UNIVERSITY OF SOUTHAMPTON

A Neuropharmacological Study of Central Dopamine Receptors

by

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CONTENTS

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ABSTRACT

FACULTY OF SCIENCE

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Doctor of Philosophy

A NEUROPHARMACOLOGICAL STUDY OF CENTRAL DOPAMINE RECEPTORS

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The experiments in this thesis describe the actions of some novel dopamine agonists and antagonists on the neuronal activity of extracellularly recorded cells in the substantia nigra zona compacta in vivo and in vitro.

The dopamine agonists, apomorphine, piribedil, S-3608, RU24213 and RU24926 all potently inhibited the neuronal activity of the substantia nigra neurones, when given intravenously. Piribedil and S-3608 were short acting agonists, whereas RU24213 and RU24926 were long acting, both with a two phase recovery. A second dose of either RU24213 and RU24926 or apomorphine produced a tachyphylactic effect, which reduced the inhibitory agonist responses.

The dibenzoyl ester of ADTN, synthesised as a lipid soluble prodrug of ADTN, failed to have any dopaminergic agonist activity on the substantia nigra cells, when given intravenously, at extremely high doses.

In addition, when tested on the hyperactivity induced by dopamine agonists, directly injected into the nucleus accumbens, S-3608 and RU24213 did not induce any locomotor activity. While RU24926 produced a weak and short stimulation of locomotor activity compared with ADTN.

A newly proposed dopamine antagonist, zetidoline, given intravenously, antagonised the inhibitory effect of apomorphine whereas the dopamine antagonist, sulpiride, at very large doses, failed to antagonise apomorphine inhibitions. Applied iontophoretically, sulpiride and zetidoline antagonised the responses to iontophoretically applied dopamine and noradrenaline, but zetidoline did not affect the responses to the neurotransmitters, glutamate, GABA and glycine. On cerebellar neurones, sulpiride failed to antagonise the responses to dopamine and noradrenaline. Zetidoline applied iontophoretically was directly depressant on cerebellar neurones.

In the in vitro nigral brain slice, zetidoline was a competitive antagonist of the inhibitory actions of dopamine with a pA_2 of 7.02.

In contrast to its potent and selective activity in inhibiting dopamine responses on substantia nigra neurones, zetidoline, in the ADTN-induced locomotor test for dopaminergic activity, was weakly active as an antagonist. Sulpiride, however, was a potent antagonist in this test.

Acknowledgements

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Abbreviations

A	Amphere
ACh	Acetylcholine
AChE	Acetylcholine esterase
ADTN	2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene
AMPT	α-methylparatyrosine
Apo	Apomorphine
C	Centigrade
cAMP	Cyclic adenosine 3',5'-monophosphate
CAT	Choline acetyltransferase
CCK	Cholecystokinin
CNS	Central nervous system
COMT	Catechol-o-methyltransferase
c.s.f.	Cerebrospinal fluid
DA	Dopamine
DBADTN	2-amino-6,7-dibenzoyl-1,2,3,4-tetrahydronaphthalene
DBisoADTN	2-amino-5,6-dibenzoyl-1,2,3,4-tetrahydronaphthalene
DOPAC	
EC_{50}	Concentration required to stimulate by 50%
EOS	Ethanolamine-o-sulphate
EPSP	Excitatory postsynaptic potential
GABA	γ-amino butyric acid
HRP	Horseradish peroxidase
5-HT	5-hydroxytryptamine
HVA	Homovanillic acid
Hz	Hertz
IC_{50}	Concentration required to inhibit by 50%
i.m.	Intramuscular
i.p.	Intraperitoneal
IPSP	Inhibitory postsynaptic potential

Additional Abbreviations

DOPAC	Dihydroxy phenyl acetic acid
GBL	Gamma buterolactone
RU24213	N-n-propyl-N-phenylethyl-p-(3-hydroxyphenyl)-ethylamine
RU24926	N-n-propyl-di-β-(3-hydroxyphenyl)-ethylamine
S-3608	4(5-coumaranyl methyl)-1-(2-thiazolyl) piperazine
SKF 38393	2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine
YM 091512	cis-N-(1-benzyl-2-methyl-pyrrolidin-3-yl)-5-chloro-2-methoxy-4-methyl amino benzamide

isoADTN	2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene
i.v.	intravenous
L-DOPA	L-dihydroxyphenylalanine
MAO	Monoamine oxidase
MFB	Medial forebrain bundle
3-MT	3-methoxytyramine
n	number of animals used
NA	Noradrenaline
NACl	Sodium chloride
6-OHDA	6-hydroxydopamine
3-PPP	3-(3-hydroxyphenyl)-N-n-propylpiperidine
PRL	Prolactin
SEM	Standard error of the mean
SN	Substantia nigra
SNC	Substantia nigra zona compacta
SNL	Substantia nigra zona lateralis
SNR	Substantia nigra zona reticulata
U.V.	Ultraviolet
Vol	Volume
VTA	Ventral tegmental area

The standard abbreviations for units used in this thesis are in accordance with Biochemical Pharmacology (1984) Vol. 33, iii-iv.

Chapter 1

1 Introduction

1.1 Dopamine as a neurotransmitter

The idea of neurochemical transmission was first postulated by Elliot (1904; 1905), who suggested that adrenaline might be "the chemical stimulant liberated on each occasion that a nerve impulse arrives at the periphery", and that the liberated adrenaline might cause a change in tension of a muscle fibre. Since then the concept of neurochemical transmission has been developed and is now widely accepted with a variety of transmitter substances proposed.

The first suggestion that dopamine (DA) could be a transmitter was made by Blasckho (1957) on the basis of its abundance in the periphery. However, before classifying a substance as a neurotransmitter, certain criteria must be fulfilled. These criteria were outlined by Werman (1966). Thus, a proposed neurotransmitter must (i) be present and stored within the nerve endings; (ii) have its synthesising enzymes nearby; (iii) be released on nerve stimulation and cause its physiological effect; (iv) mimic the actions of the natural transmitter; (v) have an inactivation mechanism; (vi) be manipulated pharmacologically and correlate with the endogenous transmitter.

These criteria have been mostly fulfilled for dopamine.

1.1.1 Presence in nerve endings

Dopamine was found to be present in the brain in approximately equal amounts to noradrenaline (NA) (Montagu, 1957; Wiel-Malerbe and Bone, 1957). Later, DA was found to have a different regional distribution to NA, DA being most concentrated in the corpus striatum (Bertler and Rosengren, 1959; Carlsson, 1959). This supported the suggestion that DA may have a separate neurotransmitter role in the brain, rather than being solely a precursor of NA and adrenaline synthesis.

The development of histochemical techniques (Eränko, 1955) for the visualisation of catecholamines in the adrenal medulla, was extended for use on nervous tissue (Falck, Hillarp, Thieme and Torp, 1962). This technique was used to demonstrate that the catecholamines were located

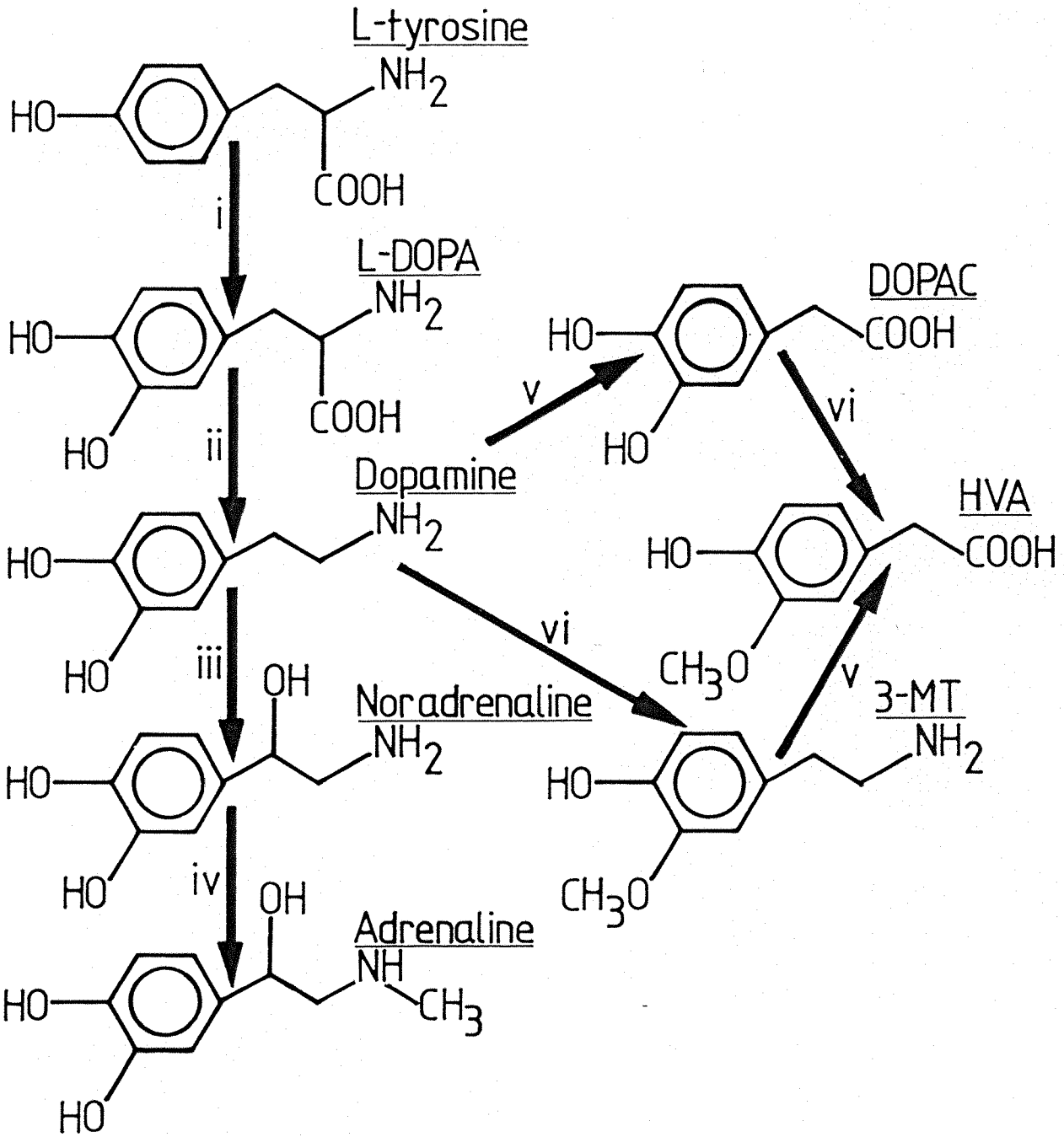


Figure 1.1 The major synthetic and metabolic pathways of catecholamines

(i) tyrosine hydroxylase, (ii) L-dopa decarboxylase, (iii) dopamine-β-hydroxylase, (iv) phenylethylamine-N-methyl transferase, (v) monoamine oxidase (vi) catechol-o-methyl transferase.

DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid;

3-MT = 3-methoxytyramine.

intraneuronally (Carlsson, Falck and Hillarp, 1962; Dahlstrom and Fuxe, 1964) and especially within axonal terminals. In addition, they showed that reserpine, which depletes the catecholamines in storage granules, abolishes the fluorescence in these areas.

1.1.2 Biosynthesis and metabolism

The synthetic pathway for catecholamines was first proposed by Blasckho (1939). The precursor of the synthesis is the amino acid, L-tyrosine, which is actively transported into dopaminergic neurones. This is hydroxylated by tyrosine hydroxylase to form L-dihydroxy phenyl alanine (L-DOPA) (see figure 1.1). This enzyme is thought to be the rate limiting step in the synthesis of dopamine; high levels of dopamine causing end-product inhibition of the enzyme (Levitt, Spector, Sjoerdema and Undenfriend, 1965). Dopamine is thought to compete with the pterin co-factor required by this enzyme. L-DOPA is then hydroxylated by L-DOPA decarboxylase to dopamine, which is then stored in storage vesicles. Both these enzymes are present in the cytoplasm of dopamine neurones. In noradrenergic neurones, dopamine is further hydroxylated at the β carbon atom by dopamine β-hydroxylase. This enzyme is membrane bound and requires dopamine to be present in storage granules before it is converted. In dopaminergic neurones this enzyme is absent and so dopamine is stored in these granules in a physiologically inactive form, chelated with ATP and magnesium. McGeer and McGeer (1974) have thoroughly reviewed this area.

When DA is released into the synaptic cleft and causes its postsynaptic effect, it must be inactivated. Dopamine is subject to a specific reuptake mechanism by the neurones (see review Horn, 1978), or it can be metabolised by either of two major degradative pathways (figure 1.1). Metabolism may be intraneuronal by monoamine oxidase (MAO), which oxidises the ethylamine side chain to give 3,4-dihydroxy phenylacetaldehyde, followed by conversion to 3,4-dihydroxy phenylacetic acid (DOPAC). Alternatively it can be metabolised by the extraneuronal enzyme, catechol-o-methyltransferase (COMT), which methylates the 3-hydroxyl group to form 3-methoxytyramine (3-MT). Both DOPAC and 3-MT can be metabolised further by COMT or MAO respectively to produce homovanillic acid (HVA). In addition, DA can be conjugated with glucuronic acid or sulphuric acid.

1.1.3 Release and neuronal responses

The release of a putative transmitter is an important prerequisite that must be satisfied before a substance can be considered a neurotransmitter. McLennan (1964,1965), using push-pull cannulae implanted into the striatum, showed that electrical stimulation of the substantia nigra could increase the dopamine output over its resting levels. Von Voigtlander and Moore (1971) confirmed this finding by showing that the amount of [^{3}H]-dopamine released into the cerebral ventricles increases on stimulation of the substantia nigra zona compacta and that this effect was frequency dependent. Additional indirect evidence came from observations on the changes in the metabolites of dopamine following lesions or the administration of drugs that alter neuronal impulse flow along dopamine neurones. Thus, γ-buterolactone, which blocks impulse flow, results in a decrease in the amounts of DOPAC detected in the striatum (Roth, Walters and Aghajanian, 1973), while neuroleptic which cause increases in the firing rate of nigrostriatal neurones also result in an increase in the dopamine metabolites found in the striatum (Bunney, Walters, Roth and Aghajanian, 1973).

Another key criterion to be demonstrated is that of identical action. Thus, the putative transmitter must evoke the same synaptic response as the endogenous transmitter. Connor (1970) showed in cats, that stimulation of the substantia nigra depressed the firing frequency of neurones in the caudate nucleus, as measured by a post-stimulus histogram. These cells were consistently inhibited by microiontophoretically applied dopamine. Dopamine had previously been found to be inhibitory when applied by microiontophoresis (Bloom, Costa and Salmoiraghi, 1965).

1.2 The anatomy of dopaminergic pathways in the CNS

Following the discovery of dopamine and other biogenic amines intraneuronally, studies were undertaken to establish the organisation of the central dopaminergic pathways. Using the Falck-Hillarp technique, the fluorescence in serial sections could be followed through the brain (Dahlström and Fuxe, 1964). In combination with mechanical and chemical lesioning techniques, using 6-hydroxydopamine (6 OHDA), the accumulation and disappearance of the fluorescence provided further

information about these pathways in the CNS (Anden, Dahlstrom, Fuxe, Larsson, Olson and Ungerstedt, 1966; Ungerstedt, 1971).

The introduction of a more sensitive method, employing glyoxylic acid to induce the fluorescence, in combination with a vibrotome sectioning system (Lindvall and Bjorklund, 1974), revealed previously unknown dopamine neuronal pathways. Several other techniques have been developed in recent years for neuroanatomical studies. These include horseradish peroxidase (HRP) tracing (Nauta, Pritz and Lasek, 1974), Fink-Hiemer staining of degenerating axons (Heedren and Chalmers, 1972) and the immunohistochemical detection of tyrosine hydroxylase using a specific antiserum (Hokfelt, Johansson, Fuxe, Goldstein and Park, 1976). These techniques have identified and confirmed the central dopaminergic pathways in the brain. This area has been fully reviewed by Lindvall and Bjorklund, 1978; Moore and Bloom, 1978.

The dopaminergic pathways are organised into three major functional systems in the forebrain (Ungerstedt, 1971; Lindvall and Bjorklund, 1978; Moore and Bloom, 1978); these are the (i) nigrostriatal, (ii) mesolimbic, (iii) mesocortical systems. Although the divisions are apparent functionally, they are not as strict as were first thought (see below). Figure 1.2 shows the major dopaminergic pathways in the brain.

The nigrostriatal pathway was first demonstrated by Andén, Carlsson, Dahlström, Fuxe, Hillarp and Larsson (1964) and originates from the cell bodies located in the substantia nigra zona compacta (SNC), A_9 and A_{10} cell groups of Dahlström and Fuxe (1964). These cells contain approximately 70% of the brain's DA. The pathway ascends through the lateral hypothalamus, the internal capsule and globus pallidus to innervate the caudate-putamen (striatum) (Ungerstedt, 1971). These neurones are thought to terminate onto small cholinergic interneurones (McGeer, Grewaal, and McGeer, 1974; McGeer and McGeer, 1976). However, not all the nigrostriatal neurones contain DA (Feltz and de Camplain, 1972; Beckstead, Domesick and Nauta, 1979).

The mesolimbic pathway arises from the ventral tegmental area (VTA) or A_{10}. These axons ascend in the dorsal portion of the medial forebrain bundle (MFB) immediately ventromedially to the nigrostriatal pathway and project to the nucleus accumbens.

Figure 1.2 The origins and projections of the major dopaminergic pathways in the rat brain

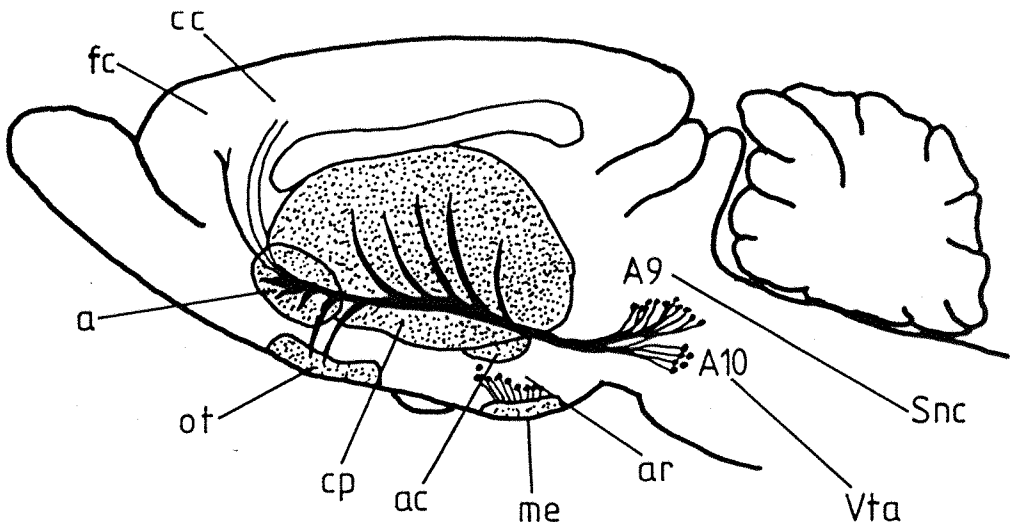
(a) a diagram of the rat brain in the sagital plane, indicating the distribution of the main central dopaminergic neuronal pathways. The stippled area indicates the major nerve terminal areas.

a = nucleus accumbens; ac = central amygdaloid nucleus; ar = arcuate nucleus; cc = cingulate cortex; cp = caudate/putamen, fc = frontal cortex; me = median eminence; ot = olfactory tubercle; snc = Substantia nigra zona compacta; Vta = ventral tegmental area.

(b) A schematic representation of the dopaminergic pathways:

(1) the nigrostriatal pathway; (2) the mesolimbic pathway; (3) the mesocortical pathway; (4) the tuberohypophyseal pathway.

a.


cc
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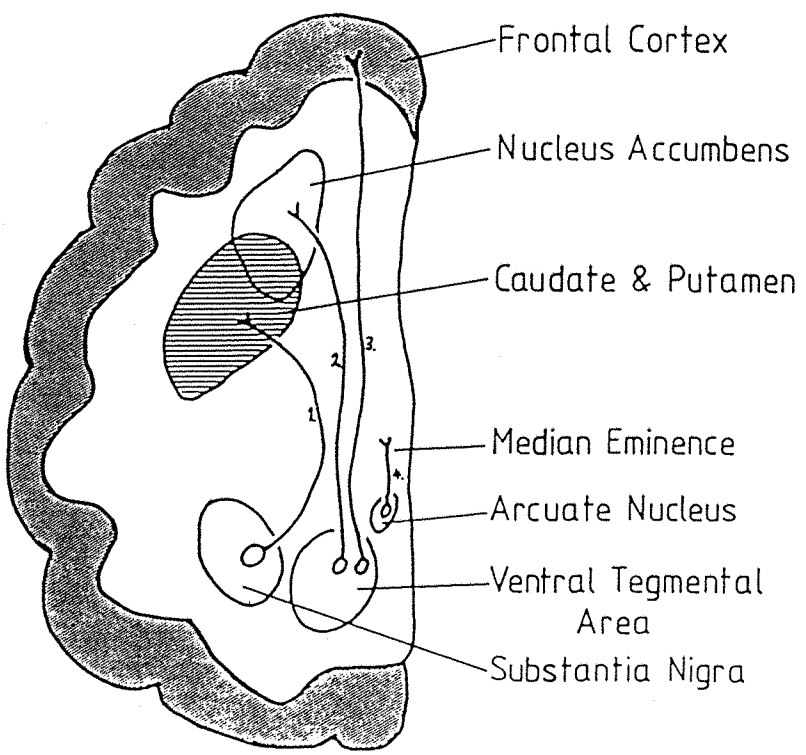
b.


Frontal Cortex
Nucleus Accumbens
Caudate & Putamen
1.
2.
3.
4.
Median Eminence
Arcuate Nucleus
Ventral Tegmental Area
Substantia Nigra

The mesocortical pathway. This term is used to define the neuronal pathway that originates from the cell bodies of the VTA and medial substantia nigra, that innervate all cortical areas other than the basal ganglia. Its projections are divided into two subdivisions: (i) the allocortical pathway, (ii) the neocortical pathway. The mesocortical system has recently been reviewed by Bannon and Roth (1983).

However, recent evidence suggests that the mesencephalic dopamine cells form a continuum of cells and the distinction between the different pathways is not as sharp as originally thought. Biochemical, histochemical and HRP tracing techniques have demonstrated a topographical organisation between the mesencephalic dopaminergic cells and their terminal projections in the basal ganglia and cortical areas (Moore, Bhatnagar and Heller, 1971; Fallon and Moore, 1978a; 1978b; Simon, Le Moal and Calas, 1979). Nevertheless, the distinction between the SNC and VTA remains useful and is supported by a wealth of evidence from behavioural studies that the projections from the SNC and VTA cell groups are involved in different aspects of motor function (Kelly, Seviour and Iversen, 1975).

There are other dopamine containing nervous pathways. The tubero-hypophyseal system which arises in the arcuate and part of the periventricular nuclei. Their axons project to all parts of the median eminence, stalk of the neural lobe and pars intermedia of the adenohypophysis (Bjorklund, Falck, Hrousek, Owman and West, 1970). This pathway exerts an inhibitory effect on the release of prolactin from the pituitary. The retinal system located in the amacrine layer of the retina (Ehringer and Falck, 1969) provides localised innervation. The periventricular system has cell bodies and fibres located in the periventricular, periaqueductal gray of the medulla oblongata and pons (Lindvall and Bjorklund, 1974). There is also an incertohypothalamic system with cell bodies in the zona incerta and periventricular hypothalamus which send projections to the preoptic-anterior hypothalamic region, suggesting an involvement with neuroendocrine mechanisms (Bjorklund, Lindvall and Nobin, 1975). Finally, there are periglomerular neurones located within the olfactory bulb (Dahlström, Fuxe, Olson and Ungerstedt, 1965).

1.3 Dopamine and brain dysfunction

Much of the current interest in DA in the CNS results from the discovery that DA is involved in the aetiology of Parkinson's disease and schizophrenia. Thus, the potential therapeutic use of dopamine mimetic or antagonist drugs to control these diseases is of great clinical importance.

1.3.1 Parkinson's disease

Parkinson's disease, first described by James Parkinson in 1817, is a disorder of the basal ganglia that manifests itself in a number of clinical symptoms. The three major symptoms being (1) tremors of the muscles at rest; (2) akinesia or difficulty in initiating or modifying ongoing motor activity; (3) muscular rigidity or stiffness of the skeletal muscles.

