoint has been discussed in several papers (Konorski & Miller, 1937a, 1937b; Skinner, 1937). An important difference between the two types of conditioning is that in most cases classically conditionable responses are 'involuntary' responses of the autonomic nervous system and that instrumentally

conditionable responses are 'voluntary' responses of the central nervous system.

Methods used to study the conditioned avoidance response

The growing requirement for tranquillisers and neuroleptics in clinical psychiatry led to the improvement of evaluative behavioural techniques already in use in physiological studies. The behavioural techniques considered were those that involve the quantitative measurement of a degree of deconditioning of a particular induced behaviour brought about by psychotropic drugs (Courvoisier, Fournel, Ducrot, Kolsky & Koetschet, 1953; Cook & Weidley, 1957).

Several behavioural techniques are used to study conditioned avoidance responses. These methods include:

- (a) shuttle box i.e. horizontal route of escape
- (b) pole-jump i.e. vertical escape responses
- (c) avoidance responses derived from free operant behaviour.

These methods will be described briefly.

(a) The shuttle box, which makes use of a horizontal route of escape was first described by Warner (1932), as the "barrier crossing response" which requires the animal in a controlled situation to move from one part of the cage to avoid punishment. The cage was a two compartment box with a grid floor, the warning signal a buzzer, and the electric shock was given through the grid floor. This was the unconditioned stimulus. The animal was taught to avoid shock by jumping from one compartment across a low hurdle dividing the box.

The shuttle box, sometimes modified, has been used by many workers. Gellhorn, Kessler & Minatoya (1943), and Kessler &

Gellhorn (1953) modified the system by replacing the hurdle by a wall with an opening and using a visual conditioning stimulus instead of an ordinary stimulus. In this thesis the shuttle box was chosen as the behavioural technique to study conditioned avoidance responses and the subsequent effect of haloperidol and trazodone on the conditioned behaviour.

(b) Vertical escape responses (pole jump)

This method involves the training of rats to climb a rope after an auditory conditioned stimulus in order to avoid an electric shock received from the gridded floor of the cage. This method was described by Winter & Flataker (1949; 1951) where rats were trained to climb a rope in order to obtain food and Courvoisier et al (1953) modified the method to test chlorpromazine.

(c) Free operant behaviour modified to produce avoidance responses

This involves the modification of Skinner's operant behaviour in which, instead of working for a reward, animals are warned to avoid a shock by pressing a bar or turning a wheel (Dinsmoor & Hughes, 1956; Sidman, 1953). Harrison & Tracy (1955) employed a high frequency auditory stimulus which was constantly present unless lever pressing produced a period of silence. These techniques have been adapted by Owen, Verhave & Robbins (1957) for pharmacological studies on drugs such as chlorpromazine and morphine.

Specificity of action of drugs

What is the value of the conditioned avoidance response to test a specific drug effect? One specific drug effect is the inhibition of the conditioned avoidance response whilst an

unconditioned response (i.e. escape from an aversive stimulus) is retained, specific because only certain types of drugs are able to inhibit the conditioned avoidance response e.g. neuroleptics. Cook & Weidley (1957) tested active substances such as chlorpromazine and reserpine. Drugs such as barbiturates failed to produce any inhibition of the conditioned avoidance response.

The effects of neuroleptics on the conditioned avoidance response have been studied by many investigators and extinction is considered an essential characteristic of these drugs.

Maffii (1959) expressed this specificity numerically as ratios of drug doses which inhibit conditioned and unconditioned responses by 50% (ID50's) and presented another means of classification of these drugs. Clinical findings strongly favour the concept of specific inhibition, as drugs such as chlorpromazine and reserpine are in some instances antipsychotics if not uniformly antischizophrenic, whereas the barbiturates are not antipsychotic, but sedative.

As with most concepts, the validity of the concept of specific inhibition in contrast to sedative effects has been doubted. Nieschulz, Hoffmann, Pependiker & Schaumacher (1957), Nieschult, Hoffmann & Pependiker (1958), as cited by Herz (1960) using the wheel-turning method, could not find a dose which inhibited the conditioned avoidance response without affecting the unconditioned responses, and produced results to show that there were no significant differences between the chlorpromazine group of drugs and the barbiturates. In 1959, as cited by Herz (1960), Klupp & Kieser, using a modified Skinner's box and both positive (reward) and negative (punishment) reinforced responses found no significant difference between the two groups of drugs.

Another argument put forward is the possibility of a motor deficit component, perhaps by weakness or reduction of muscle

tone, in the inhibition of the conditioned avoidance response by these compounds (Ader & Clink, 1957; Miller, Murphy & Mirsky, 1957; Jenny & Healy, 1959). However, if the escape response remains intact, motor deficit cannot be causing the inhibition of the avoidance response. In spite of the above discrepancies in the data with respect to conditioned avoidance inhibition, the bulk of evidence shows consistently that drugs like chlorpromazine, trazodone and haloperidol have a specific drug action. Numerous studies using different methods and many different conditions of investigations show a definite difference between specific and non-specific drugs (Cook & Kelleher, 1963). Some reports suggest that inhibition of conditioned avoidance response may be due to a fear-reducing effect of the drug (Mowrer & Lamoreaux, 1946) but there is overwhelming evidence contrary to this fear reduction hypothesis.

1. The ease with which the animals perform the responses renders it unlikely that fear and anxiety would be the motivator (Nieschulz et al, 1958)
2. In the experiments of Jacobsen & Sonne (1955) on stress induced behaviour, the reduction in tension is accompanied by an increase in response to the conditioned stimulus.
3. Meprobamate does not inhibit the conditioned avoidance response although it is used widely in man for anxiety and tension reducing effects.
4. The fact that the performance of a positively reinforced conditioned response is also decreased argues against the fear hypothesis (John, Wenzel & Tschirgi, 1958;

Riopelle & Pfeiffer, 1958). Murphee, Jenny & Pfeiffer (1959), as cited by Herz (1960), associated the inhibition of the conditioned avoidance response with anti schizophrenic potency of drugs. They stated that "one should note, that of all the pharmacological tests used for the discovery of anti schizophrenic drugs, the inhibition of the conditioned avoidance response is the most quantitative". Courvoisier (1956) also suggested a close relationship between deconditioning activity and usefulness of these drugs in the clinic.

Extinction

If the conditioned stimulus (buzzer) continues to be presented to conditioned animals, unaccompanied by the reinforcer (shock), the conditioned response progressively decreases - this is called extinction. The loss of the conditioned response is not instantaneous; conditioned responses slowly diminish with time. Conditioned pecking responses in pigeons have been shown to be maintained for four years (Skinner, 1950) and the conditioned motor response in sheep has been shown to be retained for two years (Liddel, James & Anderson, 1934). Conditioned avoidance response in Wistar rats have been shown to be maintained for the entire period of their lives (unpublished observations).

2.2 Materials and methods

Materials

1. Rat strain

- i) Wistar - albino strain male, 250g
- ii) Long Evans - hooded strain. " "

The rats of both strains were in-bred, specified pathogen

free (SPF) equivalent to category 3 star of the laboratory animal centre accreditation scheme with reference to the accreditation and recognition scheme for suppliers of laboratory animals, (MRC Manual Series No. 1 (1974). MRC Carlshalton, Surrey, U.K).

The rats were maintained under conventional housing during the experimentation period. A maximum of six rats per cage were housed in plastic cages, size 56 x 38 x 18 cms with stainless steel wire tops and stainless steel gridded floors. The cages were cleaned weekly.

Food and water were provided ad libitum. The food consisted of standard commercially supplied pellets (Labsure, Agrarian House, Castle Street, Poole).

The rooms were kept at a temperature of $22 \pm 1^{\circ}\text{C}$, under ordinary lighting with 12 hours on and off phase. The humidity was maintained at 50% with 15-20 ventilation changes per hour.

2. Shuttle-box (Ugo. Bastile, Milan) (Figure 6)
3. Ethanol 10% solution
4. Haloperidol (5mg/ml ampoules) SERENASE
5. Trazodone (100 mg capsules) MOLIPAXIN

Protocol for the conditioned-avoidance behavioural schedule

Rats were trained to avoid an electric shock in an automatically controlled two-compartment shuttle box (Fig. 6) by moving from one compartment to the other during 3.8 seconds of buzzer warning. If the rat did not respond to the buzzer it was given an electric shock provided through the grid floor of the box. The shock (unconditioned stimulus) terminated immediately upon the movement of the rat into the other compartment. The experimental session consisted of 10 trials and lasted for a period of 5 minutes. A timed protocol is shown (Fig. 7).

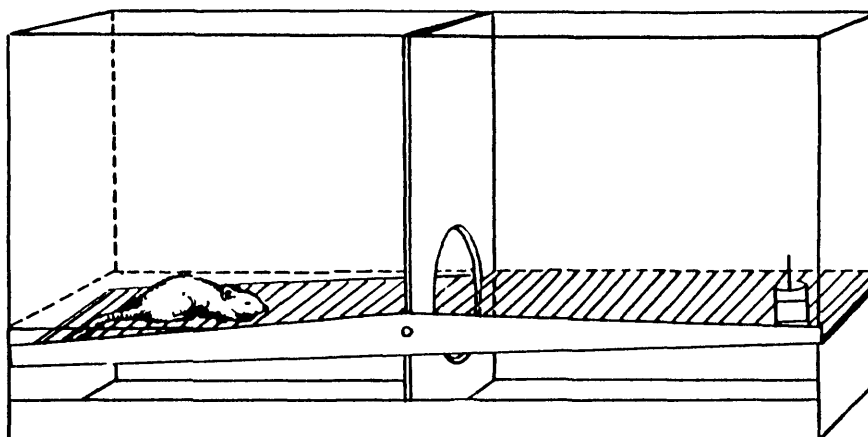


FIG 6: The Ugo Bastile Shuttle Box.

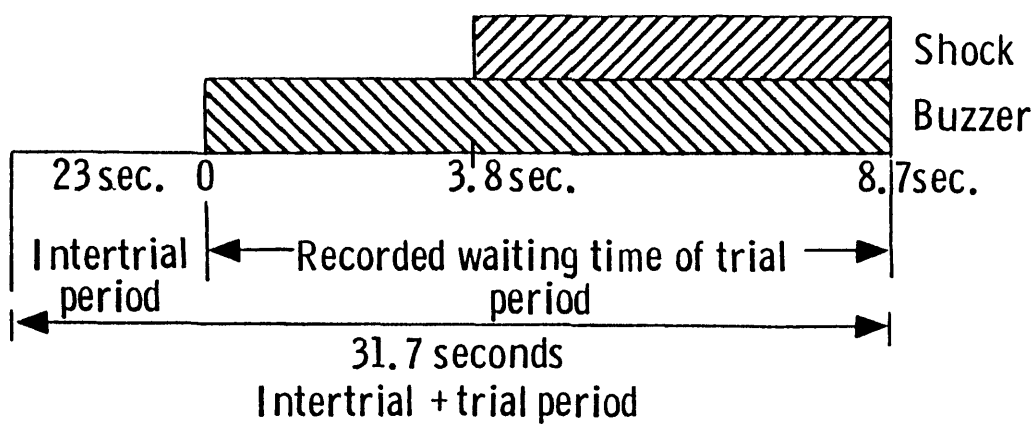


FIG 7: Timed protocol for the conditioned avoidance trial schedule.

1. The rat was placed into the shuttle-box and allowed to get accustomed to the environment for a period of 5 minutes.

2. The apparatus was switched on, the required shock (0.2 mA) and acoustic stimuli were chosen. A total of 10 trials were carried out each day.

3. Each trial lasted a period of 3.8 seconds and the shock that followed lasted for 4.9 seconds. The trial sequence has been illustrated in Figure 7.

The parameters scored on a time basis were:

1. Conditioned avoidance response (CAR) if the rat responded to the buzzer, before the electrical stimulus came on.

2. Unconditioned escape response (UER) if the rat moved to the other compartment during the shock period.

3. Escape failure (EF) if the rat failed to escape the shock.

4. Non signal movement (NSM) if the rat moved without prior stimulus.

5. Urination and defaecation were recorded

At the end of the experimental session the shuttle-box was thoroughly cleaned with ethanol (10%). Daily records were kept of the food and water intake per cage of six rats. Individual rat body weights were also recorded throughout the experiment. Each rat was brought into the training room separately in order for them not to be disturbed by the sound of the buzzer.

Dosing procedures

Haloperidol was administered in 0.5 ml of saline intra peritoneally. Trazodone was dissolved into saline and 0.5 ml of the required dose administered intra peritoneally.

Procedure for the selection of conditioned rats

A rat was selected as being conditioned after 14 days of conditioning, on the basis of performing the conditioned avoidance responses within an average of 3.8 seconds (average of 10 trials) or less.

2.3. Experimental procedure

2.3. 1. Selection of rat strain for conditioning experiments

Initial experiments were carried out to select a rat strain which would be suitable for training on the conditioned avoidance behavioural system. Twelve Long Evans and 8 Wistars were trained for a period of 14 days.

2.3. 2. Acute treatment of conditioned Wistars with haloperidol

Experiments were carried out on 7 conditioned Wistars to study the influence of haloperidol on the conditioned avoidance response. A dose-response effect of haloperidol on the conditioned response was performed in order to find a dose that would extinguish the conditioned response whilst leaving the escape response intact.

Rats were injected with haloperidol (i.p) in the dose range of 0.01 mg - 0.15 mg/kg body weight. The dose of haloperidol given per day was randomised starting with higher rather than lower doses so a 'build-up' effect of haloperidol was prevented. Each dose of haloperidol was given after two days of non-drug

treatment.

After drug treatment rats were tested first for avoidance and then for escape behaviour at 0, 1, 2, 4, 6, 8 and 24 hours. When rats were tested for avoidance for a period of 10 trials the electric stimulus was switched off, leaving the buzzer signal on. When tested for escape response for a period of 2 trials, the buzzer signal was switched off, leaving the electric stimulus on. The waiting times for avoidance and escape responses for each rat were recorded.

2.3.3. Chronic treatment of conditioned Wistars with haloperidol (0.1 mg/kg)

Seven conditioned Wistars were treated with 0.1 mg/kg of haloperidol (i.p.) over a period of 26 days. After the first 21 days of drug treatment, the treatment was stopped for a period of 6 days and resumed on the 28th day for a further 5 days.

Rats were tested for avoidance and escape behaviour 2 hours after drug administration and this was done periodically during the experimental session.

2.3.4. Acute treatment of conditioned Wistars with trazodone

After 14 days of training, 10 conditioned rats were acutely treated in a dose-dependent manner with trazodone (5 mg - 140 mg/kg). The experiments were carried out to study the influence of trazodone on the conditioned avoidance response. Rats were injected i.p. with incremental doses of trazodone and each day of drug treatment was followed by a "rest" day.

Rats were tested for conditioned and escape responses at 0, 1, 2, 4, 6 and 24 hours after each treatment.

2.3.5. Chronic treatment of conditioned Wistar with trazodone (100 mg/kg)

Ten conditioned Wistars were chronically treated with trazodone (100 mg/kg) for a period of 32 days. Rats were tested for avoidance (10 trials) and escape (2 trials) behaviour 1 hour after drug administration and this was done periodically during the experimental session (see Fig. 12).

2.3.6. Effect of haloperidol (0.1 mg/kg) and trazodone (100 mg/kg) on the food and water consumption of the conditioned rats

Daily food and water intake of the rats was recorded over the whole experimental period. The first 14 days of the experimental period were drug-free i.e. when the rats were being conditioned. Chronic treatment with either trazodone (100 mg/kg) or haloperidol (0.1 mg/kg) commenced on day 15.

2.4. Results and discussion

2.4.1. Rat strain selected for conditioning

Initially 8 Wistars and twelve Long Evans were trained for a period of 14 days in the conditioned avoidance system. These experiments produced results to show that 80% of the Wistars could be conditioned within 14 days of training whereas only 50% of Long Evans could be successfully conditioned within the same time period, therefore Wistars were selected for all subsequent conditioning studies.

Figure 8 (p103) shows the results of experiments in which 60 rats were successfully trained over a 14-day period. Rats which failed to acquire conditioned behaviour after 12 days of training were eliminated from the schedule. In the analysis of the larger

group of Wistar rats trained, it was found that 80% or more were successfully conditioned.

The conditioned response was acquired after 9 days of training and was maintained throughout the life^(3yrs) of all rats which were not subjected to any drug treatments. It is worth noting that those rats which failed to learn could be identified within the first few days of the training schedule as their behaviour differed in several respects from their successful counterparts. Over the experimental period, even though the genetic strain of rats was constant, there were batch to batch variations with respect to success rates. This was thought to be due to operator factors, and therefore one operator conducted the training for specific schedules.

2.4.2. Acute treatment with haloperidol

In Figure 9 (p104) and Table 2 (p105) the results of a dose-response effect of haloperidol on the conditioned response are shown. Seven rats were randomly injected (i.p.) with varying doses of haloperidol and tested (i.e. without the shock) for the conditioned response at 2 hour intervals over a 24 hour period. Results show that the extinction of the conditioned responses correlated directly with the drug dose. Results also show that the extinction of the conditioned response is only temporary, that the conditioned response returns within the 24 hour period at all dose levels tested. The maximum effect of the drug seemed to be reached by the eighth hour and to disappear by the twenty fourth hour. SEM were not included in Figure 9 for the purposes of clarity. Table 2 shows the SEM of the waiting times. Each rat was tested for 10 trials and the data show the variation among 7 rats. At no dose levels of haloperidol was the unconditioned escape response affected (Table 3 p106).

Discussion

The entire experiment was conducted over 14 days. Each day, 7 rats were dosed, not incrementally, but randomly with the drug, followed by a "rest" day. This experimental design was fortuitous; what was aimed for was a suitable dose which did not sedate the animals. However, at no dose were the rats sedated as measured by unconditioned escape response successes, therefore low, medium and high doses were randomly given and 0.1 mg/kg selected. (Subsequent dose response curves for trazodone were conducted simply on a dose incremental basis.)

Haloperidol impairs the conditioned avoidance response in rats in a dose-related manner but does not affect the unconditioned escape response, which remains intact at all dose levels. Thus a deconditioning effect is achieved which is specific for avoidance responding. That the extinction of avoidance is only temporary and returns within 24 hours was also reported by Fregnan & Chieli (1980). It is clear that the effect of haloperidol in conditioned rats was on the recall process. Conditioning itself i.e. the learnt behaviour, was permanent as the rats did not lose avoidance responding over the entire duration of their lives.

2.4.3. Chronic haloperidol treatment

As shown in Figure 10 (p107) daily dosing with haloperidol (0.1 mg/kg) over 32 days resulted in a graded increase in the average waiting time signifying that the rats are responding more slowly to the conditioning stimulus with chronic drug treatment. As with acute treatment this deconditioning was temporary as shown in Figure 10. After drug withdrawal the conditioned response returns until dosing was recommenced. However, after resumption of dosing the extinction of the conditioned response

was enhanced when compared with average waiting times achieved on day 1.

Discussion

During the course of daily treatment with haloperidol the effect on the avoidance response gradually became greater and reached a maximum at 22 days. This maximal response was increased after 4 days of interruption of the treatment, when the animals were re-challenged with further treatment with the drug. This potentiated effect on the conditioned avoidance response may be due to a supersensitive response to haloperidol. This suggestion was made by Fregnan & Chieli (1980) who found the same effect of haloperidol on rats conditioned as in this system.

These results are in contrast to results obtained by Danneskiold-Samsøe & Pedersen (1976), and Møller Nielsen, Fjalland, Pedersen & Nymark (1974). Danneskiold-Samsøe & Pedersen (1976) found that conditioned rats pretreated with haloperidol (10 mg/kg) when tested for avoidance responding showed a weakened inhibition of the avoidance response when compared with rats not pre-treated with haloperidol. Therefore more of the drug was required in order to produce a greater degree of the inhibition of the CAR.

Rats non-pretreated with haloperidol showed 92% inhibition of the CAR, whereas after 12 days of pre-treatment with haloperidol (10 mg/kg) this inhibition was reduced to 60%.

These authors however, do not show the effect of such a high dose on the UER. It is possible that the inhibition of the CAR may be due to the rats being sedated, at larger doses used. The contrasting concept of tolerance is explained as being due to some pharmacological activity of the neuroleptic upon repeated administration, or to a supersensitivity of the dopaminergic

receptors towards specific agonists during the withdrawal period following prolonged daily treatment with the neuroleptic, and therefore requiring more of it to produce the same amount of inhibition.

2.4.4. Acute treatment with trazodone

In Figure 11 (p108) and Table 4 (p109) the results of a dose-response effect of trazodone on the conditioned avoidance response are shown. Ten rats were injected i.p. with incremental doses of trazodone ranging from 5 mg/kg - 100 mg/kg. The effect of the drug on the conditioned response at all dose ranges was maximal at 1 hour. Results show that the extinction of the conditioned response correlated directly with the drug dose. The extinction was temporary and the conditioned response returned by the sixth hour.

At all dose ranges the unconditional escape response remained intact and this is shown in Table 5 (p110). Table 4 shows the effect of trazodone on the mean conditioned avoidance response $\pm$ SEM, which are excluded from Figure 11 for clarity.

Discussion

This experiment, conducted over 16 days, showed a similar inhibition of the CAR seen with acute haloperidol dosing. Gatti (1974) reported an inhibition of CAR by oral trazodone (100 mg/kg). In this work, oral dosing failed to produce a temporary loss of the learned response. However, it was not known when the water was consumed and therefore the time chosen to test for this response might have been inappropriate, especially as deconditioning occurs 1 hour after i.p. dosing (oral administration of 50 mg of trazodone in fasting human volunteers produced peak serum levels of the drug within 30

minutes, McLean Baird et al, 1976 unpublished report). The temporary loss of the learned response is similar to that achieved with haloperidol. It appears that recall of the learned response is affected by trazodone.

2.4.5. Chronic treatment with trazodone

A group of 10 rats were treated (daily) with trazodone 100 mg/kg i.p. over a 32 day period. As shown in Figure 12 (p111) an increase in the average waiting time occurred, signifying the loss of the conditioned avoidance response. The unconditioned escape response remained intact in trazodone treated rats. The conditioned response was maximally extinguished from the start of the experiment and remained constant over the course of treatment.

Discussion

This effect of trazodone was unlike that of haloperidol as it potentiated the extinction of CAR throughout the period of treatment. This effect may be due to sedation at 100 mg/kg dose level, but this seems unlikely as the UER was not affected. A tolerance towards neuroleptics which appears with high doses of some drugs, i.e. haloperidol (Danneskiold-Samsoe & Pedersen, 1976) is not shown by trazodone. Trazodone, like the neuroleptics, has the ability to inhibit the CAR at doses which do not alter the unconditioned response. This effect of trazodone was also shown by Gatti (1974) using the modified discriminated continuous lever pressing avoidance technique of Sidman. The authors showed a 55% inhibition of the avoidance response and 20% decrease of the UR using 100 mg/kg of trazodone (oral route).

2.4.6. Effect of haloperidol (0.1 mg/kg) and trazodone
(100 mg/kg) on the food and water consumption of the
conditioned rats

Results

During the 14 days of training, the recorded food and water intake of the rats was not altered by chronic treatment with haloperidol (0.1 mg/kg) and trazodone (100 mg/kg) Figure 13 (p112).

Results show that the drugs were not affecting the normal bodily functions of the rats.

Conclusions

Wistar rats were most effectively conditioned in this operant avoidance/escape system. Acute drug treatment showed that the extinction of the learned response was dose dependent but temporary, and that by 24 hours after dosing the conditioned response had returned. The unconditioned escape response remained intact irrespective of drug or dose level. Patterns of extinction with chronic haloperidol and trazodone differed. The neurochemical basis for the interpretation of these observations will be discussed later.

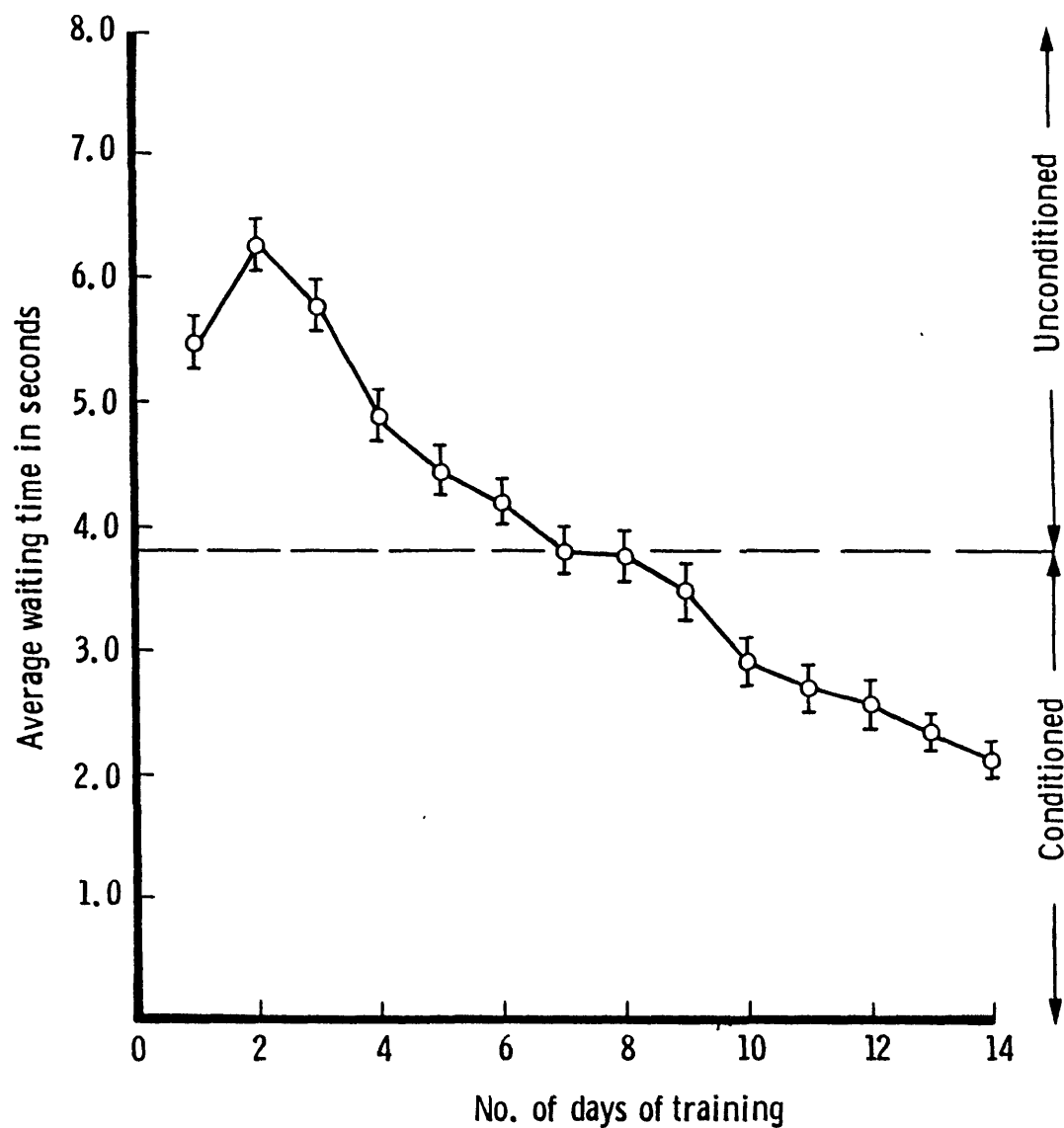


FIG 8: Conditioned avoidance response profile of Wistars successfully conditioned. Abcissa scale: number of days of training. Ordinate scale: average waiting time of 10 trials per rat per day. Each point $\bigcirc$ is the mean $\pm$ s.e. of 60 Wistar rats.