Destruction of the cells in the SN was recognised as the anatomical basis for the disease by Blocq and Marinesco (1893). However, it was not until much later that Parkinsonian patients were discovered to have drastically reduced DA and HVA levels (Ehringer and Hornykiewicz, 1960; Berheimer and Hornykiewicz, 1965) as well as reduced L-DOPA decarboxylase activity (Lloyd and Hornykiewicz, 1970). When the nigrostriatal pathway was discovered and that it utilised dopamine as its transmitter (Anden, Carlsson, Dahlstrom, Fuxe, Hillarp and Larsson, 1964) a greater understanding of the disease was possible.

The introduction of L-DOPA in the treatment of Parkinson's disease followed from experiments that showed reserpine, which depletes DA stores in the brain, causes Parkinsonian-like symptoms and that these could be reversed by administration of L-DOPA (Carlsson et al., 1957). L-DOPA can cross the blood brain barrier (which dopamine cannot) and is converted into DA, thus elevating brain DA content.

The characteristic degeneration of the dopamine nigrostriatal neurones in Parkinson's disease occurs before symptoms are seen. Indeed, a 50% decrease of nigral cells, with age, is normal, but a loss of 75% of these neurones and a striatal DA depletion of 60-80% is required for symptoms to become apparent (Bernhiemer, Birkmayer, Hornykiewicz, Jellinger and Seitelberger, 1973). The degree of DA depletion correlates well with the amount of nigrostriatal destruction. As idiopathic Parkinson's disease is a slow degenerative disorder, adaptive mechanisms

compensate for the loss by the remaining nigrostriatal neurones (see review by Schultz, 1982). In post-mortem brains of Parkinsonian patients an increased amount of [^{3}H]-haloperidol binding is seen in untreated versus treated patients, the treated patients having normal values (Lee, Seeman, Rajput, Farley and Hornykiewicz, 1978). Supersensitivity of striatal dopamine receptors has been suggested as one compensatory effect. In favour of this is the finding that mild cases of Parkinson's disease do not respond as well as severe cases to L-DOPA therapy (Birkmayer, Danielczyk, Neumayer and Riederer, 1975). The pathophysiology and chemotherapy have been fully reviewed by Hornykiewicz (1975) and Riederer, Reynolds and Jellinger (1984).

1.3.2 Schizophrenia

Schizophrenia is a disorder of mental functions involving disturbances of thought processes, emotion, delusions, hallucinations and a withdrawal from interactions with people. Both the striatum and limbic system have been implicated as the cause of the brain dysfunction (Mettler, 1955; Torrey and Petersen, 1974). Evidence implicates dopaminergic system hyperactivity in the pathophysiology of schizophrenia.

Neuroleptics, used to treat schizophrenics, were found to increase DA metabolites in the rat brain (Carlsson and Lindquvist, 1963). Later the discovery that all clinically effective antipsychotics antagonises DA receptor mediated responses gave support to the dopamine hyperactivity hypothesis. Neuroleptic treatment was found to be enhanced by α-methyl paratyrosine (AMPT) treatment (Carlsson, Roos, Walinder and Skott, 1973), but L-DOPA exacerbated psychotic symptoms in schizophrenics (Sathananthan, Angrist and Gershan, 1973). Other supportive evidence came from observations of amphetamine addicts, where a psychotic state indistinguishable from acute paranoid schizophrenia can develop (Connell, 1958), which has been successfully treated with neuroleptics (Angrist, Lee and Gershan, 1974).

The search for a biochemical explanation of schizophrenia has met with little success. In post-mortem brains of schizophrenics, no changes in dopamine turnover have been found (Owen, Cross, Crow, Longden, Poulter and Riley, 1978). However, dopamine receptor binding studies have revealed increased neuroleptic binding in post-mortem brains of schizophrenics in the caudate nucleus and nucleus accumbens (Mackay,

Bird, Iversen, Spokes, Creese and Snyder, 1980). Schizophrenia involves complex changes in behaviour which may be the result of one or more pathological disorder. The evidence for the dopamine hyperactivity hypothesis of schizophrenia has been assessed by Metler and Stahl (1976), Hornykiewicz (1978) and Alpert and Friedhoff (1980).

1.4 The substantia nigra

1.4.1 The morphology and the cytology of the substantia nigra

The substantia nigra was first described by Vicq D'Azyr in 1786, the name reflecting its dark pigmentation seen in primates but not in other species (Marsden, 1961). It extends through the midbrain as two bilateral bands of cells between the cerebral peduncle and the tegmentum (see figure 1.3). The substantia nigra has been divided into three morphological zones, the pars or zona compacta (SNC); zona reticulata (SNR) and zona lateralis (SNL). Three main classes of cells have been identified and are distinguished on the basis of the cell body size, thickness and branching of dendrites and the extent of the dendritic field (Gulley and Wood, 1971; Juraska, Wilson and Groves, 1977). Large (45-75 μm), medium (19-46 μm) and small (11-26 μm) neurones are found in all three areas. The large and medium cells in the SNR and SNL are possibly the source of efferents to other brain areas, their axons are seen to branch on occasions, which is not seen with the medium sized cells in the SNC (Faull and Carmen, 1968; Rinvik, 1975; Faull and Mehler, 1978), see figure 1.4. Small neurones found in all parts of the nucleus are presumed to be inter-neurones. Their dendrites are small and delicate with no directional arrangement to the dendritic field or preferred orientation for their axons, which seem to synapse around other nigral dendrites (Juraska et al., 1977; Gulley and Wood, 1971).

Synaptic contacts are characterised by longitudinal axodendritic arrangements (Schwyn and Fox, 1974). Consequently, numerous contacts can be made "en passage" with a single dendritic process within the pars reticulata, but relatively few made on the soma (Rinvik and Grofova, 1970). Some 90% of synaptic endings comprise large vesicles without dense cores; however, there are many endings that contain dense core vesicles, possibly related to dopamine neurone collaterals or raphé

projections. Axoaxonic synapses have been described on the axon hillock or on other synaptic endings within the substantia nigra (Gulley and Smithberg, 1971).

1.4.1.1 Zona compacta. This area is a densely packed band of cells lying on the dorsomedial aspect and consists primarily of medium sized cells, most of which contain dopamine. These cells can be distinguished from SNR cells by their dendritic branching which form four to six dendrites emerged from random points around the cell body (Gulley and Wood, 1971; Juraska et al., 1977; Grace and Bunney, 1983). Each neurone characteristically sends one or two dendrites perpendicularly into the SNR, where they often run parallel to reticulata dendrites and show varicosities (see figure 1.4). Other basilar dendrites in the zona compacta frequently divide once or twice and run parallel to the surface of the SNC for some distance. These dendrites course in all directions forming a disc-like dendritic field. The axons arise from the major dendrite passing dorsally initially, then taking a sharp turn medially (Juraska et al., 1977; Grace and Bunney, 1983). Small cells in the zona compacta represent approximately 20% of the cellular content.

1.4.1.2 Zona reticulata. Forms the bulk of the nucleus and has an organised neuropil but with relatively few cells (Gulley and Wood, 1971; Schwyn and Fox, 1974), although the majority of the afferents terminate here. Fonnum, Grofova, Rinvik, Storm-Mathiesen and Walberg (1974) calculated that 11.5% of the tissue volume of the SNR is occupied with synaptic boutons compared with 5.9% in the zona compacta. The dendrites of the large and medium cells course in an anteroposterior direction and also dorsoventrally, giving the appearance of a plate running the length of the nucleus (see figure 1.4). There is considerable overlap along the antero-posterior plane, but there is restricted overlap in the medio-lateral plane. Medium/large cells found near to the crus cerebri have dendritic fields which run lengthwise along the crus cerebri. In the peri peduncular region, dendrites run primarily in the coronal plane and parallel to the brainstem surface (Juraska et al., 1977). In both large and medium cells the axons arise from the cell bodies or primary dendrites. Of the SNR cells, some 30-40% have been categorised as small neurones. These are identical with the small cells found in the zona compacta.

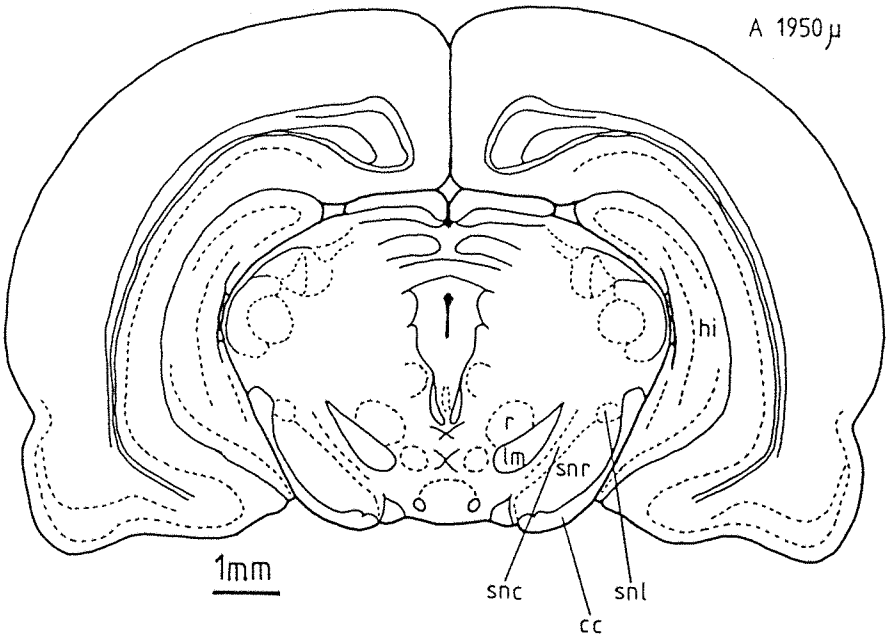
Figure 1.3 A coronal section through the rat brain at the level of substantia nigra

snr = substantia nigra zona reticulata; snc = substantia nigra zona compacta; snl = substantia nigra zona lateralis; r = red nucleus; cc = crus cerebri; lm = lemniscus medialis; hi = hippocampus.

Figure 1.4 A composite diagram of the substantia nigra neurones to show the relationships between their dendritic fields

(a) in a sagittal section; (b) in a coronal section (from Juraska et al., 1977).

Cc = crus cerebri; Lm = medial lemniscus; Snc = substantia nigra zona compacta; Snl = substantia nigra zona lateralis; Snr = substantia nigra zona reticulata; Vta = ventral tegmental area.


A 1950 µ
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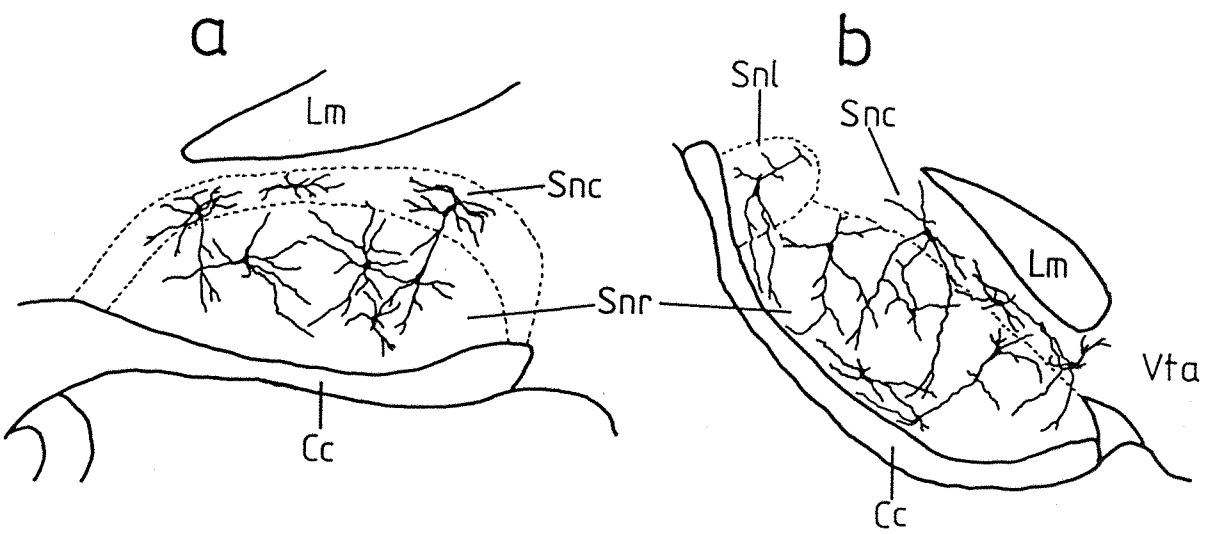
cc


a
Lm
Snc
Snr
Cc
b
Snl
Snc
Lm
Vta
Cc

1.4.1.3 Zona lateralis. All three cell types have been observed in this region. Large/medium cells, identical to those in the SNR, send long dendrites ventrally into the SNR where they run parallel to the crus cerebri; other dendrites remain within the area (Juraska et al., 1977). Small neurones, identical to those in the SNC, are found throughout this area.

1.4.2 Neuronal connections of the substantia nigra

This area has been reviewed by Carpenter (1976) and Dray (1979 and 1980). Pathways of the substantia nigra are summarised in figure 1.5.

1.4.2.1 Efferent pathways. Nigro-thalamic fibres arise from large neurones of the SNR and project to all parts of the thalamus (Faull and Mehler, 1978). Electrophysiological studies have shown that about 11% of nigro-thalamic neurones project bilaterally (Deniau, Hammond, Riszk and Feger, 1978). Nigro-collicular fibres originate from SNR cells immediately adjacent to the cerebral peduncle (Faull and Mehler, 1978). This is mainly an ipsilateral projection although contralateral fibres have been identified (Deniau et al., 1978). Kainic acid lesions have implicated γ-aminobutyric acid (GABA) as the transmitter in this region (Vincent, Hattori and McGeer, 1978).

The nigro-amygdaloid pathway arises from dopamine containing neurones in the dorsal layer of the SNC (Fallon, Riley and Moore, 1978). Other smaller pathways have been shown; nigro-raphe projections come from the SNC and SNR (Sakai, Salvert, Tauret and Jouvet, 1977). Bilateral nigro-cortical fibres from the SNC, that may be dopaminergic, have been shown by Avendano, Reinoso-Suarez and Llamas (1976). Nigro-locuscoeruleus fibres identified from the SNR (Sakai et al., 1977), and nigro-cerebellar projections demonstrated from the SNC, that are possibly dopaminergic (Chan-Palay, 1977). There is also a major output to the striatum (see section 1.4.3).

1.4.2.2 Afferent pathways. A number of afferent pathways have been identified: a pallido-nigral pathway projecting to both the SNR and SNC regions (Bunney and Aghajanian, 1976a; Carter and Fibiger, 1978); this pathway is possibly GABAergic (Fonnum, Grofova and Rinvik, 1978), but may also contain a proportion of substance-P containing neuronal fibres (Kanazawa, Emson and Cuello, 1977). Raphé-nigral fibres from the dorsal and medial raphé were clearly shown to exist (Bunney and

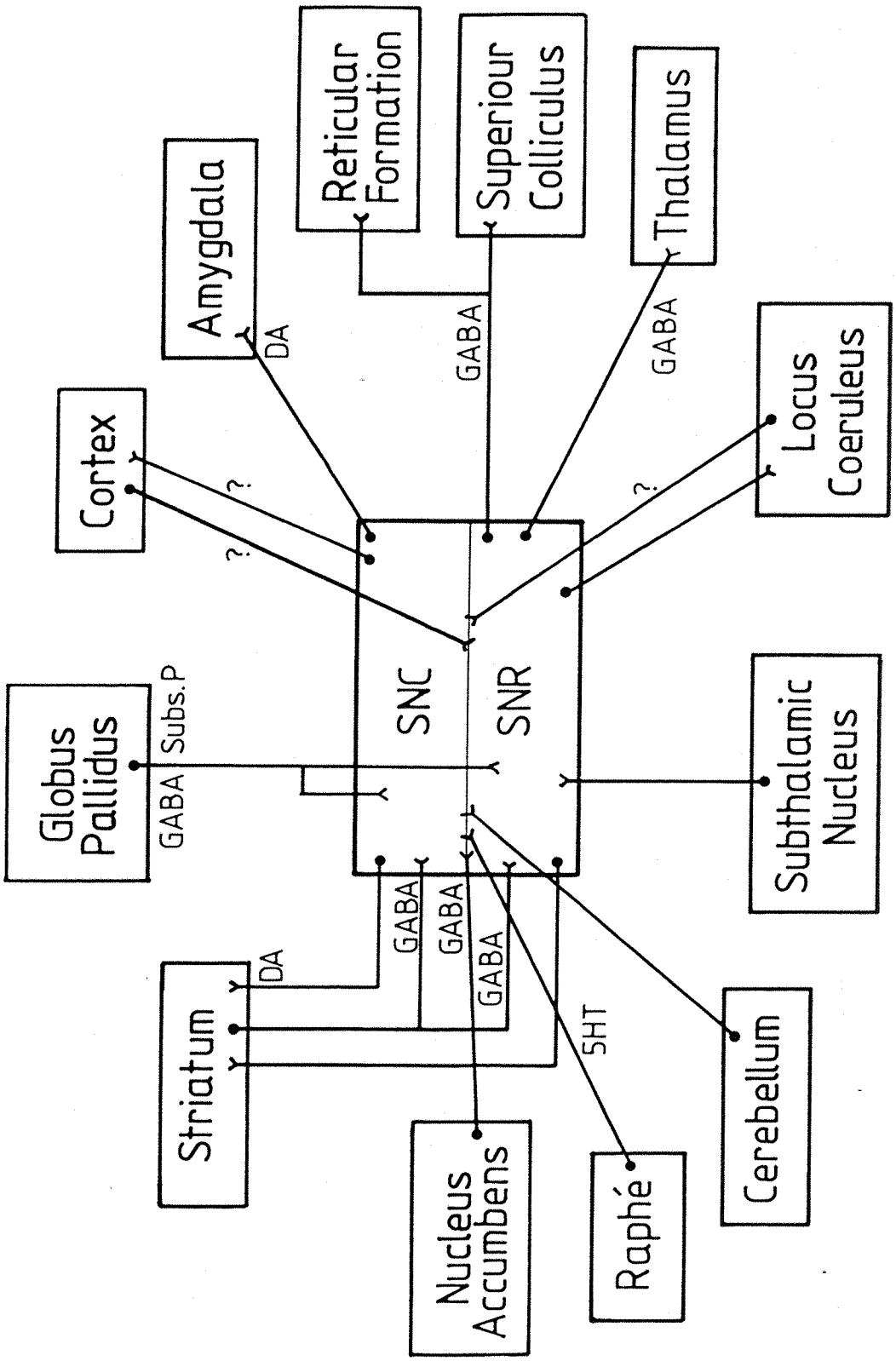


Figure 1.5 The afferent and efferent neuronal pathways of the substantia nigra, with probable transmitters shown.

Aghajanian, 1976; Fibiger and Miller, 1977). As the raphe nucleus comprises of serotonin containing neurones, the likely transmitter is 5-hydroxytryptamine (5-HT). A subthalamo-nigral pathway has been suggested following anatomical and electrophysiological studies (Kanazawa, Marshall and Kelly, 1976; Deniau et al., 1978). These project mainly to the SNR, but some have been identified in the SNC. An ipsilateral nucleus accumbens-nigral pathway that projects topographically to both the SNC and SNR has been identified (Swanson and Cowan, 1975; Nauta et al., 1978), terminating in the mediolateral SNC, SNL and some in the SNR. Cerebellar-nigral fibres were seen from degeneration studies in the medial and dorsal substantia nigra (Snider, Maiti and Snider, 1976).

A large proportion of the medium sized neurones in the striatum make contacts with nigrostriatal projection neurones (Somogyi and Smith, 1979; Bolam, Powell, Totterdell and Smith, 1981; Somogyi, Bolam and Smith, 1981). Electrophysiological studies have shown excitatory/inhibitory postsynaptic potential sequences (Feltz, 1971; Dray, Gonge and Oakley, 1976) suggesting two distinct pathways. GABA is implicated as the inhibitory transmitter (Crossman, Walker and Woodruff, 1973; Dray et al., 1976). Substance-P has been proposed as the excitatory transmitter (Dray and Straughan, 1976, 1978; Ljungdahl, Hokfelt, Nilsson and Goldstein, 1978).

1.4.3 The nigrostriatal pathway

The existence of a nigrostriatal pathway has been confirmed many times using a number of anatomical techniques (see section 1.2). The axons of this projection undergo extensive branching within the neostriatum, each branch containing numerous small varicosities. Anden, Fuxe, Hamberger and Hokfelt (1966) estimated that each dopamine neurone innervating the striatum has axons between 55-77 cm in length and that these contained approximately half a million varicosities, though there is now good evidence that dopamine is the transmitter of this pathway (section 1.2). The precise effect of DA on striatal neurones has been difficult to evaluate and is subject to debate (see review by Dray, 1979, 1980). The predominant effect of DA on striatal neurones is inhibitory (McLennan and York, 1967; Davies and Tongroach, 1978) yet some excitatory responses were reported by these authors. Other studies have reported predominantly excitatory responses (Spencer and

Haverlicek, 1974; Bevan, Bradshaw and Szabadi, 1975). Stimulation of the substantia nigra evoked inhibitions that showed a significant correlation with the inhibitory responses to iontophoretically applied DA (McLennan and York, 1967; Connor, 1970; Gonzalez-Vegas, 1974). In these same studies, excitatory responses were detected to SN stimulation and iontophoretically applied DA, but these could not be correlated as the responses were not always in the same cell. Intracellular recordings of striatal neurones have shown transient EPSP's preceding prolonged IPSP's after SN or MFB stimulation (Fuller, Hall and Buchwald, 1975; Kocsis, Sugimori and Kitai, 1977). Iontophoretic DA produces depolarizations but with a cessation of the background neuronal firing (Bernardi, Marciani, Morocutti, Pavone and Stanzione, 1978).

Not all nigrostriatal fibres are dopaminergic in nature: 6 OHDA lesions of the nigrostriatal tract that deplete striatal tyrosine hydroxylase by 93%, reduce anteriograde transport by 80% (Fibiger, Purdritz, McGeer and McGeer, 1972). By combining retrograde tracing and histofluorescence together, cells retrogradely labelled from the striatum in the SNC were found not to contain catecholamines (Ljungdahl, Hokfelt, Goldstein and Park, 1975; Guyenet and Crane, 1981). Two nigrostriatal pathways are also implicated from electrophysiological studies. Guyenet and Aghajanian (1978) found two types of fibres with different conduction velocities. The identity of the transmitter substance for these non-dopaminergic neurones is unknown. The existence of the non-dopaminergic pathway supports the idea that this pathway could be excitatory and may account for the dual responses seen in the striatum. It is difficult to judge which of the excitatory or inhibitory responses are mediated through dopamine because of the complex anatomical connections in the striatum.

Although this projection is largely ipsilateral, recent evidence suggests a small contralateral component exists (Gerfen, Staines, Arbuthnott and Fibiger, 1982). Histofluorescence techniques have double-labelled these crossed fibres, which were shown to arise from SNC cells, containing DA as well as CCK (Fallon, Wang, Kim, Canepa Loughlin and Seroogy, 1983). A small number of crossed fibres have been antidromically stimulated from the contralateral caudate nucleus (Collingridge, 1982).

1.5 Neuropharmacology of the substantia nigra

The substantia nigra contains a number of putative neurotransmitters in high concentrations (see table 1.1). Furthermore, the enzymes required for the synthesis and catabolism of some of these candidate transmitters have been identified in the substantia nigra (SN).

Most of the dopamine in the SN is found in the SNC, but approximately one third is located in the SNR (Fahn, 1976), mostly within the dendritic processes that penetrate deep into the SNR and some dopamine containing cell bodies. Calcium dependent DA release has been shown to occur from dendrites in the SN, in vitro by K^+ induced depolarisations or in vivo by electrical stimulation (Geffen, Jessell, Cuello and Iversen, 1976; Korf, Zieleman and Westerink, 1976). The mechanism of release is different from that at axonal nerve endings, since there are no storage vesicles in the dendrites (Hattori, McGeer and McGeer, 1979). Dopamine appears to be stored in smooth endoplasmic reticulum. Tetrodotoxin (which blocks Na^+ channels and nervous transmission) reduces striatal but not substantia nigra release of dopamine (Nieoullon, Cheramy and Glowinski, 1977). A dopamine sensitive adenylate cyclase is present, especially in the SNR (Kebabian and Saavedra, 1976), apparently located presynaptically, since dopamine sensitive adenylate cyclase activity remains after 6 OHDA lesions of the substantia nigra but disappears after lesioning the striatonigral tract (Gale, Guidotti and Costa, 1977; Spano, Trabbuchi and DiChiara, 1977). The uptake processes exhibit different pharmacological sensitivities: nigral uptake can be blocked by desipramine and benztropine while striatal uptake is blocked only by benztropine (Bjorklund and Lindvall, 1975).

The DA released from the dendrites of the SN has been proposed to act on dopamine "autoreceptors" located on the dopaminergic neurones and/or their dendrites, providing a mechanism for self-regulation of release (Aghajanian and Bunney, 1974; Groves, Wilson, Young and Rebec, 1975). Dopamine released from the dendrites would be expected to reduce the amount of DA released from nigrostriatal nerve terminals (Nieoullon et al., 1977). This mechanism has been proposed to account for the changes in dopamine metabolism produced when dopamine receptor agonists and antagonists are administered. The antagonists (neuroleptics)

Table 1.1 Putative neurotransmitters in the substantia nigra, their concentrations and effect on single substantia nigra neurones

Putative Neurotransmitter	Concentration per g tissue		Effect on single neurones		Source of transmitter
	Human	Rat	SNC	SNR	
Dopamine	3.2 nmol[†]	9.1 nmol[‡]	-	±	Cell bodies of nigrostriatal pathway
Noradrenaline	0.24 nmol[†]		-	±	Terminals of locus coerulens-nigral pathway?
Serotonin (5HT)	5.8 nmol[†]	10.9 nmol	0	±	raphe-nigral terminals
Acetylcholine		8.8 nmol[o]	0	+	Intrinsic and in terminals of unidentified afferents
GABA	5.3 µmol[*]	11.0 µmol	-	-	Intrinsic and in terminals of striatonigral pallidonigral pathway
Glycine	2.3 µmol[*]	1.2 µmol	-	-	Intrinsic?
Glutamate	6.01 µmol[*]		+	+	Intrinsic?
Taurine	1.2 µmol[*]	3.5 µmol	-	-	Intrinsic?
Substance P	45.6 nmol[x]	16.6 nmol[x]	+	+	Terminals of striatonigral and pallidonigral pathways
Enkephalin	0.66 ng/mg prt.[x]		0	0	Cell bodies and fibres
CCK	28.0 µmol[●]		+	0	Co-exists in SNC dopaminergic cells

[†]Lloyd and Hornykiewicz (1974); [*]Perry, Hansen and Kloster (1973); [x]Emson (1979); [o]Cheney, Le Fevre and Racagni (1975); Dray and Straughan (1976); [‡]Anden, Fuxe, Hamberger and Hökfelt (1966); [●]Stadler, Javoy-Agid, Cesselin, Legrand and Agid (1982).

increase the firing of SNC and striatal cells and increase DA turnover, while agonists have the opposite effects (Bunney, Aghajanian and Roth, 1973a; Bunney, Walters, Roth and Aghajanian, 1973b; Groves et al., 1975). These changes have been considered to be secondary to striatal postsynaptic dopamine receptors mediating a feedback loop to the nigra (Carlsson and Lindqvist, 1963; Carlsson, Kehr, Lindqvist, Magnusson and Atack, 1972). This pathway seems an unlikely explanation, since lesions of the striatonigral tract do not modify the metabolic changes of dopamine metabolism by dopamine receptor agonists and antagonists (DiChiara, Porceddu, Fratta and Gessa, 1977; Garcia-Mundez, Nicolaou, Tulloch, Wright and Arbuthnott, 1977). The release of dopamine from dendrites produces reciprocal changes in DA release from the ipsilateral striatum and contralateral SN (Nieoullon, Cheramy and Glowinski, 1978). Thus, dendritic release of dopamine may function to integrate the two nigrostriatal systems so they become interdependent upon each other.

The regulation of dopaminergic neurones has been reviewed fully by Moore and Wuerthele (1979). The pharmacology of dopamine on the SNC neurones will be dealt with in section 1.8.5.

Noradrenaline is found in nerve terminals within the SN, possibly derived from the locus coeruleus (Collingridge, James and McLeod, 1979), and is taken up into nigral neurones (Gulley and Smithberg, 1971; Farley and Hornykiewicz, 1977) by a Na^{+} and Ca^{++} dependent process (Collingridge et al., 1979). Iontophoretically applied NA causes a depression of neurones on the SNC while those of the SNR also elicit excitatory responses (Crossman, Walker and Woodruff, 1974; Collingridge and Davies, 1981). The inhibitory responses of NA on SNC neurones is possibly caused by the activation of DA receptors. Bunney and Aghajanian (1977) found that the α-adrenoreceptor agonist, clonidine, had no effect, whilst the application of the β-adrenoceptor agonist, isoprenaline, caused slight inhibitory actions on neuronal firing. The α-adrenoceptor antagonist, piperoxane, and the β-adrenoceptor antagonist, sotalol, had no antagonistic activity on these neurones (Aghajanian and Bunney, 1977).

Serotonin (5HT) is derived principally from the raphé nucleus; stimulation of the medial raphe produces predominantly inhibitory responses in the SN, although excitatory responses have been elicited (Dray, Davies, Oakley, Tongroach and Vellucci, 1978) and these synaptic

responses can be blocked by methiothepin (Dray and Oakley, 1977). The iontophoretic application of 5HT onto SN neurones produces both excitatory and inhibitory responses and these responses to 5HT can be blocked by either methiothepin (Dray and Oakley, 1977) or methysergide (Davies and Tongroach, 1978). However, on identified SNC cells, 5HT had no effect on the firing rate (Collingridge and Davies, 1981). Lesions of the raphé produce increases in the SN DA turnover and could be tonically inhibitory on dopamine neurones (Dray et al., 1978; Garcia-Mundez, Arbuthnott and Eccleston, 1979). Dopamine also has an inhibitory influence on the release of 5HT from the SN (Hery, Soubrie, Bourgoin, Motastrue, Artaud and Glowinski, 1980). Unilateral injections of 5HT into the SN produce ipsiversive turning which can be blocked by methysergide (Costall and Naylor, 1974; James and Starr, 1980). These effects were enhanced by DA receptor agonists, nialamide or uptake blockers, and abolished by haloperidol (Costall, Naylor, Marsden and Pycock, 1976; James and Starr, 1980).

The highest concentration of γ-aminobutyric acid (GABA) in the brain is found in the SN, especially on the zona reticulata-compacta border (Tappez, Brownstein and Kopin, 1977). Iontophoretically applied, GABA inhibits both SNC and SNR neurones, the SNR neurones being twenty times more sensitive to GABA than the SNC neurones (Grace and Bunney, 1978; Waszczak, Eng and Walters, 1980). Inhibitions produced by GABA can be blocked by the iontophoretic application of bicuculline (Dray et al., 1976; Olpe and Koella, 1978) and picrotoxin (Crossman et al., 1974). Muscimol, a GABA agonist, inhibits both SNC and SNR neurones, when applied iontophoretically, which can be blocked by bicuculline in both areas (Olpe and Koella, 1978; Waszczak et al., 1980). However, given intravenously, muscimol increases the firing of SNC cells, but still inhibits firing in SNR neurones (Walters and Lakoski, 1978; McNeil, Gower and Saymanska, 1978; Waszczak et al., 1980). The inhibitory effects of intravenous muscimol on the SNR cells were blocked by intravenous bicuculline or picrotoxin, whereas the excitation of the SNC cells were not affected by these GABA antagonists. When applying GABA iontophoretically into the SNR, a simultaneous recording of SNC neurones revealed an excitatory response which was antagonisted by intravenous picrotoxin. Iontophoretically applied glutamate into the SNR produced an inhibitory response in the SNC neurones and this response could also

be blocked by picrotoxin (Grace and Bunney, 1979). Unilateral microinjections of GABA mimetic drugs or GABA antagonists have been used to manipulate the activity of the nigrostriatal pathway. The results from such experiments have produced contradictory data. Some reports find increased nigrostriatal activity when GABA mimetics are directly applied to the SN, resulting in either increased release of DA in the ipsilateral striatum (Cheramy, Nieoullon and Glowinski, 1978) or evoking contraversive turning behaviours (Oberlander, Dumont and Boissier, 1977; Olpe, Schellenberg and Koella, 1977; Martin, Papp and Bicino, 1978). This turning behaviour has been blocked by picrotoxin (Oberlander et al., 1977). However, picrotoxin was found to be ineffective in this turning by Martin et al. (1978). Contraversive turning has been elicited following intranigral injections of picrotoxin (Scheel-Kruger, Arut and Magelund, 1977) and bicuculline (Olpe et al., 1977), but others found bicuculline did not produce rotational activity (Oberlander et al., 1977; Martin et al., 1978).

The GABA transaminase inhibitor, ethanolamine-o-sulphate (EOS) increases nigral GABA concentrations. When unilaterally injected into the substantia nigra, EOS caused contraversive rotation (Koob, Del Fiqcco and Iversen, 1978), while in combination with intraperitoneal amphetamine or apomorphine, ipsilateral rotations were evoked (Dray, Fowler, Oakley, Simmonds and Tanner, 1977). The conflicting experimental data was resolved by a study of the site of the microinjections. Thus, Kilpatrick and Starr (1981) showed that muscimol injected into the SNR caused contraversive turning (in agreement with previous reports), while injections into the SNC produced ipsilateral rotation positioning of the injection site in the SN. These data suggest that an inhibitory neurone exists that is sensitive to GABA receptor agonists and antagonists in the SNR and which makes synaptic contacts with SNC neurones. This would account for the paradoxical excitations by GABA agonists on the SNC neurones.

Glycine depressed neuronal activity of the SN neurones when applied iontophoretically. This effect was blocked by the antagonist strychnine (Crossman et al., 1974; Dray et al., 1976), yet Feltz (1971) found glycine to be ineffective. Dray et al. (1977) have suggested that glycine interneurones might tonically inhibit nigrostriatal neurones. Support for this hypothesis came from intranigral injections of glycine,

which reduced DA release in the striatum which was blocked by strychnine (Cheramy et al., 1978). Unilateral injections of glycine caused ipsiversive turning, while strychnine caused contraversive turning in rats (James and Starr, 1979). However, the neurotoxin kainic acid did not produce the expected decrease in glycine concentration that would be expected if a glycinergic neurone exists (James and Starr, 1979).

Acetycholine (ACh), together with its synthetic and catabolic enzymes, choline acetylase (CAT) and acetylcholine esterase (AChE) respectively, are found in the SN, suggesting the presence of a cholinergic pathway (Fonnum, Grofova, Rinvik, Storm-Mathisen and Walberg, 1974; Cheney, Le Fevre and Racagni, 1975). In addition, a calcium-dependent release of ACh occurred from SN slices (Massey and James, 1978). A cholinergic pathway from the striatum was proposed by Oliver, Parent, Simard and Poirier (1970). However, striatal lesions failed to show a decrease in the CAT levels in the nigra. Thus, a cholinergic system must be assumed to be intrinsic until a cholinergic pathway is demonstrated (Fonnum et al., 1974).

Substantia nigra neurones are excited by ACh, the neurones in the SNR being particularly sensitive (Dray et al., 1976; Kemp et al., 1977), yet some inhibitory responses have been detected (Kemp et al., 1977; Pinnock, Woodruff and Turnbull, 1983). However, on identified SNC neurones, ACh had no effects (Collingridge and Davies, 1981; Pinnock et al., 1983). Microinjections of cholinergic drugs suggested a net inhibitory function on dopaminergicneurones. Atropine infused into the SNR produced an increase in striatal DA release while carbacol attenuated this release (Javoy, Agid, Bouvet and Glowinski, 1974). These inhibitory responses can be blocked by neuroleptics (James and Massey, 1978), but since the cholinergic agonist activates release of DA in vitro from SN slices (Straughan and James, 1978) these effects are possibly indirect.

L-Glutamic acid has been found to be a strong excitant of nigral neurones on iontophoretic application (Feltz, 1971; Collingridge and Davies, 1981). L-Aspartic acid, Kanic acid, N-methyl-D-aspartate and D-L-homocysteic acid were excitatory on both SNC and SNR neurones (Collingridge and Davies, 1981; Pinnock et al., 1983). All these excitatory amino acids caused a decrease in spike amplitude which is proposed to be due to overdepolarisation of the cell membrane. These

excitatory responses have been reversed by D-α-adipic acid, D-α-aminosuberate or GABA (Collingridge and Davies, 1981). The highest concentration of substance P in the brain is found in the SN (Brownstein, Mroz, Kizer, Palkovitz and Leeman, 1976). This peptide was reported to be released from nerve endings by depolarisation (Iversen, Jessel and Kanazawa, 1976). Substance P has been shown to excite SN neurones (Davies and Dray, 1976; Walker, Kemp, Yajima, Kitagawa and Woodruff, 1976), although the effects are greater on SNR than on SNC neurones (Pinnock and Dray, 1982). Unilateral injections of substance P into the SN resulted in contralateral rotation (Olpe and Koella, 1977; James and Starr, 1979) and increased the release of DA and the concentration of DA metabolites in the striatum (Michelot, Leviel, Nieoullon, Glowinski and Kerdelhu, 1978). These behavioural responses to substance P were blocked by pretreatment with haloperidol and exaggerated by nialamide, suggesting that these actions are mediated through increased activity of the nigrostriatal pathway (James and Starr, 1979).

Enkephalin immunoreactivity has been found associated with SN neurones in the SNL and SNR (Hokfelt, Elde, Johansson, Terenius and Stein, 1977; Wamsley, Young and Kuhar, 1980). Some reports have suggested that opiate receptors are located on the SNC dopamine containing neurones (Pollard, Llorens, Schwartz, Gros and Dray, 1978). However, Reisine, Nagy, Beaumont, Fibiger and Yamamura (1979) found no opiate binding to nigral dopaminergic cell membranes.

In electrophysiological recordings of single units, systemically administered morphine resulted in an increasing of firing of SNC neurones, which was blocked by naloxone (Iwatsubo and Clouet, 1977; Finnerty and Chan, 1981). Conversely, naloxone depressed the activity of SNR neurones. Iontophoretically applied morphine caused excitations of SNR neurones, but had no effects on SNC cells (Collingridge and Davies, 1982). It has been proposed that the excitatory effects of morphine on SNC cells is indirect since met-enkephalin has no effects on the firing rate of both SNC and SNR neurones (Collingridge and Davies, 1982). In the substantia nigra, immunohistochemical techniques have revealed that cholecystokinin (CCK) can co-exist with DA in neurones of the SNC, which represent a sub-population of neurones in the mesencephalon (Hokfelt, Rehfeld, Skirboll, Ivemark, Goldstein and Markey, 1980).

Single unit responses to CCK, given intravenously in low doses, caused an inhibition of firing, but at higher doses excitation of firing was seen (Skirboll, Grace, Homer, Rehfeld, Goldstein, Hokfelt and Bunney, 1981). The inhibitory effects of CCK could be blocked by haloperidol, but the excitatory responses were unaffected. Iontophoretically-applied CCK produced only excitatory responses (Skirboll et al., 1981). These single unit responses to CCK were only found in CCK containing areas of the mesencephalon.

From the profile described above, the SNC and SNR neurones represent two distinct cell populations, both physiologically and pharmacologically. In consequence, care must be taken to identify the location of neurones in neuropharmacological experiments of this region.

Recently the importance of oestrogens in influencing the activity of dopaminergic neurones has been recognised. Chiodo and Caggiula (1980, 1983) have shown that 17-β-oestradiol can influence the sensitivity of dopaminergic neurones to apomorphine and DA. This has relevance in comparing results obtained from animals of different sex, and from animals in different phases of the oestrus cycle.

1.6 The nucleus accumbens

Because of its location, similar cytoarchitecture and development, the nucleus accumbens has been regarded as a ventromedial extension of the striatum (Swanson and Cowan, 1975; Heimer and Wilson, 1979). The definition as to what constitutes the nucleus accumbens, if it is a separate structure, remains unclear at present (Chronister and De France, 1981; Domesick, 1981). Figure 1.6 shows the nucleus accumbens as defined in the rat brain atlas of Konig and Klippel (1963). Cytologically, it is remarkably similar to the striatum, with no defined structure, but containing 'clusters' or 'aggregations' of cells. The neurones in the nucleus accumbens are classified by their size and presence or absence of spiny processes on their dendrites. The cells are, however, smaller and more densely arranged than in the striatum (Heedreen, 1981).

Functionally, the nucleus accumbens differs from the striatum, being more like the septum (Conrad and Pfaff, 1976). It modulates an animal's tendency to move (i.e. random locomotion), whereas the striatum

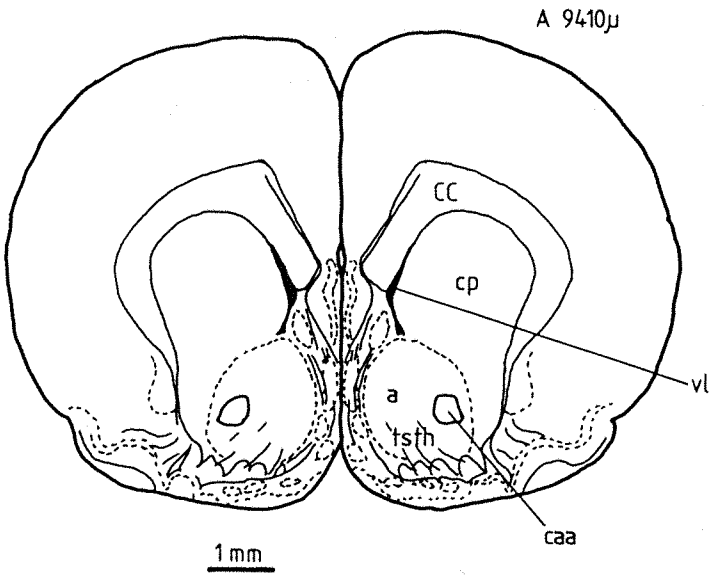


Figure 1.6

A coronal section through the rat brain at the level of the nucleus accumbens. a = nucleus accumbens; caa = anterior commisure; cc = corpus callosum; cp = caudate/putamen; tsth = septohypothalamic tract; vl = lateral ventricle.

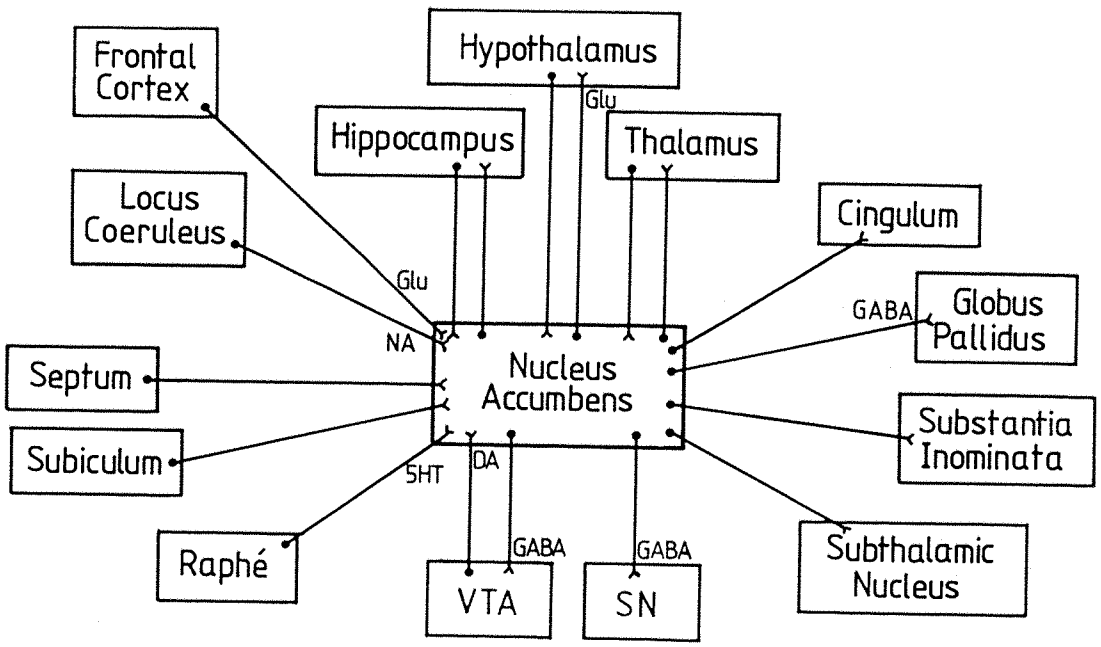


Figure 1.7

The afferent and efferent connections of the nucleus accumbens, with probable transmitters indicated.

is involved in complex motor movements like stereotyped behaviours (e.g. grooming). Mogenson, Jones and Yim (1980) suggested that the nucleus accumbens constitutes a transitional zone between the basal ganglia and limbic structures, whereby motivation can be translated into actions.

1.6.1 Afferent and efferent connections of the nucleus accumbens

The afferent and efferent connections of the nucleus accumbens are complex and will be briefly summarised in this present review (see figure 1.7).

The nucleus accumbens contains many of the 'classical' transmitters and their synthetic and degradative enzymes, as well as a number of the centrally active peptides (see review by Johansson and Hokfelt, 1981). High levels of substance P and enkephalin are present in similar levels to those found in the striatum. Neurotensin and somatostatin are the only substances that differ largely in their localisation between the nucleus accumbens and the striatum (Johansson and Hokfelt, 1981).

A major dopaminergic afferent pathway originates from the ventral tegmental area (A_{10}) and is termed the mesolimbic pathway. This was identified by the histofluorescent techniques and forms a dense network of nerve terminals in the nucleus accumbens (Andén et al., 1966; Dahlstrom and Fuxe, 1964; Lindvall et al., 1974). However, not all the neurones of this pathway are catecholaminergic since 6 OHDA lesions do not eliminate all the neurones projecting to the accumbens (Wang, 1981). Electrophysiological evidence suggests that this pathway is mono-synaptic being antidromically stimulated from the nucleus accumbens and has similar extracellular action potentials and properties to the SNC neurones (Mogenson and Yim, 1979; Wang, 1981). It has been suggested that the mesolimbic system is involved in the therapeutic action of antipsychotic drugs and the pathogenesis of schizophrenia (Matthysse, 1973).

A projection has been identified from the raphé nucleus and is presumed to be serotoninergic in nature (Newman and Winnans, 1980). High levels of cholineacetyltransferase and acetylcholine are found in the nucleus accumbens, but cholinergic neurones are thought to be intrinsic as kanic acid lesions of the nucleus accumbens destroys the cholinergic structures (Fonnum and Walaas, 1981). In addition,

afferents are received from the amygdala, thalamus, septum, hipocampus and from cortical areas (Swanson and Cowan, 1975; Raisman, 1966; Newman and Winnans, 1980). The transmitter substances of these pathways are as yet undetermined.

Efferents from the nucleus accumbens go largely to the ventral putamen and globus pallidus (Williams, Crossman and Slater, 1977; Nauta, Smith, Faull and Domesick, 1978). The globus pallidus projection has been proposed to be GABAergic (Pycock and Horton, 1976). They showed that EOS injections or electrolytic lesions of the globus pallidus abolished the hyperactive response to dopamine injections into the nucleus accumbens. This is supported by electrophysiological evidence (Dray and Oakley, 1978; Jones and Mogenson, 1980). A number of neurones in the globus pallidus were inhibited by electrical stimulation of the nucleus accumbens; these inhibitory effects were blocked by iontophoretic application of picrotoxin. Other efferents have been described which include the thalamus, hypothalamus, VTA and SN. The connections of the nucleus accumbens are described in greater detail by Domesick (1981).

1.7 Studying the dopamine receptor

Subsequent to the proposal that DA had a neurotransmitter role, methods for the study of dopamine's action were required. Two of the earliest models developed to study specific dopamine receptor-mediated events were the isolated brain of the snail, Helix aspersa (Woodruff and Walker, 1969) and the vasodilations of the dog renal vasculature (Goldberg, Sonneville and McNay, 1968). Subsequently, a number of other biochemical, behavioural, peripheral and electrophysiological methods were developed.

1.7.1 The Helix aspersa model

The isolated brain of the Helix aspersa contains a number of large neurones (some greater than 100 μm in diameter) that can be used for intracellular recording of the neuronal activity. Responses to drugs whether applied by microiontophoresis or by the addition to the bath perfusate, can be measured by monitoring the transmembrane potential or changes in conductance. Dopamine depressed the firing rate of some identifiable neurones in the Helix brain (Kerkut and Walker, 1961).

The structure-activity requirements of the dopamine receptor for agonist activity were found to be rigid (Woodruff and Walker, 1969). Using a large number of phenylethylamine derivatives, these investigations found an absolute requirement for two hydroxyl groups at position 3 and 4 of the phenolic ring and the presence of a terminal nitrogen atom on the side chain. Substitutions of the amino group with a methyl group (n-methyl-dopamine; epinine) did not affect activity, but further ethyl or propyl substitutions considerably reduced activity (Woodruff and Walker, 1969). The structural requirements of this model are distinctly different from those of either α or β-adrenoceptors.