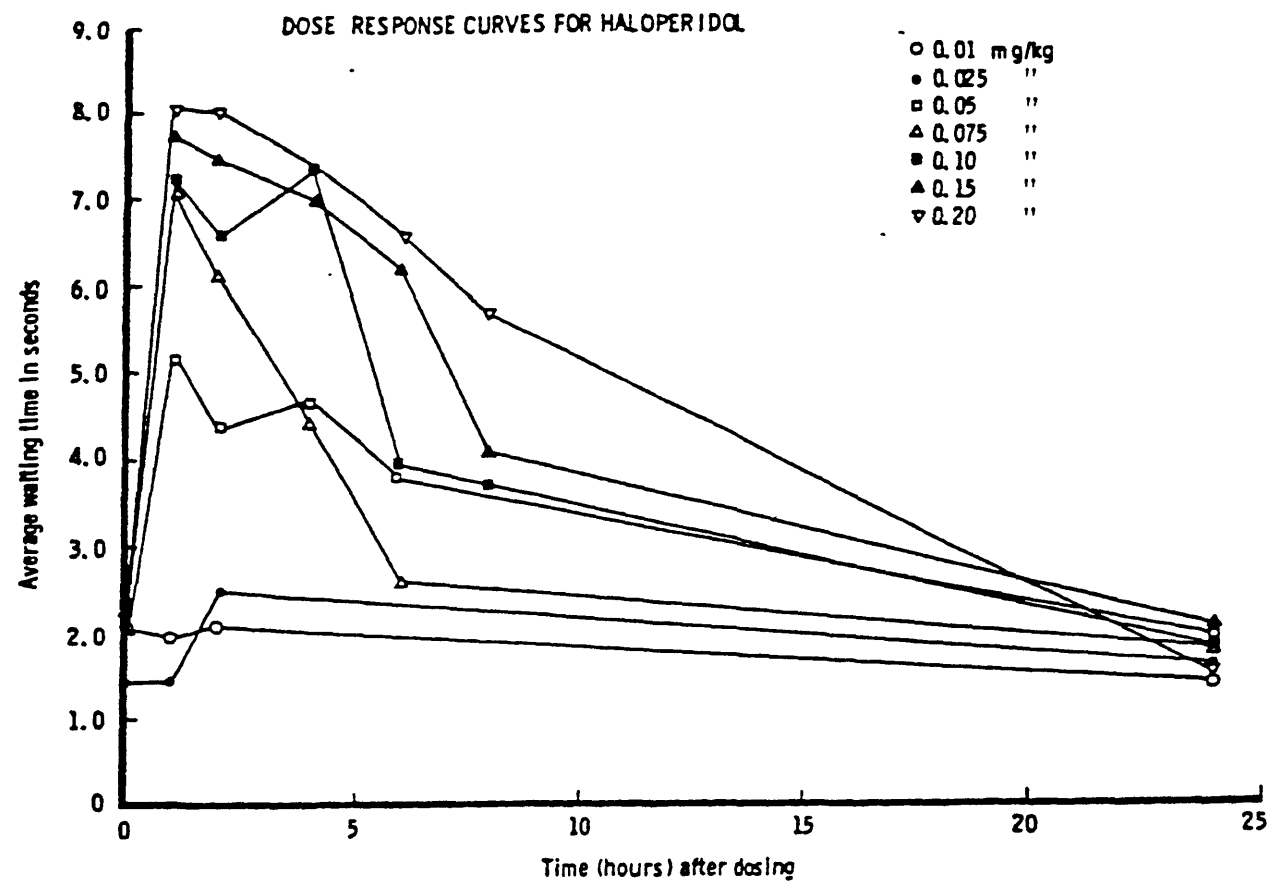


FIG 9: Dose response curve for haloperidol. Each point is the average mean waiting time (i.e. of 10 trials per rat) for a total of 7 rats. s.e.m. has been omitted for clarity and have been shown in Table 2.

TABLE 2

Dose of haloperidol	Number of hours after dosing						
	0 hour	1 hour	2 hours	4 hours	6 hours	8 hours	24 hours
0.1 mg/kg	2.07 $\pm$ 0.49	1.93 $\pm$ 0.28	2.05 $\pm$ 0.26	-	-	-	1.40 $\pm$ 0.38
0.025 mg/kg	1.41 $\pm$ 0.10	1.44 $\pm$ 0.15	2.00 $\pm$ 0.22	-	-	1.49 $\pm$ 0.14	1.62 $\pm$ 0.10
0.05 mg/kg	1.69 $\pm$ 0.15	5.22 $\pm$ 0.38	4.32 $\pm$ 0.91	4.69 $\pm$ 1.04	3.77 $\pm$ 1.25	-	1.95 $\pm$ 0.16
0.075 mg/kg	1.58 $\pm$ 0.17	7.34 $\pm$ 0.30	6.08 $\pm$ 0.79	4.40 $\pm$ 0.95	2.59 $\pm$ 0.36	2.00 $\pm$ 0.10	1.80 $\pm$ 0.16
0.1 mg/kg	1.95 $\pm$ 0.19	7.22 $\pm$ 0.26	6.56 $\pm$ 0.78	7.31 $\pm$ 0.83	3.90 $\pm$ 1.04	3.71 $\pm$ 0.91	1.80 $\pm$ 0.15
0.15 mg/kg	1.79 $\pm$ 0.24	7.72 $\pm$ 0.04	7.44 $\pm$ 0.31	6.97 $\pm$ 0.66	6.16 $\pm$ 0.90	4.06 $\pm$ 0.83	2.55 $\pm$ 0.20
0.20 mg/kg	1.81 $\pm$ 0.23	8.07 $\pm$ 0.14	8.02 $\pm$ 0.04	7.39 $\pm$ 0.55	6.61 $\pm$ 0.42	5.66 $\pm$ 0.87	1.50 $\pm$ 0.12

TABLE 2: Effect of varying doses of haloperidol on the conditioned response of the rats. Each figure in seconds is the average waiting time (10 trials per rat) for a total of 7 rats $\pm$ s.e.m.

Dose of haloperidol	Number of hours after dosing					
	0 hour	1 hour	2 hours	4 hours	6 hours	8 hours
0.1 mg/kg	-	-	-	-	-	-
0.025 mg/kg	-	-	-	-	-	-
0.05 mg/kg	-	4.35 ± 0.19	4.71 ± 0.40	4.20 ± 0.20	4.05 ± 0.03	-
0.075 mg/kg	-	4.32 ± 0.16	4.51 ± 0.19	-	-	-
0.1 mg/kg	-	4.62 ± 0.27	4.28 ± 0.49	4.62 ± 0.27	4.01 ± 0.01	4.18 ± 0.06
0.15 mg/kg	-	4.54 ± 0.26	5.37 ± 0.39	4.61 ± 0.35	4.28 ± 0.21	4.18 ± 0.10
0.20 mg/kg	-	4.54 ± 0.26	4.80 ± 0.27	5.05 ± 0.27	4.60 ± 0.32	4.64 ± 0.35

TABLE 3: Effect of varying doses of haloperidol on the ^{unconditioned} escape response of rats (in seconds). Each point in seconds is the mean of two escape responses measured in seconds for 7 rats ± s.e.m.

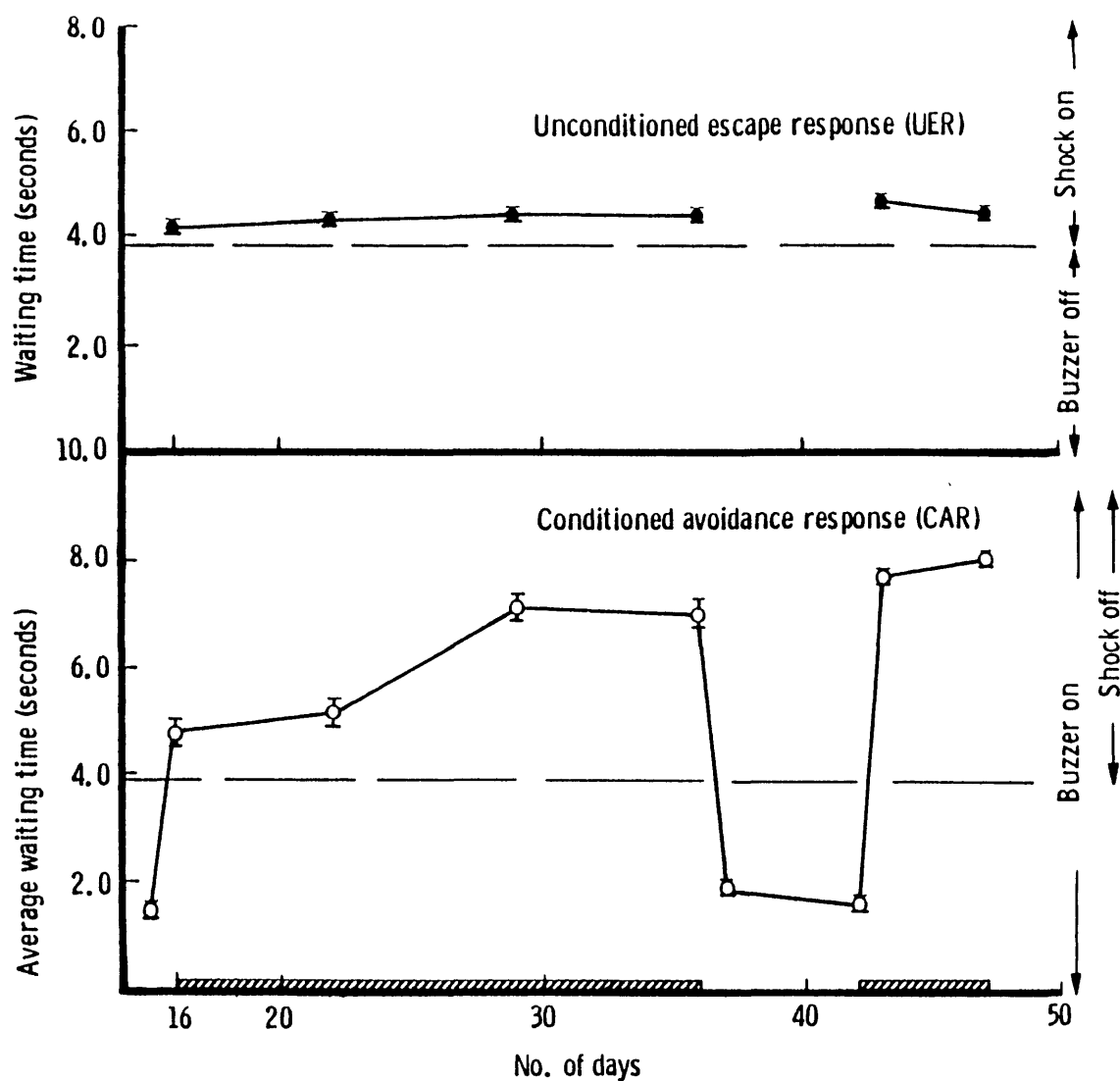


FIG 10: Effect of haloperidol (0.1 mg/kg i.p) on CAR and UER tested 2 hours after drug administration. Abcissa: number of days of haloperidol treatment////. Each point O is the average mean waiting time of 10 trials per rat for a number of 7 rats $\pm$ s.e.m. Each point ● is the mean $\pm$ s.e. of 7 rats.

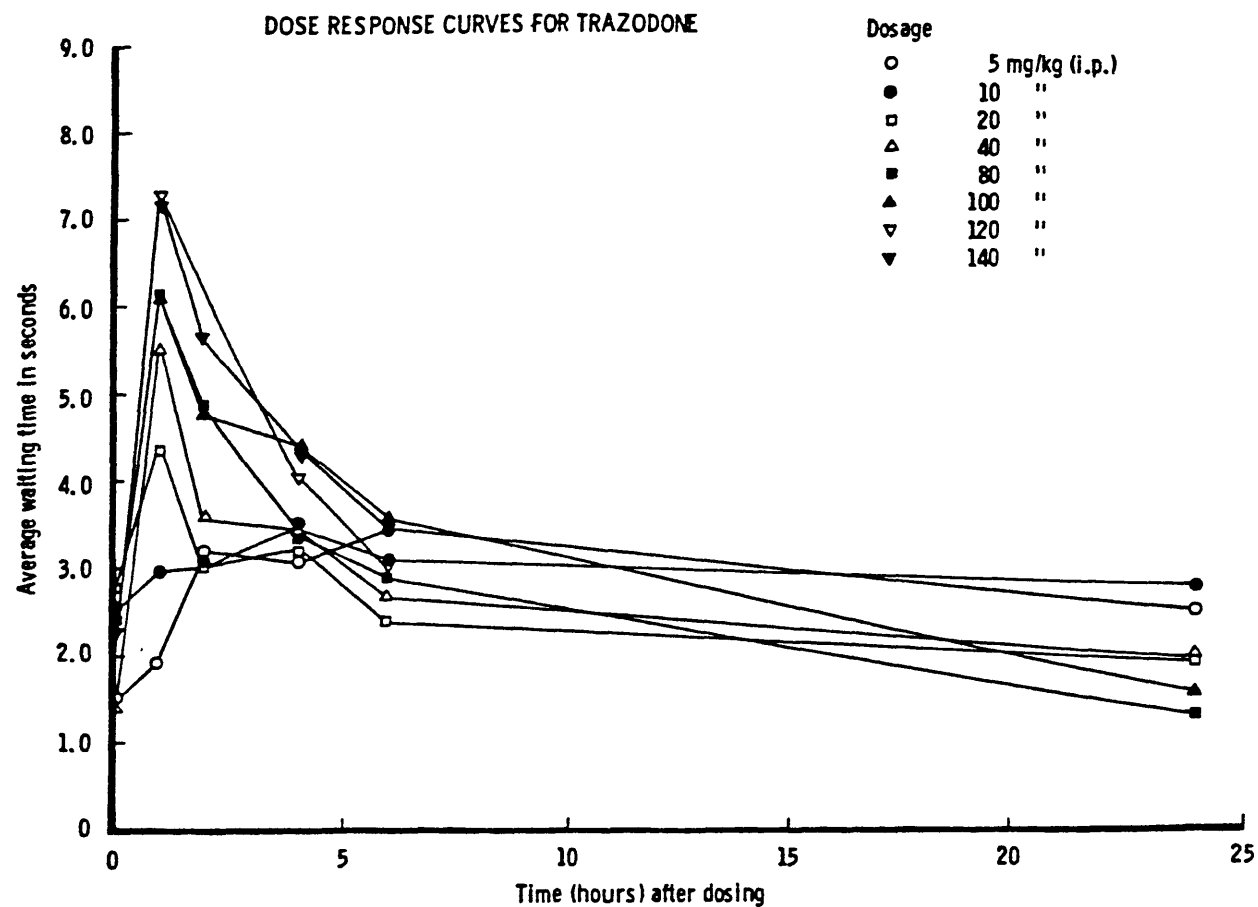


FIG 11: Dose response curve for trazodone. Each point is the average waiting time (i.e. 10 trials per rat per day) for a total of 10 rats. s.e.m. has been omitted for clarity and have been included in the data in Table 4.

Dose of haloperidol	Number of hours after dosing					
	0 hour	1 hour	2 hours	4 hours	6 hours	24 hours
5 mg/kg (i.p.)	1.45 ± 0.19	1.91 ± 0.21	3.18 ± 0.89	3.05 ± 1.02	3.47 ± 1.22	2.50 ± 0.12
10 mg/kg (i.p.)	1.52 ± 0.13	2.45 ± 0.975	3.01 ± 0.95	3.44 ± 1.02	3.13 ± 1.10	2.85 ± 0.12
20 mg/kg (i.p.)	1.95 ± 0.20	3.91 ± 0.24	3.03 ± 1.17	3.23 ± 0.80	2.27 ± 0.30	1.30 ± 0.20
40 mg/kg (i.p.)	1.88 ± 0.17	6.21 ± 0.96	4.85 ± 1.17	3.77 ± 1.08	2.87 ± 0.89	1.95 ± 0.16
80 mg/kg (i.p.)	1.38 ± 0.09	5.60 ± 0.96	3.55 ± 0.93	3.34 ± 1.05	2.66 ± 0.18	1.30 ± 0.10
100 mg/kg (i.p.)	1.96 ± 0.21	6.99 ± 0.59	4.67 ± 0.77	4.43 ± 1.10	3.55 ± 0.81	1.55 ± 0.10
120 mg/kg (i.p.)	1.55 ± 0.12	7.26 ± 0.35	6.03 ± 0.88	4.08 ± 0.88	2.94 ± 0.40	1.75 ± 0.20
140 mg/kg (i.p.)	1.61 ± 0.07	7.20 ± 0.52	5.60 ± 0.81	4.39 ± 0.99	3.48 ± 0.44	2.25 ± 0.15

TABLE 4: Effect of varying doses of trazodone on the conditioned response of the rats. Each figure (in seconds) is the average mean waiting time (10 trials per rat) for a total of 10 rats ± s.e.m.

Dose of trazodone	No. of hours after dosing				
	0 hour	1 hour	2 hours	4 hours	6 hours
5 mg/kg	-	-	-	-	-
10 mg/kg	-	-	-	-	-
20 mg/kg	-	-	-	-	-
40 mg/kg	-	4.80 ± 0.17	-	-	-
80 mg/kg	-	4.26 ± 0.11	-	-	-
100 mg/kg	-	4.45 ± 0.18	-	-	-
120 mg/kg	-	4.65 ± 0.16	4.46 ± 0.32	-	-
140 mg/kg	-	4.21 ± 0.13	4.33 ± 0.18	-	-

TABLE 5: Effect of varying doses of trazodone on the escape response of rats. Each point is the mean escape response (in seconds) of 10 rats ± s.e.m.

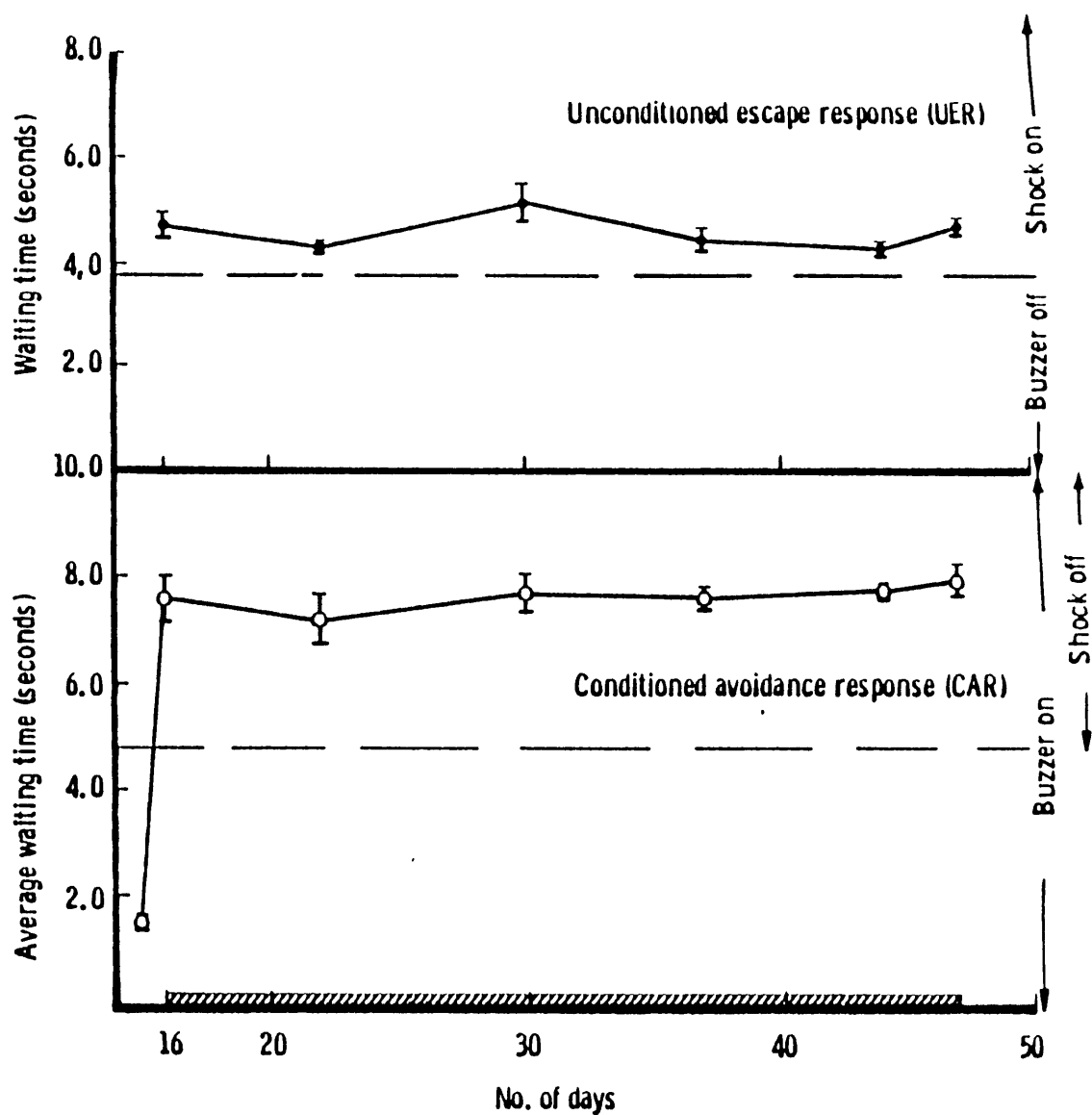


FIG 12: Effect of trazodone (100 mg/kg i.p) on CAR and UER tested after 1 hour of drug administration. Abcissa: number of days of drug treatment. Each point $\bigcirc$ is the average waiting time of 10 trials per rat for a number of 10 rats $\pm$ s.e. Each point $\bullet$ is the mean $\pm$ s.e of 10 rats.

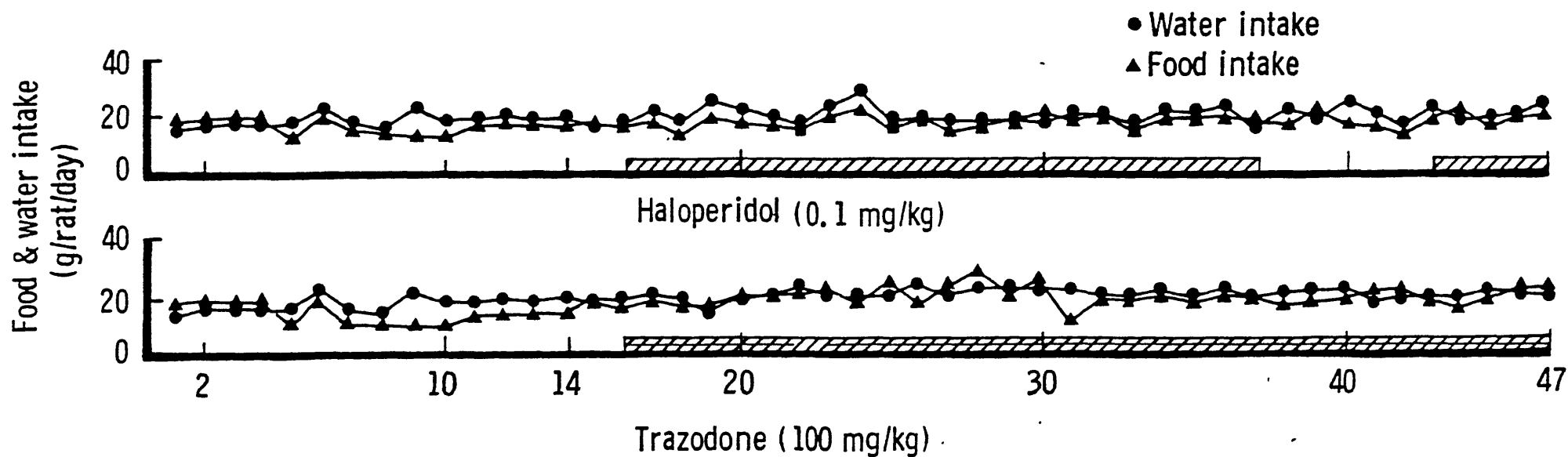


FIG 13: Effect of haloperidol (0.1 mg/kg) and trazodone (100 mg/kg) on the food and water intake of conditioned rats. Number of rats on trazodone treatment were 10, those on haloperidol treatment were 7. Abcissa scale shows the number of days of haloperidol treatment////, and trazodone treatment////. s.e.m. were omitted for clarity.

SECTION 3: STUDY OF TRANSMITTER RELEASE USING SLICES PRELOADED
WITH RADIOLABELLED AMINES

3.1. Introduction to methods

In order to establish a procedure for the study of endogenous release of neurotransmitters, initial experiments were carried out by preloading brain tissue slices with a radiolabelled amine and subsequently releasing the radiolabel by electrical stimulation. This technique was first described by Baldessarini & Kopin (1966) and modified on several occasions since (Dismukes & Mulder, 1977). The procedure in this thesis employed ^3H -dopamine for preloading the slice sections during a preliminary incubation period, superfusion of the slice sections to remove excess label and subsequent release of the tritium by electrical stimulation. The slice sections were electrically stimulated on two occasions and the superfusate fraction collected at 1 minute intervals throughout the experimental period.

Initial experiments were carried out in the presence and absence of CaCl_2 in the superfusion media to establish the calcium ion dependency of ^3H -dopamine release.

The drugs trazodone and haloperidol, were under test in the conditioned avoidance behavioural system. Parallel in vitro studies on brain slices were designed to measure any possible effect of these drugs on electrically stimulated release of preloaded tritiated neurotransmitters. Subsequently comparisons could be made between drug effects on preloaded neurotransmitter release and drug effects on endogenous neurotransmitter release, release in both cases being achieved by electrical stimulation.

Technetium ($\text{Tc}^{99\text{m}}$), a γ -emitting isotope with a half life of 6 hours, was added to the superfusion media in order to monitor the volume of superfusate in each 1 minute collection. This is fully explained in the section on analysis of data p121.

3.2. Materials and method

All chemicals purchased were Analar grade. Substances purchased from British Drug House (B.D.H.) Chemicals Ltd., Poole, England were: calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), potassium chloride (KCl), sodium chloride (NaCl), magnesium sulphate ($\text{Mg}/\text{SO}_4 \cdot 7\text{H}_2\text{O}$), potassium dihydrogen orthophosphate ($\text{KH}_2 \text{PO}_4$), D-glucose, L-ascorbic acid, and magnesium chloride (MgCl_2). Sodium hydrogen carbonate (NaHCO_3) was purchased from Hopkin & Williams.

Pargyline was purchased from the Sigma Chemicals Company, Micellar NE240 scintillation cocktail from G & G Chemicals and technetium from the Department of Nuclear Medicine, Charing Cross Hospital. Superfusate apparatus consisted of a Grant Instrument water bath Type SB2X, Grant Instruments, Barrington, Cambridge, size 48.5 x 24 x 2.5 cm; Watson and Marlow peristaltic pump; transfer bath; Fenton Lewis double channel stimulator, two 500 ml reservoir flasks; 8-channel constant current stimulator adaptor; oscilloscopes; 0.2 ml volume gold electrode chambers; silicone tubing.

Materials for the acrylic mould were De Trey Zelgan alginate and rapid repair acrylic obtained from Cottrell & Co., Charlotte Street, London.

Method: Superfusion system

A diagrammatic representation of the apparatus for superfusion of brain slices is shown in Figure 14 (p123).

The electrode chamber (made by Charing Cross Hospital workshop) and a cross section representation are shown in Figure 15 (pl24).

The electrode chamber consists of two electrodes 10 mm apart with a chamber volume of 0.2 ml. Gold was used as the electrode material due to the problems associated with platinum and silver, (see p71) which are alternative materials used in electrophysiological experiments.

The electrode chambers are clamped and lowered horizontally into the Grant's water bath maintained at 37°C.

The Kreb's bicarbonate saline contained in 500 ml flasks, was delivered to the electrode chambers via silicone rubber tubing by means of a Watson-Marlow microperfusion pump. The flow rate was maintained at 0.4 ml/min. This flow rate was accurately determined by the use of technitium in the superfusion media. Each 1 minute sample was collected, counted and the flow per minute determined using a computer programme (described in detail in the section on analysis of data).

Superfusion media

The physiological medium used was Kreb's bicarbonate saline at pH 7.4 and composed of NaCl (120 mM), KCl (4.75 mM), KH_2PO_4 (1.2 mM), CaCl_2 (2.6 mM), NaHCO_3 (25 mM), MgSO_4 (1.2 mM), glucose (10 mM), 2mCi per ml of $\text{Tc}^{99\text{m}}$ added to 5 litres of superfusing media.

During the incubation of slices prior to superfusion ascorbic acid (0.1 mg/ml) an antioxidant and pargyline (10^{-7}M) as a metabolic inhibitor were added. The medium was pre-gassed with 95% O_2 /5% CO_2 for 45 minutes before commencing the experiment. During the experiment Kreb's bicarbonate saline was gassed with 95% O_2 /5% CO_2 , bubbled into the reservoir and maintained

during the superfusion period.