As a direct result of these studies, Woodruff (1971) postulated a model of the dopamine receptor and predicted that a rigid analogue of dopamine, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) (figure 1.8), would behave as a dopamine agonist. ADTN contains, within its structure, the structure of dopamine in its extended β-rotameric form; two other rigid analogues that also contain the structure of dopamine in different conformations are norsalsolinol and 2-amino-5,6-dihydroxy-1.2,3,4-tetrahydronaphthalene (iso-ADTN) (figure 1.8). These have been shown to be less active in a variety of different model systems. Thus, it was suggested that dopamine at its receptor acts in the extended β-rotameric form (see reviews, Woodruff, 1978, 1982). ADTN contains an assymetric carbon atom and thus exists in two enantiomers (+) and (-). These have been resolved into their absolute configuration; the R-(+)-ADTN being a hundred times more potent than the S-(-)-ADTN on both snail neurones (Batta, Walker and Woodruff, 1979) and on their ability to stimulate the DA sensitive adenylate cyclase (Davis, Poat and Woodruff, 1978). Although this model has proven useful in stimulating other pharmacological studies, there are a number of discrepancies which suggest that it may not be a good model of mammalian dopamine receptors.

The classical neuroleptics, haloperidol and chlorpromazine, had no antagonistic activity on the dopamine induced inhibitions (Woodruff, Walker and Kerkut, 1971) while fluphenazine and cis-flupenthixol were weak antagonists (Batta, Walker and Woodruff, 1978) although sulpiride was found to be stereospecifically active on dopamine inhibitions in this model (Batta et al., 1979). Furthermore, apomorphine, a potent dopamine agonist in other model systems, was an antagonist on the Helix neurones.

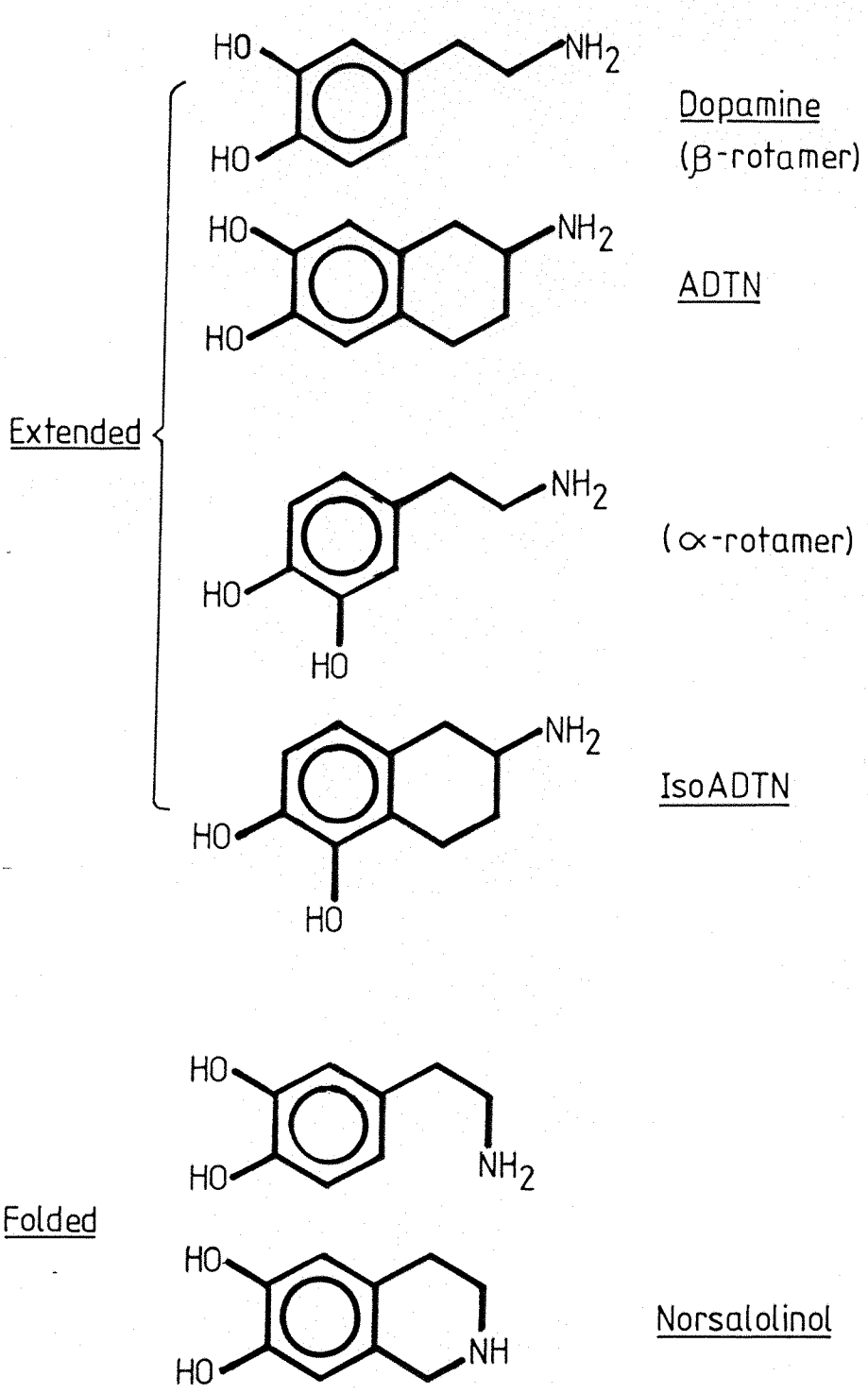


Figure 1.8

The conformations of dopamine and their rigid structural analogues.

1.7.2 Peripheral dopamine receptor models

A large number of in vitro and in vivo preparations using peripheral tissues were demonstrated to be sensitive to DA in a variety of species (Goldberg, 1972; Goldberg, Volkman and Kohli, 1978). These are mostly associated with the vasculature and sympathetic nerves that innervate the vascular beds. Only a few of these preparations will be mentioned here.

Dopamine produces a vasodilation of the renal artery of the phenoxybenzamine treated dog, resulting in an increased renal blood flow. The structure-activity requirements for dopamine mimetic activity were shown to be the same as the Helix model (Goldberg, Sonneville and McNay, 1968). Substitutions of the terminal amino group larger than a methyl yielded an inactive compound, epinine being equipotent with dopamine. Some N,N-di-substituted analogues of dopamine smaller than propyl were inactive, whereas N,N-dipropyldopamine and larger substitutions had agonist activity, as were apomorphine and ADTN (Volkman, Kohli, Goldberg, Cannon and Lee, 1977; Kohli, Weder, Goldberg and Ginos, 1980). Again, ADTN was approximately equipotent with DA while isoADTN was inactive in this preparation (Volkman et al., 1977). A detailed description ofthe structure-activity requirements of this model is contained in the review by Goldberg (1978). The antagonist profile is similar to other models; haloperidol is a potent antagonist of DA responses (Yeh, McNay and Goldberg, 1969). However, the active isomer of sulpiride was claimed to be the R-(+)-sulpiride which is the inactive isomer in other model systems.

In addition to the in vivo preparations, in vitro techniques for the peripheral dopamine receptors have been developed in order to eliminate some limitations of the in vivo preparations. In the intact animal, vasodilation can occur as a result of non-receptor mechanisms. Drugs that affect blood pressure may affect the blood flow measurements: an in vitro model using helical strips of the renal vasculature (Goldberg and Toda, 1975) given supportive evidence for specific DA receptors. The strips, pretreated with phenoxybenzamine, responded to DA, epinine and ADTN, while propanolol was without effect. Another useful model is the dog femoral vascular bed. Here, DA receptors are located presynaptically on noradrenergic nerve endings. Dopamine is suggested to act on the presynaptic DA receptors, which inhibit NA

release, resulting in vasodilation and an increased femoral blood flow (Bogaert, De Schaepdryver and Willems, 1977).

Other peripheral models that have been studied include a presynaptic dopamine receptor located on the cardio-accellerator nerve in cats and dogs (Ilhan, Long and Cannon, 1975), the rabbit ear artery (McCulloch, Rand and Story, 1978) and the isolated rabbit splenic artery (Hilditch and Drew, 1981).

1.7.3 Behavioural models

Several dopamine mediated behaviours have been distinguished. Although these models are not a direct measure of receptor mediated activity, since they do not separate effects due to pre or postsynaptic stimulation, or between the effects produced by other neurotransmitter systems, they are important techniques for monitoring the effect of drugs in conscious animals as well as assessing a drug's potential for clinical use, for they possess the pharmacodynamic properties apposite to the clinical situation. The methods used to assess dopaminergic induced behaviours have been reviewed by Costall and Naylor (1980).

1.7.3.1 Stereotypy. It is well documented that dopamine agonists, such as α-amphetamine or apomorphine, can induce stereotyped behaviours (Randrup and Munkvad, 1967). These behaviours consist of repetitive sniffing, licking, chewing, biting, rearing and grooming, different characteristic behaviours being elicited at different threshold doses (Fray, Sahakian, Robbins, Koob and Iversen, 1980). This stereotyped behaviour is often assessed visually by an arbitrary rating scale.

All classes of neuroleptics antagonised the stereotypic behaviours except sulpiride, which was inactive (Costall and Naylor, 1975). This test has disadvantages because it has failed to detect the neuroleptic potential of clozapine and thioridazine (Janssen, Niemegeers and Schellekens, 1965; Ljungberg and Ungerstedt, 1978).

1.7.3.2 Catalepsy. Catalepsy is defined as an 'inability to correct an imposed position whilst maintaining a righting reflex' and is a frequently used test for assessing neuroleptic potential. This is distinct from catatonia where rigidity of the postural muscles is encountered. Catalepsy is produced after an acute administration of a neuroleptic, the intensity of which is assessed by the use of a scoring system (for example, Costall, Fortune and Naylor, 1978).

There is good correlation between potency of almost all classes of neuroleptics (following peripheral administration) in this test and their clinical potency (Costall and Naylor, 1980). Even so, some spurious results have become evident. The narcotics and metoclopramide can induce catalepsy, but are not antipsychotics clinically (Borestein and Bles, 1965). Sulpiride has no cataleptic activity when given peripherally (Costall and Naylor, 1973), yet shows antipsychotic activity in the clinic (Costall and Naylor, 1975; Lewis, Bond and Curry, 1983). This anomaly can be explained by sulpiride's poor lipid solubility and poor penetration into the brain. Intracerebral ventricular administration of sulpiride induces a cataleptic state, being equipotent to haloperidol (Honda, Satoh, Shimomura, Satoh, Noguchi, Vehida and Kato, 1977).

1.7.3.3 Circling behaviour. Circling behaviour was first reported by Anden, Dahlstrom, Fuxe and Larsson (1966) following unilateral disruption of the nigrostriatal pathway, which resulted in asymmetric body posturing and circling. Later, Ungerstedt (1968) described a technique based on the injection of 6 OHDA into the substantia nigra to lesion the catecholamine containing cells. After allowing time for the nigrostriatal pathway to degenerate, injections of dopamine and dopamine agonists elicited circling behaviours (Ungerstedt, 1971b,c). This circling behaviour was thought to be due to an imbalance in the dopaminergic transmission induced by lesioning of the nigrostriatal tract (Ungerstedt, 1971c). This model is particularly useful in that it can distinguish between directly and indirectly acting dopamine agonists. Directly acting agonists, such as apomorphine, cause turning away from the lesioned side (contraversive turning). This can be explained as a result of postsynaptic supersensitivity of the dopamine receptors that develops in the striatum on the lesioned side, as a result of denervation (Ungerstedt, 1971c). Stimulation of dopamine receptors in both striata results in an imbalance of the two nigrostriatal pathways. Indirectly acting agonists, such as amphetamine, cause the release of dopamine from the intact nigrostriatal pathway, which stimulate the dopamine receptors on the intact side of the brain, which results in turning towards the lesioned side (ipsiversive turning). Circling behaviour is quantified by counting the number of rotations made in a given time period (Ungerstedt and Arbuthnott, 1970). This behaviour has been antagonised by all the classes of neuroleptics including the

benzamides (Dolphin, Jenner, Marsden, Pycock and Tarsy, 1975; Pycock, Tarsy and Marsden, 1975; Honda et al., 1977).

1.7.3.4 Locomotor activity. The central stimulant, d-amphetamine, was shown to cause increased locomotion in animals; an effect which was suggested to be mediated via dopamine receptor activation (van Rossum and Hurkmans, 1964). The nucleus accumbens was first implicated in the initiation and control of locomotor activity by Pijnenberg, Woodruff and van Rossum, (1973). Bilateral injections of ergometrine into permanently implanted cannulae in the nucleus accumbens caused an intense and long lasting stimulation of locomotor activity. This stimulation was blocked by pimozide and haloperidol but not by α-methyl-paratyrosine (Pijnenberg et al., 1973). Following pretreatments with nialamide (a monoamine oxidase inhibitor), bilateral injections of DA into the accumbens evoked locomotor activity (Pijnenberg and van Rossum, 1973). Noradrenaline stimulated locomotor activity, although it was much less active than DA. Activity induced by either DA or NA was antagonised by haloperidol but not by phentolamine or propanolol (Pijnenberg, Honig and van Rossum, 1975). Subsequently, a variety of phenylethylamine and isoquinoline derivatives were tested for agonist activity in this system (Costall, Naylor and Pinder, 1976a, 1976b; Pijnenberg, Honig, van der Heyden and van Rossum, 1976). These studies should be interpreted with caution considering that some were conducted using nialamide pretreatments which may enhance indirect actions of these compounds.

However, ADTN, when given either via the cerebral ventricles (Woodruff, Elkhawad and Pinder, 1974) or by bilateral injections into the nucleus accumbens (Elkhawad and Woodruff, 1975) produced a long-lasting stimulation of locomotor activity. Here again, ADTN was more potent than isoADTN and R-(+)-ADTN was the active enantiomer (Woodruff, Davies, Andrews and Poat, 1979). Electrolytic lesions of the nucleus accumbens of rats significantly reduced the locomotor activity elicited by ADTN, whereas lesions of the caudate nucleus had no effect (Woodruff, Kelly and Elkhawad, 1976).

ADTN activity was antagonised by haloperidol and pimozide (Elkhawad and Woodruff, 1975), and more recently by fluphenazine, cis-flupenthixol and sulpiride; the S-(-)-sulpiride being the active isomer (Andrews and Woodruff, 1978; Woodruff and Andrews, 1979).

The involvement of DA in locomotor activity has been reviewed recently by Fishman, Feigenbaum, Yanai and Klawans (1983).

1.7.4 Biochemical models

Biochemical methods have been used to detect the activity or changes in activity of dopaminergic systems or dopamine receptor mechanisms as a result of receptor stimulation either in vitro or in vivo.

Changes in the levels of dopamine and its metabolites (HVA and DOPAC) are considered to reflect the changes in the dopaminergic activity (Vogt, 1969) as measured in the striatum. Dopamine agonists and antagonists can change the turnover of DA in a dopaminergic brain area; neuroleptics increasing and agonists decreasing the levels of the metabolites. This was originally thought to act via a neuronal 'feedback loop' (Carlsson and Lindqvist, 1963), although Groves, Wilson, Young and Rebec (1975) considered this effect to be due to a self-inhibitory mechanism of the dopaminergic neurone mediated via their autoreceptors.

The release of endogenous or preloaded radiolabelled dopamine from nerve endings can be detected in the perfusate from the tissue used. In vivo release of DA can be elicited by electrical stimulation of nerve pathways (McLennan, 1965; Von Voigtlander and Moore, 1971), whereas in vitro release can be produced by either electrical stimulation or by depolarisation with high concentrations of potassium (Chiueh and Moore, 1974; Raiteri, Angelini and Levi, 1974). Dopamine agonists or antagonists can alter the release of dopamine - or other transmitters - by acting on pre or post-synaptic DA receptors (Arbilla, Kamal and Langer, 1980; Scatton, 1982). The area of presynaptic regulation of release has been reviewed by Starke (1981).

Prolactin (PRL) is tonically inhibited by dopamine released into the hypopyseal portal capillaries by the tuberoinfundibular dopaminergic neurones (Ojeda, Harms and McCann, 1974). Dopamine and dopamine agonists inhibited PRL secretion (Mueller, Simpkins, Moore and Mietes, 1976) both in vitro as well as in vivo, while neuroleptics elevated plasma prolactin levels (MacLeod and Robyn, 1977). The regulation of prolactin secretion is thoroughly reviewed by MacLeod (1976).

Dopamine receptors have been extensively studied by radioligand binding techniques (see review by Seeman, 1980) using a wide variety of agonists and mainly antagonists. Ligand binding studies have shown a potency profile of activity similar to that described for other models. Again, ADTN has a high potency, while the antagonists potency profile correlate well with the clinical potency used to reduce psychoses. However, the introduction of ligand binding techniques led to the description of a bewildering array of binding sites. These sites were identified by their binding characteristics rather than by a biological response. The confusion that has arisen is possibly due to variations in methodology and possibly the subunit structure of the receptor complex.

Kebabian, Petzold and Greengard (1972) discovered that dopamine could stimulate the production of adenosine 3',5'-monophosphate (cAMP) in striatal tissue. Subsequently, dopamine sensitive adenylate cyclase activity was found in other dopamine containing regions of the brain (Clement-Cormier and Robinson, 1977) such as the nucleus accumbens and substantia nigra.

Structure-activity studies of dopamine and related analogues revealed different structural requirements for activity from α or β-adrenoceptors (Miller, Horn, Iversen and Pinder, 1974), but correlated with other dopamine receptor models (Kebabian, 1978). ADTN was equipotent with dopamine in stimulating the striatal adenylate cyclase (Miller et al., 1974; Munday, Woodruff and Poat, 1974) but more potent in the nucleus accumbens (Watling, Woodruff and Poat, 1978); the R-(+)-ADTN isomer being 100 times more potent than the S-(-)-ADTN isomer and isoADTN being 50-350 times less potent than ADTN (Woodruff et al., 1979) in agreement with other model systems. Interestingly, apomorphine, a potent agonist behaviourally, was a partial agonist on this model (Kebabian et al., 1972).

The neuroleptic profile on the adenylate cyclase revealed some important anomalies compared with other systems. The buterophenones were less active at antagonising the dopamine stimulation of adenylate cyclase activity than was predicted from other pharmacological models (Karobath and Leitch, 1974), while sulpiride and other benzamides were inactive (Trabucchi, Longoni, Fresia and Spano, 1975).

1.7.5 Electrophysiological systems

Following the Helix aspersa model discussed earlier, studies on central mammalian dopamine were required. The technique of microiontophoresis (Curtis, 1964; Kozhechkin, 1980) has provided a method by which the action of drugs on the neuronal activity of a single cell can be studied. Drugs in solution bear a net charge (which can be altered by changing the pH of the solution). By passing a current down an electrode of the same polarity as the net charge of the drug in solution, the drug is expelled from that microelectrode into the surrounding environment (the quantity of drug expelled is dependent on equation 1; see appendix). Drugs applied by this method into the microenvironment surrounding the electrode tip will only influence a small number of cells which include the neurone being recorded.

Dopamine was first shown to inhibit neuronal firing in the cat caudate nucleus (Bloom et al., 1965). This was later extended to include a variety of mammalian species and in several brain areas (see York, 1975; Woodruff, 1978). Some investigators found that a proportion of the cells recorded from the caudate nucleus were excited by dopamine (McLennan and York, 1967; Connor, 1970); this proportion was as high as 50% (Spencer and Haverlick, 1973; Bevan et al., 1975), although other authors found no such excitations (Woodruff, McCarthy and Walker, 1976). Feltz (1969) and Connor (1970) showed that cells that were inhibited by nigral stimulation were also inhibited by dopamine iontophoresis. In addition, monosynaptic excitatory responses were not affected by dopamine iontophoresis (Feltz, 1969; Feltz and McKenzie, 1969). However, it is generally found that dopamine inhibits spontaneously active or iontophoretically driven cells (using either glutamate or D,L-homocysteic acid) in all areas that receive dopaminergic innervation. Dopamine agonists such as ergometrine and ADTN mimicked the actions of dopamine on these cells. These responses were diminished by all the classes of neuroleptics (Woodruff et al., 1976; Bunney and Aghajanian, 1976; Skirboll, Grace and Bunney, 1979; Akaike, Susa and Takaori, 1983).

1.7.5.1 The dopamine autoreceptors on the SNC neurones. The dopamine containing neurones of the SNC were initially used to measure the effect of systemically applied drugs on the firing rate of these neurones. It had been proposed that the activity of these cells were

under a neuronal inhibitory feedback mechanism, to account for unusual biochemical changes seen after neuroleptic treatments (Carlsson and Lindqvist, 1963). Aghajanian and Bunney (1973) discovered that dopamine applied iontophoretically had inhibitory effects on the dopaminergic cells of the SNC, suggesting that the cell bodies and dendrites contained dopamine receptors, termed 'autoreceptor' by Carlsson (1975).

Monitoring the activity of these neurones provides a useful preparation for the study of the dopamine autoreceptors. The effect of drugs applied systemically (by either intravenous, intraperitoneal or subcutaneously) or iontophoretically can be monitored for their effects on the dopamine autoreceptors, as measured by changes in the neuronal activity. This method has the advantage of measuring a direct physiological response and allows an assessment of the ability of the drug to penetrate the blood brain barrier. Intravenously applied dopamine agonists such as apomorphine, piribedil, amphetamine and ergometrine inhibit the firing rate, while isoapomorphine has no effect on these cells (Bunney, Aghajanian and Roth, 1973a; Bunney, Walters, Roth and Aghajanian, 1973b; Woodruff and Pinnock, 1981). The response to these agonists can be antagonised by the classical neuroleptics, given systemically. A disadvantage of this technique is that some drugs like ADTN are unable to cross the blood brain barrier in adequate quantities to have an effect; in addition the effects of systemically applied drugs are not restricted to the single unit being recorded.

Iontophoretic studies on the dopamine autoreceptors have produced similar results to other model systems. The dopamine autoreceptors are potently inhibited by DA, apomorphine, piribedil and ADTN; ADTN being exceptionally potent and desensitising the receptor (Aghajanian and Bunney, 1977; Walters et al., 1975; Woodruff and Pinnock, 1981). The action of dopamine agonists applied iontophoretically can be blocked by the classical neuroleptics: haloperidol, chlorpromazine and fluphenazine (Aghajanian and Bunney, 1973,1977) as well as the atypical neuroleptic sulpiride (Pinnock, Woodruff and Turnbull, 1979), S-(-)-sulpiride again being the more active isomer (Woodruff and Pinnock, 1981).

Although the dopamine autoreceptors on the cell bodies of the SNC neurones appear to have a similar pharmacological profile to other model systems for the dopamine receptor, the number of compounds tested

is not adequate enough to equate the receptors on these models as identical. There is, however, evidence to suggest that these somato-dendritic dopamine receptors are more sensitive to dopamine and apomorphine than the postsynaptic dopamine receptors in the striatum (Skirboll et al., 1979).

The recent development brain slice techniques from which the neuronal activity can be recorded in vitro (see Kerkut and Wheal, 1981) has been recently applied to the substantia nigra. The in vitro nigral slice developed by Pinnock (1983a) allows known concentrations of drugs to be applied to the solution bathing the brain slices. These drugs may alter the activity of the dopaminergic neurones in the SNC. This method can allow quantitative measurements of an agonist's pharmacological efficacy in inhibiting the spontaneous firing rate or of an antagonist's potency to attenuate agonist responses. Quantitative measurements from such experiments can be directly compared with other model systems.

1.8 Multiple dopamine receptors

Multiple dopamine receptors were first suggested by Cools and van Rossum (1976) to account for the different effects of dopamine receptor agonists in electrophysiological experiments (recently reviewed by Cools and van Rossum, 1980; Cools, 1981). They proposed that the DA receptors could be classified into excitatory (DA_e) and inhibitory (DA_i) receptor subtypes. The DA_i receptors are proposed to be located postsynaptically and mediate inhibitory responses, whereas DA_e receptors are located presynaptically on non-dopaminergic nigrostriatal neurones and mediate excitations of neuronal activity.

An alternative classification system proposed by Goldberg and Kohli (1979) is based on experimental evidence from peripheral tissues. They suggest that there are two receptor subtypes: DA_1 and DA_2. Goldberg and Kohli based this classification on the differential potencies of a series of agonists and antagonists in two model systems (see review by Goldberg and Kohli, 1983). The relevance of this classification system to the central nervous system is at present uncertain.

Another system proposed for central dopamine receptors was based on the dopamine sensitive adenylate cyclase model. Initial studies showed that dopamine and dopamine agonists stimulated the adenylate cyclase in striatal tissue and that the classical neuroleptics were competitive antagonists on this system. Although the buterophenones, while being potent antagonists of dopamine agonist responses in vivo, displayed only weak antagonism of dopamine in the adenylate cyclase assay compared with the phenothiazines and thioxanthenes (Miller et al., 1974; Leysen and Laduron, 1979). Furthermore, the substituted benzamides displayed no ability to antagonise dopamine in this system (Trabbuchi, Longoni, Fresia and Spano, 1975).

Dopamine and other agonists like apomorphine and the dopaminergic ergots were effective on dopaminergic systems such as the inhibition of PRL secretion in nanomolar concentrations, but were only agonists on the dopamine stimulated adenylate cyclase in the micromolar range of concentrations (Kebabian et al., 1972; Caron Beaulieu, Raymond, Gagne, Drouin, Lefkowitz and Labrie, 1978). The dopamine agonist SKF 38393 was found to stimulate the DA sensitive adenylate cyclase, but was without effect on PRL secretion, emesis in dogs or stereotypy in rats (Setler, Sarau, Zirkle and Saunders, 1978). Radioligand binding studies produced some anomalies; for example, binding sites had low affinities and shallow atypical displacement curves for some antagonists (Tietler, Weinreich, Sinclair and Seeman, 1978).

Kebabian and Calne (1979) proposed a classification system to explain these observations. They proposed that there were two receptor subtypes, one being linked to an adenylate cyclase that mediated increases in intracellular cAMP levels (the D-1 receptor), the second (D-2) receptor was not linked with an adenylate cyclase. They suggested that bovine parathyroid contained D-1 receptors, while the mammalian anterior pituitary contained D-2 receptors. Cis-flupenthixol was proposed as a selective D-1 antagonist and sulpiride as a selective D-2 receptor antagonist.

Although many people have interpreted their results in the light of this theory, there are a number of discrepancies which do not give support to the hypothesis. It is claimed that ^{3}H-cis-flupenthixol labels a cyclase-linked population of receptors which are distinct from those labelled by buterophenones (Hytell, 1978), yet Schwarcz, Creese,

Coyle and Snyder (1978) concluded that at least one third of the receptors labelled by ^{3}H-haloperidol were linked with the adenylate cyclase. In ^{3}H-sulpiride binding experiments, cis-flupenthixol was found to be a potent displacer of binding (Freedman and Woodruff, 1981), being more potent that (-)-sulpiride and molinidone. The benzamide, YM-091512, a potent antagonist of dopamine on the adenylate cyclase (Usada et al., 1981) inhibits the binding of ^{3}H-sulpiride binding (Jenner et al., 1981). The dopamine agonist, SKF 38393, is classified as a selective D-1 agonist. However, SKF 38393 caused locomotor activity when injected directly into the nucleus accumbens of the rat and this effect was antagonised by sulpiride (Freedman, Wait and Woodruff, 1979).

An anatomical location was suggested for the receptor subtypes; the D-1 receptor being located on intrinsic neurones in the striatum, while the D-2 receptors were located on the cell bodies SNC neurones and presynaptically on nigrostriatal and corticostriatal nerve endings. This was based on lesioning studies (Schwartz et al., 1978; Garau et al., 1978). However, cortical ablation reduced the so-called D-2 binding sites by between 30-50% (Schwartz et al., 1978; Freedman et al., 1982), confirming that D-2 receptors were located on the corticostriatal pathway. But kainic acid lesions of the striatum reduced ^{3}H-sulpiride binding by 37% suggesting that some D-2 binding sites were located on intrinsic neurones, which is contrary to the Kebabian and Calne hypothesis.

Receptor linkage to an adenylate cyclase has been suggested to be guanine nucleotide sensitive (Zalinisser and Molinoff, 1978). Thus, the D-2 (non-cyclase linked) receptor will be unaffected by the presence of guanine nucleotides. Yet, Freedman, Poat and Woodruff (1981a,1981b) found that agonist affinity to displace ^{3}H-sulpiride binding was decreased in the presence of guanine nucleotides. Thus, the hypothesis is not supported by the evidence.

The D-2 binding site has been proposed as the only relevant dopamine binding site (Seeman, 1980) since it shows a good correlation between potency of neuroleptics to displace D-2 binding and the clinical dose used to reduce psychoses. As there is no attributable biological function to the adenylate cyclase linked 'receptor' its relevance to dopamine remains in doubt.

Chapter 2

2. Materials and Methods

2.1 Electrophysiology in vivo

2.1.1 Preparation of animals for recording

Male Wistar rats weighing between 150-180 g were anaesthetised with chloral hydrate (350 mg/kg). The right femoral vein was cannulated, if drugs were to be administered intravenously. The head was secured in a David Kopf stereotaxic frame to allow the accurate positioning of electrodes into the required brain area. Anaesthesia was then changed to a 1-2% halothane with O_2 mixture inhaled from a face mask; this allows a light level of anaesthesia to be maintained for prolonged periods of time. Body temperature of the animal was maintained at between 36-38°C by means of a homoeothermic blanket. A sagital suture was made on the scalp and the muscles parted to expose the skull. Micromanipulators were brought into position and zeroed on the skull landmark Lambda. Using a dental drill, holes were drilled approximately 3 mm in diameter above the area to be investigated. The underlying dura were pierced and carefully removed to reveal the underlying cortical surface which was covered with a 0.9% NaCl solution to prevent the exposed brain from drying.

The co-ordinates used in this study were taken from Konig and Klippel (1963). For a 150 g rat, the substantia nigra zona compacta, the following co-ordinates were used: A.1.7-2.2; L.1.6-2.2; D.6.6-8.5; depth (D) being measured from the surface of the brain. A stimulating electrode was placed in the caudate nucleus for identification of the neurones in the nigrostriatal tract. The exact positioning of the electrode tip took account of the topographical innervation of the candate nucleus determined by Guyenet and Aghajanian (1978). The stimulating electrode was inserted at an angle of 20° in the sagital plane, so that the electrode tip was located within the co-ordinates: A.8.0-9.6; L.2.5-4.0; D.2.0-5.0.

2.1.2 Recording of neuronal activity

Extracellular recordings were made by either single or eight-barrelled electrodes for intravenous or iontophoretic administration of drugs respectively. Eight-barrelled electrodes, for iontophoresis, were

constructed by a modification of the method described by Crossman, Walker and Woodruff (1974). Prefibred pyrex glass capillary tubing (Clark Electromedical GC. 150-15) with 1.5 mm outside diameter, 0.86 mm inside diameter were cut into 7 cm lengths. Seven pieces of glass were then held together using heat shrink tubing (R.S. 4.8 mm) as shown in figure 2.1a. These glass bundles were mounted in a Narishige, vertical electrode puller. The centre portion of the glass bundle was heated to softness by the surrounding heater coil. The bottom chuck was then rotated through 180°, while the heater remained on, placing a twist in the electrode blank. This ensures that all the barrels pull to one tip and gives greater strength to the final electrode. The coil was then switched off to allow the electrode blank to cool. The electrode was then pulled in the usual manner adjusting the heating and magnetic pulling currents to obtain an electrode with a shaft approximately 1 cm in length that will allow recordings from deep in the rat brain. The seven barrelled electrode was held together permanently by applying super epoxy resin (Plastic Padding Ltd.) around the base of the shaft (figure 2.1b). The electrode tip was then broken back under microscopic control to an approximate tip size of 6-10 μm diameter by means of a clean glass rod. The shafts of the six outside barrels were then bent outwards to accommodate the iontophoretic leads (figure 2.1c). A single electrode pulled from the same type of glass then had two bends placed in it near the shaft, over a fine flame, so that the single electrode approximated the outline of the seven barrelled electrode (figure 2.1d). Both electrodes were placed in micromanipulators, brought close together so that the shafts were approximately parallel (this is checked in two planes). The tips were adjusted so that the tip of the single electrode protrudes from the tip of the multi-barrelled electrode by between 7-15 μm. The tips of the electrode were secured by applying an ultra-violet (U.V.) light setting glue (Locktite, 350 and 358) (Brown, Mayer and Arbuthnott, 1980). These glues have considerable advantages over conventional adhesives; the glue does not set in the air unless exposed to U.V. light; the glue when applied to the shafts is fairly fluid and will flow along to the tips without causing blockage of the multi-barrelled pipettes. Once the glue has been applied, the positioning of the electrode shafts and tips can be checked and repositioned if necessary. Re-alignment can be made without the risk of the glue setting during the procedure. When satisfied with the alignment, the glue is cured by illuminating the assembly with U.V. light of 350 nm wavelength using a high power lamp (Phillips HPR; 125 W) for

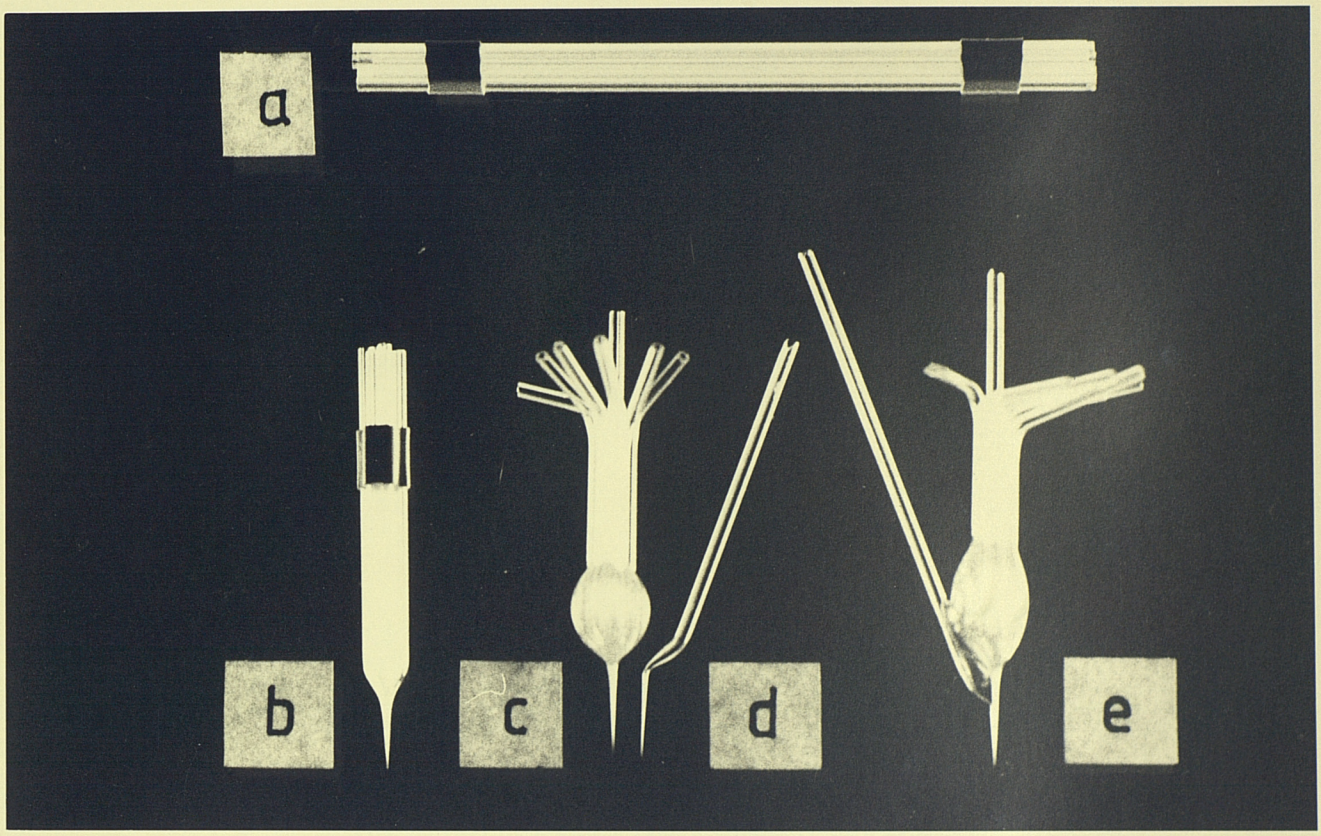


Figure 2.1

The stages in the construction of the multi-barrelled microelectrodes (described in detail in the text).

approximately 30 seconds. Extra strength was given to the electrode assembly by applying more glue to the base of the shafts and illuminating for 2 minutes. These electrodes can be used immediately, although they were usually made 24 hours beforehand (figure 2.1e).

Figure 2.2 is a block diagram of the equipment used to record and display the action potentials recorded. Extracellular recordings were made through the protruding tip of multi-barrelled or single electrodes filled with 2M NaCl containing 2% pontamine sky blue. A silver wire was inserted into the electrodes and connected to a Neurolog NL100 head stage (Digitimer Ltd.); a second indifferent electrode was placed in the fluid covering the brain to allow differential recordings to be made. The signal was amplified (NL104), filtered (NL125) to eliminate much of the background noise. The filtered signal was then passed to a Medlec FOR-4.2 oscilloscope and to an audio output to assist in locating the neurones. A third output was to a Spike trigger (NL200); individual action potentials were counted by a pulse integrator (NL601) and the analogue output displayed on a Servoscribe pen recorder as a continuous ratemeter recording. The signal could also be passed to a Racal Thermionic Store 4 tape recorder, where the extracellular potentials recorded could be processed when convenient. Permanent records of individual oscilloscope beam sweeps or spike records were made using the fibre optic recording facility of the oscilloscope. These records were made onto Kodak Linagraph direct print paper (type 1801), which can be either photodeveloped in normal light or processed photographically using standard photographic techniques.

2.1.3 Stimulation and identification of neurones

Bipolar stainless steel co-axial electrodes (Rhodes Medical Instruments, SNEX 100) were stereotaxically positioned in the caudate nucleus as described in section 2.1.1. Stimulation pulses were generated from a Grass S8 stimulator and fed to a stimulation isolator and connected to the stimulation electrode (see figure 2.2). Pulses were delivered either as single, or trains, of pulses, at regular intervals. Stimulation pulses triggered the oscilloscope beam so the effect of stimulation could be monitored on the oscilloscope. The stimulating current was measured indirectly by recording the stimulation voltage and dividing it by the stimulating electrode's resistance. Single stimulation pulses were given at a rate of 1-2 Hz, of between

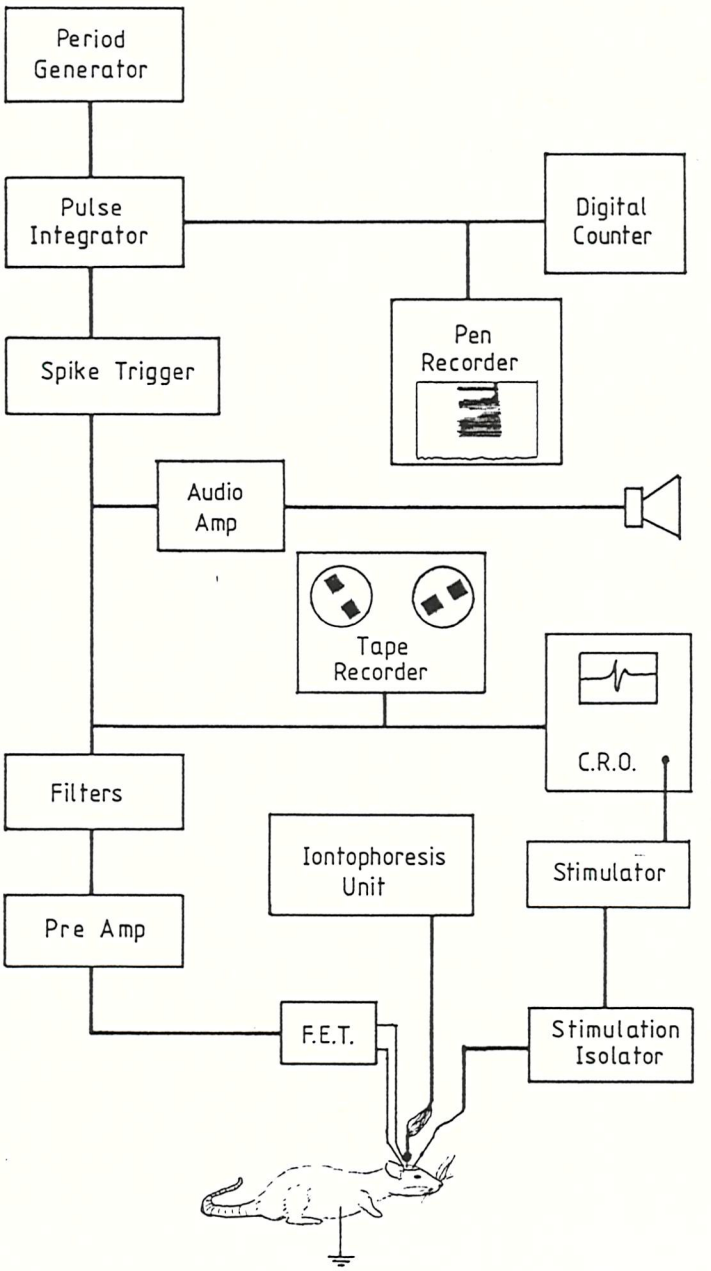


Figure 2.2

Block diagram of the equipment used to record, stimulate and iontophorese drugs onto the SNC cells.

0.3-1.0 mseconds in length at currents between 0.1-1 mA. Trains of pulses were delivered in a series of 2 or 3 pulses per train at a frequency of 100-300 Hz and at a rate of 0.5-0.3 trains per second.

Electrodes were lowered into the brain by means of a stepping motor microdrive (David Kopf). The dopamine containing neurones of the SNC were identified by monitoring for the characteristic electrophysiological properties, of size and shape of the action potentials, the rate of firing and their sensitivity to either intravenously administered apomorphine or iontophoretically applied dopamine, and by antidromic stimulation of the SNC cells from the ipsilateral caudate nucleus, as previously described by Bunney, Walters, Roth and Aghajanian (1973), Guynet and Aghajanian (1978) and Grace and Bunney (1980). Identified SNC neurones were monitored for at least 5 minutes to obtain a stable baseline firing rate. Recordings in the cerebellum from Purkinje cells were attempted by observing the electrical activity of recorded units and by histological examination subsequent to the experiment. The firing pattern of Purkinje cells have been identified (Woodward, Hoffer and Lapham, 1969). Purkinje cell firing is characterised by high frequency bursts of activity and irregular singlet spike firing.

2.1.4 Drug application

Drugs for intravenous administration were dissolved as follows: apomorphine, RU24926 and RU24213 were made up in 8 mM tartaric acid; sulpiride, in the minimum tartaric acid required to dissolve it; haloperidol in a 1% lactic acid in 0.9% NaCl. Dibenzoyl ADTN was dissolved in a mixture of ethanol, water and polyethylene glycol 400 (3:3:4 vol/vol). The remainder of the compounds were dissolved in 0.9% NaCl. Drugs were injected into the animals in a volume of between 0.025-0.3 ml.

Drugs for iontophoretic application were dissolved in distilled water with the exceptions of DA and NA which were dissolved in 8 mM tartaric acid to prevent oxidation of the molecules, and sulpiride which was dissolved in the minimum tartaric acid required for solvation. The pH of drug solutions was adjusted with hydrochloric acid to between pH 3-5. Table 2.1 shows the drugs used in this study, the concentrations used for iontophoresis and the pH of the experimental solution. All drugs were ejected as cations except for L-glutamate. One barrel of the electrode assembly always contained 1M NaCl which was used as a balance

Table 2.1

Drugs used in this study are listed below, their structures are given in the appendix.

Drug	Concentration used for iontophoresis	pH	Source
ADTN HBr	-	-	Calbiochem-Behringer
Apomorphine HCl	-	-	McFarlan Smith
Chloral Hydrate	-	-	B.D.H.
Dopamine HCl	200 mM	3-3.5	Koch-Light
DB ADTN	-	-	Dr. A.S. Horn
Fluphenazine HCl			Squibb
GABA	200 mM	4-5	B.D.H.
l-glutamate	200 mM	4-5	B.D.H.
Glycine	200 mM	4-5	B.D.H.
Haloperidol			Searle
Noradrenaline HCl	200 mM	3-3.5	Koch-Light
Piribedil methane sulphonate	-		Servier
d,l-propanolol HCl	200 mM	4-5	Sigma
RU24213			Roussel UCLAF
RU24926			Roussel UCLAF
(±)-sulpiride	100 mM	4-5	Ravizza (Italy)
Taurine	200 mM	4-5	B.D.H.
Halothane	-	-	I.C.I.
S-3608 chlorohydrate			Servier
Zetidoline HCl	100-200 mM	4-5	Lepetit (Italy)

channel to eliminate current artifacts (Salmoiraghi and Weight, 1967). Iontophoretic currents were produced by a Neurophore iontophoretic unit (Digitimer Ltd.) with automatic current balancing. The resistance of the iontophoretic electrodes usually varied between 20-120 MΩ with some even greater than this. Extremely high resistances were taken to indicate that a barrel was blocked. During experimentation, drugs were prevented from leaking out of the microelectrodes by using a 10 nA retaining current opposite in charge to the ejection current.

2.1.5 Analysis of data

The electrophysiological data obtained from these experiments were presented in three forms, either as (1) single or combined sweeps of the oscilloscope beam showing action potentials or evoked potentials following stimulations; (2) continuously moving film, spike records. Both of these were obtained directly from the oscilloscope fibre optics recording system; (3) a continuous rate meter recording in which the number of spikes counted per 10 second period is shown on the vertical scale.

2.1.6 Verification of recording and stimulating electrode positions

Neurones were initially identified in the manner described in section 2.1.3. At the end of each successful experiment, pontamine sky blue dye was ejected as an anion by passing a 2-5 μA current for 5-10 minutes, from the recording electrode (Hellon, 1971; Boakes, Bramwell, Briggs, Candy and Tempesta, 1974). The brain was then fixed by perfusing the rat through the aorta with 50 ml of 10% formyl saline and then extracted. The position of the dye was verified histologically by examining serial sections of the brain cut on a freezing microtome.

Verification of the position of the stimulating electrode was made by examining the position of the electrolytic lesion made at the electrode tip by passing a current of 1 mA for 10 seconds.

2.2 In vitro nigral slices

2.2.1 Preparation of slices

Male Wistar rats (150 g) were killed by a blow to the neck and their brains rapidly, but carefully, removed in order to minimise dissection damage to the brains. A block of tissue containing the substantia nigra was cut and glued onto a glass cutting stage (rostral side uppermost). This stage was then secured in the bath of a vibrotome (Oxford Instruments)

which contained artificial cerebro-spinal fluid (csf) (see table 2.2) bubbled with 95% O_2/5% CO_2 and maintained at a temperature of 37°C. Coronal sections 300 µm thick were cut taking care that the tissue block did not squash while cutting as this can result in a loss of activity in the slice. Slices were transfered to the recording bath (figure 2.3) and were maintained at an oxygenated artificial csf, humidified 95% O_2/5% CO_2 interface at 37°C. The slice was perfused by the csf at a rate of approximately 0.25 ml/second.

2.2.2 Recording and identification of the substantia nigra zona compacta neurones

Extracellular recordings were made from single barrelled electrodes as described in section 2.1.2. Neurones visually identified as being located in the zona compacta of the substantia nigra (figure 2.4a) were also monitored for the characteristic electrophysiological properties of the dopamine containing cells described by Guyenet and Aghajanian (1978) and Grace and Bunney (1980). Amplified, filtered signals were displayed on an oscilloscope, passed to a tape recorder or to an audio output to assist in locating cells. The signal was passed to a Digitimer D130 spike processor where individual impulses were counted. This was displayed as a continuous ratemeter recording on a Gould, Brush pen recorder.

2.2.3 Experimental procedure

Identified neurones were monitored for at least 5 minutes before drug application to obtain a stable baseline firing rate. Drugs were dissolved in the artificial csf containing 1 mM ascorbic acid and diluted to the required concentrations with the artificial csf. Drugs were applied to the bath until plateau responses were obtained. The drugs were then washed out by returning to the original artificial csf. Dose response curves were obtained to dopamine in the presence of zetidoline at different concentrations. At concentrations of dopamine greater than 3 mM the osmotic balance of the solution is compromised; thus this was the maximal concentration used. Only one cell per slice was analysed.

2.2.4 Analysis of data

Ratemeter recordings were made of the firing rate of the cells and the percentage inhibition of the cell firing rate, when the response had plateaued, was measured. The results are expressed as the mean ± the standard error of the mean. From the dose response curves of dopamine

Table 2.2 Composition of the artificial cerebrospinal fluid used in the in vitro slice experiments

Salt	Concentration (mM)
NaCl	124.0
KCl	2.0
KH_2PO_4	1.25
$MgSO_4$	2.0
$CaCl_2$	2.0
$NaHCO_3$	25.0
Glucose	11.0

The solution was bubbled with a 95% O_2/5% CO_2 gas mixture and then adjusted to pH 7.4.