Methodology for the preparation of brain slices

Preparation of brain slices

(a) Principle

The stereotaxic atlas of Thompson (1978) modified from the Massopust atlas, established that electrocoagulative lesions in selected regions of the rat brain, caused a deficit of active avoidance behaviour in conditioned animals.

Slices were selected from those regions of the brain established as responsible for the conditioned avoidance behaviour (Thompson, 1978).

Using the inferior colliculus as a zero reference point (see Figure 16) three slices were obtained (See Figures 17, 18, 19), stained with Sudan black and compared with slices in the Massopust Atlas (Massopust, 1961) which have a corresponding reference designation (see below). These slices were labelled S9, S13 and S14.

A modified brain slicer (Brown & Halliwell, 1981) was used to cut reproducible coronal slices from specific regions of the brain.

Slice thickness

Slices used were 600 μ . This thickness was found to be appropriate for electrophysiological experiments. The thicker the slice, the more difficult it is to ensure a sufficient O₂ supply to all cells within the slice. Slices of 700 μ are still physiologically viable and have been used successfully where long term activity of the slice has been recorded (White, Nadler & Cotman, 1978).

Brain slicer

The brain slicer (Figure 20 p125) was modified by designing a guillotine attached to steel poles (34 cm high) alongside the base, with a screw gauge with 0.72 mm pitch. This permits accurate slicing a specific distance away from a preset zero reference point. The guillotine was dropped on to a brain supported by an agar mould from a distance of 11 cm. This avoided damage to the tissue as the blade cut straight through the agar without bouncing back as it would if a McIlwain brain chopper were used.

Preparation of the agar mould

1. Several acrylic models of fresh brains (rats weighing 150g-300g) were prepared from a 20% solution of alginate. This solution was poured into a flexible plasticine container and the brain was embedded in the alginate with the dorsal surface upwards. When the alginate had solidified the brain was removed. Rapid repair acrylic was then poured into the impression made by the brain in the alginate. A small steel handle was held just projecting into the acrylic solution until the acrylic solidified. After 5 minutes the acrylic brain mould was removed and subsequently used to make an impression in the agar.
2. This model was used to make an agar mould of the lower half of the brain, which acts as a support for subsequent slicing of fresh brains.
3. The agar mould was prepared by dissolving 4g of agar into 100 ml distilled water contained in a beaker (4% solution). The solution was heated for 15 minutes whilst stirring. The

dissolved agar solution was then poured into a rectangular plasticine container (size 8 x 3 x 3 cm) to a depth of 2.5 cm. The acrylic brain mould of the appropriate size (attached to a handle) was held 1 cm above the base of the container until the agar solidified. When the agar mould had solidified, the acrylic brain mould was gently removed from the block using a fine spatula to cut around the edge of the acrylic mould. The removal of the acrylic mould leaves an imprint of the ventral part of the brain, which acts as a support for the fresh brain and hence for the subsequent slicing procedure. The agar block was then fixed onto the brain slicer using warm liquid agar solution placed into a ring on the base of the slicer (Figure 20 pl25).

Slicing procedure

Dissection of the rat brain

Rats were decapitated by a guillotine and their brains were dissected according to McIlwain (1975). The skin above the skull was removed with scissors to expose the cranium and a cut made along the sagittal suture. The occipital bone was removed with bone forceps by a series of short nibbling cuts as far forward as the parieto-occipital suture. The parietal bones were removed by prising upwards until a break occurred along the sagittal suture of the cranium and the frontal bones were removed in a similar manner. By means of a spatula the brain was gently removed from the base of the cavity starting at the rostral end and the optic tracts and cranial nerves cut. The brain was then placed on the agar mould fixed on the base of the brain slicer.

Slicing

The blade was lined up with the midpoint of the pineal gland

and a three-quarter turn of the micro gauge lined the blade with the midpoint of the inferior colliculus. This was the zero reference point designated $F = 0$ (see Figure 16 pl26). Three slices (0.6 mm thick) were selected for subsequent neurotransmitter release studies. The slices were designated S9, S13 and S14, S9 being the corticolimbic slice and S13 and S14 being the corticostriatal slices (see Fig. 17, pl27; Fig 18, pl28; Fig 19, pl29). Slices were removed from the blade by a spatula moistened by Krebs' and then placed in a petri dish containing Krebs'. During this procedure the supporting agar detached itself.

	Reference in the present studies	No. of turns of the screw guage from the zero point $F = 0$	Massopust reference
S9	$F = 5.7$ mm	8 turns	$F = 4.5$ mm
S13	$F = 7.7$ mm	9 $7/8$ turns	$F = 6.5$ mm
S14	$F = 8.4$ mm	10 $3/4$ turns	$F = 7.2$ mm

Each slice was then cut along the midline to obtain two half sections, each half was weighed on a thin glass slide prior to release studies. Using this technique of slicing it was possible to obtain slices accurately from the desired region.

Preloading and superfusing brain slices

The ^3H -dopamine release studies were carried out on corticostriatal slices S13 and S14. Each slice was cut along the midline and designated Section 3 and Section 4 for the portions obtained from S13 and Section 5 and Section 6 for those portions obtained from S14. Throughout this work the term "section" refers to a brain slice cut along the midline to obtain

two halves. Prior to superfusion, slice sections were incubated in 5 ml of Krebs' bicarbonate saline containing cold dopamine 10^{-7}M , pargyline, (10^{-7}M), ^3H -dopamine (25 μCi) and ascorbic acid (0.1 mg/ml) at 37°C for 45 minutes, being constantly gassed with 95% O_2 /5% CO_2 .

After 45 minutes of incubation slice sections were transferred to four electrode chambers with a fine spatula and superfused for a further 40 minutes to remove excess label on the surface of the slices. Slice sections were then observed under different conditions.

3.3. Experimental procedures

3.3.1. Ca^{2+} ion dependency of release

The basal release of ^3H -dopamine in the absence and presence of Ca^{2+} ions in the superfusion media was studied. Subsequently the stimulated release of ^3H -dopamine in the absence and presence of Ca^{2+} in the superfusion media on first (S_1) and second (S_2) stimulations was observed.

3.3.2. The stimulated release of ^3H -dopamine in the absence (S_1) and in the presence (S_2) of trazodone (10^{-6}M) in the superfusion medium was studied.

3.3.3. The release of ^3H -dopamine in the absence (S_1) and presence (S_2) of haloperidol (10^{-7}M) in the superfusion medium was studied.

Slices were stimulated for a two minute period using square wave pulses of alternating polarity (2ms, 10Hz and 20mA). The voltage across each electrode was monitored with oscilloscopes.

Each minute a superfusate sample was collected and first counted for Tc^{99m} and then five days later for tritium after the addition to the scintillation vial of 5 ml of Micellar NE 240 scintillation cocktail. The radioactivity of the superfusate was determined by liquid scintillation counting using a liquid scintillation spectrometer.

At the end of the experiment each brain slice section was removed from the electrode chamber, homogenised in 1 ml of PCA 0.2N and 0.2 ml of the homogenate placed into a scintillation vial containing Micellar NE 240 scintillation cocktail and counted for tritium activity.

3.4. Methods for analysis and presentation of release data

Results of the preloaded ^3H dopamine release experiments are expressed as follows:

$$\frac{\text{cps released upon stimulation-basal release}}{\text{cps remaining in the tissue}} \times 100 = \% \text{ of total radioactivity released upon the stimulation}$$

The results are graphically presented as efflux curves plotted against time (abscissa).

One minute superfusate fractions were collected. The exact volume collected was estimated by counting Tc^{99m} in each vial. The volume of superfusate collected per minute was calculated from the known counts of 1 ml standard Tc^{99m} in the superfusion medium. A computer programme was devised in order to perform the calculations, taking into consideration the decay correction for each one minute sample collected over a 40 minute period for each slice section. After a 7 day period, samples were then counted for tritium. The computer programme (II) was then used to

calculate tritium counts per second per unit volume of superfusate (see appendix for computer programmes).

Each slice section (when stimulated) was stimulated twice. The first 2 minutes period of stimulation termed S_1 , and the second 2 minutes period of stimulation termed S_2 . The effect of a drug was always tested out on the second stimulation period. The results of S_1 and S_2 were then expressed as a ratio of S_2/S_1 .

Background values for the scintillation counter were estimated by counting 0.5 ml of Kreb's bicarbonate saline, this value was subsequently subtracted from all radioactivity readings.

The total tissue radioactivity at the onset of stimulation was estimated by calculating the total fractional radioactivity released and adding this to the remaining tissue activity in the slice at the end of the experiment.

Correction of superfusate volumes using Tc^{99m}

Figure 21 (p130) shows an example of data obtained from a slice for a release experiment before and after correction for volume. In the present studies it was found that the correction for volume was necessary due to the slightly differing flow rates from each electrode chamber and the presence of 'bubbles' in the outflow of the superfusion system which might cause variation in volume in each one minute sample collection (0.4 ml approx).

Methodological Results

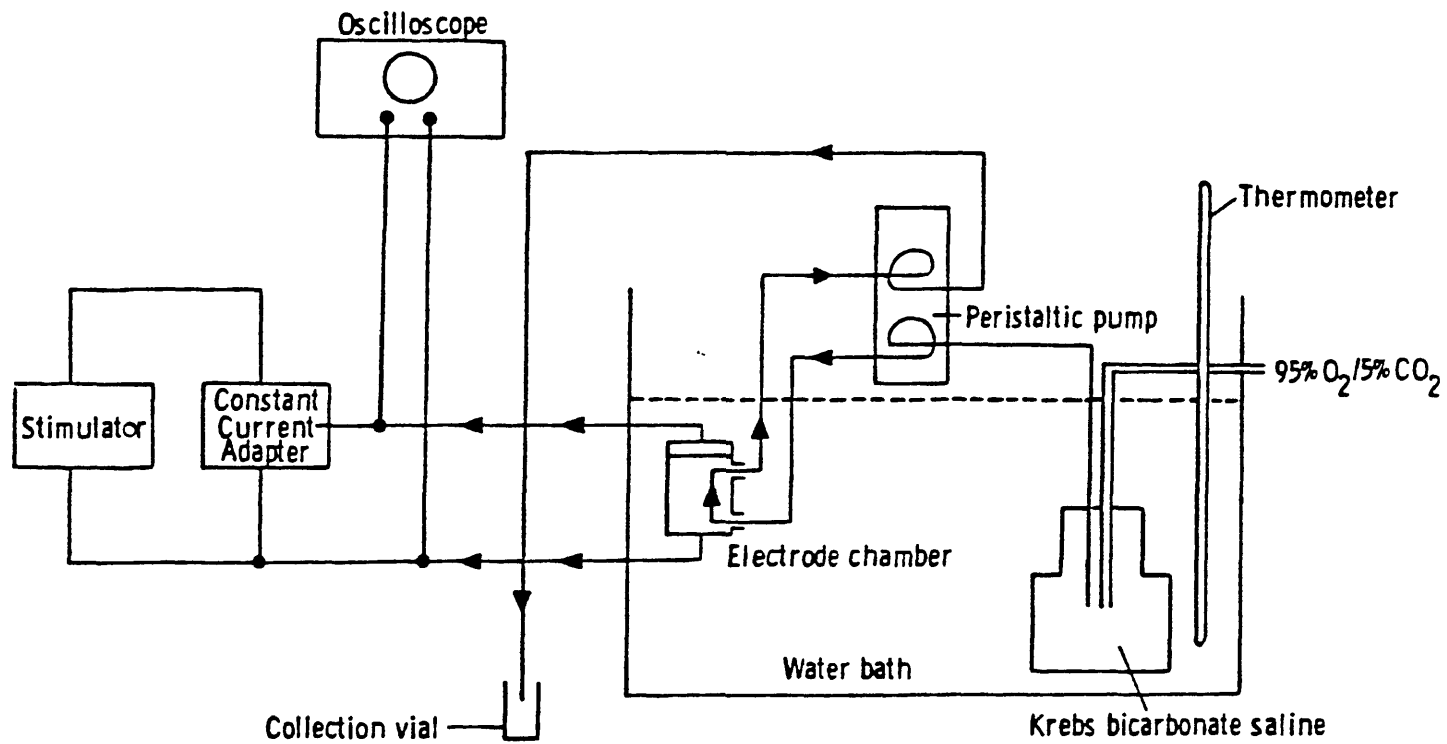


FIG 14: Diagrammatic representation of the superfusion apparatus.

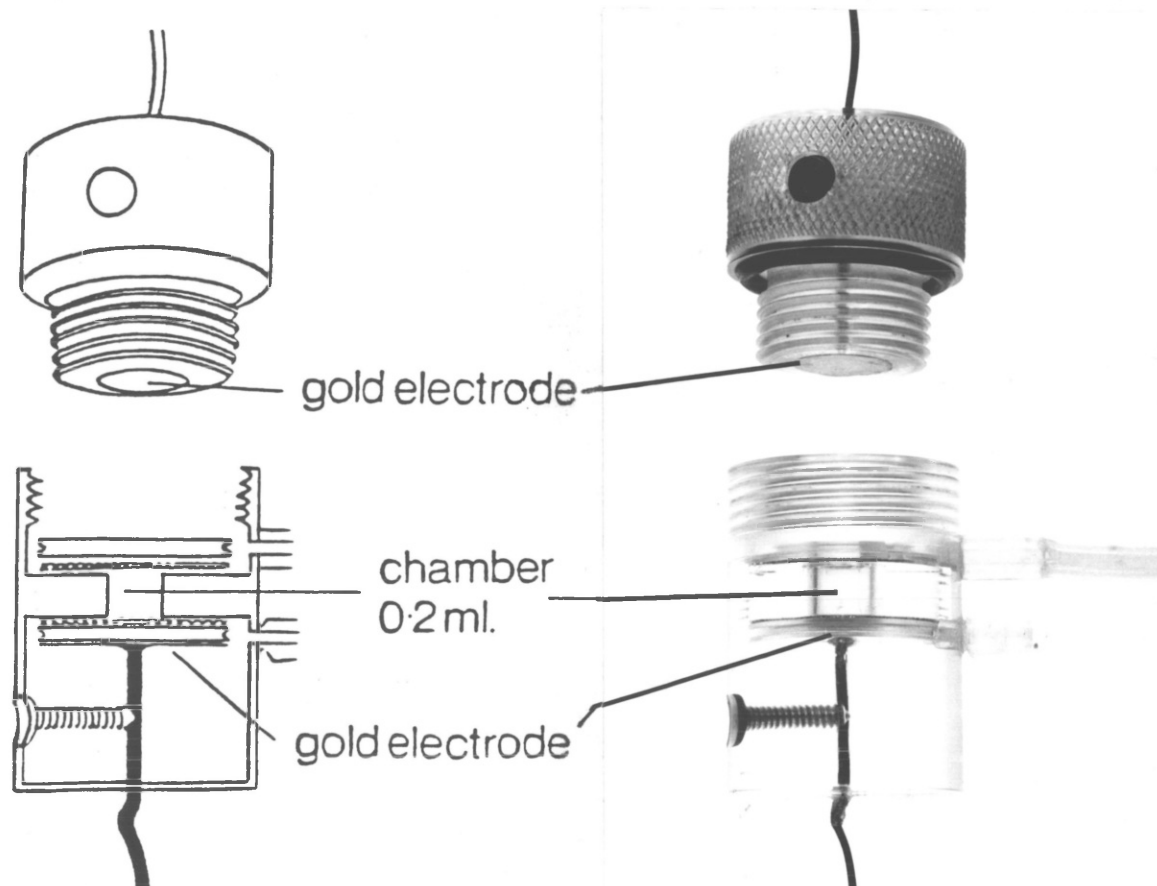


FIG 15: Gold electrode chamber.

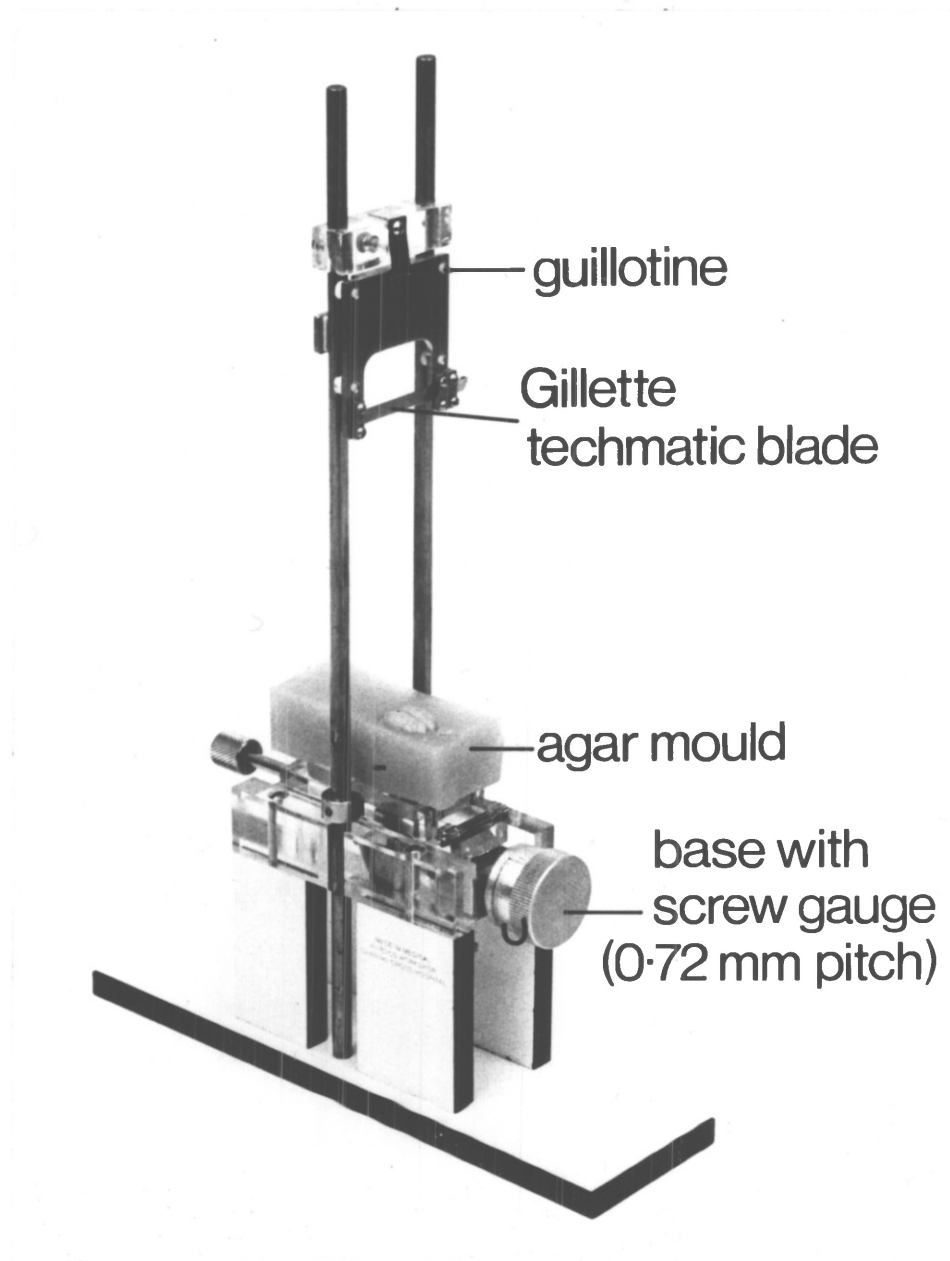
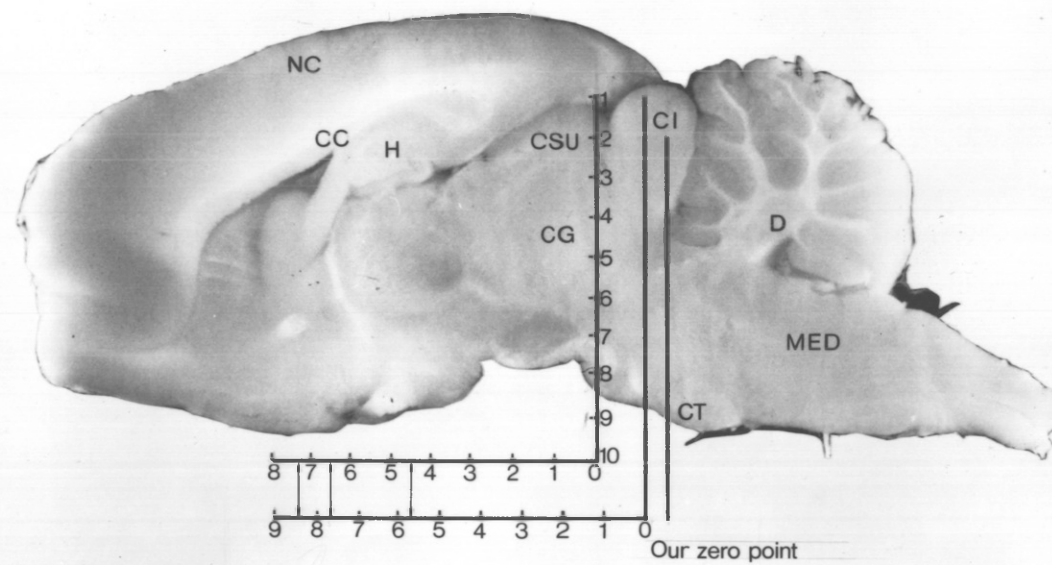


FIG 20: Brain slicer.

Fig 16 A PARASAGITTAL SECTION OF THE ENTIRE BRAIN
(RAT WEIGHT 200g) 1mm LATERAL TO THE MIDLINE



Key to abbreviations

AB	Basilar nucleus of the amygdala
AC	Central nucleus of the amygdala
AL	Lateral nucleus of the amygdala
AM	Medial nucleus of the amygdala
CAA	Anterior commissure, anterior division
CAT	Anterior commissure, temporal division
CAU	Caudate nucleus
CC	Corpus callosum
CF	Column of the fornix
CG	Central grey matter
CHO	Optic chiasm
CI	Inferior colliculus
CIN	Internal capsule
CLA	Clastrum
CN	Cingulum
CO	Cortical nucleus of the amygdala
CSU	Superior colliculus
CT	Trapezoid body
D	Dentate nucleus of cerebellum
DB	Diagonal band of Broca
DM	Dorsomedial hypothalamic nucleus
FBL	Medial forebrain bundle, lateral division (See FBM)
FBM	Medial forebrain bundle
FE	Endorhinal groove
FF	Fields of Forel
FRH	Rhinal fissure
H	Hippocampus
HL	Lateral habenular nucleus
M	Medial nuclear group of the thalamus
MED	Medulla
NAC	Nucleus accumbens of the septum
NC	Neocortex
NL	Reticular nucleus of the thalamus
PC	Precommissural spinal fibers
PYC	Pyramidal cortex
S	Septal region
TO	Olfactory tubercle
TOL	Lateral olfactory tract
V	Ventral nuclear group of the thalamus
VDA	Mammillothalamic tract
VL	Lateral ventricle
VM	Ventromedial hypothalamic nucleus

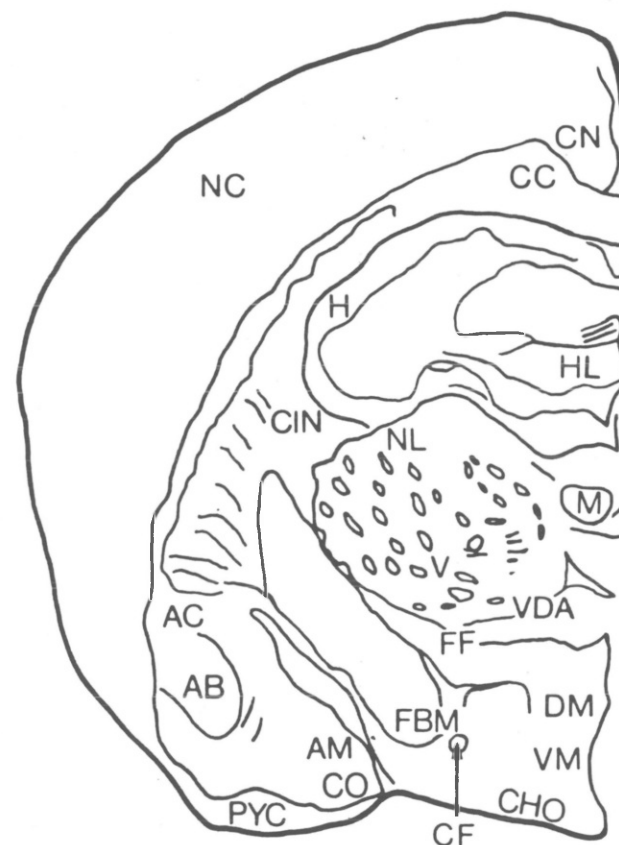
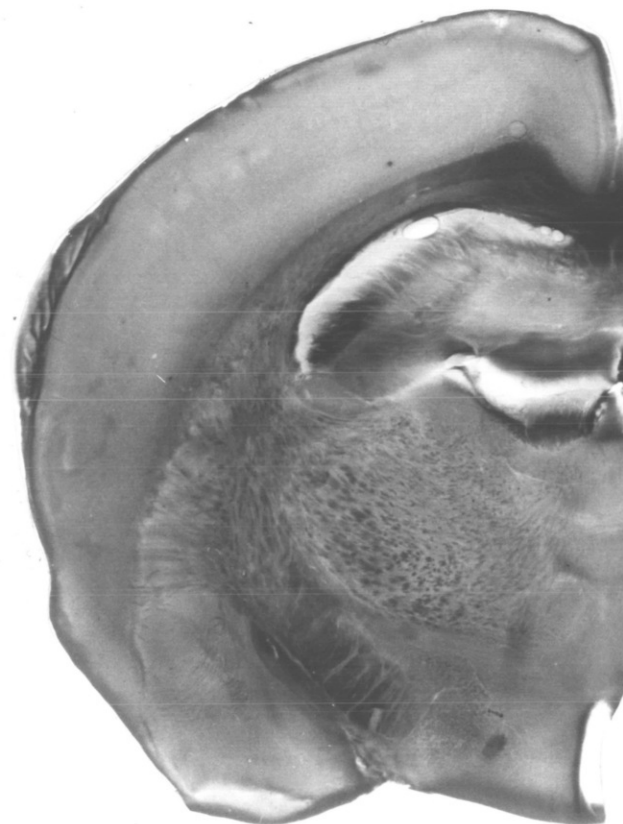


FIG: 17 RAT BRAIN SLICE (S 9)

Key to abbreviations

AB	Basilar nucleus of the amygdala
AC	Central nucleus of the amygdala
AL	Lateral nucleus of the amygdala
AM	Medial nucleus of the amygdala
CAA	Anterior commissure, anterior division
CAT	Anterior commissure, temporal division
CAU	Caudate nucleus
CC	Corpus callosum
CF	Column of the fornix
CG	Central grey matter
CHO	Optic chiasm
CI	Inferior colliculus
CIN	Internal capsule
CLA	Clastrum
CN	Cingulum
CO	Cortical nucleus of the amygdala
CSU	Superior colliculus
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FBL	Medial forebrain bundle, lateral division (See FBM)
FBM	Medial forebrain bundle
FE	Endorhinal groove
FF	Fields of Forel
FRH	Rhinal fissure
H	Hippocampus
HL	Lateral habenular nucleus
M	Medial nuclear group of the thalamus
MED	Medulla
NAC	Nucleus accumbens of the septum
NC	Neocortex
NL	Reticular nucleus of the thalamus
PC	Precommissural spinal fibers
PYC	Pyriform cortex
S	Septal region
TO	Olfactory tubercle
TOL	Lateral olfactory tract
V	Ventral nuclear group of the thalamus
VDA	Mammillothalamic tract
VL	Lateral ventricle
VM	Ventromedial hypothalamic nucleus