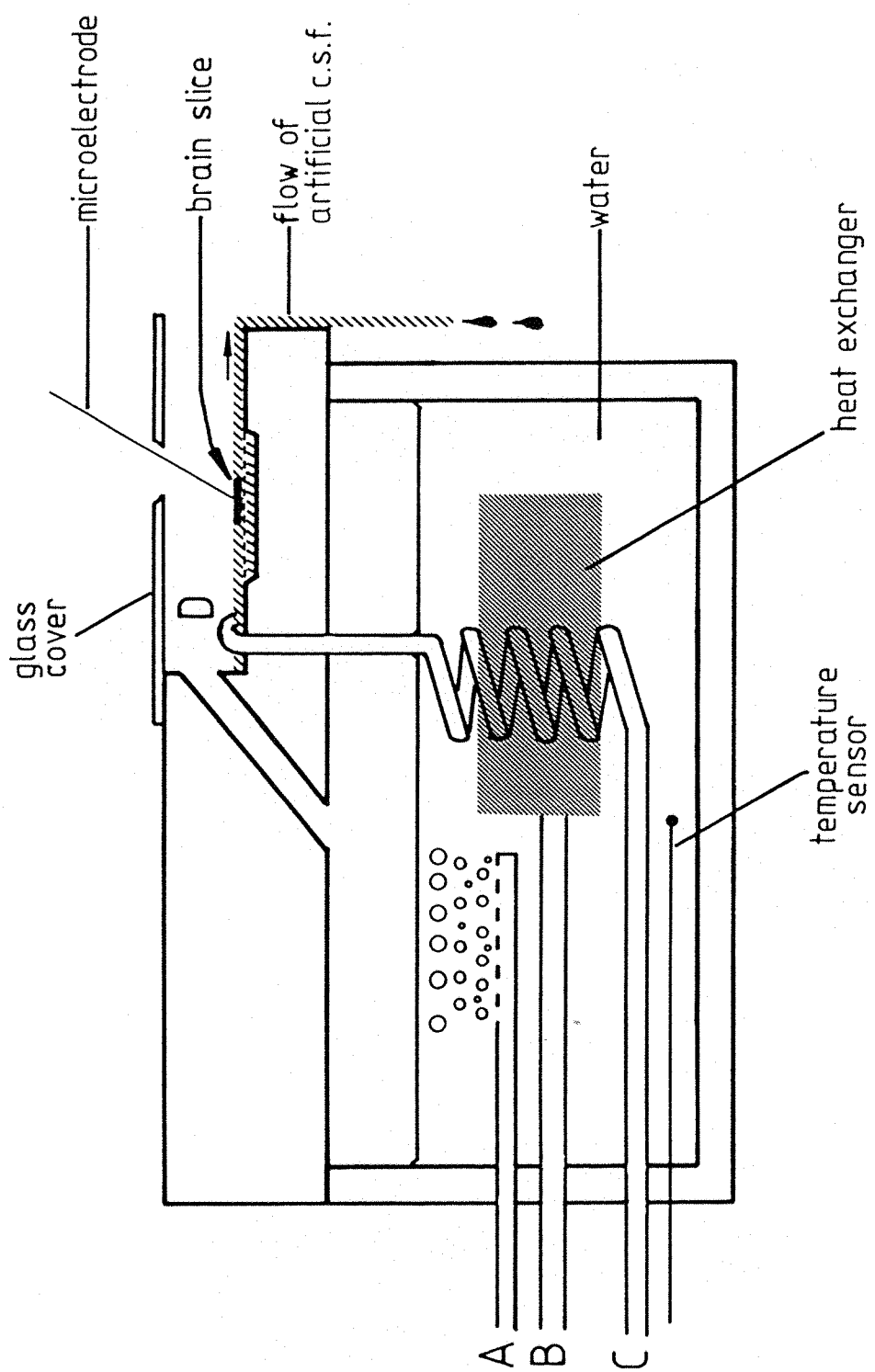


Figure 2.3 Cross-section through the experimental bath used to record the neuronal activity of the SNC cells in brain slices.

A, inlet for 95% O_2/5% CO_2 gas mixture; B, circulating hot water to maintain bath at 37°C; C, inlet for oxygenated artificial cerebrospinal fluid; D, inlet for warmed and humified O_2/CO_2 gas mixture and for warmed artificial cerebrospinal fluid.

Figure 2.4

Serial coronal sections through the rat brain at the levels of: (a) the substantia nigra; (b) the nucleus accumbens. (from Konig and Klippel, 1963).

a = nucleus accumbens; caa = anterior commisure; cl = claustrum; cp = caudate/putamen; ip = interpeduncular nucleus; lm = medial lemniscus; r = red nucleus; rcc = corpus callosum (radiations); snc = substantia nigra zona compacta; snl = substantia nigra zona lateralis; snr = substantia nigra zona reticulata; tob = basal optic tract; tol = lateral olfactory tract; tsth = septohypothalamic tract; vl = lateral ventricle.

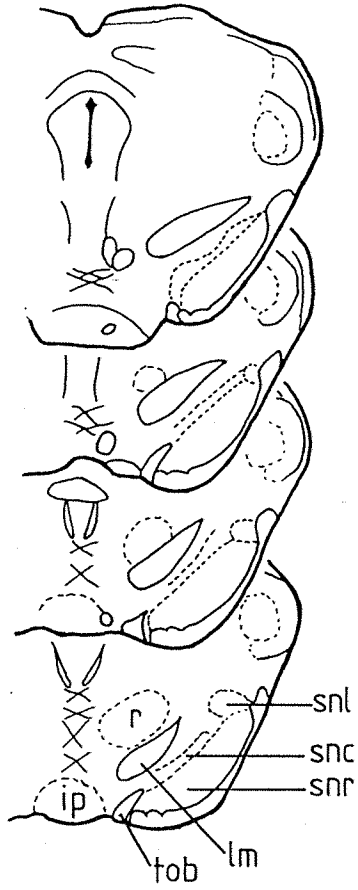
a

2420µ

2180µ

1950µ

1760µ



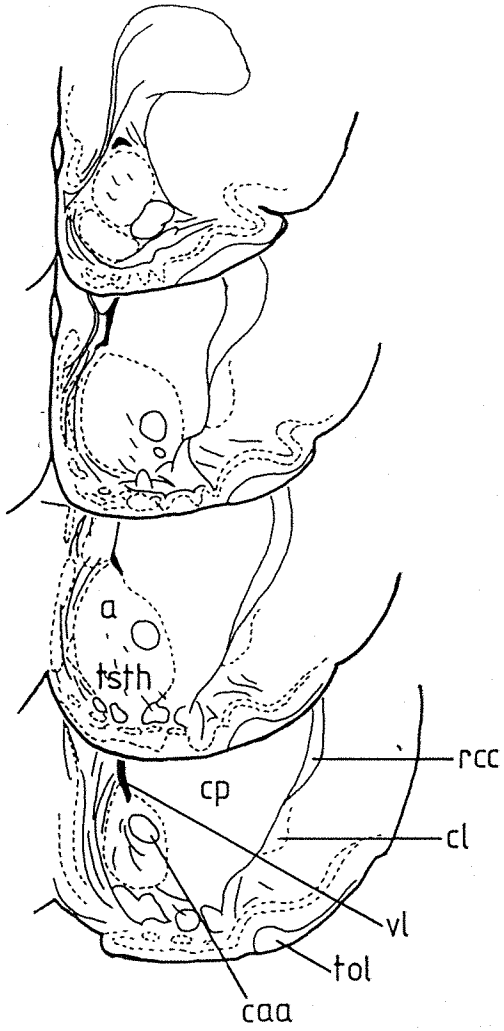
b

10050µ

9650µ

8920µ

8380µ



in the absence and in the presence of zetidoline, the ED_{50} for dopamine at each antagonist concentration was determined. The agonist dose ratio for each concentration of antagonist was calculated, a Schild plot drawn and a pA_2 value obtained (Schild 1947a,b).

2.3 Behavioural experiments

2.3.1 Preparation of animals for chronically implanted cannulae and intracerebral injections

Male Wistar rats weighing 180-200 g at the time of surgery were used. Two stainless steel guide cannulae (figure 2.5) were implanted bilaterally into the brain above the nucleus accumbens and permanently fixed in place. The animals were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and their heads secured in a stereotaxic frame (David Kopf, 900). A sagital incision was made in the scalp and the skin reflected to expose the skull. The overlying muscles and connective tissue were removed to expose the bone, which was cleaned with 70% ethanol and allowed to dry. Two holes were drilled in the skull for the introduction of the cannulae at the following co-ordinates: A=9.2; L= ±2.4, taken from Konig and Klippel (1963) using Lambda as the stereotaxic zero. The cannulae were lowered into the brain at an angle of 12°; this aims the cannulae at the nucleus accumbens but also misses the lateral ventricles and bulk of the caudate nucleus (see figure 2.4b). Two other burr-holes were made into the skull slightly posteriorly to the cannulae for two small stainless steel screws to be fixed into them. After the screws were secured, dental acrylic cement (De Trey) was applied around these screws and around the cannulae to make a firm and secure attachment to the skull. The stylets were fitted into the cannulae and the incision closed with suture silk. The animals were injected with an antibiotic (Ampiclox 40 mg/kg i.m.) to prevent infection of the wound and allowed to recover in their cages for a week. During this time the rats were accustomed to being handled before intra-accumbens injections were started. Figure 2.6 shows a fully recovered rat with bilaterally cannulated nucleus accumbens, six weeks after surgery.

2.3.2 Intra-accumbens and intra-peritoneal injections

Drugs were injected into the nucleus accumbens bilaterally via the permanently implanted cannulae. The injections were given to conscious,

manually restrained animals, by a Hamilton 5 µl glass microsyringe fitted with a 30 gauge needle. Each cannulae protruded into the brain ending short of the nucleus accumbens. Each cannulae was calibrated and short plastic sleeves (Portex ppl0 tubing) cut and slipped over the injection needle so that the tip of the needle protrudes 6.5 mm into the brain (figure 2.5). Thus, injections could be made directly into the nucleus accumbens. Drugs for intra-accumbens injections were dissolved in sterilised 0.9% saline except for ADTN and sulpiride which were dissolved with the aid of tartaric acid. RU24213 and RU24926 were dissolved in the minimum amount of N,N-dimethyl formamide and then adjusted to volume with 0.9% sterile saline. Drugs were injected into the brain in a volume of 1 µl per side over a period of approximately 30 seconds. The inner stylets were replaced after injection was completed.

Intra-peritoneal injections were given in a volume of 1 ml/kg of body weight, the drugs being dissolved in sterile 0.9% NaCl. Following the experiment animals were left for at least 2 days before another injection was given.

2.3.3 Measurement of locomotor activity

The locomotor activity of the rats was measured in activity cages obtained from I.C.I. The cages containing the animals are placed on top of the detectors. The detectors are a series of photoelectric sensors arranged in a 6 x 8 grid underneath the cage (figure 2.7). The cages are illuminated from above by an ordinary 100 watt light bulb. Animal movement in the cage interrupts the incident light on the sensors below. Every five minute period the number of interruptions detected in each cage is printed. Prior to injections the animals are placed in the experimental conditions for half an hour to familiarise them with the surroundings. These experiments were always started between 10 a.m. and 12 noon and took place in a quiet experimental room in the animal house.

2.3.4 Analysis of data

In all experiments the total number of interruptions made by each animal for every half-hour period were combined and the results expressed as a mean ± the standard error of the mean. Collation of the data was undertaken with the aid of a Commodore PET 4000 computer using a program written in conjunction with Dr. P.G. Dorey.

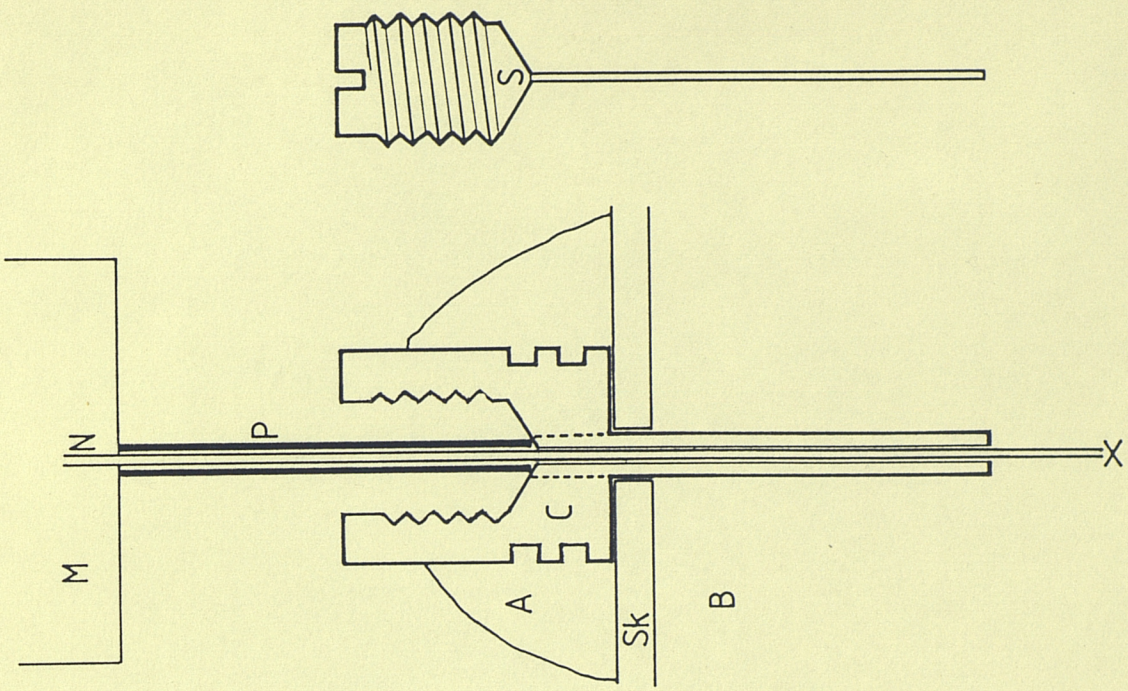
Figure 2.5

Diagram of the guide cannulae and the equipment used to inject drugs directly into the nucleus accumbens.

A = acrylic cement; B = brain tissue; C = cannulae; M = microsyringe;
N = needle; P = plastic sleeve; S = stylet; Sk = skull;
X = injection site in the brain.

Figure 2.6

Photograph of a rat fitted with bilateral guide cannulae above the nucleus accumbens.


S
N
P
X
M
C
A
Sk
B

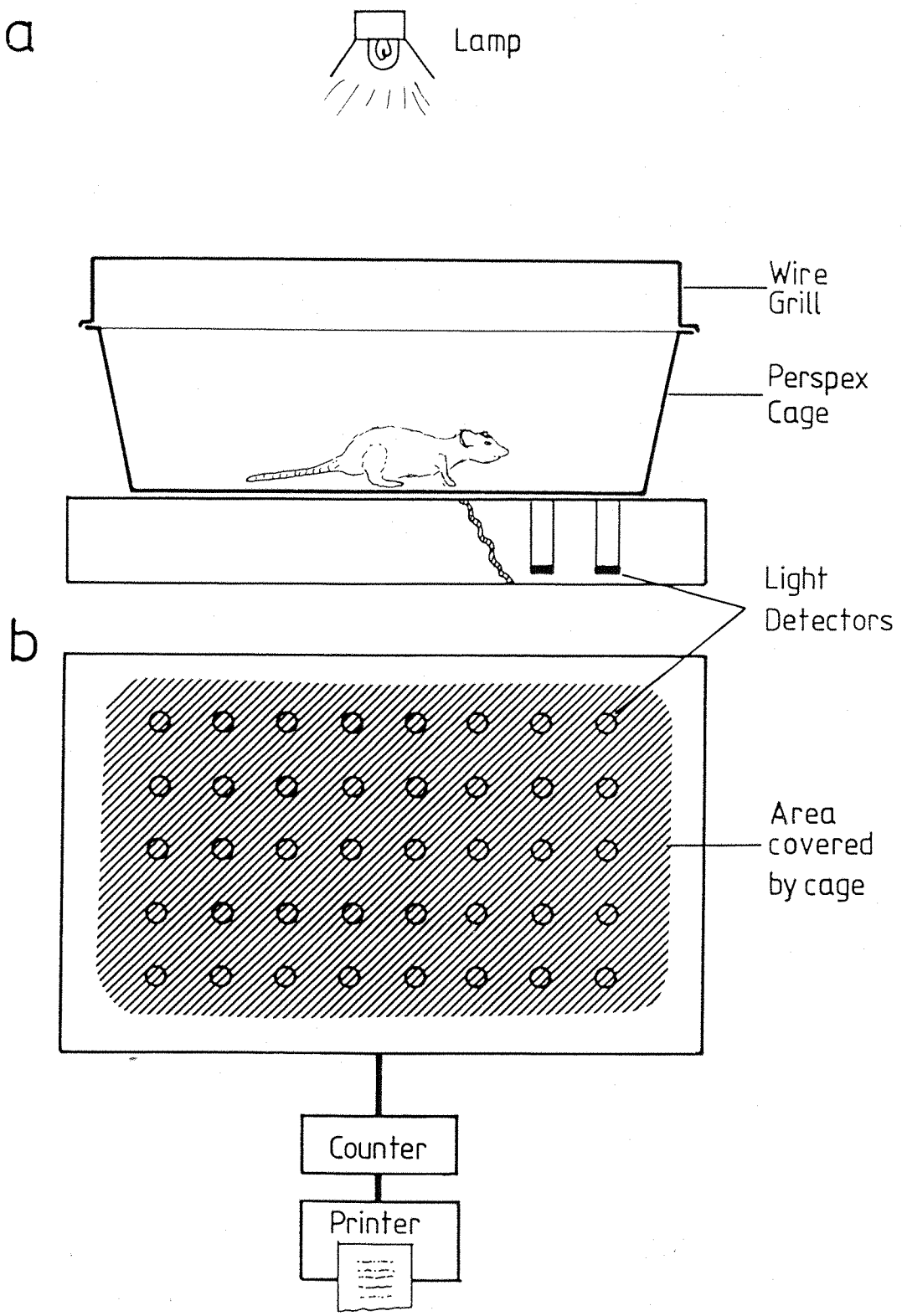


Figure 2.7

Diagram of the apparatus used to measure the locomotor activity of the rat.

2.3.5 Verification of the nucleus accumbens injection site

At the end of a series of experiments, the animals were killed by sodium pentobarbitone anaesthesia. The animals' brains were then fixed by perfusing with 50 ml of 10% formyl saline via the aorta. The brains were removed and sectioned on a freezing microtome; serial sections (20-30 μm thick) were analysed to see the exact position of the injection needle tip. If verification could not be made then the results were discarded.

Chapter 3

3. Results

3.1 Electrophysiology and identification of the SNC neurones

The neurones contained within the SNC are mostly spontaneously active, although some are quiescent and can be activated by iontophoretically applied excitatory amino acids and CCK (Bunney, Grace, Homer and Skirboll, 1982). In the present study, only spontaneously active neurones were studied.

Extracellularly recorded SNC cells had stable firing rates; a single unit rarely varied by more than 10% when not influenced by drugs. These cells fired at a rate of between 1 and 8 Hz and had characteristic wide, triphasic potentials, as shown in figure 3.1a, approximately 4-5 msec wide, often with a pronounced initial segment. Some cells fired with a slow bursting pattern, as can be seen in figure 3.1b; each burst showed a gradual decrease in the spike amplitude. These cells usually had the higher spontaneous firing rates.

By listening to the audio output, the SNC cells can be distinguished from other cells in the area: the SNC cells make a lower pitched sound than the SNR cells, which have a much shorter action potential (2-3 msec) and generally have faster firing rates (between 0.1-60 Hz). Despite the high density of cells in the SNC it was usual to record no more than two SNC units in a single penetration of the electrode. These electrophysiological characteristics are similar to those described by Bunney et al. (1973), Guyenet and Aghajanian (1978) and Grace and Bunney (1980).

The SNC neurones were further identified by antidromic stimulation of the ascending axons from these cells in the nigrostriatal tract. Stimulation of the pathway at either the medial forebrain bundle or in the striatum causes antidromic potentials to invade the cell soma. Figure 3.1c shows the antidromic responses elicited by stimulating the striatum. These stimulating pulses usually produced only the initial segment of the action potential, but occasionally a full action potential developed, as can be seen in the fourth sweep in figure 3.1c. The latent

Figure 3.1 Photographs taken from the oscilloscope.

A. A single action potential recorded extracellularly from a substantia nigra zona compacta neurone. The action potentials are triphasic, being approximately 5 mseconds in duration, and often show an initial segment on the first positive phase.

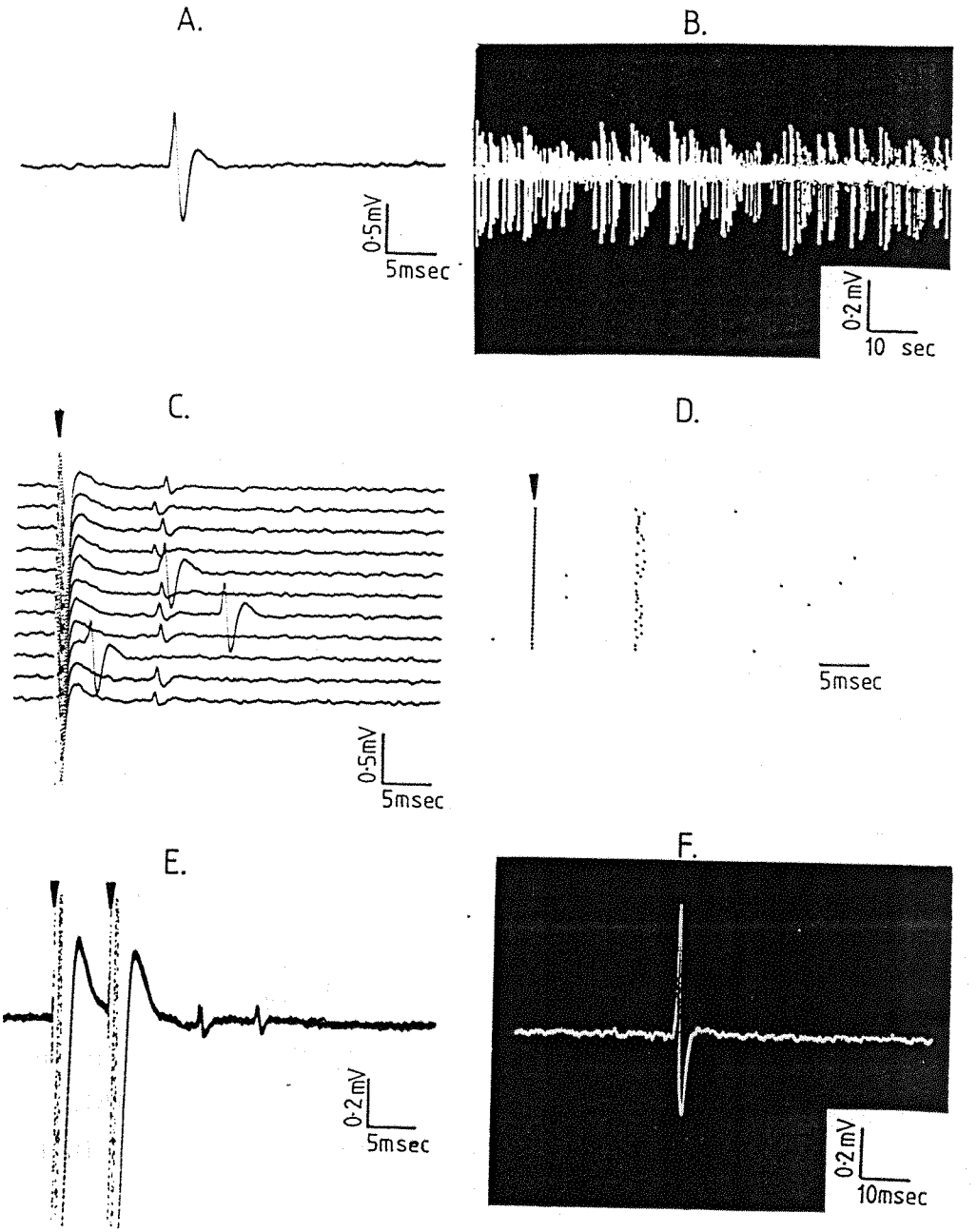
B. A continuous spike record of the neuronal activity of a single substantia nigra zona compacta neurone, showing the slow bursting activity with a decrease in the spike amplitude during the bursts.

C. Antidromic stimulation of a substantia nigra zona compacta neurone following the delivery of an electrical stimulation pulse to the ipsilateral striatum. In the eleven consecutive oscilloscope sweeps a stimulation pulse of 0.2 mA and 0.5 msec duration was given 5 mseconds after the oscilloscope beam is triggered. The initial segments are evoked on most of the stimulation pulses, although sometimes a full action potential occurs, as shown on the fifth sweep. Sometimes a convenient spontaneous action potential occurs soon after the stimulating pulse, as can be seen in the ninth sweep. The antidromic and spontaneous action potentials, travelling in opposite directions, show collision in the axon and consequently the antidromic potential is absent from the expected position in this sweep.

D. A raster dot display of the antidromic stimulation of substantia nigra zona compacta neurones following the electrical stimulation of the striatum. The arrow head shows the dots due to the stimulation artifact, while the other dots represent action potentials. This shows that there is a slight variation of approximately 1 msec in the latency between the stimulation pulse and antidromic potential recorded at the SNC cell body.

E. Antidromic stimulation of a substantia nigra compacta neurone following two stimulation pulses of 0.3 mA of 0.5 msec duration, given at a frequency of 200 Hz. This trace is the result of ten superimposed sweeps and shows that the neurone can be stimulated at high frequency and show frequency following of the antidromic potentials.