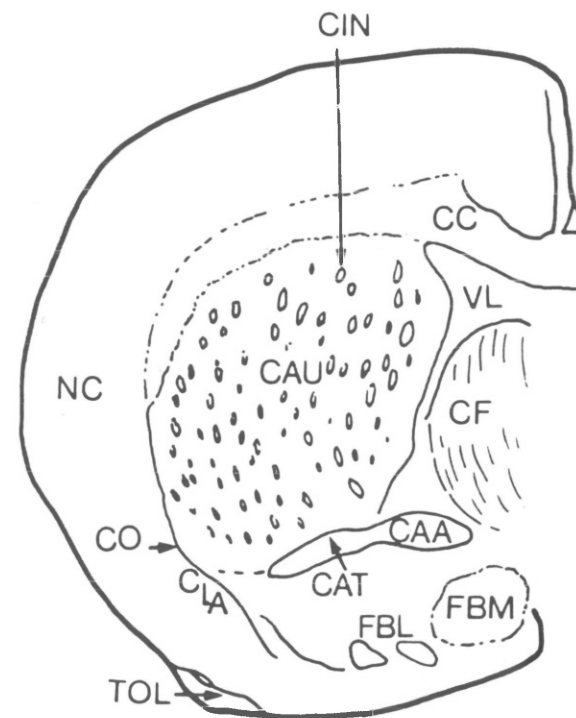
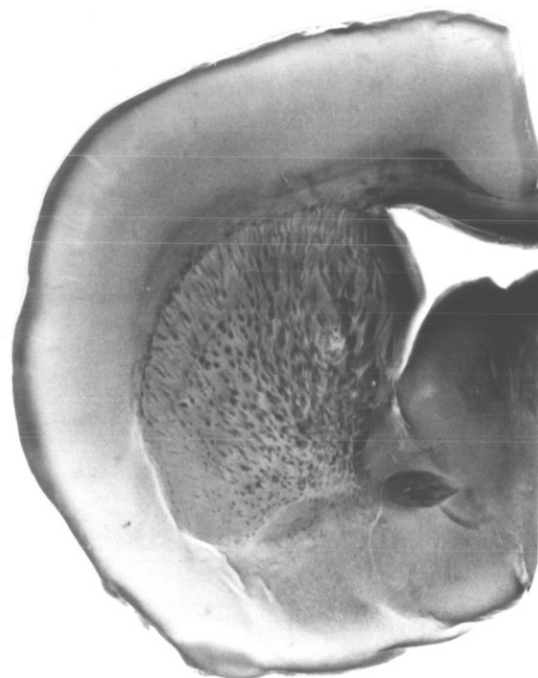


FIG:18 RAT BRAIN SLICE (S 13)

Key to abbreviations

AB	Basilar nucleus of the amygdala
AC	Central nucleus of the amygdala
AL	Lateral nucleus of the amygdala
AM	Medial nucleus of the amygdala
CAA	Anterior commissure, anterior division
CAT	Anterior commissure, temporal division
CAU	Caudate nucleus
CC	Corpus callosum
CF	Column of the fornix
CG	Central gray matter
CHO	Optic chiasm
CI	Inferior colliculus
CIN	Internal capsule
CLA	Clastrum
CN	Cingulum
CO	Cortical nucleus of the amygdala
CSU	Superior colliculus
CT	Trapezoid body
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FBM	Medial forebrain bundle
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PC	Precommissural spinal fibers
PYC	Pyriform cortex
S	Septal region
TO	Olfactory tubercle
TOL	Lateral olfactory tract
V	Ventral nuclear group of the thalamus
VDA	Mammillothalamic tract
VL	Lateral ventricle
VM	Ventromedial hypothalamic nucleus

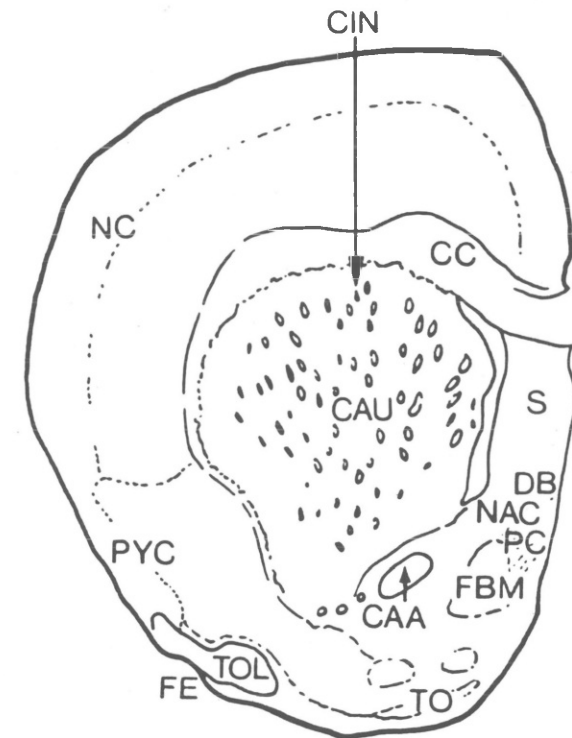
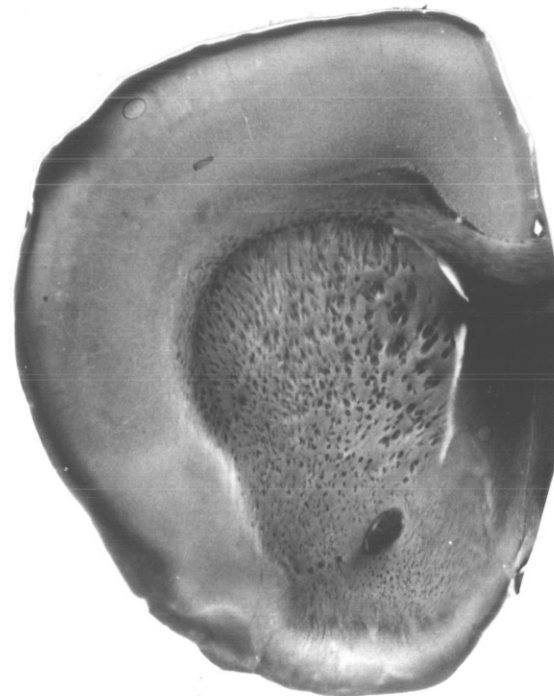


FIG 19 RAT BRAIN SLICE (S 14)

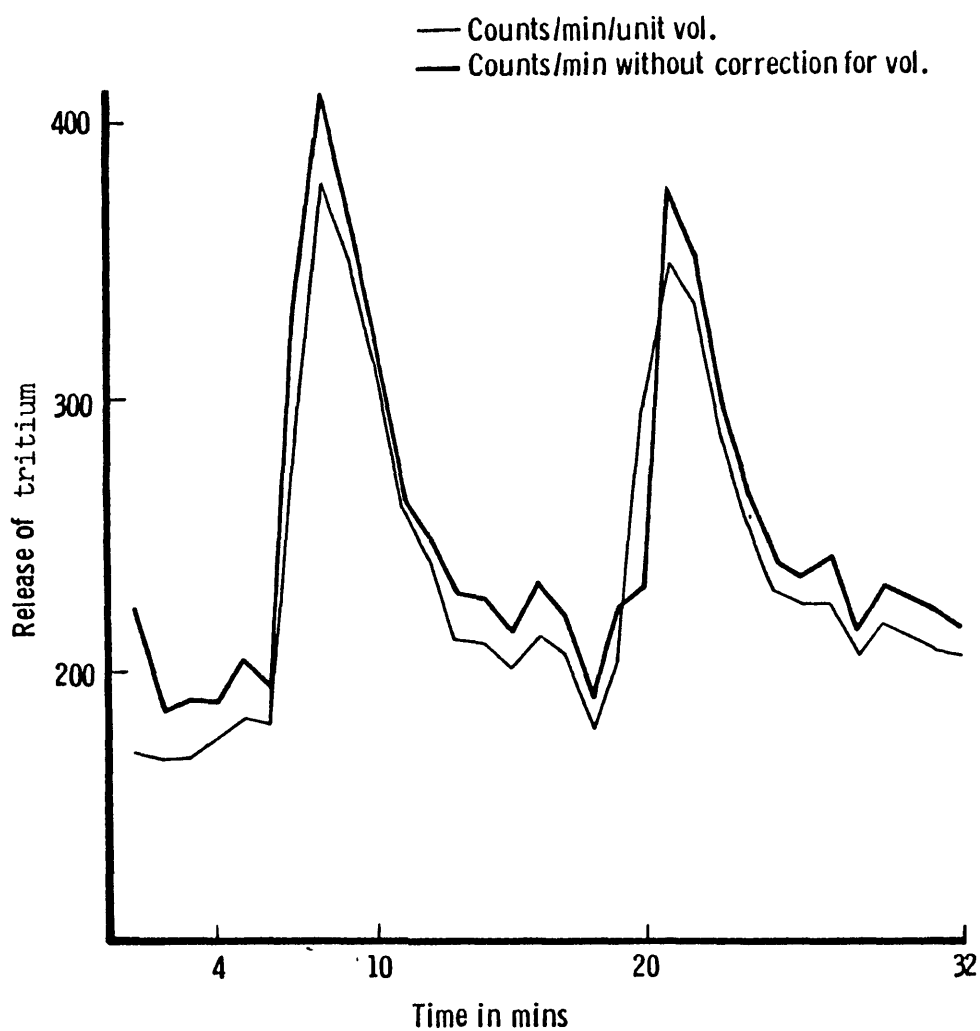


FIG 21: Graph demonstrating release data before and after correction for volume using Tc^{99m} in the superfusion medium.

3.5. Results

3.5.1. Ca²⁺ ion dependency of release

Figure 22 (pl33) illustrates the Ca²⁺ ion dependency of ³H-dopamine release. In the absence of Ca²⁺ in the superfusion media on electrical stimulation there was no release of ³H-dopamine above the basal. Release of tritium in the absence of Ca²⁺ was found to be of the order of 0.001% (n = 9). Figure 23 (pl34) shows the release of preloaded ³H-DA from corticostriatal slices upon electrical stimulation in the presence of Ca²⁺ ions, in the superfusate. The release is plotted as counts/second on a unit volume basis calculated from Tc^{99m} data.

Table 6 (pl35) shows that upon electrical stimulation the release on first (S₁) and second (S₂) stimulations is significantly greater than the basal release figures. Furthermore under control conditions, there is no significant difference between the first and second electrical stimulations.

n = 12.

Thus, subsequent experiments to test the effect of drugs on stimulated release were conducted by addition of the drug to the superfusing medium just prior to S₂.

3.5.2. The effect of trazodone 10⁻⁶M on ³H-dopamine release

Table 6 shows the results of the effect of trazodone on the S₂/S₁ ratios. Trazodone was superfused over the slices prior to the second stimulation. The data shows that there is a significant difference (p<0.001) between the first and second stimulations. The ratios of S₂/S₁ in the presence of normal Krebs' and in the presence of trazodone were also significantly different (p<0.001). Figure 24 (pl36) shows the effect of

trazodone on electrically stimulated release of ^3H -dopamine.

3.5.3. The effect of haloperidol (10^{-7}M) on ^3H -dopamine release

Table 6 shows the preliminary data for the influence of haloperidol on ^3H -DA release. Initial results show that there is a decrease in the release of tritium in the presence of haloperidol. These results are consistent with those obtained by Seeman & Lee (1975). However, no final conclusion can be drawn from these data until further experiments are conducted.

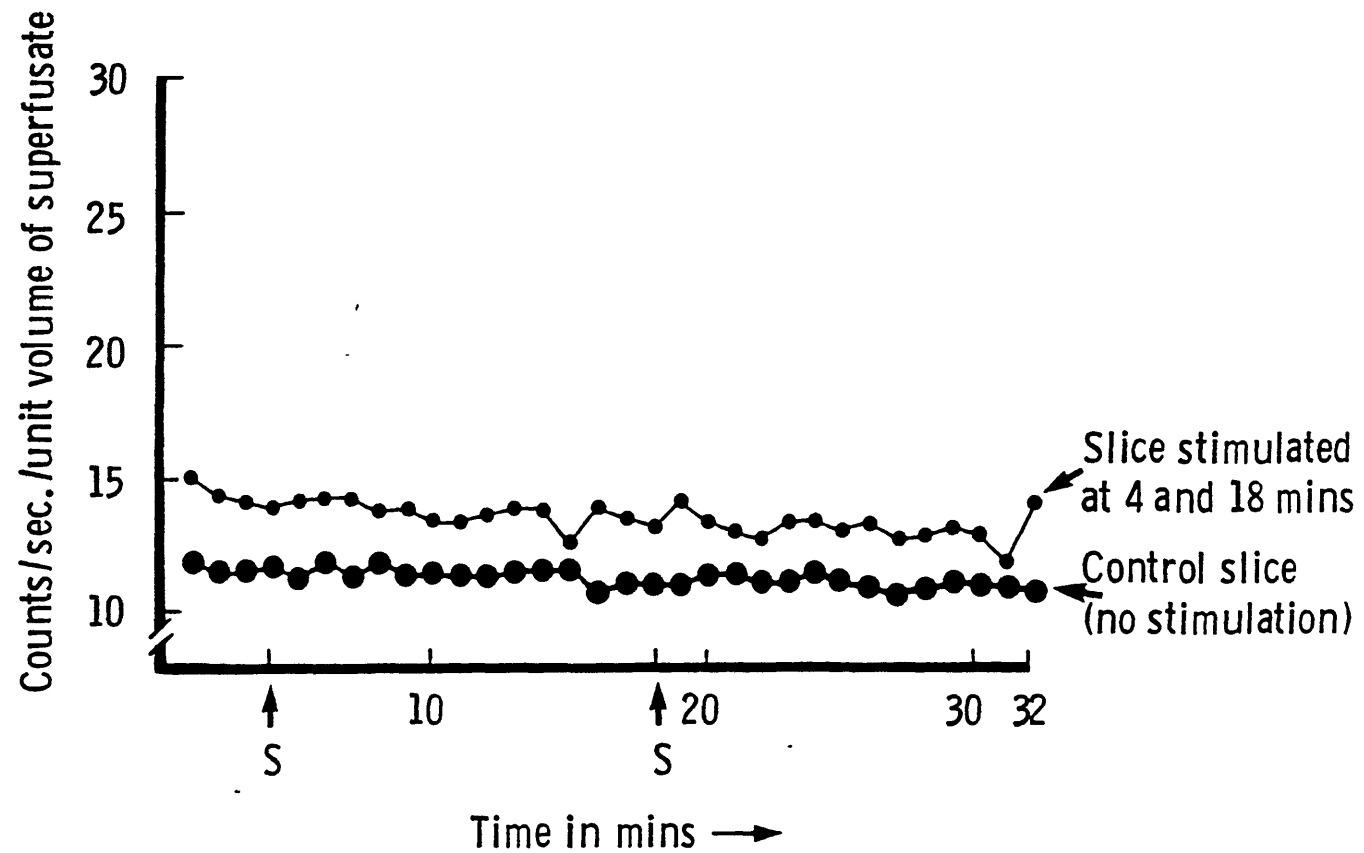


FIG 22: Rat brain corticostriatal slices preloaded with ^3H -dopamine and superfused with Ca^{2+} free Krebs' bicarbonate saline.

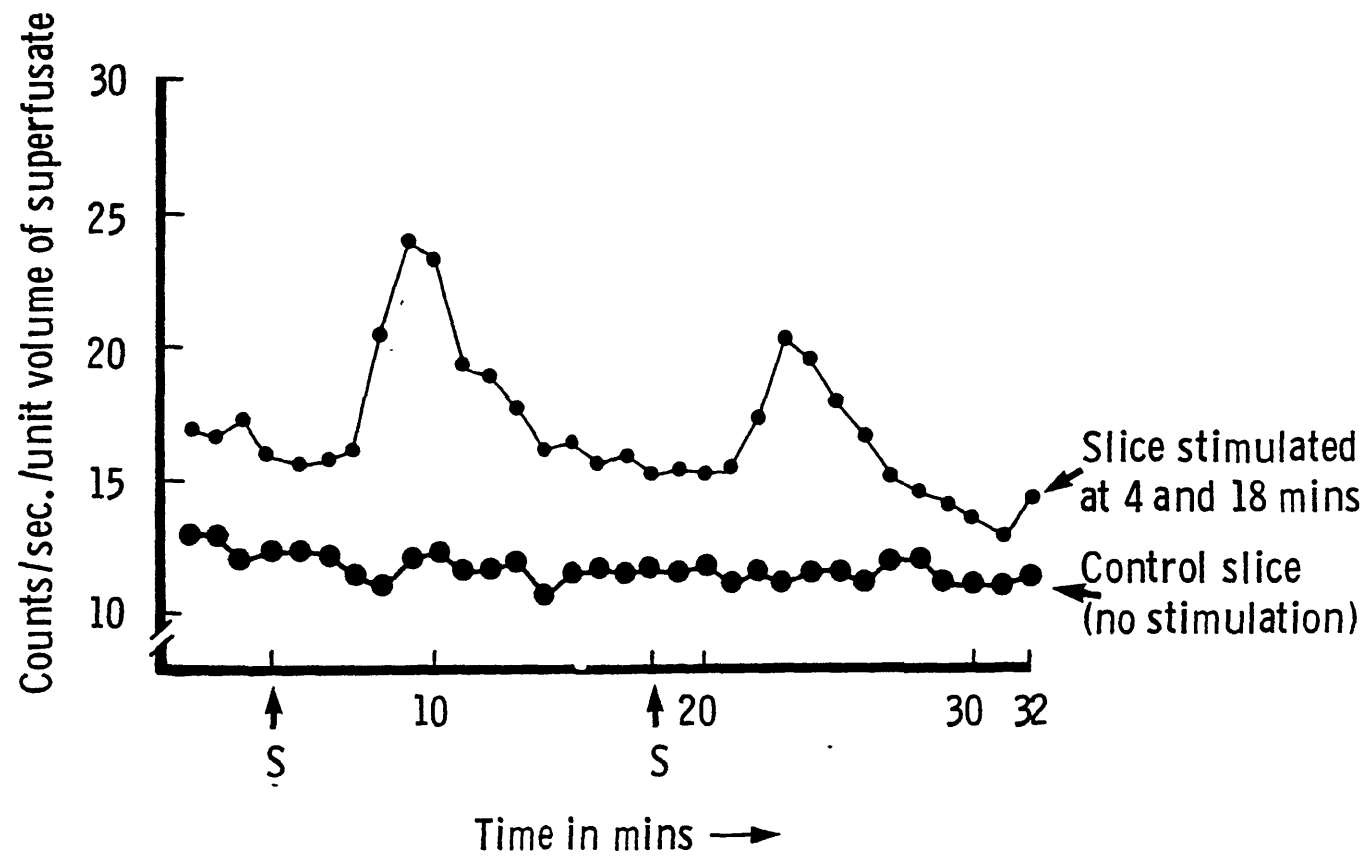


FIG 23: Rat brain corticostriatal slices preloaded with ^3H -dopamine and superfused with Kreb's bicarbonate saline containing Ca^{2+} .

TABLE 6

Release of ^3H -dopamine on electrical stimulation in the presence of calcium ions, trazodone and haloperidol

S_1 = the percentage radioactivity released at the first two minutes period of stimulation (10Hz, 2 ms, 20 MA). This figure was calculated with respect to total tissue radioactivity at the onset of stimulation.

S_2 = the percentage radioactivity released at the second two minutes period of stimulation. This figure was also calculated with respect to total tissue radioactivity at the onset of stimulation.

Basal release figures are in the order of 0.0014 ± 0.0005 ($n = 15$)

CONDITION	S_1	S_2	S_2/S_1
Slices superfused with normal Krebs'	n = 12 x = 1.446 SEM = 0.19	n = 12 x = 1.271 SEM = 0.18	n = 12 x = 0.94 SEM = 0.08
Slices superfused with normal Krebs' containing trazodone	n = 6 x = 0.724 SEM = 0.17	n = 6 x = 5.239 SEM = 1.38	n = 6 x = 5.388 SEM = 1.02
Slices superfused with normal Krebs' containing haloperidol	n = 3 x = 0.559	n = 2 x = 0.35	n = 2 x = 0.47

Student t - test shows that there is a highly significant difference $p < 0.001$ between the ratios of S_2/S_1 in the presence of normal Krebs' and the ratios of S_2/S_1 in the presence of Krebs' containing trazodone.

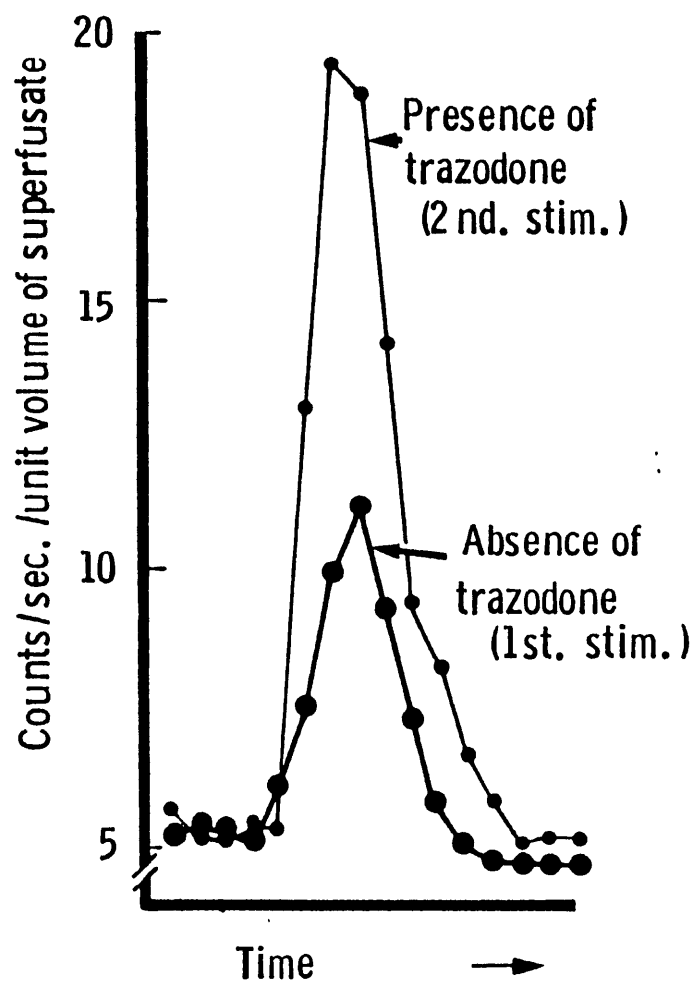


FIG 24: Rat brain corticostriatal slice, preloaded with ^3H -dopamine and superfused with Krebs' bicarbonate saline with and without trazodone (10^{-6}M).

3.6. Discussion

Electrical stimulation greatly enhances the "overflow" of radioactivity from brain slices preincubated with radioactively labelled monoamines (Baldessarini & Kopin, 1967; Farnebo & Hamberger, 1971). The stimulation-induced overflow is Ca^{2+} dependent and sensitive to tetrodotoxin, which blocks propagation of nerve impulses (Farnebo, 1971; Kao, 1966). There is therefore reason to believe that the stimulation-evoked release is similar to that occurring under physiological conditions. This technique has been used to study the mode of action of psychoactive drugs.

The aim of the ^3H -DA release experiment was to establish a methodology for the release of endogenous amines and to evaluate the effects of trazodone and haloperidol on the release of radiolabel from slices in the system. The results from these experiments depend on the variability of each constituent of the system namely:-

- (a) the slice
- (b) an effective electrical stimulus
- (c) superfusate volume.

A slice thickness of 0.6 mm was chosen for these experiments. Reproducible intact slices were obtained which could be cut into half sections, weighed and subsequently stimulated. Damaged slice sections characteristically showed massive efflux of radiolabel and an irregular baseline value. Slice S9 from the corticolimbic area of the brain, was the most difficult to cut due to the ventricular spaces in the brain, and

0.6 mm thickness was the minimum with which it was possible to work, so for consistency this thickness was also chosen for corticostriatal slice studies.

Evidence provided by Cohen (1974) showed that cut slices had 0.17 mm of damaged tissue; slices 0.35 mm or more thick would have an inner core of undamaged tissue, which was biochemically and electrophysiologically viable.

A major problem encountered in electrical stimulation of slices were small "bubbles" which accumulated in the electrode chamber and prevented the passage of the current. Various attempts were made to eliminate these (T pieces in the perfusion tubing, effective temperature control, miniaturised sintered glass aerators), none of these totally eliminated the problem. Indeed the miniaturised glass sintered aerators exacerbated the situation! Finally the chambers were connected to an oscilloscope which monitored the voltage across each one and showed whether the slice was effectively stimulated. Those slices not stimulated were abandoned.

The superfusate volume was monitored using TC^{99m} as slight differences in the flow rate and small bubbles in the outflow might contribute to variations in the volume. All the preloaded radiolabelled amine release data was corrected to unit volume.

In the study of basal release and stimulated release in the presence and absence of Ca^{2+} , it was clearly established that release was calcium ion dependent (Patel & Turnbull, 1982b). This is one criterion that, this is vesicular release, and not non-specific release from other stores. Furthermore if this is the case, it is more than possible that the preloaded radiolabelled dopamine is exchanging with endogenous vesicular dopamine.

It is possible that the release of preloaded radiolabel is

dependent on the frequency of stimulation. The stimulation parameters chosen in these ^3H -DA release studies were 10 Hz, 2 ms and 20 mA. It was suggested by Rand, Story, Allen, Glover & McCulloch (1973) that an increase in frequency (0.1-1.6 Hz) produced a decrease in release per pulse.

It is possible that release of ^3H -DA in the presence of haloperidol (10^{-7}M) is not enhanced in these present experiments because of the high frequency of stimulation used. However, experiments carried out by Farnebo & Hamberger (1971), who also used high frequencies of stimulation, found that they had an enhanced release in the presence of haloperidol. If frequency dependence was an important factor in the modulation of release, it is difficult to explain why the release of ^3H -DA in the presence of trazodone is greatly enhanced. It would appear that frequency of stimulation is not the principal factor controlling release.

Further confusing evidence was reported by Kalsner (1980) and Arbilla, Langer & Lehman (1981). Kalsner's studies on the release of ^3H -DA from guinea-pig vas deferens suggest that release of ^3H -DA is not frequency dependent (p60 of the thesis). However, as mentioned previously, Arbilla et al (1981) found that the release of ^3H -DA from cat caudate slices in the presence of the neuroleptic sulpiride was less effectively facilitated at 1 Hz than at 3 Hz because at the higher frequency there would be more dopamine released.

Rand et al (1973) also found that phenoxybenzamine did not increase the efflux of tritium after single pulse administration in the guinea-pig atria (p59 of the thesis).

To what extent frequency plays a role in the modulation of ^3H -DA release in these slice studies is under review. Currently experiments investigating the role of varying frequency in

relation to release are under test.

The other possible explanation of the result may relate to the observation by Starke, Reimann, Zumstein & Hertting (1978) that haloperidol is retained in the silicone tubing used in the superfusion apparatus. Starke et al (1978) found that silicone tubing retained haloperidol and interfered with subsequent experiments. Using ^3H -haloperidol they verified the contamination of silicone tubings. They perfused silicone tubing with ^3H -haloperidol, washed them subsequently for 18 hours and identified the radioactive contaminant as haloperidol by thin layer chromatography. The authors suggest that part of the discrepancies in the literature concerning dopaminergic modulation of DA release may be due to the retention in superfusion tubing of neuroleptic drugs which are subsequently extracted by slow elution and then interfere with subsequent experiments. The authors showed that this effect does not occur with Vitron tubing. They observed that when silicone tubes had been previously exposed to haloperidol the S_2/S_1 for ^3H -DA release from caudate slices in the presence of apomorphine was 0.96 ± 0.06 , whereas if the experiment was repeated with tubing exposed only to haloperidol solvent the S_2/S_1 ratio was 0.24 ± 0.04 . Thus haloperidol retained in the tubing prevented the observation of the effect of apomorphine on the release of ^3H -DA.

The results showed that trazodone enhanced the stimulated release of preloaded ^3H -DA in corticostriatal slice sections. Stefanini, Fadda, Porceddu & Gessa (1976) showed that trazodone is capable of increasing DA turnover possibly by increasing DA synthesis. At dose levels of 25-100 mg/kg given i.p. the concentration of both DOPAC and HVA were increased without affecting the concentration of dopamine in the brain (i.e.

increased turnover). Thus, in vitro studies showed that trazodone affects dopamine synthesis and turnover. In vitro studies also showed that trazodone enhances the release of dopamine on electrical stimulation of slices preloaded with ^3H -DA (Patel & Turnbull, 1982a). These results may be explained by the presynaptic dopamine receptor blockade theory. This would account for the highly significant release of tritium by trazodone.

Explanations for the influence of antipsychotics on the release of tritiated dopamine

There are basically two types of theory explaining neuroleptic drug actions.

These are the "dopamine receptor blockade" and the "coupling blockade" hypothesis. Carlsson & Lindqvist (1963) observed that antipsychotics such as chlorpromazine and haloperidol increase the turnover of dopamine in brain tissue.

Their experiments were carried out on mice; haloperidol or chlorpromazine given one hour after an injection of nialamide to inhibit monoamine oxidase activity, were shown to enhance the accumulation of the O-methylated metabolites normetamphetamine and 3-methoxytyramine, without influencing the total levels of the catecholamines themselves. The authors suggested that a possible mechanism for chlorpromazine and haloperidol was the blockade of monoaminergic receptors in the brain, resulting in the compensatory activation of monoaminergic neurons (i.e. increased firing). This activation would then lead to an increased synthesis and release of monoamines and as a consequence to increased formation of monoamine metabolites. Later, Bunney, Walters, Roth & Aghajanian (1973) showed that the increased firing rate of DA cells in the substantia nigra was reversed by

the dopamine agonist amphetamine. Other workers showed that amphetamine given alone decreased the firing rate of dopaminergic cells and this is consistent with the evidence that amphetamine promotes presynaptic release and blocks reuptake of DA (Carlsson, Fuxe, Hamberger, Lindqvist, 1966). By causing a postsynaptic accumulation of dopamine at receptor sites, a feedback inhibition of dopamine neurons might occur.

Apomorphine, a DA agonist, decreases the stimulated release of ^3H -DA and this effect may also be explained by receptor blockade hypothesis (Starke, Reimann, Zumstein & Hertting, 1978).

This theory however, does not take into account the fact that there is no direct quantitative relationship between any of these neuroleptic actions and their antipsychotic potencies in the clinic (Janssen, Neimegeers & Schellekens, 1965). The butyrophenones, haloperidol and spiroperidol are 20-100 times more potent clinically than chlorpromazine (CPZ), yet are equal to or weaker than CPZ in blocking dopamine sensitive adenylate cyclase (Kebabian, Petzold & Greengard, 1972).

Seeman & Lee (1975), in their coupling-blockade hypothesis of neuroleptic action, provided evidence showing a correlation between the clinical potency of neuroleptics and their ability to inhibit the stimulus-induced release of dopamine in rat striatal slices.

The coupling-blockade hypothesis of neuroleptic action recognises that neuroleptics are fat soluble and surface active drugs which readily accumulate in cell membranes (Seeman & Baily, 1963). This would lead to membrane expansion and hence the blocking of nerve membrane impulses (Courvoisier et al, 1953).

The above effect would displace the membrane associated Ca^{2+} and enhance the spontaneous release of transmitter and modulate the coupling between impulses and neurosecretion (Seeman, 1972;

Seeman & Lee, 1974; Quastel, Hackett & Okamoto, 1972).

Seeman & Lee (1974) studied the electrically stimulated release of ^3H -DA on rat neostriatal slices using a modified method of Farnebo & Hamberger (1971). Their results showed that neuroleptic inhibition of the stimulated release of ^3H -dopamine, directly correlated with their clinical antipsychotic effect. They concluded that the possible mechanisms underlying neuroleptic inhibition of impulse-triggered release of ^3H -DA may be threefold.

1. The neuroleptics might be inhibiting nerve impulse conduction. Small diameter fibres are particularly vulnerable to the blocking actions of neuroleptics (Stalman & Seeman, 1974), especially because dopaminergic neurons are small 0.2 μm (Soltelo, 1971). The threshold concentration of neuroleptic at which the ^3H -DA release inhibition commenced was the same concentration as would be found in the plasma of patients being retreated with neuroleptics (Seeman, Stalman & Chau-Wong, 1974).

Since anaesthetics actually increase the firing rate of nigral dopamine neurons (Bunney et al, 1973), impulse inhibition by neuroleptics, if it exists in striatal tissue, could occur in the small presynaptic dopaminergic neuron terminals; the neuroleptic and anaesthetic-induced increase in nigral neuron firing rate might, in fact, arise by neuronal feedback as a consequence of presynaptic depression by neuroleptics.

2. Neuroleptics may be blocking presynaptic receptors to block negative feedback mechanisms but this would cause an increase in DA release as shown by Farnebo & Hamberger (1971). The concentration of the drug which blocks presynaptic receptors however, does not correlate with the clinical potency.

3. The neuroleptics may be inhibiting the coupling between the impulse and the neurosecretion of DA by interfering with the Ca^{2+} influx in presynaptic nerve terminals (Seeman, 1972; Quastel et al, 1972).

Numerous papers have been published supporting both the receptor blockade and coupling blockade theory of neuroleptic action, but whatever the mechanism for neuroleptic inhibition of stimulated ^3H -dopamine release, a presynaptic site of neuroleptic action can explain many of the neuroleptic effects in the brain.

Seeman & Lee (1974) explain the inhibition of stimulated ^3H -DA release by the following sequence of events:

- i) The neuroleptic may first partially inhibit impulse-secretion coupling in the dopamine preterminal.
- ii) Less DA released by impulse would disinhibit tyrosine hydroxylase via presynaptic receptors whilst eliciting neuronal feedback activation of nigral neuron firing.
- iii) The firing increase would activate tyrosine hydroxylase (Zivkovic & Costa, 1974) and more dopamine per quantum would be released even though not every impulse may be reaching the final terminal. The net effect of all these actions together with the small but significant neuroleptic enhancement of spontaneous DA release, may lead to increased DA turnover (Anden, Corrodi, Fuxe & Ungerstedt, 1971; Møller-Nielsen, Fjalland, Pederson & Nymark, 1974).

The results of the present studies showing the effect of haloperidol on ^3H -DA release may be explained by the above series of events of the coupling receptor blockade theory.

It can be concluded that there is no precise explanation for the mode of action of neuroleptics. The receptor-blockade and the coupling blockade theory, both supported by numerous publications, try to provide a possible mechanism.

SECTION 4: STUDY OF ENDOGENOUS NEUROTRANSMITTER RELEASE

4.1. Introduction

One criterion to be satisfied by putative neurotransmitters is that they are released in response to (electrical) stimulation. Most release studies involve the use of a previously taken up isotopically labelled compound (Baldessarini & Kopin, 1966) because of the difficulties in measuring very small absolute amounts of endogenous transmitter released.

Although a great deal of data has been generated using pre-labelling procedures, it is not known to what extent the release of endogenous amine parallels that of the pre-labelling procedures. It is possible that pre-labelling may be non-specific. Radiolabelled amine may be taken up by neurons which do not normally use the amine as a transmitter, or may not be evenly distributed within the releasable endogenous pool. The metabolic transformation of the amine may result in release of radiolabelled metabolites, not the transmitter itself. Measurement of the release of endogenous amines by sensitive techniques such as high performance liquid chromatography (HPLC) eliminates these potential problems and allows the simultaneous estimation of noradrenaline, dopamine and DOPAC.

Endogenous noradrenaline and dopamine release studies have been carried out using potassium chloride and d-amphetamine stimulation (Kant & Meyernoff, 1978), The endogenous amines released being measured by radioenzymatic assay. As already discussed, there are a number of difficulties which arise when comparing release data using electrical and KCl stimulation. However, these authors report a significant increase of dopamine and noradrenaline released in several brain regions examined,

also the release figure in ng/g of tissue were significantly greater than those reported in this thesis. An explanation may lie in the different mechanisms of release that may be involved using the two different modes of stimulation and in the slice preparations employed.

In this thesis the endogenously released NA, DA and DOPAC were assayed using high performance liquid chromatography (p152).

4.2. Principles of catecholamine assay

Both spectrophotofluorometric assay techniques and radioenzymatic assay were tried to estimate low levels (pg amounts) of standard amines. Both techniques proved to be unsuitable for the estimation of pg amounts of amines and both techniques will be critically evaluated briefly. HPLC proved to be the most suitable technique for the estimation of pg amounts of DA, NA and DOPAC, and was used to estimate these substances in both superfusate and tissue samples.

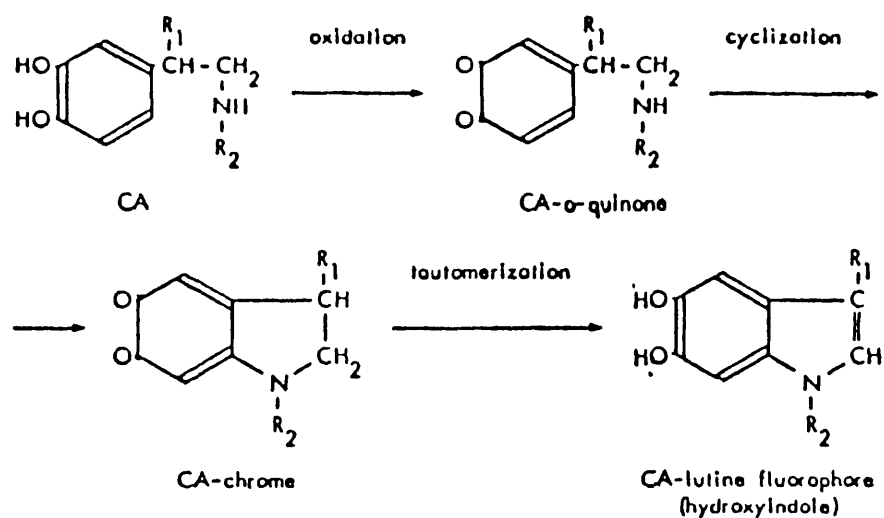
4.2.1. Spectrophotofluorometric assay

The estimation of catecholamines was based on the production of a fluorescent derivative by means of oxidation and subsequent chemical rearrangement. The two most widely used chemical procedures for estimation of catecholamines involve conversion of catecholamines to fluorescent products either by oxidation using the hydroxyindole reaction (Carlsson & Waldeck, 1958) or by condensation of oxidised catecholamines with ethylenediamine dihydrochloride so called EDD reaction (Anden, Roos, & Werdinius, 1963).

The purification and concentration of catecholamines is achieved by column separation techniques using Dowex 50 and

alumina. (Udenfriend, 1969; Sharman, 1971; Weil-Malherbe, 1971; Anton & Sayre, 1962). Once separated, fluorometric determination of individual fluorescent products can be carried out. The trihydroxyindole reaction for adrenaline and noradrenaline (Valori, Brunori, Renzini & Corea, 1970), was used in this work to estimate noradrenaline level in standard solutions.

The principle of the hydroxyindole method involves the oxidation of the catecholamines first to the corresponding chromes and then the rearrangement of fluorescent lutines in a strongly alkaline medium. Figure 25 shows the conversion of the catecholamines to their respective hydroxyindole fluorophores. The figure is a very simplified representation of the chemistry of the reaction which has been a focus of numerous investigations. The reaction is typical of a type which occurs when ferricyanide is used as oxidant. Hydroxyindole assays as a group are specific for compounds having a 2-(4-hydroxyphenyl) ethylamine nucleus and therefore a large number of compounds with certain structural similarities can be converted to fluorescent hydroxyindoles. There are however, a number of variables in the hydroxyindole assay schedule such as oxidation, pH, use of a catalyst, type of oxidant, time allowed for oxidation, types of reducing/ stabilising agents mixed with sodium hydroxide, and their concentrations, time allowed for tautomerisation, the pH at which fluorescence is recorded, the time required, all of which influence maximal development of fluorescence at the optimal wavelengths (Lavery & Taylor, 1965).



for A: $R_1 = OH$, $R_2 = CH_3$, fluorophore = 1-methyl-3,5,6-trihydroxyindole
 for NA: $R_1 = OH$, $R_2 = H$, fluorophore = 3,5,6-trihydroxyindole
 for DA: $R_1 = H$, $R_2 = H$, fluorophore = 5,6-dihydroxyindole

FIG 25: Conversion of the catecholamines to their respective hydroxyindole fluorophores.

The fluorescence of the reaction product in this reaction needs to be stabilised by the addition of an antioxidant. The antioxidant used normally and introduced by Lund (1949) was ascorbic acid. However, ascorbic acid was found to give rise to high blanks when estimating small amounts of catecholamines. This is because in alkaline solutions ascorbic acid gives rise to fluorescent oxidation products which cause the high blank readings. Valori et al (1970) found that a freshly prepared solution of 0.5% 2,3-dimercaptopropanol (BAL) in a sodium sulphite solution containing 5 gm $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ in 10 ml water gave a very satisfactory result, the lutine fluorescence was found to be as high as with ascorbic acid but the blank values were smaller and more stable. However, this preparation had drawbacks in that clear solutions of BAL could not be obtained, resulting in differing quenching and variable blank values. Finally, the authors found 10% BAL in a 25% formaldehyde solution in the absence of other antioxidants to be a suitable solution, as formaldehyde increased the solubility of BAL in water.

The method of Valori, et al (1970) was used to measure noradrenaline levels in standards. The method was found to be sensitive in the ng range.

Due to the lack of sensitivity over the range required for the estimation of monoamine release from discrete brain regions the spectrophotofluorometric assay was not used in the present work.

4.2.2. Radioenzymatic assay

Several radioenzymatic methods for the assay of catecholamines have been described in recent years. Before the advent of high performance liquid chromatography, radioenzymatic assay techniques were most popular since they allow the

measurement of catecholamines in tissue and body fluids with high accuracy and sensitivity as compared with the conventional fluorometric methods. The assay employs the 3-O-methylation of monoamines by means of radiolabelled S-adenosyl methionine in the presence of the enzyme catechol-O-methyl transferase (COMT). Engleman, Portnoy & Lovenberg (1968) and Nikodijevik, Daly & Creveling (1969) reported the first attempt to use COMT for the determination of catecholamines from plasma and tissue (heart) respectively. This approach was further developed by Coyle & Henry (1973); Cuello, Hiley & Iversen (1973), to measure catecholamines from tissue.

Principle of the assay

For dopamine, noradrenaline and adrenaline the derivatives which result from the O-methylation by COMT are 3-methoxytyramine, normetanephrine and metanephrine respectively. The enzymatic reaction which proceeds for catecholamines is shown in Figure 26.

The O-methylated derivatives from standards and biological samples obtained from the enzymatic incubation are extracted, separated and the radioactivity counted by liquid scintillation spectrometry. The initial amount of the catecholamines present as substrates in the unknown samples is inferred from the radioactivity obtained for the corresponding O-methylated product.

The radioenzymatic assay was tried as a technique to estimate catecholamines and DOPAC. The assay technique used was that of Saller & Zigmond (1978).

Several difficulties were encountered in trying to make the assay operable for the purpose of this thesis.

The difficulties were especially due to the need to assay

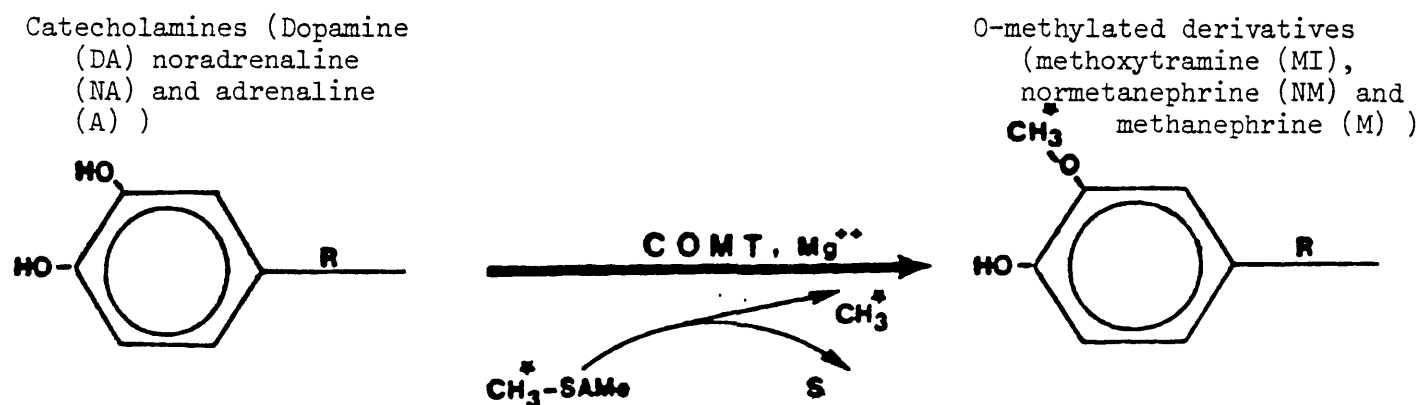


FIG 26: Principle of the radioenzymatic reaction. Conversion of catechol to o-methylated derivatives. CH_3^* , radiolabelled methyl group (^3H); S-AMe, s-adenosyl methionine; COMT; catechol O-methyltransferase; R is $-\text{CH}_2-\text{CH}_2-\text{NH}_2$ for DA and MI, $-\text{CH(OH)-CH}_2-\text{NH}_2$ for NA and NM, $-\text{CH(OH)-CH}_2-\text{NH-CH}_3$ for A and M. (Modified from Cuello, 1979.)

catechols in large volumes of Krebs' solution . The inter-assay variations were great and not in the least dependable. The complexity introduced into the assay was due to:

- i) batch to batch variation in COMT preparation;
- ii) the inhibitory action of Ca^{2+} on COMT;
- iii) variation in preparation of ^3H -S-adenosyl methionine by different firms
- iv) variation in duplicate samples.

Difficulties were met in solving a specific problem that may have contributed to a particular assay. The assay technique of Saller & Zigmond (1978) was tried using several different preparations of COMT to give maximal response, use was made of ethyleneglycoltatraacetic acid (EGTA) to take up the Ca^{2+} present in Krebs' which inhibited COMT and at the same time Mg^{2+} ions were increased, a co-factor for COMT activity. ^3H adenosyl methionine prepared by New England Nuclear, Southampton, alleged to be purer was also tried. Satisfactory results were not obtained using the radioenzymatic assay and the technique was abandoned in favour of high pressure liquid chromatography.

4.2.3. High performance liquid chromatography (HPLC)

The earliest example of a liquid chromatographic separation is credited to the Russian botanist Tswett who, in 1903, as cited by Pryde & Gilbert (1979) separated plant pigments by absorption chromatography for which purpose he used a chalk column. The first real stimulus which launched several forms of chromatography and provided the basis of modern instrumental, high efficiency methods, occurred when, in 1941 Martin & Synge published a most far-seeing paper. In this paper they described

the discovery of liquid-liquid partition chromatography and laid the foundation of gas-liquid chromatography by suggesting "the moving phase could be replaced by a gas", and that of high performance liquid chromatography (HPLC). The prediction for HPLC was "thus the smallest height equivalent to a theoretical plate should be obtainable using small particle sizes and a high pressure difference across the lengths of the column". Most of the development with respect to HPLC occurred in the mid-60s.

Chromatography is the method of separating components of a mixture which depends on the affinity of the various solute molecules for the stationary and the moving phase, whatever they may be. Classification of chromatographic methods depends on the type of stationary and mobile phases used, the form of their presentation and the different mechanisms which control their separation. For example, liquid-solid (adsorption) chromatography, in which the stationary phase is a solid adsorbent which may be packed in a column, spread on a plate, or impregnated into porous paper with fixed active sites (e.g. alumina) on to which the interactions of the solutes occur. In liquid-liquid chromatography the solute molecules distribute themselves between two immiscible liquid phases according to their relative solubilities. In gas-chromatography the moving phase is replaced by a gas.

In HPLC the stationary phase involves the use of smaller particles and a liquid mobile phase; suitable flow rates are achieved by pumping under pressure. Appropriate detection systems are employed to show when the separated components elute off the column. With the development of new microparticulate resins for either ion exchange or reversed-phase chromatography, HPLC has become one of the most widely used methods for the determination of catecholamines and their metabolites.

Reverse phase chromatography is the term used when the stationary phase is non-polar and the mobile phase is polar. In ion exchange chromatography, the chromatographic support contains ions which are capable of being exchanged with ionic solutes in the mobile phase.

Most of the HPLC methods use either electrochemical detection directly (Keller, Oke, Mefford & Adams, 1976; Kissinger, 1977) or fluorescence detection after derivatisation. For catecholamines derivatisation may be achieved with orthophthalaldehyde (Davies, Gehrke, Gehrke Jr., Cunningham, Kuo, Gehardt, Johnson & Williams, 1978), oxidation to the trihydroxyindoles (Yui, Itokawa & Kawai, 1980), or the native fluorescence of the compounds (Anderson & Purdy, 1979).

Liquid chromatography with electrical detection provides a rapid, sensitive and specific technique for the assay of neurotransmitter monoamines and their metabolites. Electrochemical techniques were first applied to chromatographic detection by Kemula (1952) as cited by Mefford (1981). Little use was made of the technique until it was refined and applied to the analysis of catecholamines and ascorbic acid by Adams & co-workers (Kissinger, Refshauge, Dreiling & Adams, 1973).

Electrochemical detection can be achieved by means of direct oxidation or reduction, but in practice for the analysis of biogenic amines only oxidative processes are used.

Principles of electrochemical detection

A compound must have electrochemical activity for it to be amenable to electrochemical detection, that is the compound must be able to rearrange in such a way as to accommodate the loss or gain of one or more electrons. The rearrangement needs to occur within certain potential limits which are determined by the

electrode material and the bulk properties of the solvent in which the electrode is placed. In theory, the electrochemical oxidation can be carried out at any inert electrode. Platinum, gold and carbon are typical inert materials used for this purpose. However, platinum and gold undergo fairly easy surface oxidation and this involves the passage of electrochemical currents, even in the absence of analysable species in solution. This is known as the residual current, which, whilst being small, can amount to a noise level far in excess of the current representing oxidation of brain tissue monoamines. Carbon paste electrodes which are made of high purity graphite mixed into a paste with an inert liquid or glassy carbon-electrodes serve as electrode surfaces with extremely low residual current. The potential limits for carbon electrodes in aqueous solvents are approximately + 2V to -0.8 to -10V with Ag-AgCl reference electrode. Nearly all aromatic amines and phenols are oxidised within these potential limits. The process of electrochemical oxidation converts the phenolic compounds to quinones. This is demonstrated in Figure 27 for:

(a) catecholamines

(b) O-methylated catechol metabolites.

Figure 27

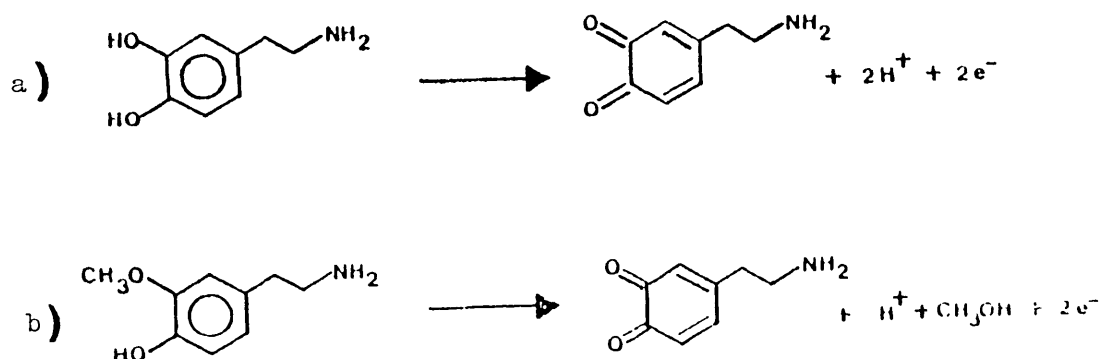


Fig. 27: Typical 2 electron oxidative mechanisms for (a) catecholamines; (b) 2 electron oxidative mechanisms for O-methylated catechol metabolites.

During the oxidation process two electrons are lost to the detector electrode, thus providing the basis for detection for these compounds. O-Methylated metabolites of catecholamines are more difficult to oxidise, since the O-methyl group makes the reaction (Figure 27) more difficult. The methoxy group must be cleaved and this requires a much higher energy of activation and therefore a higher electrochemical potential.

The current which is derived from the abstraction of these electrons is proportional to the concentration of the species in solution (see Figures 31 and 32 in Results Section).

Uses of HPLC

The determination of catecholamines in rat brain tissue was one of the earliest applications of HPLC with electrochemical detection (Kissinger et al, 1973). A modified and improved technique was later published by Keller et al (1976). Most of the analyses have depended upon an alumina extraction (Anton & Sayre, 1962) which, by absorption onto alumina, separates catechol-like compounds from any methoxylated metabolites. It is also a preconcentration step; the catechol compounds are next desorbed into a micro-volume of acid. The compounds are then separated using HPLC. HPLC is column chromatography in which very small particle size stationary phases (resins) are used so that separations may be achieved with short columns and rapid elution. Such columns do not operate with gravity flow of eluent, but always require pressure pumping. The designation HPLC is often taken to mean high pressure liquid chromatography. Most methods for HPLC use microparticulate reverse phase HPLC (Felice, Felice & Kissinger, 1978; Mefford, 1981).

Reverse phase chromatography

Reverse phase chromatography involves the use of a non-polar stationary phase in conjunction with a polar mobile phase, a technique introduced by Howard & Martin (1950). The most common method for performing these separations has been the use of a hydrocarbon bonded surface in conjunction with a relatively non-polar eluent such as methanol. The polar molecules have little affinity for the non-polar hydrophobic support, and are eluted fairly rapidly by the aqueous mobile phase. The most common bonded hydrocarbon phase are the octadecylsilyl groups ($C_{18}H_{37}Si$). The mechanism of reverse phase chromatography has not been satisfactorily worked out. Sodium octylsulphate (SOS) is used as an "ion pairing" reagent in reverse phase chromatography. The function of the ion pairing reagent is to increase the retention times of the amines presumably by providing a polar (ionic phase) by the $-SO_3^-$ groups on the non-polar C_{18} chain of the octadecylsilyl groups. This would help to retain the positively charged NH_2 of the amines and this is presumably why DOPAC is not affected by SOS. The relatively non-polar aspect of methanol would displace the non-polar aspect of the amines.

There are many advantages to the use of microparticulate reverse phase HPLC. These are better batch to batch reproducibility, and better resolutions and faster turnover of samples as a result of shorter retention times, especially with the use of smaller columns. The disadvantages of reverse phase system are that the smaller particle size requires higher pressures for operation and therefore a more costly pumping and injection system.

The difficulty in estimating catecholamine metabolites lies in the extraction procedure. Except for DOPAC, the other

metabolites are not absorbed on alumina.

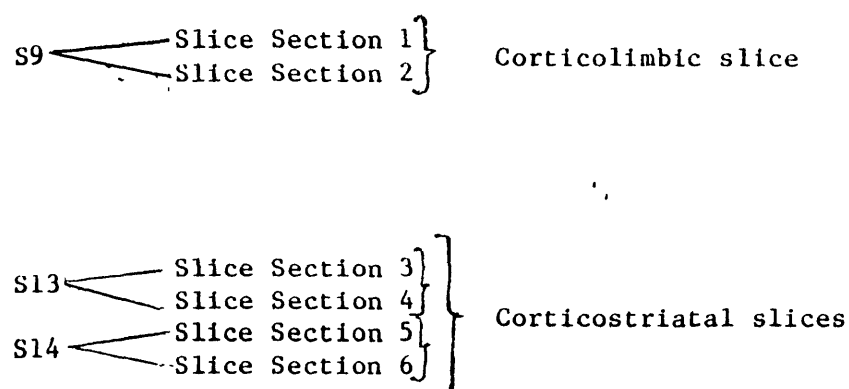
The HPLC system and the material for HPLC will be further described on page 161.

4.3. Experimental procedure for the 1) release of endogenous monoamines from rat brain slices; 2) the estimation by HPLC of DA, NA and DOPAC

Having established the methodology of ^3H -dopamine release (see Section III), the technique was applied to the study of endogenous transmitter release.

At the onset of the experiment the weight and number of the rat was recorded. The rat was guillotined and brain slices were obtained according to the procedure described in Section III.

The slices used for the endogenous dopamine and noradrenaline release studies were S9, S13 and S14. Each slice was cut along the midline into two slice sections and labelled slice Section 1-6 as follows:



Each slice section was randomly selected for treatment, in order to eliminate any changes in the release pattern due to possible differences in the left and right hemispheres. Each slice section was weighed and put into test-tubes

containing 5 ml of Kreb's solution with pargyline (10^{-7} M) and ascorbic acid (0.1 mg/ml). The slice sections were then incubated for 40 minutes at 37°C with 95% O₂/5% CO₂ bubbling through the solution. At the end of the 40 minutes, the slice sections were removed and each one placed into an electrode chamber.