F. A single action potential recorded extracellularly from a substantia nigra zona compacta neurone in a brain slice of the substantia nigra.


A.
0·5mV
5msec
B.
0·2mV
10 sec
C.
0·5mV
5msec
D.
5msec
E.
0·2mV
5msec
F.
0·2mV
10msec

period (the period between stimulus and the recorded potentials) of some cells varied slightly (by 0.5-1.0 msec) to a constant stimulation strength, as can be seen in figures 3.1c and 3.1d. Similar variations in antidromic latency of SNC neurones have been reported by Collingridge, James and McLeod (1980), which they attributed to either changes in axon excitability following the conduction of a previous orthodromic action potential or activation of different parts of a branched and twisted axon. The latent period was used to calculate the conduction velocity of the axons (0.46 ± 0.07 m/sec; n=23). This is compatible with previously reported figures (e.g. 0.58 m/sec by Guyenet and Aghajanian, 1978) and is typical of a small diameter unmyelinated fibre. The antidromic responses could be made to follow a train of stimuli at a frequency of between 100-300 Hz as can be seen in figure 3.1e. Figure 3.1c also shows that a conveniently placed spontaneous action potential collides with and eliminates the antidromically elicited potential. Thus, in this study, the criteria for antidromic stimulation of neurones (Lipski, 1981) were satisfied. Approximately 20% of the neurones tested were antidromically stimulated.

Subsequent to any successful experiment, the brains were examined histologically to confirm the placement of the recording electrode by examining that the position of pontamine sky blue dye was within the SNC, as figure 3.2a shows. Similarly, histological examination was carried out to determine the position of the lesion, produced by the stimulating electrode, laying within the striatum as shown in figure 3.2b. Where confirmation of the positions of recording or stimulating electrodes was not made, the results were rejected.

3.2 Agonist studies

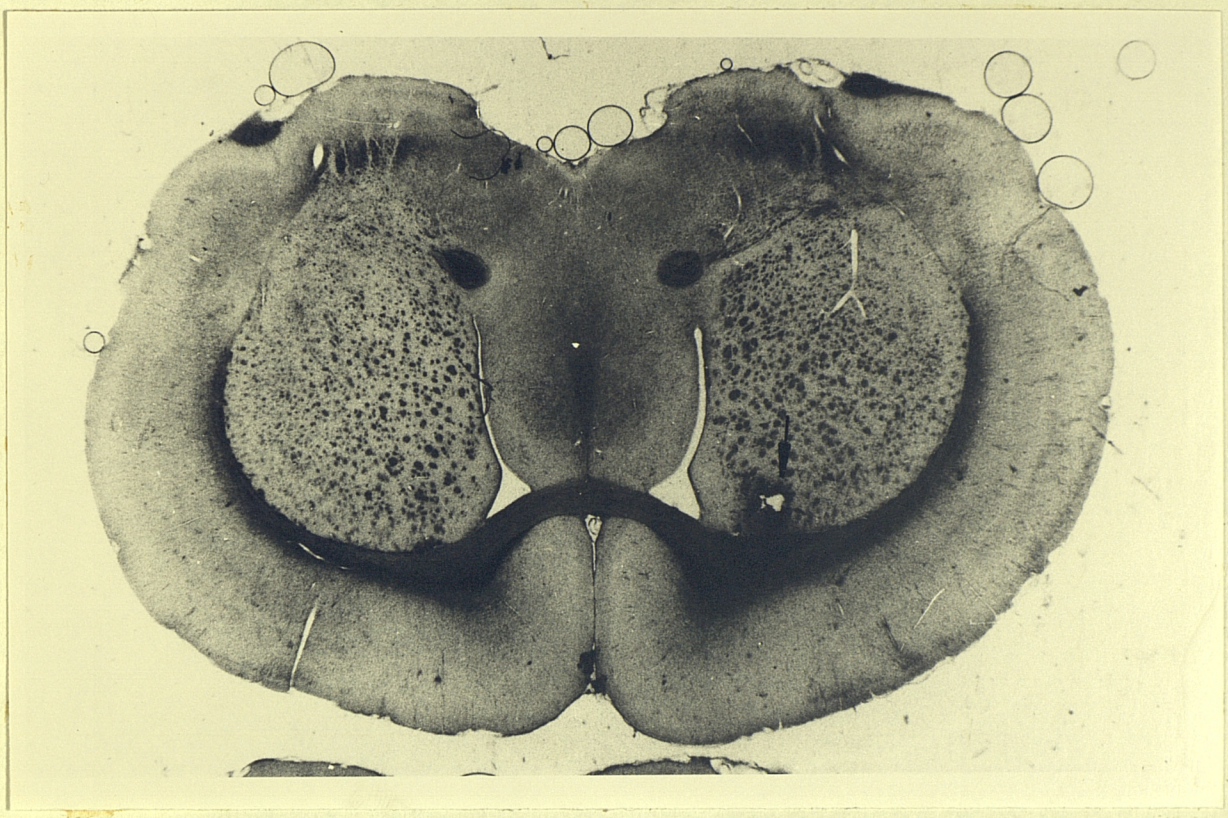
3.2.1 The effects of intravenous apomorphine, piribedil and S-3608 on the SNC neuronal firing

The dopamine agonist, apomorphine, is known to potently inhibit the firing rate of the dopamine containing neurones in the SNC when given intravenously (Bunney, Aghajanian and Roth, 1973). Any potential dopamine agonist can thus be compared with apomorphine in this system.

Figure 3.2 Coronal sections through the rat brain at the levels of (a) the substantia nigra and (b) the caudate nucleus.

(a) shows the position of the pontamine sky blue dot marking the position of the tip of an electrode used to record the neuronal activity of a SNC neurone.

(b) shows the position of the lesion in the caudate nucleus produced by the stimulating electrode used to antidromically stimulate an SNC neurone.



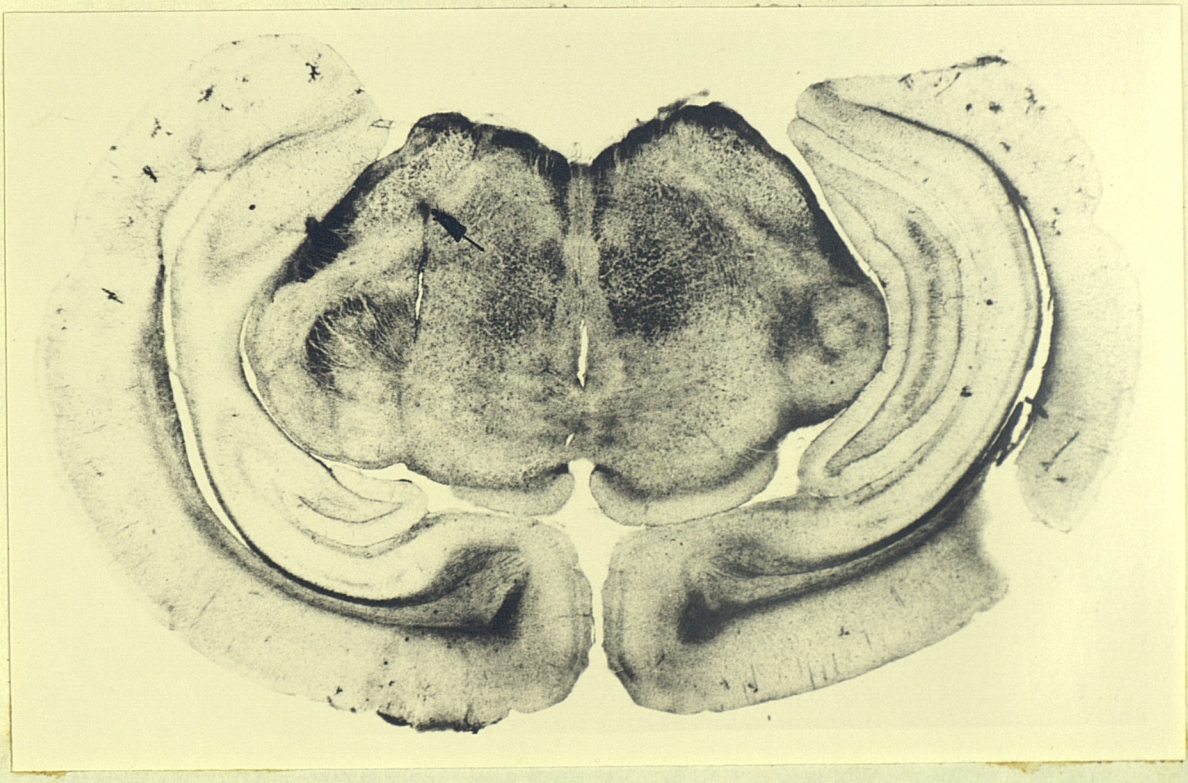
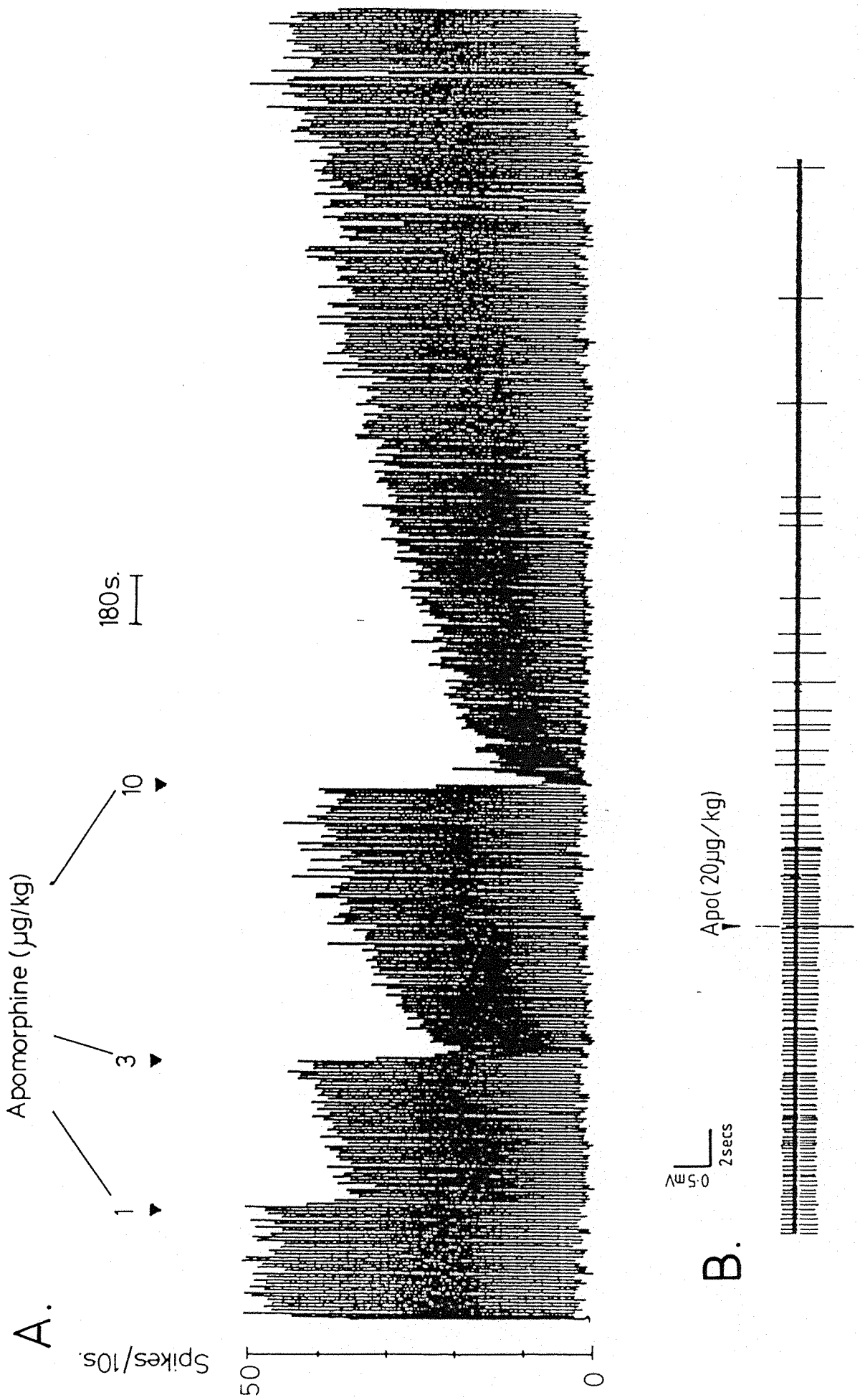


Figure 3.3

A. A rate-meter trace of a SNC cell. Apomorphine given at doses of 1, 3 and 10 µg/kg caused a dose related inhibition of the neuronal activity.

B. A spike record of the neuronal activity of a SNC neurone. Apomorphine (20 µg/kg), given intravenously (at the arrowhead), rapidly produced an inhibition of the firing rate and also caused an increase in the spike amplitude.


A.
Spikes/10s.
50
0
180s.
Apomorphine (µg/kg)
1
3
10
B.
0·5mV
2secs
Apo(20µg/kg)

The effects of apomorphine on the extracellularly recorded SNC neurones is shown in figure 3.3. On these cells, doses of apomorphine between 1 and 50 µg/kg given intravenously caused dose dependent depressions of the neuronal firing rate with an associated increase in the spike amplitude, as shown in figure 3.3b. A dose of 10 µg/kg of apomorphine generally produced an inhibition of the SNC neuronal firing of approximately 50%, while threshold responses were usually achieved at a dose of 0.5 µg/kg and a temporary cessation of firing was produced at doses of 20 µg/kg (or larger). Guyenet and Aghajanian (1978) calculated an ED_{50} for apomorphine on the SNC cells of 9.3 µg/kg, which is comparable with the present results. Depression of the neuronal firing rate started within 15 seconds of drug administration and a maximal effect usually occurred within 30 seconds. Recovery of the firing rate was generally biphasic. The initial recovery was rapid, returning to about 30% of the basal firing rate, followed by a much slower recovery rate, a dose of 20 µg/kg taking approximately 20-30 minutes to return to the basal firing levels. The fast initial phase of recovery from apomorphine only occurred at the higher doses given. This observation is in agreement with previous reports (Walters et al., 1975).

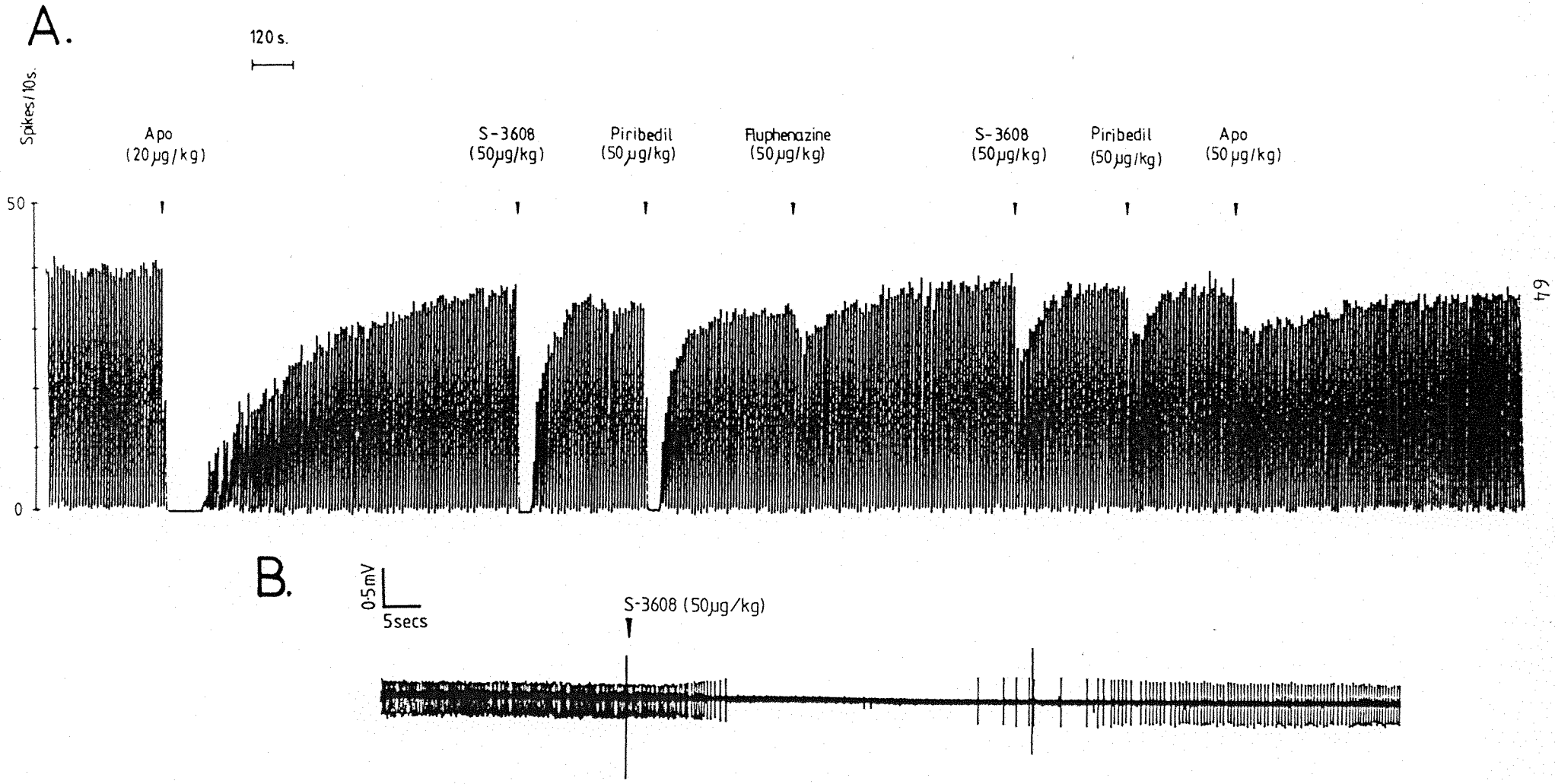
To check the assumption that the apomorphine response was mediated via dopamine receptors, the dopamine receptor antagonist, fluphenazine, was given. Figure 3.4 shows that fluphenazine given intravenously (50 µg/kg), after an initial dose of apomorphine, antagonised the actions of apomorphine. Fluphenazine blocked the actions of apomorphine on five of the six cells tested. The antagonistic action of fluphenazine took 3-4 minutes to take effect. In one experiment the responses of apomorphine (20 µg/kg), given repeatedly at intervals of 20-30 minutes, were monitored continuously for two hours following a single intravenous injection of fluphenazine (50 µg/kg). Throughout this period the inhibitory effects of apomorphine were antagonisted. Thus, fluphenazine was an extremely potent and long-lasting antagonist of apomorphine in this system.

Having demonstrated the dopamine-like activity of apomorphine on the SNC cells, a new potential dopamine agonist was tested on this system. S-3608 (4(5-coumaramyl methyl)-1-(2-thiazolyl) piperazine, HCl) was proposed as a dopamine agonist by Poignant, Gressier, Petitjean, Regnier and Canevari (1974). However, little is known about its pharmacology.

Figure 3.4

A. A rate-meter trace of an extracellularly recorded SNC neurone. Intravenously applied apomorphine, S-3608, and piribedil inhibited the neuronal firing rate. The inhibitions due to S-3608 and piribedil rapidly reversed while the inhibition by apomorphine lasted much longer (approximately 25 minutes). Intravenously applied fluphenazine antagonised the effects of further doses of apomorphine, piribedil and S-3608.

B. A spike record of an extracellularly recorded SNC neurone. S-3608 given intravenously rapidly caused an inhibition of the neuronal activity with an associated increase in the spike amplitude.


A.
120 s.
Spikes/10s.
50
0
Apo
(20 µg/kg)
S-3608
(50µg/kg)
Piribedil
(50µg/kg)
Fluphenazine
(50µg/kg)
S-3608
(50µg/kg)
Piribedil
(50µg/kg)
Apo
(50µg/kg)
64
B.
0·5mV
5secs
S-3608 (50µg/kg)

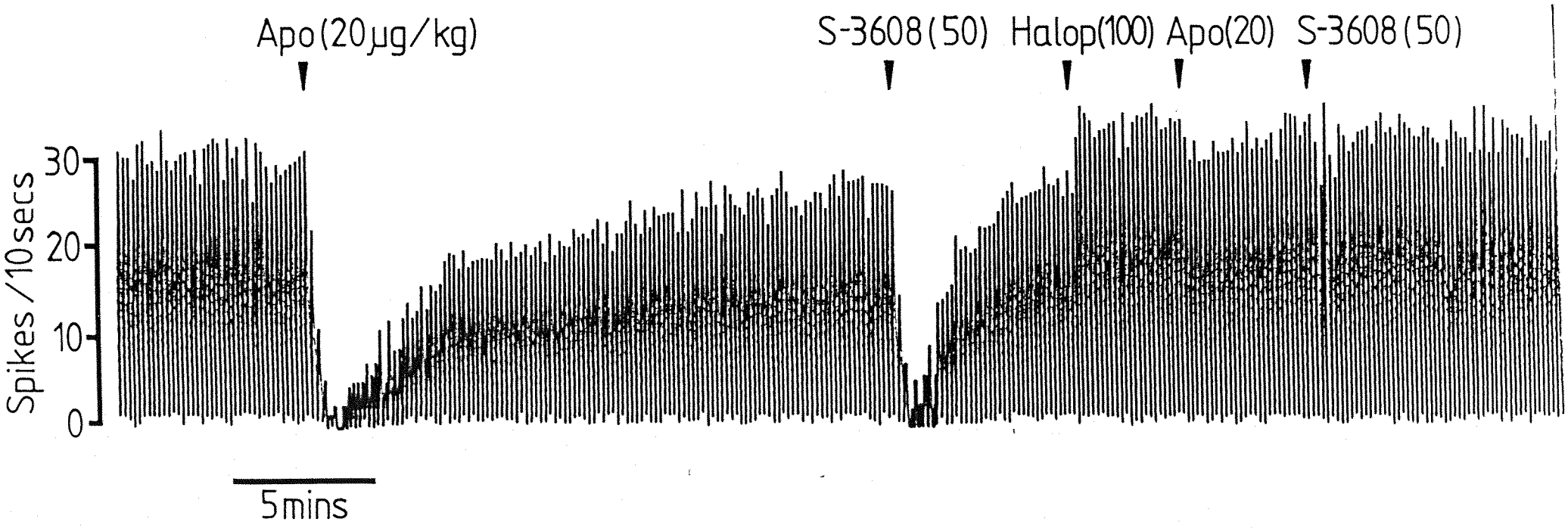
Thus, S-3608 and its structural analogue, piribedil, were administered intravenously in order to compare their effects with those of apomorphine.

Piribedil, given intravenously at doses of 5-250 µg/kg, caused dose-dependent inhibitions of the neuronal firing rate in 13 of the 14 cells tested. Figure 3.4 shows the effect of a dose of 50 µg/kg i.v. on the neuronal firing rate of the SNC cells. The onset of action was rapid, occurring within 15 seconds of drug administration, with an associated increase in the action potential's amplitude, similar to that seen with apomorphine, Recovery from the neuronal inhibition was rapid, taking approximately 4-5 minutes to return to the basal firing rate.

S-3608, applied intravenously at doses between 10-250 µg/kg, produced dose-dependent inhibitions of the neuronal activity on all 30 cells tested. Figure 3.4 shows that the inhibition of firing rate was similar to that of piribedil. Inhibitions occurred about 15 seconds after drug administration and were associated with an increase in the extracellular action potential amplitude, as shown in figure 3.4b. Recovery from the neuronal inhibition was rapid, taking about 4-5 minutes. As figure 3.4 shows, the inhibitions of neuronal activity produced by piribedil and S-3608 were equipotent in this system, both compounds having similar onset and duration of action but with approximately one third the potency of apomorphine. Recovery from an equipotent dose, which temporarily stops the neuronal firing rate, took approximately 5 minutes for piribedil and S-3608. This was shorter than the recovery from apomorphine which took about 25 minutes. Thus, piribedil and S-3608 are therefore able to rapidly cross the blood brain barrier and have an inhibitory effect on the SNC neurones within seconds. The dopaminergic nature of the responses to piribedil and S-3608 were tested using the dopamine receptor antagonists, fluphenazine and haloperidol. As figure 3.4 shows, fluphenazine, given intravenously at 50 µg/kg, antagonised the inhibitions produced by piribedil on all 4 cells tested, while the inhibitory effects of S-3608 were antagonised by fluphenazine in 6 of the 7 cells tested. The one cell on which the inhibition due to S-3608 was not antagonised was the same cell on which the apomorphine response was not antagonised by fluphenazine. Haloperidol, given intravenously at 100 µg/kg blocked the effects of S-3608 on the two cells tested, as shown in figure 3.5.