The slice sections were then superfused with oxygenated Kreb's solution for a period of 15 minutes at a flow rate of 0.3 ml per minute. The superfusion apparatus is described in detail in Section III of this thesis. At the end of 15 minutes of superfusion a 10 minute superfusate fraction was collected from each slice section prior to electrical stimulation. To each 10 minute superfusate fraction was added 60 µl of dihydroxybenzylamine (DHBA) (1 ng/50µl), the internal standard. The samples were collected in order to determine the basal release of neurotransmitters and DOPAC content in the superfusate fraction. At the end of the 10 minutes the slice sections were electrically stimulated for 2 minutes at 10Hz, 2ms and 20mA and a further 10 minute superfusate fraction collected to which was added 60 µl of DHBA (1ng/50µl). The release of neurotransmitters on first stimulation (S₁) was determined in these superfusate fractions.

Slice sections were then superfused for a further 10 minutes before the 2nd stimulation (electrical parameters as for S₁), and again a 10 minute fraction collected and to each a 60 µl of DHBA (1 ng/50µl) added.

The samples were immediately stored at -80°C until extraction on alumina. The slice sections were superfused for a further 10 minutes before the removal of the slice sections from the electrode chambers. Slice sections were then sonicated in 1 ml of 0.2N PCA. 350 µl of each homogenised slice section was

placed into an Eppendorf tube containing 100 μ l of DHBA (1 ng/50 μ l) and stored at -80°C until extraction on alumina, the estimation of NA, DA and DOPAC was then conducted using HPLC.

Endogenous DA, NA and DOPAC release studies were conducted on brain slice sections of rats taken from five treatment groups. These treatment groups were designated A-E. At the end of each release experiment the content of DA, NA and DOPAC in the slice sections was estimated. Estimations of DA, NA and DOPAC in both superfusate fractions and in the brain slice sections were carried out using HPLC (p161).

Brain slice section of naive rats (Group A)

Naive rat brains ($n = 7$) were used to study endogenous NA, DA and DOPAC release from corticolimbic and corticostriatal slice sections.

Brain slice sections from conditioned rats (Group B)

Rats ($n = 7$) were conditioned on the conditioned avoidance behavioural system. These rats were used to study the release of DA, NA and DOPAC from corticolimbic and corticostriatal brain regions.

Brain slice sections from conditioned rats chronically treated with haloperidol (0.1 mg/kg) (Group C)

Conditioned rats ($n = 7$) were treated over a 32 day period with haloperidol (0.1 mg/kg). At the end of the treatment period the rats were sacrificed 2 hours after the last drug treatment. The release of DA, NA and DOPAC was then studied from corticolimbic and corticostriatal brain regions.

Brain slice sections from conditioned rats chronically treated with trazodone (100 mg/kg) (Group D)

Conditioned rats ($n = 6$) were treated over a 32 day period with trazodone (100 mg/kg). At the end of the treatment period the rats were sacrificed 2 hours after the last drug treatment. The release of DA, NA and DOPAC was then studied from corticolimbic and corticostriatal brain regions.

Brain slice sections from naive rats chronically treated with haloperidol (0.1 mg/kg) (Group E)

Naive rats ($n = 6$) were treated with haloperidol (0.1 mg/kg) over a 32 day period. Rats were sacrificed 2 hours after the last drug treatment and DA, NA and DOPAC release from corticolimbic and corticostriatal slices was estimated.

HPLC system: materials and methods

Figure 28 shows a simple diagram of the basic components of an HPLC system. This consists of a low pulse pumping system with a pressure maximum of 4000 psi, an essentially zero dead volume sample injection system, a suitable chromatographic column, a detector system with a device to apply a potential to the detector electrode and monitor faradaic current, and a flow chart recorder.

Citric acid, sodium acetate, hydrochloric acid, and perchloric acid all analytical reagent (Analar) grade, were purchased from British Drug House (BDH) Chemicals Ltd., Poole. Methanol (HPLC grade) was purchased from Rathburn Chemicals Ltd., Peeblesshire. Sodium octylsulphate was purchased from Eastman Kodak Co., Rochester, and alumina (Woelm N-DC) from Woelm Pharma Eschwege, Germany. Chemicals purchased from Sigma Chemicals, Poole, and of Analar grade, were dopamine (3-hydroxytyramine

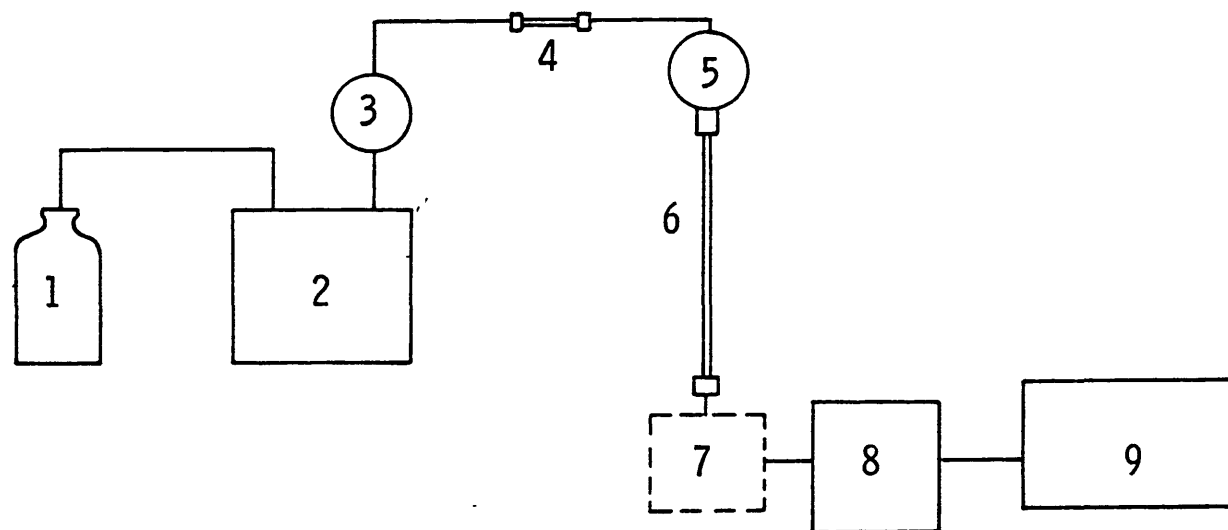


FIG 28: Flow diagram of HPLC apparatus 1) solvent; 2) pump; 3) pulse dampner; 4) pre column to clean the solvent; 5) injection port; 7) detector electrode; 8) amperometric controller (amplifier); 9) chart recorder. 6) column

hydrochloride), ethylenediaminetetraacetic acid (EDTA) disodium salt; L-arterenol (noradrenaline) bitartrate; 3,4-dihydroxyphenylacetic acid; 3,4-dihydroxybenzylamine (Sigma grade); L-epinephrine (adrenaline) and TRIS (hydroxymethyl) aminomethane (buffer reagent grade). 24 μ ODS purchased from HPLC Technology, Macclesfield, Cheshire.

Apparatus used for HPLC

Pumping system

A Constametric I (dual head) pump from Laboratory Data Control, was used. A precolumn containing 25 μ ODS was used in order to clean the solvent. This was positioned between the pump and the sample injector and also served as a pulse dampner.

Injection system

A Rheodyne 7125 injection valve purchased from Anachem was used. Injection valves operate by loading a sample loop while the mobile phase is pumped via a bypass system to the column. The Rheodyne injection valve has an external replaceable loop and by means of a lever the flow can be switched through this loop, carrying with it the sample solution onto the column. Very reproducible injections are possible with this system, a criterion which is very important in quantitative work. An excess of sample was injected to ensure that the 50 μ l loop was completely full. Figure 29 shows the operational basis for the valve injector.

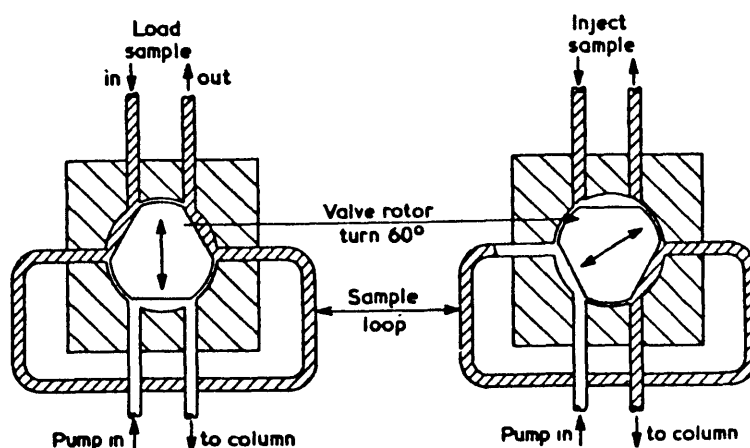


FIG 29: Valve injector for HPLC (taken from Pryde & Gilbert, 1979).

Column used

A Hypersil 3 μ Octadecylsilyl (ODS) column 100 x 5 mm was used. The hypersil resin was manufactured by Shandon, Runcorn, Cheshire and the column was packed and supplied by HPLC Technology Limited, Macclesfield, Cheshire, U.K.

Solvent system

Catecholamines and DOPAC were separated using a solvent consisting of citric acid (0.1M), sodium acetate (0.025M), sodium octyl sulphate (25mg/litre), ethylenediaminetetraacetic acid (EDTA) (50mg/ litre) and methanol (3% v/v) (pH of solvent was 2.6). A flow rate of 1.0 ml/min was maintained. Through experience it was found that each column needed slight variation in the solvent composition in order to maintain the retention times of each substance, most probably related to the packing of the column. Manipulation of the methanol and ion-pairing reagent

sodium octyl sulphate as well as pH can solve many of the problems associated with different batches of columns.

Methanol is the solvent generally used with glassy carbon electrodes. Increasing the methanol content of the solvent decreases the column retention times of all amines proportionally, generally without altering the elution order.

The use of the octyl sulphate as mentioned, allows selective manipulation of the retention characteristics of the amines and DOPAC. Increasing the amount of octyl sulphate increases the retention times of amines whilst leaving the DOPAC unaffected, in this manner elution order can be manipulated. Once the desired order of elution is attained the methanol content may be altered to change the retention times of all compounds, whilst retaining resolution and order of elution.

Detector system

An Amperometric detector was used for electrical detection. This was purchased from Bioanalytical Systems (Lafayette Ind., USA). Figure 30 shows the detector and reference electrode design. Under 1.0 ml/min flow rate and a potential of 0.7V the glass carbon electrode oxidises 1-5% of the species of interest present in the solution. The glassy carbon electrode is part of a channel wall formed by a fluorocarbon gasket between two inert plastic blocks (see Figure 30)

Chart recorder

A standard Servogor Zio supplied by Labdata Instrument Services Limited, Cambridge, England was used. The recorder recorded the voltage put out by the amperometric detector. The voltage is a function of the current produced at the electrode thereby converting NA into volts. The electronic controller

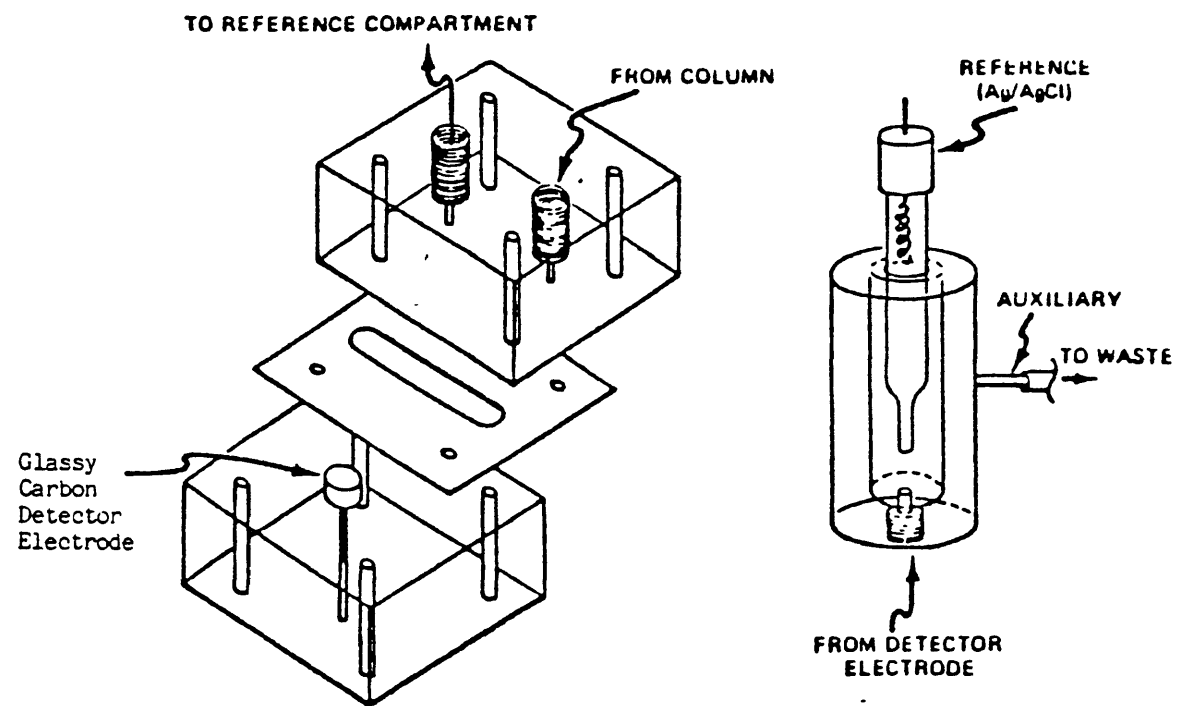


FIG 30: Diagram of the detector and reference electrode.
(Taken from Mefford, 1981)

converts this current (in the nA range) into volts which feed into the chart recorder.

Procedures used for analysis of HPLC data

Chromatographic parameters

The column capacity ratio k' or the k' value of a solute is the usual method of indicating solute retention. The k' values are obtained from the elution chromatogram by

$$k' = \frac{t_r - t_o}{t_o}$$

where t_r is the retention time of the given peak and t_o is the retention time of the solvent peak. Strictly speaking, retention volume, rather than retention times, should be used for chromatographic calculations, because they take into account any changes in the peak e.g. peak broadening. However, if constant flow rates can be assumed then retention times are convenient to use and can be obtained directly from the chromatogram.

Identification of the dopamine, noradrenaline, and DOPAC peaks

The monoamines and DOPAC peaks in the sample elution chromatogram were identified by their having the same k' values as standards. Standard curves to each substance of interest were obtained in two concentration ranges shown in Figure 31, 32. The accuracy of the HPLC system was governed by the ability to calibrate the system using the standards in the two concentration ranges. A standard solution of the amine of interest was also added to the unknown tissue sample to show that there was no distortion of the shape of the unknown peak and that the two peaks were therefore identical, this procedure was not done routinely.

Analysis of the elution chromatograms

Quantitative analysis was carried out by measuring peak height. Using the peak height of a known amount of the internal standard (dihydroxybenzylamine) DHBA simple recoveries through alumina were calculated and accounted for. The recovery through alumina was shown to be $52.59\% \pm 0.68\%$ (s.e.m.) and $n = 577$.

Preparation of samples for analysis on HPLC

Preparation of alumina

Alumina was prepared according to Anton & Sayre (1962).

Alumina (200 g) was added to one litre HCL (2M) and the mixture boiled for 20 minutes, with constant stirring. The mixture was then cooled, filtered through a sintered glass funnel and the residual alumina washed with 15 litres of deionised (double glass) distilled water over two days. The alumina was then dried at 200°C for four hours and stored at 30-50°C in a desiccator.

Sample preparation

The method for extraction of catecholamines (dopamine and noradrenaline) and DOPAC from brain slice section superfusates was according to Mefford (1981). This extraction procedure was modified for the extraction of DA, NA and DOPAC from slice section superfusates.

Following an endogenous monoamine release experiment to each 3ml superfusate fraction collected, was added 60µl of DBHA (1 ng/50 µl). These samples were then stored until subsequent analysis.

The brain slice sections were sonicated in 1 ml of 0.2N PCA. 350 µl of this homogenate was transferred into Eppendorf

tubes and 100 μ l of DHBA (1 ng/50 μ l), was added. The samples were stored at -80°C until further analysis.

Superfusate extractions

The following procedure, carried out on ice, was followed for the extraction of DA, NA and DOPAC.

1. To the 3ml brain slice superfusate fraction containing 60 μ l of DHBA (1 ng/50 μ l) 300 μ l of 1M Tris buffer pH 8.6 and 10mg of alumina (acid-washed) were added.
2. The sample was mixed by a rotary stirrer for 15 minutes to ensure complete adsorption of catecholamines and DOPAC onto the alumina.
3. The tubes were then centrifuged at 2,000 RPM (1000g) in a cooled centrifuge (MSE Scientific Cool spin), for a period of 3 minutes, and the supernatant then aspirated.
4. The alumina pellet was then washed by adding 1.5ml of deionised distilled water and stirring, pipetting into an Eppendorf tube and centrifuging again for 3 minutes. The supernatant was then removed by aspiration, taking care not to touch the alumina at the bottom of the tube.
5. To the alumina, 60 μ l of 0.2N PCA was then added to desorb the catecholamines.
6. The sample was then centrifuged at 3,500 RPM (2600g) for 15 minutes.

7. 50 μ l of the sample (i.e. the supernatant), taking care not to touch the alumina, was injected into the HPLC system using a Hamilton syringe.

Brain slice homogenates extractions

The procedure was the same as for brain slice superfusates except at:

Step 1 To 350 μ l of the homogenate containing 100 μ l of the internal standard, was added 1.0ml of 0.5M TRIS buffer pH 8.6 and 10 mg of alumina.

At step 5 The catecholamines were desorbed into 100 μ l of 0.2N PCA and 50 μ l injected into the HPLC system. All the steps in the extraction procedures for both superfusate and brain slice homogenate samples are shown in the flow chart on p171.

Analysis of data

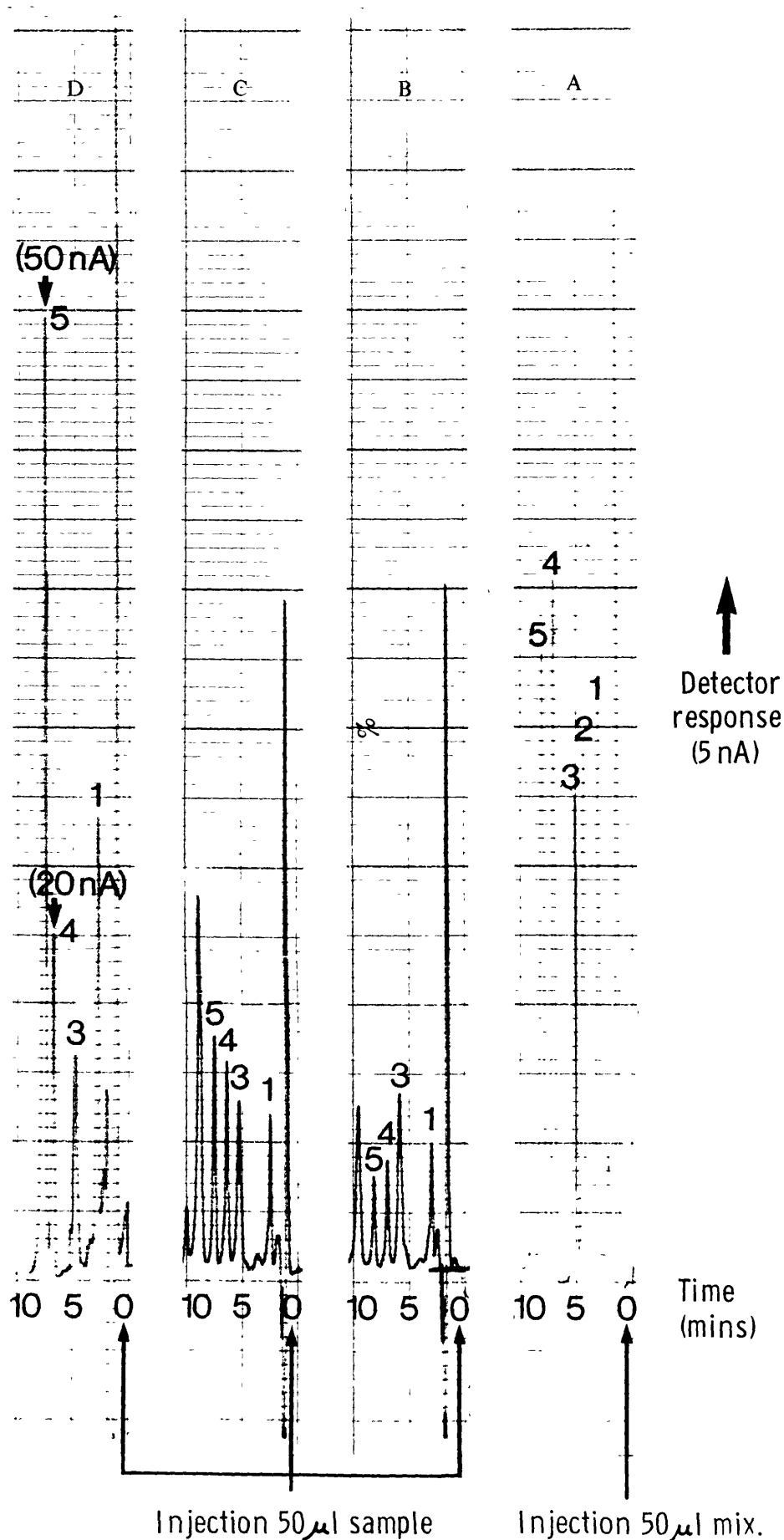
The amines, AD (adrenaline) NA, DA, and the metabolites DOPAC and the internal standard DHBA were identified according to their retention times (See chromatogram below Figure 33.33b)

Once identified the peak height of each substance was measured. The peak height axis was calibrated to correspond to the picoamperes of current generated.

On the Amphotometric Detector the current in the range of 1 nA to 100 nA for 1 volt could be set. 1 nA on the detector is equivalent to 1 volt full scale deflection (FSD) on the chart recorder. Due to the need to increase sensitivity (2 fold) the FSD on the detector was 0.5 volt, this meant that 1 nA range on the detector would be 0.5 nA FSD on the chart recorder.

The responses of amines and DOPAC were as follows: DA 187.7 pA/pmole NA 287.5pA/pmole DOPAC 247 pA/pmole DHBA 170.2 pA/pmole.

Fig 33: Chromatographic resolution of (A) 1 ng standard solution of monoamines, DOPAC and internal standard DHBA; (B) brain slice superfusate (basal release); (C) brain slice superfusate (stimulated release); (D) brain slice homogenate. 1) Noradrenaline (2.5'). 2) Adrenaline (4.3'). 3) Dihydroxybenzylamine (5.0'). 4) Dihydroxyphenylacetic acid (7.0') and 5) Dopamine (8.6'). Figures in brackets are retention times in minutes.



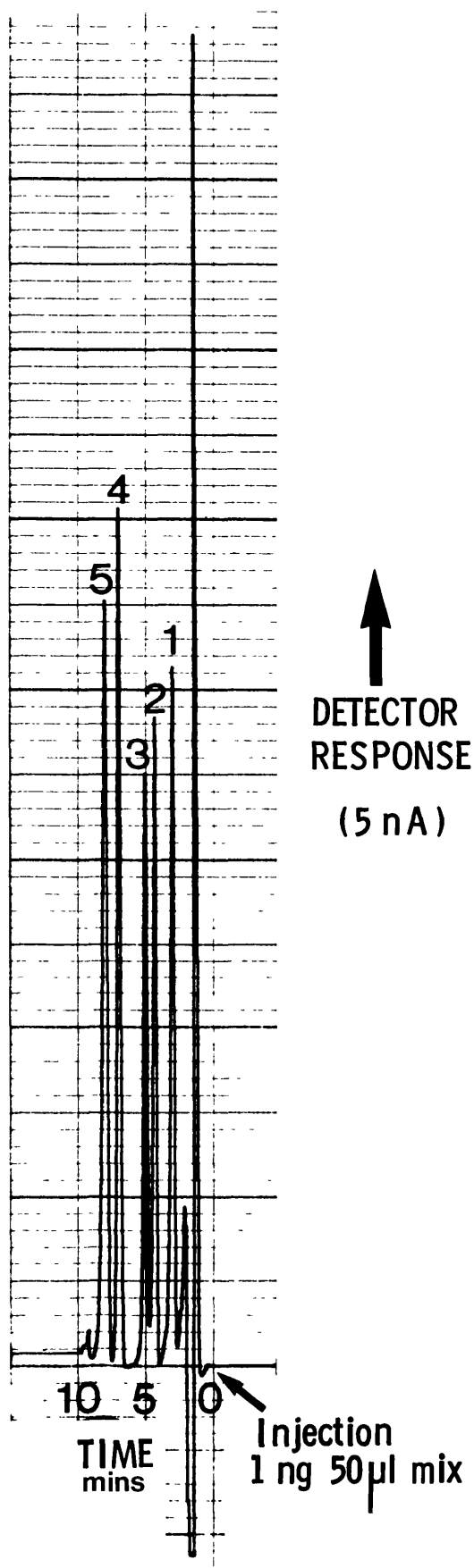


FIG 33b Chromatographic resolution of 1 ng standard solution of monoamines, DOPAC and internal standard DHBA. 1) Noradrenaline (2.5'). 2) Adrenaline (4.3'). 3) Dihydroxybenzylamine (5.0'). 4) Dihydroxyphenylacetic acid (7.0') and 5) Dopamine (8.6'). Figures in brackets are retention times in minutes.

Before analysis of superfusate and tissue samples, dose response curves to DA, NA, DOPAC and DHBA was carried out (Figures 32, 33). The dose response curves were obtained in two ranges 1-1000 pg and 1-10 ng. This was to ensure that the HPLC system could assay substances both in the sensitive (for superfusate estimations) and normal tissue ranges.

The internal standard DHBA was added to each superfusate and tissue sample to account for percentage recovery through alumina.

Before analysis of samples, a standard 1 ng/50 µl mix of AD, NA, DA, DHBA, DOPAC was always chromatogrammed, this was done regularly after analysis of 12 samples.

The current generated by an amine in the sample was then divided by the current generated by the standard 1 ng of the amine to give the concentration of the amine in the sample before accounting for recovery through alumina.

$$\frac{\text{pA current generated by DA in a sample}}{\text{pA current generated by DA in 1 ng standard}} = \text{ng of DA in sample}$$

The ng of DA in the sample could then be converted to the actual DA in the sample after accounting for recovery through alumina obtained using DHBA in the sample. Therefore:

$$\frac{\text{ng of DA in sample}}{\% \text{ of recovery through alumina}} = \text{actual DA in the sample}$$

4.4. Results

1. Significant release on electrical stimulation

Electrical stimulation of rat brain slice sections from corticolimbic and corticostriatal areas show that for most slice sections and for all conditions highly significant release of noradrenaline and dopamine occurred and that basal release

figures were constant throughout the period of the experiment (Figures 34 a-d). Two consecutive periods of stimulation S_1 and S_2 were carried out. Release in all the figures and tables are presented for both periods of stimulations. It should be noted that in Figure 34 (a) for treatment C, that S_1 figures were not significantly different from basal levels whereas as expected S_2 figure showed a significant difference.

2. Comparisons of the release of endogenous NA and DA from brain slice sections of naive and conditioned rats

The release of endogenous dopamine and noradrenaline was measured in brain slice superfusates from naive and conditioned rats. Figure 35 shows the results obtained after electrical stimulation of brain sections expressed on a tissue weight basis. It was seen that in conditioned animals significant reduction in release of both neurotransmitters from both regions is apparent. For convenience Table 7 shows release figures on 1st stimulation only. Student t-tests indicate a significant decrease in neurotransmitter release from slices of conditioned rat brains ($p < 0.001$). It appeared that the conditioning programme influenced the amount of neurotransmitter released.

3 Total neurotransmitter concentrations in brain slice sections

Total neurotransmitter concentrations remaining in all the slice sections and for all treatments are shown in Table 8. Several points emerged from this table.

A. The dopamine content of the corticostriatal slice sections was five times that of corticolimbic slice sections in all treatments.

B. In comparing NAIVE with CONDITIONED animals the total tissue value for neurotransmitters NA and DA are not significantly different after treatment when slice sections from the same areas are compared.

4. Percentage release figures for DA and NA calculated with respect to total amine content in slice section of naive and conditioned rats

When % release figures are examined (Table 9) several points emerged.

The percentage of dopamine released from corticostriatal slice sections was smaller than that released from corticolimbic slices, even though there was significantly more dopamine present (Table 8).

When comparisons are made between conditioned and naive rats percentage release figures in Table 9, a lower percentage of neurotransmitter was released from the conditioned rats, when compared with release figures from naive rats brain slice sections. This holds for both areas of rat brain.

It appears that the conditioning programme resulted in a decrease in the percentage of neurotransmitter released, that is, the total amine figure remained constant but less neurotransmitter was mobilised when rats had been subjected to a conditioning programme.

5. Results for the effect of chronic haloperidol treatment of conditioned rats (Treatment C)

When conditioned rats were treated with haloperidol this resulted in temporary extinction of the learned response. The effect of in vivo drug treatment on in vitro neurotransmitter release patterns is shown in Table 10. One clear cut result

emerged. Haloperidol significantly reduced dopamine release in corticolimbic slices ($p < 0.001$). Neurotransmitter release profiles in corticostriatal slices showed no significant differences between conditioning and haloperidol treatment. The release of NA from corticolimbic slices upon 1st and 2nd stimulations yielded an equivocal result.

6. Treatment E (naive + haloperidol treated) compared with treatment A (naive)

Table 11 shows the results obtained in studies in naive rats treated chronically with haloperidol. No significant differences in release patterns were obtained except in corticostriatal slice sections where a significant decrease in noradrenaline release was obtained.

The noradrenaline release patterns were susceptible to haloperidol treatment in naive rats whereas dopamine release patterns were susceptible to haloperidol treatment in conditioned rats (Figure 34)

7. Treatment D (conditioned rats treated with trazodone)

When conditioned rats were treated with trazodone this resulted in temporary extinction of the learned response. The effect of in vivo drug treatment on in vitro neurotransmitter release is shown in Figure 36. This result shows that trazodone significantly increases the release of noradrenaline and dopamine from both regions of the brain. The effect of trazodone on the release of endogenous dopamine parallels the effect of this drug on the release of preloaded radiolabelled dopamine. The release of ^3H -DA was significantly enhanced by trazodone (Section III). Further examination of the data suggests that trazodone treatment reestablishes the release pattern shown by naive rats' brain

slice sections (treatment A). Figure 36.

6. Results of DOPAC release

DOPAC estimations in brain superfusates from all treatments are shown in Table 12. The most important observation was that the increase in superfusate DOPAC levels as a result of S_1 and S_2 stimulation periods was highly significant. There was a very large variation in the figures for the release of DOPAC (see SEM Table 12) unlike SEM for NA and DA. The release of DOPAC from corticostriatal slice sections is greater than from corticolimbic slice sections.

The total levels of DOPAC in the corticostriatal slice sections in all treatments are also higher than in the corticolimbic slice sections (Table 12).

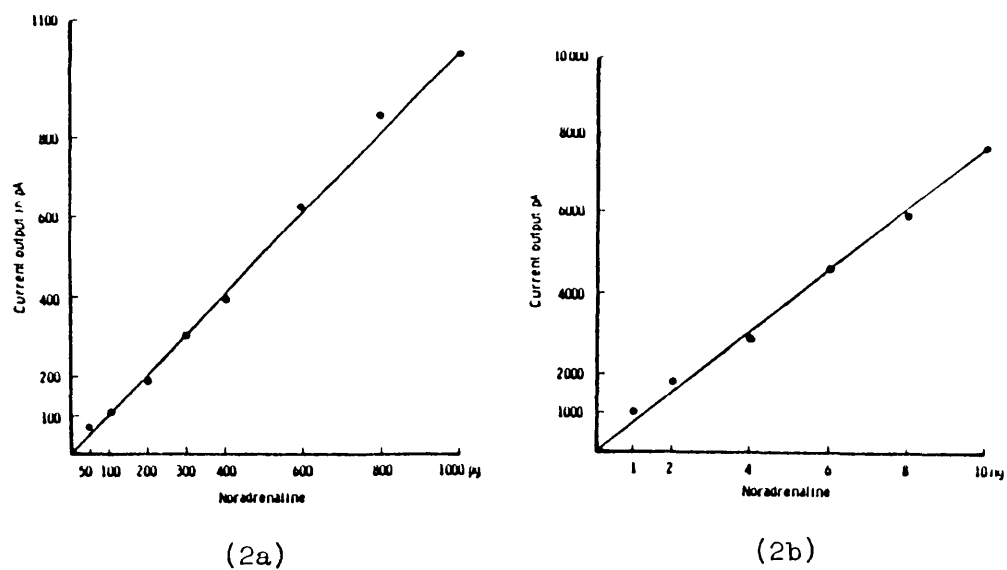
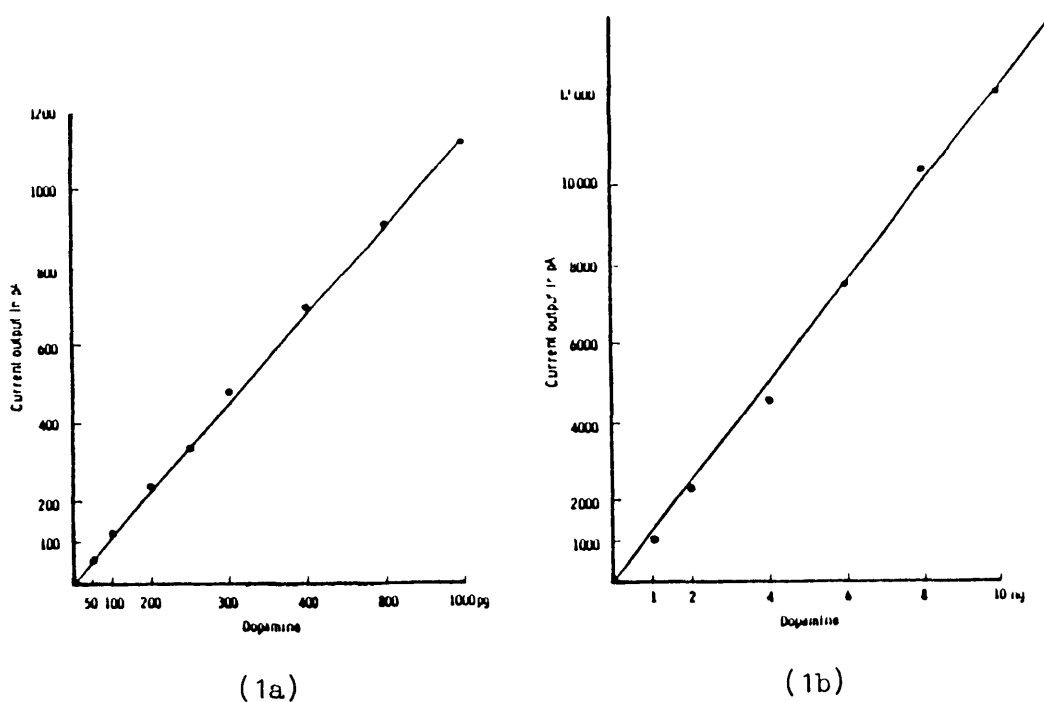
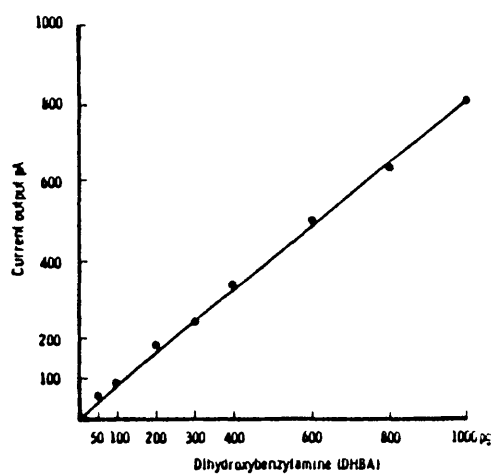
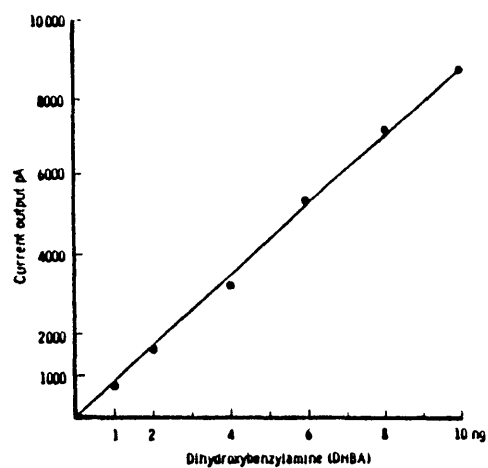


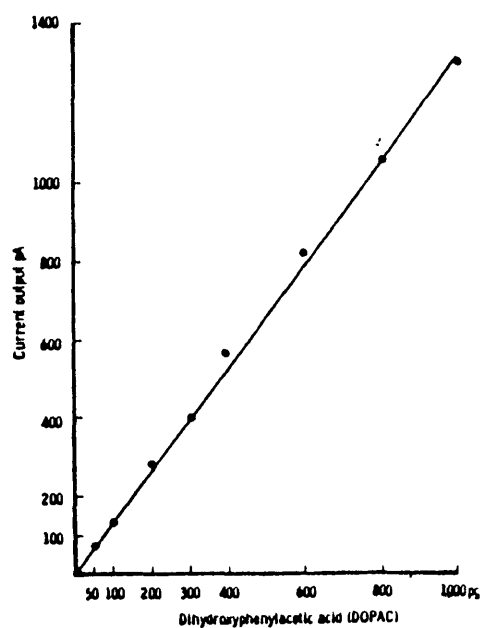
FIG 31: Standard curves to dopamine (1a; 1b) and noradrenaline (2a; 2b) in two concentration ranges (a) 50-1000 pg (b) 1-10 ng.



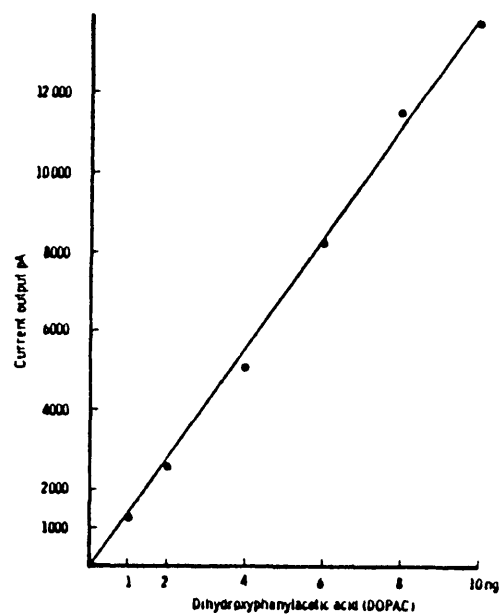
(3a)



(3b)



(4a)



(4b)

FIG 32: Standard curves to DHBA (3a; 3b) and DOPAC (4a; 4b) in two concentration ranges (a) 50-1000 pg (b) 1-10 ng.

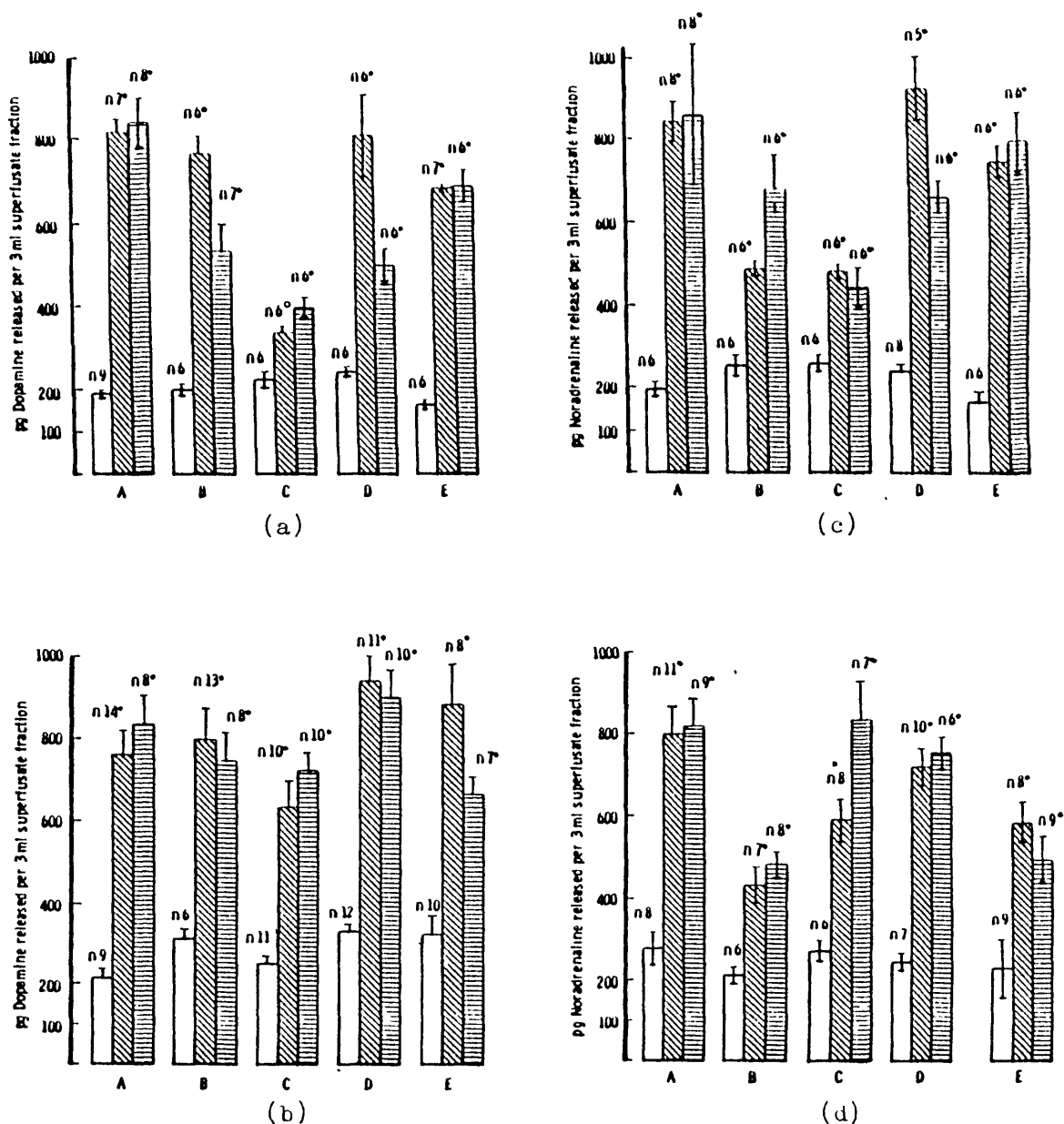


FIG 34 (a-d): The release of endogenous neurotransmitters (noradrenaline and dopamine) from corticolimbic and corticostriatal slice sections from treatment groups A-E.

A = Brain slice sections from naive rats.

B = Brain slice sections from conditioned only rats.

C = Brain slice sections from conditions rats chronically treated with haloperidol (0.1 mg/kg).

D = Brain slice sections from conditioned rats chronically treated with trazodone (100 mg/kg).

E = Brain slice sections from naive rats chronically treated with haloperidol (0.1 mg/kg).

Basal release = $\square$, 1st stimulation = diagonal lines , 2nd stimulation = horizontal lines .

The probability of the differences between basal and stimulated were calculated using student t-test $p < 0.001$.

(a) Dopamine release from corticolimbic slice sections.

(b) Dopamine release from corticostriatal slice sections.

(c) Noradrenaline release from corticolimbic slice sections.

(d) Noradrenaline release from corticostriatal slice sections.

Treatment of rats	Brain region	Amount of DA released in relation to the total expressed as a percentage		Amount of NA released in relation to the total expressed as a percentage	
		1st stimulation	2nd stimulation	1st stimulation	2nd stimulation
A Naive	Corticolimbic	2.45% (n=8)	2.71% (n=6)	2.71% (n=8)	4.61% (n=6)
	Corticostratial	0.50% (n=14)	0.64% (n=8)	3.32% (n=15)	3.63% (n=9)
B Conditioned	Corticolimbic	1.52% (n=7)	1.10% (n=7)	0.83% (n=6)	1.00% (n=6)
	Corticostratial	0.26% (n=10)	0.23% (n=8)	0.33% (n=8)	1.71% (n=7)

TABLE 9: Amount of DA and NA released from slice sections in relation to the total slice amine content expressed as a percentage.

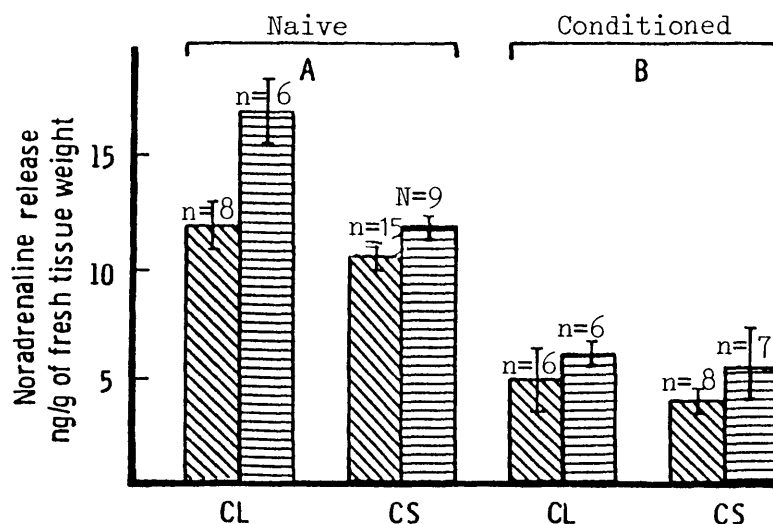


Fig 35a) Release of endogenous noradrenaline from corticolimbic (CL) and corticostriatal (CS) slice sections from naive (A) and conditioned (B) rat brains. Bars represent s.e.m. ▨ = release on first stimulation. ▩ = release on second stimulation.

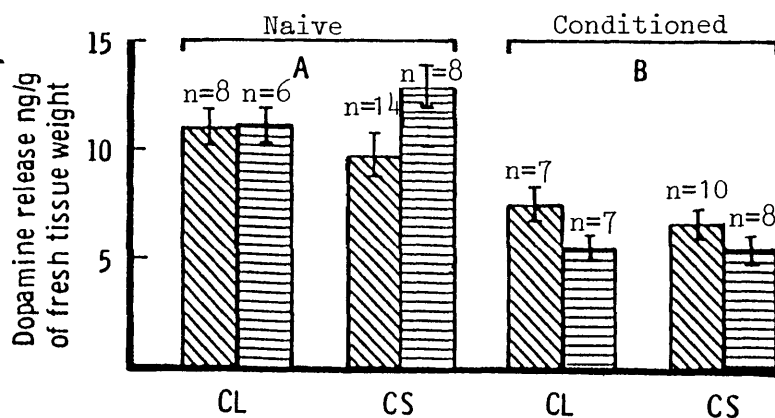


Fig 35b) Release of endogenous dopamine from corticolimbic (CL) and corticostriatal (CS) slice sections from naive (A) and conditioned (B) rat brains. ▨ = release on first stimulation. ▩ = release on second stimulation. Bars represent s.e.m.

	Brain region	ng/g of dopamine released after subtraction of the basal release	ng/g of noradrenaline released after subtraction of the basal release
Naive rats	Corticolimbic	10.40 ± 0.90 n = 8	11.48 ± 1.50 n = 8
	Corticostriatal	10.12 ± 1.00 n = 14	10.46 ± 0.86 n = 21
Conditioned rats	Corticolimbic	7.45 ± 0.46 n = 7p*	4.83 ± 0.45 n = 6p*
	Corticostriatal	6.23 ± 0.89 n = 10p*	3.60 ± 0.90 n = 13p*

TABLE 7: Endogenous release of noradrenaline and dopamine from brain slices of naive and conditioned rats. Each number is a mean ± s.e.m. of n (number) slices. P* is the significance of the difference in the release of amines from brain slices of conditioned rats when compared with the brain slices from naive rats. $p^* < 0.001$

TABLE 8

Substance	Brain region	(A) Naive	(B) Conditioned	(C) Conditioned + haloperidol treated (0.1 mg/kg)	(D) Conditioned + trazodone treated (1.00 mg/kg)	(E) Naive + haloperidol treated (0.1 mg/kg)
Dopamine	Corticolimbic	425 ± 42 (n = 9)	489 ± 47 (n = 6)	476 ± 69 (n = 7)	521 (n = 4)	409 (n = 4)
	Corticostriatal	1992 ± 199 (n = 19)	2362 ± 157 (n = 10)	1812 ± 138 (n = 16)	2427 ± 200 (n = 8)	2563 ± 198 (n = 8)
Noradrenaline	Corticolimbic	423 ± 29 (n = 8)	589 ± 53 (n = 8)	459 ± 64 (n = 7)	532 ± 60 (n = 6)	556 ± 38 (n = 6)
	Corticostriatal	323 ± 19 (n = 12)	337 ± 48 (n = 7)	422 ± 28 (n = 14)	478 (n = 5)	468 ± 38 (n = 10)
DOPAC	Corticolimbic	76 ± 12 (n = 6)	152 (n = 4)	136 ± 12 (n = 6)	27 (n = 3)	40 (n = 4)
	Corticostriatal	170 ± 20 (n = 15)	205 ± 21 (n = 11)	170 ± 25 (n = 6)	184 ± 29 (n = 8)	186 ± 25 (n = 8)

TABLE 8: The total DA, NA and DOPAC content in corticolimbic and corticostriatal slices, in ng/g of fresh tissue ± sem.

	Treatment B Conditioned	Treatment C Haloperidol treated
DOPAMINE RELEASE	(n = 7)	(n = 6)
Corticolimbic slice sections	7.45 ± 0.44 (S ₁) 5.44 ± 0.60 (S ₂)	1.47 ± 0.16 (S ₁)* 2.80 ± 0.35 (S ₂)*
NORADRENALINE RELEASE	(n = 6)	(n = 6)
Corticolimbic slices	4.86 ± 0.45 (S ₁) 6.45 ± 0.64 (S ₂)	4.23 ± 0.57 (S ₁)** 3.63 ± 0.44 (S ₂)**

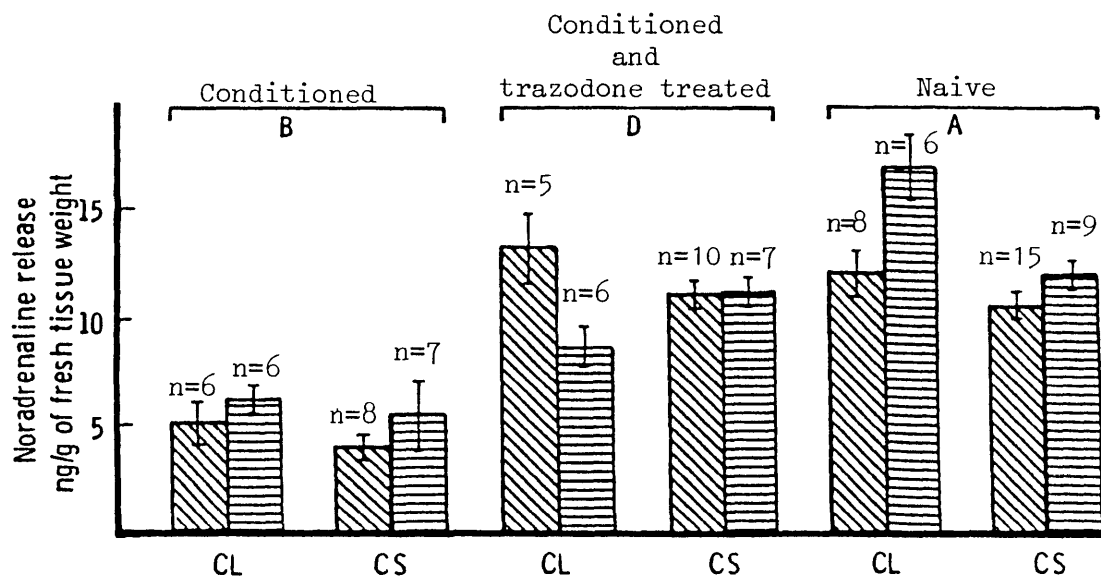
TABLE 10: Dopamine and noradrenaline released from corticolimbic slice sections from treatment groups conditioned (B) and conditioned and haloperidol (C) treated. Each figure is the mean ± s.e.m. expressed in ng/g of fresh tissue weight. S₁ and S₂ are 1st and 2nd stimulations respectively.

**The variation in S₁ and S₂ figures yield an equivocal results.

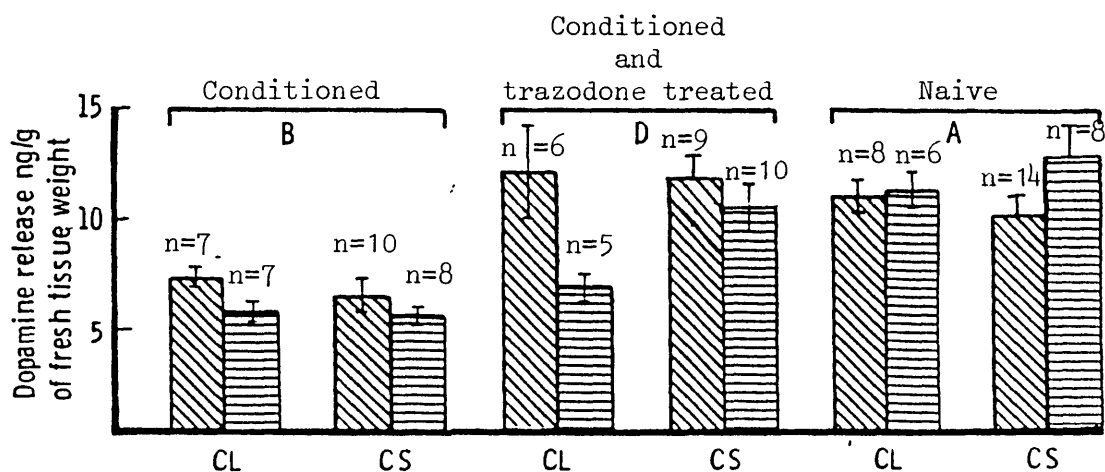
* Significant decrease in release calculated using Students t-test
p= <0.001.

Treatment of rats	Brain region examined	ng of dopamine released per g of fresh tissue		ng of noradrenaline released per g of fresh tissue	
		1st stimulation	2nd stimulation	1st stimulation	2nd stimulation
A naive	Corticolimbic	10.4 ± 0.90 (n=8)	11.55 ± 0.99 (n=6)	11.48 ± 1.54 (n=8)	16.98 ± 1.60 (n=6)
	Corticostriatal	10.12 ± 1.00 (n=14)	12.92 ± 1.50 (n=8)	10.46 ± 0.86 (n=15)	11.73 ± 0.91 (n=9)
E naive + haloperidol treated 0.1 mg/kg	Corticolimbic	11.31 ± 2.40 (n=5)	8.66 ± 0.18 (n=6)	10.50 ± 1.30 (n=7)	12.88 ± 1.94 (n=6)
	Corticostriatal	11.99 ± 1.55 (n=7)	8.67 ± 0.80 (n=9)	4.98 ± 0.39 (n=6) * ↓	5.48 ± 0.71 (n=8) * ↓

TABLE 11: Release of endogenous dopamine and noradrenaline from brain slice sections of naive (A) and naive and haloperidol treated (E) rats. Each figure is a mean ± sem. * = p < 0.001. ↓ Arrow denotes a decrease.



(a)



(b)

Fig 36a) Release of endogenous noradrenaline from corticolimbic (CL) and corticostriatal (CS) slice sections of conditioned (B), conditioned and trazodone treated (D) and naive (A) rat brains.

Fig 36b) Release of endogenous dopamine from corticolimbic (CL) and corticostriatal (CS) slice sections of conditioned (B), conditioned and trazodone treated (D) and naive (A) rat brains.

▨ = release on first stimulation.

▤ = release on second stimulation.

Bars represent s.e.m.

TABLE 12

Slice region	DOPAC basal release ng/g of tissue	1st stim. ng/g of tissue	2nd stim. ng/g of tissue	Treatment groups of rats
Corticolimbic	0.087 ± 0.007 n = 7	10.09 ± 1.83 n = 13	6.76 ± 1.54 n = 11	(A) Naive
Corticostriatal	0.21 ± 0.03 n = 19	25.40 ± 3.44 n = 19	23.87 ± 5.96 n = 20	
Corticolimbic	0.43 ± 0.19 n = 5	15.66 ± 5 n = 11	15.21 ± 8.00 n = 9	(B) Conditioned
Corticostriatal	0.20 ± 0.02 n = 5	18.71 ± 3.15 n = 15	14.88 ± 3.46 n = 11	
Corticolimbic	0.11 ± 0.016 n = 7	3.44 ± 0.89 n = 5	3.10 ± 1.25 n = 5	(C) Conditioned + haloperidol treated (0.1 mg/kg)
Corticostriatal	0.29 ± 0.05 n = 11	26.02 ± 6.36 n = 18	26.55 ± 6.73 n = 24	
Corticolimbic	0.11 n = 5	7.40 n = 4	4.90 ± 0.84 n = 5	(D) Conditioned + trazodone treated (100 mg/kg)
Corticostriatal	0.20 ± 0.025 n = 5	34.70 ± 12.96 n = 11	24.35 ± 6.41 n = 14	
Corticolimbic	0.11 ± 0.02 n = 5	7.26 ± 2.73 n = 9	5.74 ± 0.64 n = 5	(E) Naive + haloperidol treated (0.1 mg/kg)
Corticostriatal	0.35 ± 0.06 n = 9	28.18 ± 5.30 n = 17	20.22 ± 4.64 n = 15	

DOPAC levels in corticostriatal and corticolimbic slices in all treatment groups.

4.5. Discussion

Literature on the electrically stimulated release of endogenous amines from small slices of brain tissue is scarce. There were problems of detection prior to the advent of sensitive techniques (such as HPLC) for the estimation of picogram amounts of amines, especially in large superfusate fractions. A further complicating factor was, and is, the difficulty associated with electrical stimulation procedures. The release of endogenous amines from brain slices by high potassium chloride concentrations has been reported from various laboratories. Assay techniques include HPLC and radioenzymatic assay on slice perfusates. The table below compares data from three groups of workers. Basal release and stimulated release figures for dopamine only are shown, as the areas from which NA release was studied are not comparable between the three groups.

Authors	Slice region	Stimulus	Basal release	Stimulated release
Kant & Meyernoff (1978)	Striatal	55 mM KCl	280 µg/g	4 fold increase
Bennett et al (1981)	Striatal	55 mM KCl	169 µg/g	17 fold increase
This work	Corticostratial	Electrically stimulated	3.7 ng/g	4 fold increase

The order of basal release and stimulated release of dopamine is different in the electrically stimulated mode. It is possible that the KCl releases DA from compartments which are different from those mobilisable by electrical stimulation. These are analyses from regions of the brain which are partially included in the present slices. It is difficult to compare data in this thesis with figures obtained by other workers because the slice characteristics, namely method of slicing, area of the brain studied, thickness, superfusion techniques, are so different. Slices obtained by Kant & Meyerhoff (1978) were 0.3 mm x 0.3 mm, cut using a McIlwain chopper, whereas those of Bennett, Marsden, Sharp & Stolz, (1981) were 0.5 mm x 0.5 mm obtained using an 'egg slicer' technique. Slices used in this

study were 0.6 mm thick and the sections approximately 5 mm in diameter. Explanations for apparent differences may lie in the type of stimulus and in the period of stimulation. In naive rats, total amine measurements in the two areas of the brain showed that although there was 5 times more DA in the corticostriatal slice than in the corticolimbic slice, less DA was mobilised (0.5% approximately as compared with 2.5% Table 9). This suggests that there may be stores of DA not mobilisable by electrical stimulation.

Conditioning significantly reduced DA and NA released from the corticolimbic slices (Table 9). As shown in Table 8, the total amine figures for naive and conditioned treatment groups are not significantly different from one another, therefore conditioning in some way appear to alter the ease with which these amines can be mobilised. The data for noradrenaline are not as convincing as that for dopamine, when the corticostriatal data are examined, and further work needs to be done to more tightly characterise the regions of the brain examined in the corticostriatal slices by coring S13 and S14 slices and repeating this experiment.

The reduction of amines released after conditioning treatment may be related to the observation that rats, once conditioned, remain conditioned indefinitely without reinforcement. It is apparent that some permanent alteration in the CNS needs to occur for this to happen. It would be shortsighted to assume that this alteration relates to the releasability of NA and DA only. There may be several other factors which are influencing this release. The effect could be due to neuromodulation by substances such as gamma aminobutyric acid (GABA) or an alteration in the 5HT levels, or even peptides, now considered to be involved in memory processes.

When conditioned rats were treated with haloperidol, (and their behaviour extinguished) a decrease in the release of DA from rat brain corticolimbic slices was observed. Total amine levels in the slices did not appear to be significantly altered by drug treatment. The drug thus may be said to influence "releasability", in the same direction as was observed when naive and conditioned rat brain slice release figures were compared i.e. a reduction in released amine occurs.

The results from corticostriatal slices after haloperidol treatment showed no change in DA or NA releasability, but as this slice appears to have a significant "unreleasable" pool of DA, a more defined slice is desirable before any discussion of release pattern changes subserving conditioning, and subsequent modification by drugs (which extinguish such conditioned behaviour), can be justified.

That haloperidol is acting on a "modified releasable pool" is supported by the lack of effect of haloperidol on DA release from naive untreated rats. An explanation of haloperidol effects in terms of receptors cannot utilise the receptor blockade theory. It is possible that haloperidol is affecting release indirectly as it is already acting on a release pattern modified by conditioning. The effect of haloperidol on NA release shows an anomaly (see Table 9). The NA results were equivocal for technical reasons, associated with S₁ and S₂ stimulations; modifications to the superfusion system and small electrode chamber designs are underway to eliminate the problems of 'bubbles' which are a major cause of distortion of figures for S₁ and S₂.

The increase observed in DA and NA released from brain slice sections of trazodone treated rats shows a dramatically different drug effect from that reported for haloperidol. Both

drugs extinguish conditioning, but their effects at the molecular level appear to be opposite. The ability of trazodone to enhance release of DA shows the same effect on endogenous amine release, as was reported for preloaded ^3H -DA release (p131).

It is possible that the blocking effect of trazodone on presynaptic dopamine receptors increased DA and NA release from their respective presynaptic sites.

After trazodone treatment the release patterns which emerged reflected the original release pattern shown by naive rats. The deconditioning effect of trazodone may re-establish the releasable pools of neurotransmitter which have been suppressed or inhibited as a result of the conditioning process.

(Haloperidol appears to act differently.) The effect of trazodone on naive rats' brain release patterns is currently under test.

The DOPAC results presented in Table 12 (p188) showed that basal release was consistently very low from all slice sections and all treatments. As the slice sections were preincubated with the irreversible monoamine oxidase inhibitors (MAOI), pargyline little change in DOPAC levels had been expected. However upon electrical stimulation, DOPAC appeared to be "released" in association with DA and NA into the superfusate fractions, although variations in SEM were very wide (unlike those for NA and DA). Furthermore, significantly more DOPAC was released from corticostriatal slice sections which were seen to possess 5 times more DA than in corticolimbic slice sections. It is possible that electrical stimulation under these conditions may result in a dissociation of the MAOI from its enzyme. Elevated DOPAC values may result from the re-uptake of released DA and its subsequent conversion to DOPAC by the MAO. In future, other MAOI will be tested and their efficacy monitored by measuring DOPAC

and HVA levels in superfusates. Another modification intended is the addition of MAOI to the superfusing fluid throughout the experiment.

Future studies

1. In order to verify that electrically stimulated release of endogenous amine is strictly analogous to in vivo synaptic release, the dependence of endogenous NA and DA release on calcium ions needs to be established.
2. In the particular technique of slicing as described in this thesis, inevitably swelling occurs. It would be preferable for release data to be expressed on a slice protein basis rather than fresh slice weight basis.
3. It has been postulated that conditioning resulted in a 'reduced releasable pool' of neurotransmitters and that trazodone re-established the pattern of release found in the naive animal. The effect of trazodone on the release of NA and DA from naive brain slice sections is under investigation in order to define releasability more tightly.
4. The cortical tissue contribution to the slices from specific areas of rat brain has complicated the interpretation of results. A suitable coring device is now available and future studies will be conducted on slices with a more highly defined neuroanatomical constitution.
5. With extended HPLC facilities further release studies will be carried out with reference to 5HT which plays an intimate part in the neurotransmitter balance in limbic and striatal systems.

Trazodone is known to block 5HT receptors, and therefore a deeper understanding of its mode of action necessitates the inclusion of 5HT estimations in future release studies.

SECTION 5: GENERAL DISCUSSION

Many of the drugs influencing human mental functions and animal behaviour appear to act by interfering with central monoamine neurotransmission. Over the past 20 years evidence has accumulated implicating these monoamines as neurotransmitters in the nervous system. In this thesis an original approach was adopted relating neurotransmission to conditioned behaviour and how this may be modified using psychoactive drugs.

An ideal antipsychotic drug would be one which would antagonise psychotic behaviour, but not normal behaviour, so that there would be an elimination of psychotic behaviour and a replacement of it by normal behaviour. For this reason the effect of drugs on both normal and abnormal (conditioned) rat behaviour has been studied.

The use of the conditioned avoidance behavioural system to condition rats induced a learned response which was apparently permanent. This learned response was induced in a highly circumscribed environment under the control of the operator and each individual rat had to respond within the same environmental confines. This artificially induced learned behaviour can be scheduled exactly as required and this may be done by altering the timing of each stimulus. A random intertrial period might be an appropriate modification to ensure that any unrecognised stimulus was randomised. The controlled conditioned response of the rat was superimposed upon its natural response, which is to escape. Under these conditions, a behavioural conditioned response was induced which was affected specifically by neuroleptics which modify the artificially induced behaviour, without affecting the natural escape response. These drugs must be affecting the microenvironments of those regions of the brain

which have been permanently adjusted in order to attain a specific behaviour which is imprinted from inception. These patterns of behaviour may be mediated by different functions. There was disparity between the effects of chronic haloperidol and chronic trazodone treatment on avoidance responding. Haloperidol treatment resulted in a graded increase in the average waiting time signifying that the rats were responding more slowly to the conditioning stimulus, whereas trazodone treatment resulted in the conditioned response being maximally extinguished from the start of the experiment and remaining so over the period of treatment. This might relate to differences in the bioavailability of the two drugs in the CNS. (100 times more trazodone is required to extinguish the response, but the extinction is achieved within a much shorter period of time). The mechanisms by which the drugs modify learned behaviour may be different (for example, it is known that trazodone is a powerful 5HT blocker).

In this thesis the areas of the brain investigated were those shown by Thompson (1978) to be associated with a learned response. He lesioned specific areas of the brains of animals which had attained a learned response and showed that learning was subsequently lost. These lesioning studies may have wiped out the 'memory' of the learned response, whereas in this study only recall was temporarily affected. This is an important distinction to be made. Furthermore it is not possible to say if memory and recall are subserved by the same chemical correlates which interact to produce the conditioned avoidance response.

The release of endogenous amines was measured in conditioned rats chronically treated with the drug; these rats were sacrificed at the time of total extinction of the recall of learned behaviour. Conditioned rats acutely treated with drugs

were not studied. Such a study of the chemical correlates underlying behaviour from the time of extinction through the 24 hours before total recall re-emerges, would shed light on the correlation between behaviour and releasability of specific transmitters. Thus any effect resulting from drugs accumulating in the CNS would be avoided.

To analyse the influence of the two drugs on the release of neurotransmitters from specific areas of the brain, two neurotransmitter release systems were employed, namely the preloaded radiolabelled neurotransmitter studies and the endogenous DA, NA and DOPAC release studies. Most of the studies carried out to evaluate the mechanism of psychoactive drugs on transmitter release have utilised preloaded labelled amines prior to stimulation. Using these techniques, theories for the modulation of amine release have been suggested. However the interpretations of the data using preloaded tissue require assumptions which are not encountered in endogenous amine release studies. These assumptions are that the labelled amine mixes uniformly and proportionately with all pools within the nerve terminals and that the labelled amine is taken up selectively by its own terminal. There are data which suggest that labelled amines may be taken up by neurons that do not normally contain the amine (Snyder & Coyle, 1969). These studies suggest that the release of labelled amines may not be comparable to that of the endogenously released amines. However, as with all concepts, alternative evidence has been produced for example by de Langden and Mulder (1979) who showed that ^3H -DA is only taken up in vitro by structures which other studies suggest contain dopaminergic terminals. Their studies showed that in striatal homogenates only the synaptosomal fraction would accumulate ^3H -DA, furthermore, 90% is not metabolised even in the absence of

monoamine oxidase inhibitors, suggesting that it is contained in a pool of 'bound' vesicular dopamine rather than in a pool of free cytoplasmic dopamine.

In the preloaded ^3H -dopamine release studies trazodone produced a highly significantly increase in release of tritium whereas haloperidol produced a decrease in tritium release. It is not possible to make direct comparisons between these results and those obtained for the release of endogenous DA from brain slices of conditioned rats chronically treated with haloperidol or trazodone because ^3H -DA may not be taken up into dopaminergic terminals and therefore not comparable to endogenous DA release. Secondly as the results show, the naive rat cannot be compared with the conditioned rat as their releasability patterns are different. This interesting new finding needs further investigation.

The results from endogenous neurotransmitter release studies showed that conditioning alone changed the release pattern of both DA and NA (decreased release). It could be argued that the learning process has produced a 'permanent' change in the microenvironment of specific parts of the brain. However, trazodone (which extinguished the learned response) re-established the naive pattern from both areas of rat brain (increased release). The trazodone results, in both release systems, follow the same pattern (enhanced release of DA). Haloperidol, however, selectively reduced DA release in corticolimbic areas (CF trazodone which increased DA release).

It is clear that no general statement can be made about the relationship between the effects of these drugs on conditioned behaviour and release patterns in brain slices, but it can be stated that learning may be reflected in an alteration in release patterns which can be reversed by trazodone treatment when naive

patterns are re-established. With haloperidol the further inhibition of endogenous release appears to amplify changes brought about by conditioning. It would be naive to believe that only DA and NA release were influenced by conditioning and drug treatment. Other transmitters such as 5HT and modulators such as GABA may be playing an active role. These original observations have led to further studies on the conditioned brain.

In the earlier part of the thesis the concept of presynaptic receptors and the control of neurotransmitter release has been introduced. It seems clear that besides the chemical postsynaptic receptors which mediate effector responses there may also be present on the presynaptic elements receptors which act to modulate presynaptic neurotransmitter release, this ultimately producing the effector response (i.e. conditioned behaviour). It may be that the observed changes in release patterns brought about by conditioning or drug treatment are modulated via presynaptic receptors. Using cored slices experiments are intended to observe the neurotransmitter release patterns over the 14 day period of training starting from day 1, to establish whether the decrease in neurotransmitter release occurs as the learning skill is acquired.

Studies are at present being carried out in this laboratory to see if the actual conditioning 'pathway' process may be traced using the 2-deoxyglucose (2DG) technique (Sokoloff, Reivich, Kennedy, DesRosiers, Patlak, Pettigrew, Sakurada & Shinohara, 1977). This technique involves the tail vein injection of radioactive 2DG which, once it enters the active site in the rat CNS is prevented from being metabolised any further and therefore could be detected in the brain sections by autoradiography. With this technique it may be possible to localise those areas of the brain involved in conditioning if these regions are more

metabolically active than normal.

Results from the conditioned avoidance system poses the problem whether the use of positive reinforcement or reward training procedures would produce similar release patterns. If 'learning' in a operant conditioning system is universal with respect to its chemical correlates, different areas of the brain more finely localised, for example hippocampal slices, might be investigated and light shed on the biochemical basis of memory and recall. Furthermore the analysis of neuroleptic drug action on release patterns in various parts of the brain will enlarge the profile of data on mechanisms of neuroleptic drug action.

In conclusion, although DA and NA release has been shown to be affected by conditioning and further modulated by psychoactive drugs, the relative importance of DA and NA for these functions needs additional clarification. Such a clarification is hampered by the interaction of these transmitters with various other transmitters and neuromodulators. In the CNS at least 20 putative transmitters have been shown to exist, many of these may have presynaptic autoregulatory functions and could take part in affecting even the simplest form of a response. It is not possible to predict how these transmitters may be interacting to induce the conditioned response and the manner in which they may be affecting the release of NA and DA from those regions of the brain.

Many studies have shown the interactions between the various transmitter systems. Some of these are enkephalins, inhibiting acetylcholine and dopamine release from the striatum (Subramanian, Mitznegg & Sprugal, Dömschke, Dömschke, Wunch & Demling, 1977), dopamine increasing GABA release from rat substantia nigra (Reubi, Iversen, Jessell, 1977), GABA stimulating NA release from occipital cortex (Arbilla & Langer,

1978), and glutamate stimulating DA release from the rat striatum (Giorguieff, Kemel & Glowinski, 1977).

The difficulties of understanding the functional interactions between neurochemical events in the brain and behaviour are immense. The association of one or two neurotransmitters with behaviour is much too restrictive. With the development of sensitive techniques for the simultaneous analysis of several neurotransmitters it may become possible to study the interactions between these substances, and correlate such interactions with behaviour.

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Appendix: Computer programmes for
analysis of release data.
(See Section III)
(Written in fortran).

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1 CH HASH 20/07/81U CPS & VOLUME WITH DECAY CORRECTION
2 PROGRAM HASH
3 DIMENSION T(4),ID(2),CH(3,4),C(4),CJ(3)
4 DIMENSION BUF(100)
5 DATA IY/1HY/
6 C DECAY FACTOR (6 HOUR HALF-LIFE, IN MINUTES)
7 X=0.693/360.
8 IP=3
9 CALL OFRW(12,10HDATA/HASH..BUF,J)
10 WRITE(1,10)
11 10 FORMAT(17HNUMBER OF SAMPLES)
12 READ(1,*)N
13 WRITE(1,15)
14 15 FORMAT(' VOLUME OF STANDARDS ')
15 READ(1,*) SVOL
16 WRITE(1,20)
17 20 FORMAT(14HCHANNEL NUMBER)
18 READ(1,*)M
19 C READ IN BACKGROUND
20 DO 6 J=1,2
21 READ(IP,50) ID,T(J),(CH(I,J),I=1,3)
22 6 C(J)=CH(M,J)/T(J)
23 BKG=(C(1)+C(2))/2.
24 C READ IN STANDARDS
25 210 DO 8 J=1,2
26 READ(IP,50) ID,T(J),(CH(I,J),I=1,3)
27 8 C(J)=CH(M,J)/T(J)
28 STD=(C(1)+C(2))/2.
29 TIME=0.
30 N=N+2
31 WRITE(9,240) BKG, STD,SVOL
32 240 FORMAT(1H1,/' BACKGROUND = ',F10.3,12X,' STANDARD = ',F10.3
33 + ', (C.P.S.)/' STD. VOL = ',F5.2, ' ML.'/'/' NO. ID C'

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34      + COUNTING TIME   C.P.S'
35      + , ' DECAY CORR.   C.P.S   VOLUME /10(8H-----))
36 C NOW READ IN THE SAMPLES AND DO CALCULATION
37      DO 100 J=1,N
38      READ(IP,50) ID,FT,(CJ(I),I=1,3)
39      50 FORMAT(2A2,F8.2,3F7.0)
40      CC=CJ(M)/FT
41      TIME=TIME+FT/60.
42      DF=EXP(-TIME*X)
43      CPS=CC/DF
44      VOL=SVOL*CPS/STD
45      WRITE(9,150) J,ID,FT,CC,DF,CPS,VOL
46      WRITE(12,155) J,ID,VOL
47      155 FORMAT(2X,I3,3X,2A2,F11.3)
48      150 FORMAT(2X,I3,3X,2A2,5X,5F11.3)
49      100 CONTINUE
50      VOL=-10.
51      WRITE(12,155) J,ID,VOL
52      WRITE(1,160)
53      160 FORMAT(' ANOTHER GROUP ON THIS TAPE ??')
54      READ(1,162) IJ
55      162 FORMAT(A1)
56      IF(IJ.EQ.IY) GOTO 200
57      WRITE(12,155) J,ID,VOL
58      ENDFILE 12
59      STOP
60      200 WRITE(1,10)
61      READ(1,*) N
62      WRITE(1,20)
63      READ(1,*) M
64      GOTO 210
65      END

```

```

1  C# HASH2  14/07/81G COMBINE H-3 AND TC-  VOLUME DATA
2      PROGRAM HASH2
3      DIMENSION BUF(200),IALN(60,4)
4      DIMENSION BUF2(200)
5      DIMENSION TC(60),CLB(60),CLM(60),VOL(60)
6      DIMENSION CJ(3),ID(2)
7      DIMENSION FRED(200)
8      CALL OFRO(12,10HDATA/HASH.,BUF,J)
9      WRITE(1,10)
10     10 FORMAT(' TRITIUM CHANNEL ?')
11     READ(1,*) IC
12  C READ BACKGROUND, AND SKIP SAMPLES 3 & 4
13     15 READ(3,50) ID,FT,(CJ(I),I=1,3)
14     BKG=CJ(IC)/FT
15     READ(3,50) ID,FT,(CJ(I),I=1,3)
16     BKG=(BKG+CJ(IC)/FT)/2.
17  C SKIP 3 & 4
18     17 DO 20 J=1,2
19     20 READ(3,50) ID
20  C NOW MOVE THROUGH SAMPLES IN GROUPS
21     K=0
22     30 K=K+1
23     READ(12,52) IN,ID,VOL(K)
24     52 FORMAT(2X,I3,3X,2A2,F11.3)
25     IF((VOL(K).LT.0.).AND.(K.EQ.1)) STOP
26     IF(VOL(K).LT.0.) GOTO 56
27     IALN(K,1)=ID(1)
28     IALN(K,2)=ID(2)
29  C READ H-3 VALUE
30     READ(3,50) ID,FT,(CJ(I),I=1,3)
31     IALN(K,3)=ID(1)
32     IALN(K,4)=ID(2)
33     50 FORMAT(2A2,F8.2,3F7.0)

```

```

34      CC=CJ(IC)/FT
35      TC(K)=CC
36      GOTO 30
37      56 K=K-3
38 C WE NOW HAVE A GROUP OF K SAMPLES
39 C
40 C  PROCESS THIS GROUP OF SAMPLES
41 C
42      SUM=0.
43      WRITE(9,110)
44      DO 60 J=1,K
45      CLB(J)=TC(J)-BKG
46      TC(J)=CLB(J)
47      60 SUM=SUM+VOL(J)
48      AVOL=SUM/FLOAT(K)
49      DO 70 J=1,K
50      IF(VOL(J).LT.AVOL) GOTO 65
51 C VOL GREATER THAN AVERAGE, PASS FORWARD
52      C=CLB(J)*AVOL/VOL(J)
53      CLB(J+1)=CLB(J+1)+CLB(J)-C
54      GOTO 66
55 C VOL LESS THAN AVERAGE, BORROW
56      65 IF(J+1.GE.K) GOTO 64
57      CBORR=CLB(J+1)*(AVOL-VOL(J))/VOL(J+1)
58      C=CLB(J)+CBORR
59      CLB(J+1)=CLB(J+1)-CBORR
60      GOTO 66
61      64 C=CLB(J)
62      66 CONTINUE
63      VOL(J+1)=VOL(J+1)+VOL(J)-AVOL
64      CLM(J)=C
65      WRITE(9,100)(IALN(J,L),L=1,4),TC(J),VOL(J),AVOL,CLB(J),CLM(J)
66      70 CONTINUE

```

```

67 110 FORMAT('1 TC-NO. H3-NO. COUNTS      VOL      MEAN V.      CO'
68      +      , 'RR C.      NET COUNTS')
69 100 FORMAT(4X,2A2,2X,2A2,7F10.3)
70 C
71 C      NOW PLOT THE GRAPH
72 C
73      CALL ORIG(200,200,BUF2,400)
74      CALL MOVE(0,600)
75      CALL JOIN(0,0)
76      CALL JOIN(800,0)
77      AMAX=-100
78      DO 105 J=1,K
79      IF(AMAX.LT.CLM(J)) AMAX=CLM(J)
80 105 CONTINUE
81      SC=450./AMAX
82      DO 120 J=1,K
83      IX=35+J
84      IY=CLM(J)+SC
85      CALL MOVE(IX-2,IY-2)
86      CALL JOIN(IX+2,IY+2)
87      CALL MOVE(IX-2,IY+2)
88      CALL JOIN(IX+2,IY-2)
89 120 CONTINUE
90      CALL DRAW
91      GOTO 17
92      END

```

A modified procedure for electrically stimulating brain slices from conditioned rats

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Putative antipsychotic drugs are commonly evaluated in rats conditioned to respond to specific stimuli. We have employed this system to correlate the effect of drugs on conditioned behaviour with neurotransmitter changes in brain slices.

A modified version of a brain slicer (Brown & Halliwell, 1981) is used to cut reproducible coronal slices from specific regions of the rat brain. Regions selected were those demonstrated by stereotaxic lesioning procedures to be involved in conditioned avoidance learning mechanisms (Thompson, 1978). Each test slice is transferred to a micro-electrode chamber, superfused with Krebs bicarbonate saline and stimulated for a 2 min period via gold electrodes, using square wave impulses of alternating polarity (10 sec, 2 msec, 20 mA). One of the released transmitters, dopamine, is estimated by radioenzymic assay.

In order to test the Ca^{2+} -dependence of this depolarization-induced release, we preloaded slices with [^3H]dopamine and carried out experiments with and without Ca^{2+} in the superfusing medium. The volume of each superfusate fraction was monitored using $^{99}\text{Tc}^{\text{m}}$. The results show that this electrically-stimulated release of [^3H]dopamine is Ca^{2+} -dependent. This technique provides a useful tool to study the influence of haloperidol and trazodone on the release of neurotransmitters from naive and conditioned rats.

Our thanks to K. Jeyasingh for advice and help with $^{99}\text{Tc}^{\text{m}}$ measurements (Department of Nuclear Medicine).

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THE EFFECTS OF TRAZODONE ON THE ELECTRICALLY STIMULATED RAT BRAIN SLICES PRELOADED WITH (³H) DOPAMINE

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Trazodone has an unusual spectrum of pharmacological activity, and there appear to be discrepancies in published data with respect to brain dopamine levels (Stefanini et al, 1976 ; Boissier et al, 1974). However, it is agreed that, in common with neuroleptics, it eliminates the conditioned response, leaving the escape response intact in the classical avoidance escape paradigm (Gatti, 1973) which suggests that dopaminergic tracts are involved.

The release of tritium from brain slices pre-incubated with ³H dopamine by electrical stimulation has been studied by several investigators, e.g. (Dismukes & Mulder, 1977). To study the effect of trazodone on electrically mobilisable radiolabel from rat striatal slices preloaded with ³H dopamine, an improved system was recently developed principally for studying the effects of neuroleptics on endogenous dopamine release in conditioned rats (unpublished material). A modified version of a brain slicer (Brown & Halliwell, 1981) was used to obtain reproducible coronal slices from striatal regions. Each test slice in a micro electrode chamber (0.2ml) received 2 min. periods of stimulation via gold electrodes by square impulses of alternating polarity (10's, 2ms, 20mA). The volume of eluate in each superfusate fraction was monitored using standard ⁹⁹Tc^m. Experiments were carried out with and without ³Ca²⁺ in the superfusing media to test the dependency of electrically induced ³H dopamine release on ³Ca²⁺, generally regarded as essential for depolarisation induced release. Results are calculated on the basis of the total tissue radioactivity at the onset of stimulation. The percentage release of tritium from slices superfused with bicarbonate saline containing ³Ca²⁺ was 0.9129 ± 0.3386 (n=9) first stimulation and 0.7006 ± 0.4553 (n=5), second stimulation. For slices superfused with calcium-free solution the figures were 0.0012 ± 0.0015 (n=4) first stimulation, and 0.0007 ± 0.0017 (n=5), second stimulation. The results strongly suggest that the release is ³Ca²⁺ dependent. To measure the effect of trazodone on slices preloaded with ³H dopamine, electrically evoked release in the absence of the drug established control release levels. Subsequently the drug was added to the superfusing medium (0.024mM) prior to the second electrical stimulation. Control release levels were 0.8855 ± 0.4586 (n=4). In the presence of trazodone the release levels were 6.024 ± 3.3945 (n=4).

Preliminary results show that in our modified system, significant release of radiolabel was calcium ion dependent and trazodone apparently increased the proportion of electrically mobilisable ³H dopamine or its metabolites. These results contribute to the puzzle of the pharmacological actions of trazodone on brain dopamine, but do not clarify the issue. The influence of drugs such as trazodone on releasable endogenous dopamine in naive and conditioned animals is currently under test.

Thanks to K. Jeyasingh for advice and help with ⁹⁹Tc^m measurements (Department of Nuclear Medicine).

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