Figure 3.5

A ratemeter recording of a SNC neurone inhibited by intravenously applied apomorphine and S-3608. Haloperidol caused a slight elevation in the baseline firing rate and antagonised the effects of further doses of apomorphine and S-3608 (the figures in brackets indicate the doses given in µg/kg).



3.2.2 The effects of intravenous RU24213 and RU24926 on SNC neuronal firing

The two N,N-diphenylethylamine derivatives RU24213 (N-n-propyl-N-phenyl ethyl-p-(3-hydroxyphenyl)-ethylamine HCl) and RU24926 (N-n-propyl-di-β-(3-hydroxyphenyl)-ethylamine, HCl), have been suggested to be potent dopamine receptor agonists (Euvrard, Farland, Di Paolo, Beaulieu, Labrie, Oberlander, Raynaud and Boissier, 1980). They are derived from tyramine and only contain one hydroxyl group on their phenolic ring. Structure-activity studies have suggested that two hydroxyl groups are required in positions 3 and 4 of the phenyl moiety. It was thus of interest to test these compounds on this system in comparison with apomorphine.

Both RU24213 and RU24926 were given intravenously at a dose of between 50-200 µg/kg and tested on 14 and 18 cells respectively. In all cells tested, an inhibition of the firing rate was observed, as figures 3.6 and 3.7 show. Inhibitions were associated with an increased spike amplitude similar to that seen with apomorphine. Figure 3.8 shows the increased spike amplitude following an intravenous injection of RU24926. The onset of action of these inhibitory responses occurred within 15 seconds of drug administration, similar to that seen with apomorphine and the other compounds. The maximal inhibitory effect occurred within a minute of the drug injection. As figures 3.6 and 3.7 show, recovery from the inhibitory effects of a single dose of either RU24213 or RU24926 was again biphasic, with an initial fast phase of recovery to approximately 40-50% of the pre-drug firing rate within 5 minutes. This is followed by a second slower phase which lasted about an hour.

Attempts were made to obtain dose-dependent responses to both compounds, but these proved impossible to obtain on the same cell. On separate cells, an initial dose of 50 µg/kg was observed to produce a smaller degree of inhibition than a dose of 100 µg/kg. However, as figures 3.6 and 3.7 show, tachyphylaxis occurred when repeated administrations of these drugs were given. The responses to apomorphine were similarly attenuated (as can be seen in figures 3.6 and 3.9) when either RU24213 or RU24926 had previously been given. In one experiment the response of apomorphine following the administration of three doses of RU24926 (cumulative dose of 150 µg/kg) were monitored for 5 hours. Repeated doses of apomorphine (20 mg/kg) at 30 minute intervals showed

Figure 3.6

Ratemeter trace of the neuronal activity of a SNC cell, showing the inhibitory effect of intravenously applied RU24213. Repeated doses of RU24213 show a reduction in the response to this drug. This tachyphylactic effect reduced the response of these cells to further doses of apomorphine (the figures in brackets indicate the doses given intravenously in µg/kg).

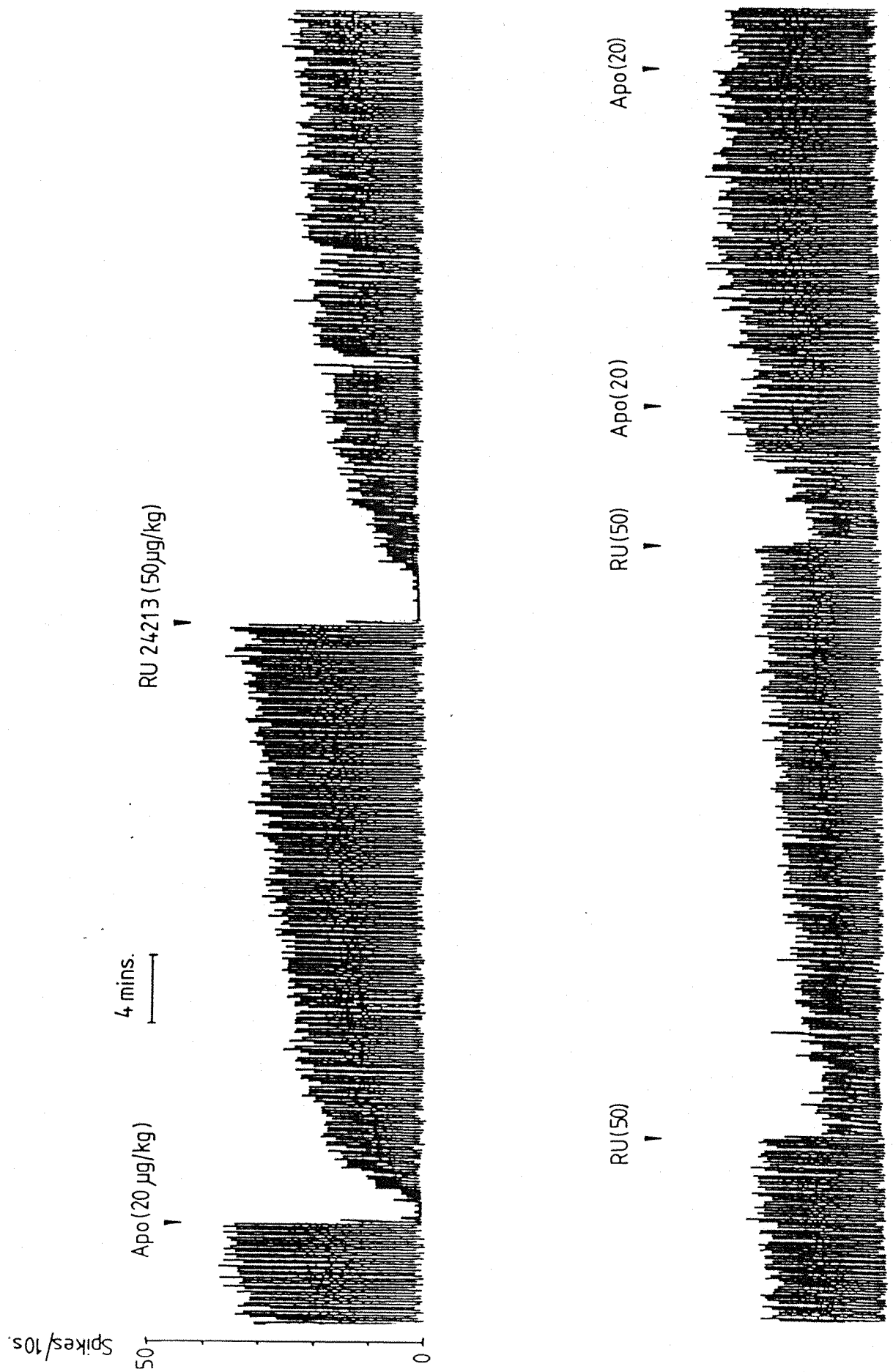
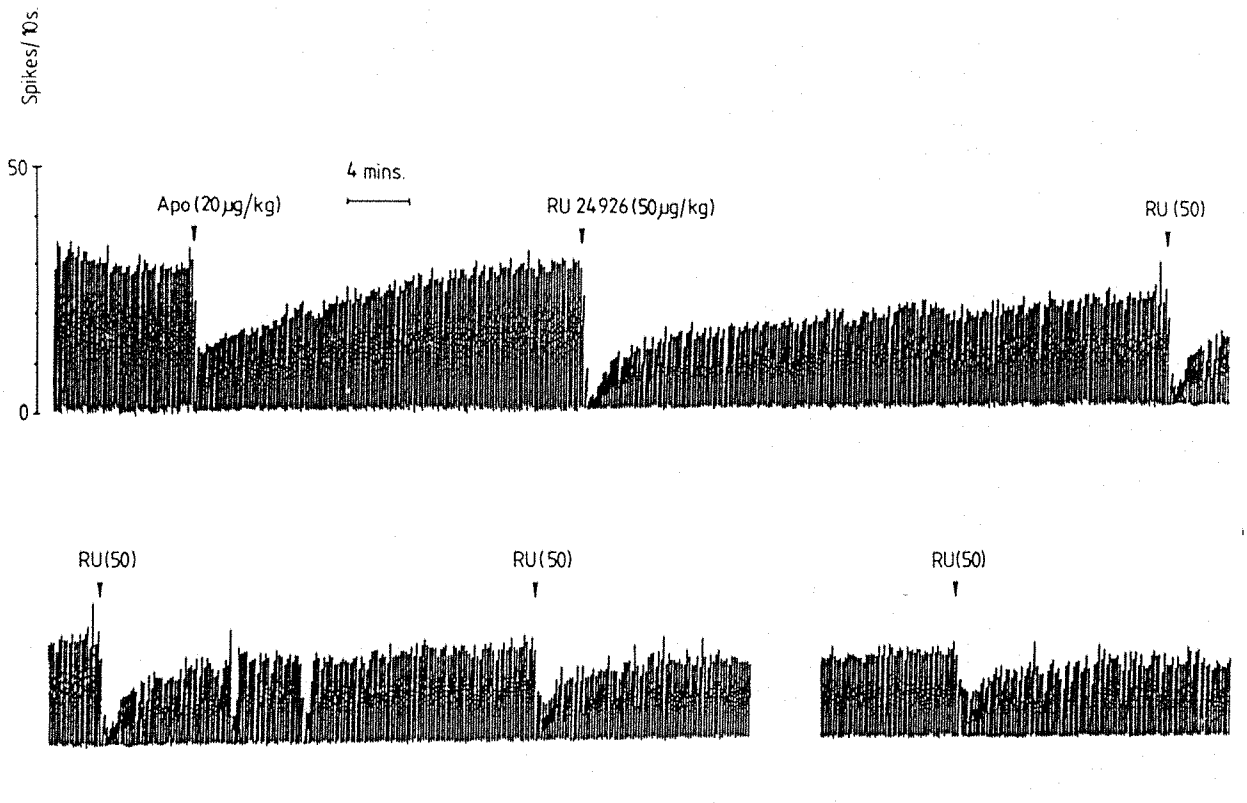

Spikes/10s
50
0
Apo(20 µg/kg)
4 mins.
RU 24213 (50µg/kg)
RU(50)
Apo(20)
RU(50)
Apo(20)

Figure 3.7

Ratemeter recording of a SNC neurone showing the inhibitory effect of intravenously administered apomorphine and RU24926. Repeated doses of RU24926 produces a reduced inhibitory effect on the neuronal activity (the figures in brackets represent the doses given in μg/kg).

Figure 3.8

A continuous spike record of the neuronal activity of an SNC neurone. RU24926 given intravenously produced a rapid inhibition of the neuronal firing rate with an associated increase in the spike amplitude. Ten minutes later (as shown in the bottom trace), the spike amplitude had returned to the original size and the firing rate had partially returned to the pre-drug levels.


Spikes/10s.
50
0
4 mins.
Apo (20 µg/kg)
RU 24926 (50µg/kg)
RU (50)
RU(50)
RU(50)
RU(50)

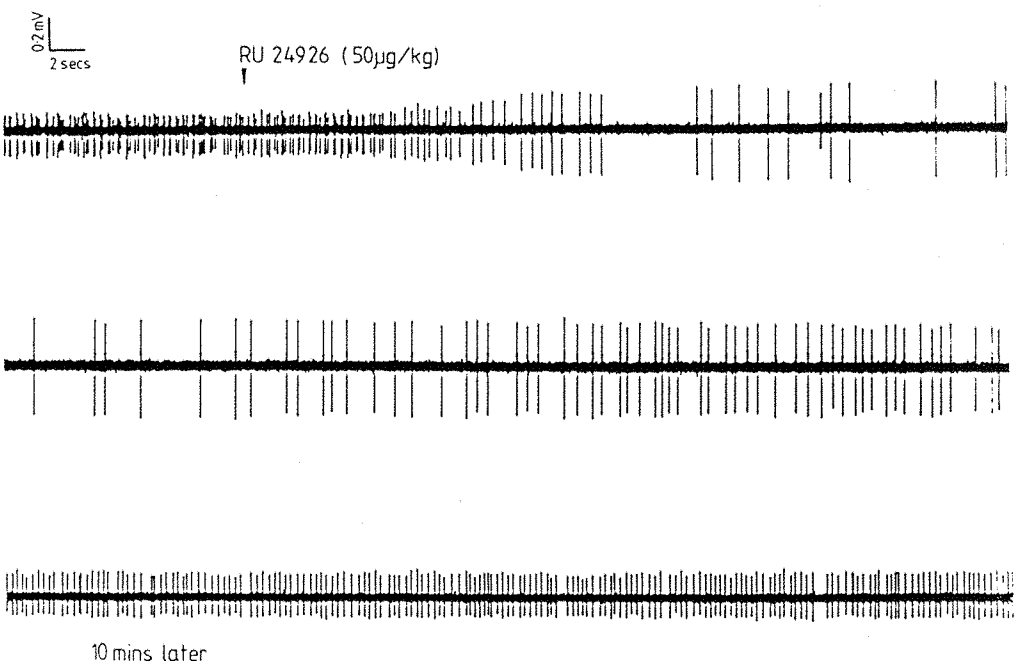
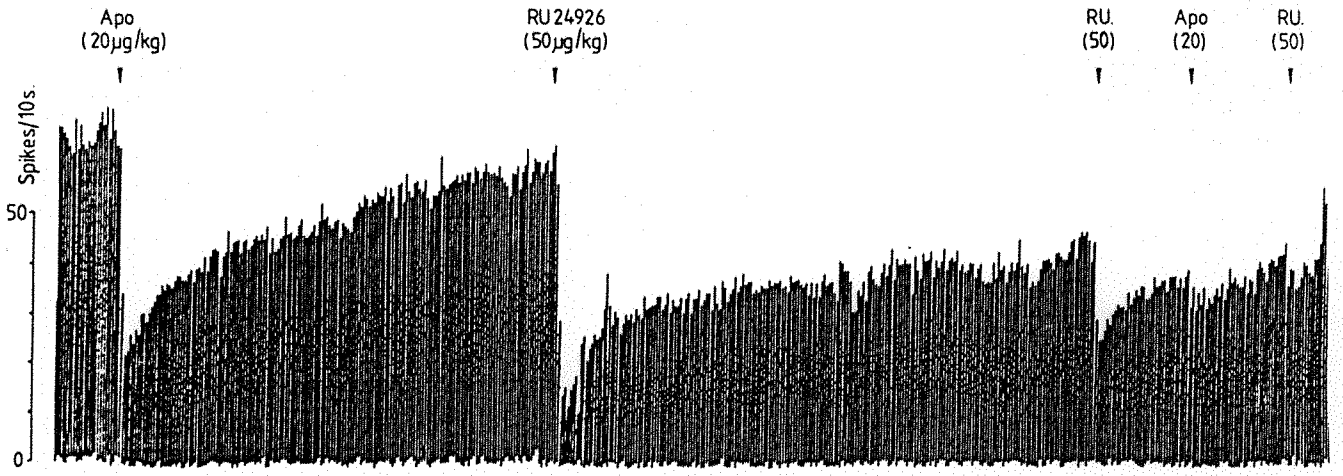

0.2mV
2 secs
RU 24926 (50µg/kg)
10 mins later

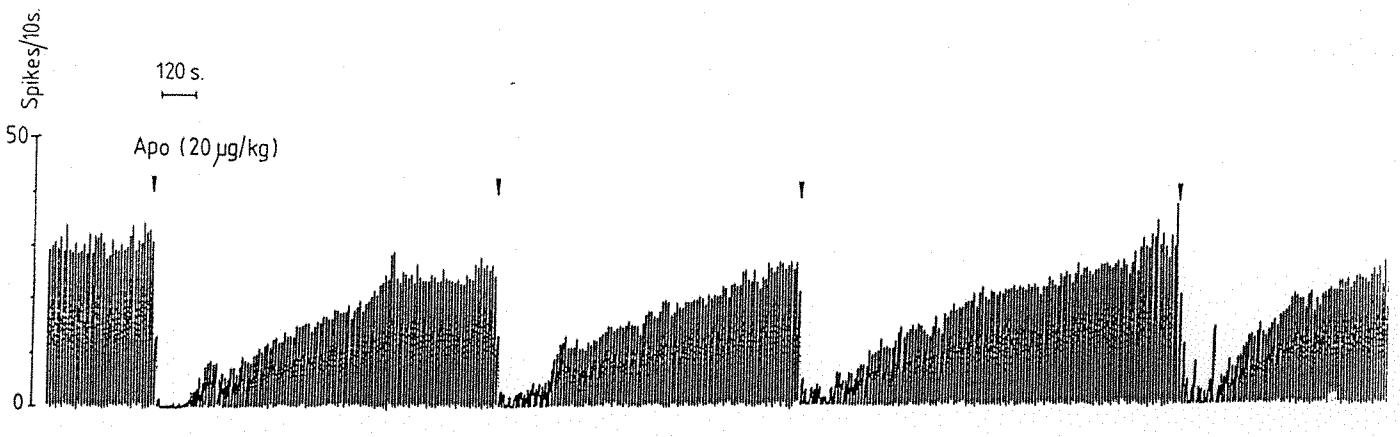
Figure 3.9

A ratemeter trace showing the inhibitory effect of apomorphine and RU24926 on the neuronal activity of an SNC cell. Following a single dose of RU24926 the effect of subsequent doses of RU24926 and apomorphine produced a reduced inhibitory action on the neuronal firing rate.

Figure 3.10

A ratemeter recording of a SNC cells neuronal activity inhibited by four equal doses of apomorphine (20 µg/kg) given at regular intervals. This shows that repetitive doses of apomorphine do not reduce the inhibitory effect of apomorphine on the neuronal activity.


Apo
(20µg/kg)
RU24926
(50µg/kg)
RU.
(50)
Apo
(20)
RU.
(50)
Spikes/10s.
50
0


Spikes/10s.
120 s.
50
Apo (20 µg/kg)
0

no significant return of the response to apomorphine. Yet, as figure 3.10 shows, repeated doses of apomorphine (20 µg/kg i.v.), given to five cells previously unaffected by either RU24213 or RU24926, showed no attenuation of the inhibitory responses.

Thus, these two compounds appear to act as dopamine receptor agonists on the dopamine autoreceptor, mimicking the effects of apomorphine. In addition, the present results confirm the suggestion that the actions of RU24213 and RU24926 are long acting. Whilst the action of both compounds are longer acting than apomorphine, they are less potent, having equal potency with each other but with approximately a third the potency of apomorphine.

3.2.3 The effect of dibenzoyl ADTN on neuronal firing rate

The semi-rigid analogue of dopamine, ADTN, is known from in vitro and in vivo studies to be a potent and long-acting dopamine agonist (see section 1.7). Peripheral administration of ADTN produces no central effects because it does not readily cross the blood brain barrier. The dibenzoyl ester of ADTN (2-amino-6,7-dibenzoyl-1,2,3,tetrahydronaphthalene; or DBADTN) is a lipophylic pro-drug proposed to cross the blood brain barrier. Once inside the CNS it is metabolised to the active form, ADTN (Horn, De Kaste, Dijkstra, Rollema, Feenstra, Westerink, Grol and Westerbrink, 1978). Experiments were performed to test the effect of this pro-drug on the SNC cells' neuronal activity.

As shown in figure 3.11, when administered intravenously at cumulative doses of up to 26 mg/kg, no effect was observed on the firing rate on all seven of the cells tested. Neuronal activity was monitored for as long as the cells could be recorded (in one case for over 2½ hours) following drug administration. The doses given were far in excess of the doses that would be expected to cause inhibition of neuronal firing when compared with the doses that reduced the levels of dopamine metabolites in the striatum (Horn et al., 1978; A.S. Horn, personal communication).

3.2.4 The effect of direct intraaccumbens injections of S-3608

Following the detection of the agonist properties of S-3608 detected on the dopamine autoreceptors in the SNC, the study was extended to test the stimulant effect on locomotor activity induced by intra-accumbens

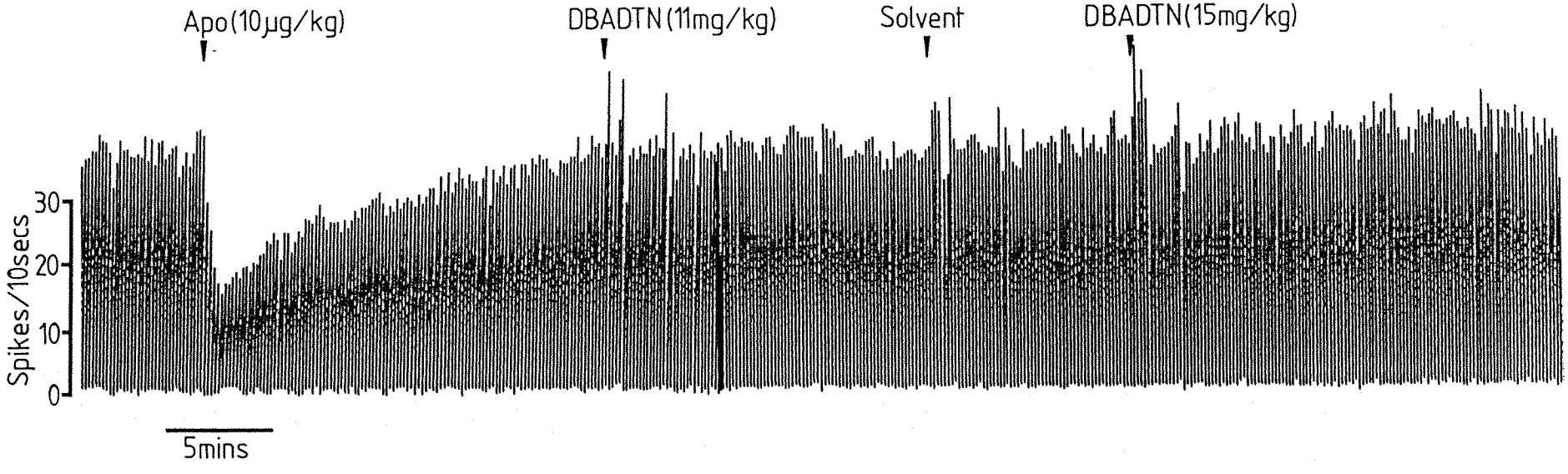


Figure 3.11

A ratemeter trace of the neuronal activity of an SNC cell inhibited by intravenously administered apomorphine. Neither of the two doses of DBADTN given intravenously (at a cumulative dose of 26 mg/kg), nor the solvent used to dissolve the drug had any effect on the neuronal firing rate.

injections of dopamine agonists. Each animal with bilateral cannulations of the nucleus accumbens, were tested for their response to (±)ADTN, to evaluate the success of the operation. ADTN is a potent stimulant of locomotor activity in the rat (Elkhawad and Woodruff, 1975). Animals that did not respond after 3 intra-accumbens injections were discarded from further experimentation.

Bilateral microinjections of (±)ADTN (100 nmol/side) directly into the nucleus accumbens of conscious animals were monitored for 24 hours. Figure 3.12b shows that a strong, continuous locomotor activity was induced which lasted for approximately 15 hours (n=10). Locomotor activity usually started within 15 minutes of a injection of ADTN. The hyperactive animals were not aggressive and could be freely handled. These results are similar to those reported by Elkhawad and Woodruff (1975) and Andrews and Woodruff (1978).

Bilateral intra-accumbens injections of S-3608 over a dose range of 100-250 nmol/side failed to elicit any locomotor activity (n=10). Figure 3.12a shows the locomotor activity of the rats following a bilateral injection of S-3608 (250 nmol/side) compared with a similar bilateral injection of 0.9% NaCl solution as a control (nor was any sedative effect seen compared with the control). Figure 3.12b is a comparison of the effects of injections of ADTN (100 nmol/side) with those of S-3608 (250 nmol/side). When the same animals were tested 3 days after the injections of S-3608, the locomotor response to ADTN was still present, indicating that the lack of response cannot be explained by a decreased sensitivity of the animals used.

The lack of activity by S-3608 was unexpected since other dopamine agonists, including the structural analogue of S-3608, piribedil, have been shown to induce hyperactivity in rats (Butterworth, Poignant and Barbeau, 1975).

3.2.5 The effect of intra-accumbens microinjections of RU24926 and RU24213

The response to bilateral intra-accumbens microinjections of RU24213 and RU24926 into conscious animals was tested in six rats that had been previously assessed for their sensitivity to ADTN (100 nmol/side). Figure 3.13a shows that RU24213, given at doses up to 200 nmol/side, had no significant effect on the locomotor activity of the six animals

Figure 3.12 Graph showing the locomotor activity measured in rats following bilateral microinjections directly into the nucleus accumbens.

(a) shows the locomotor activity produced by bilateral microinjections of S-3608 at a dose of 250 nmol per side (■—■; n = 10), compared with similar bilateral microinjections of 0.9% NaCl (●- -●; n = 4).

(b) shows the locomotor activity produced by bilateral microinjections of ADTN (100 nmol/side; □—□; n = 10), compared with the locomotor activity induced by S-3608 (250 nmol/side; ●—●).

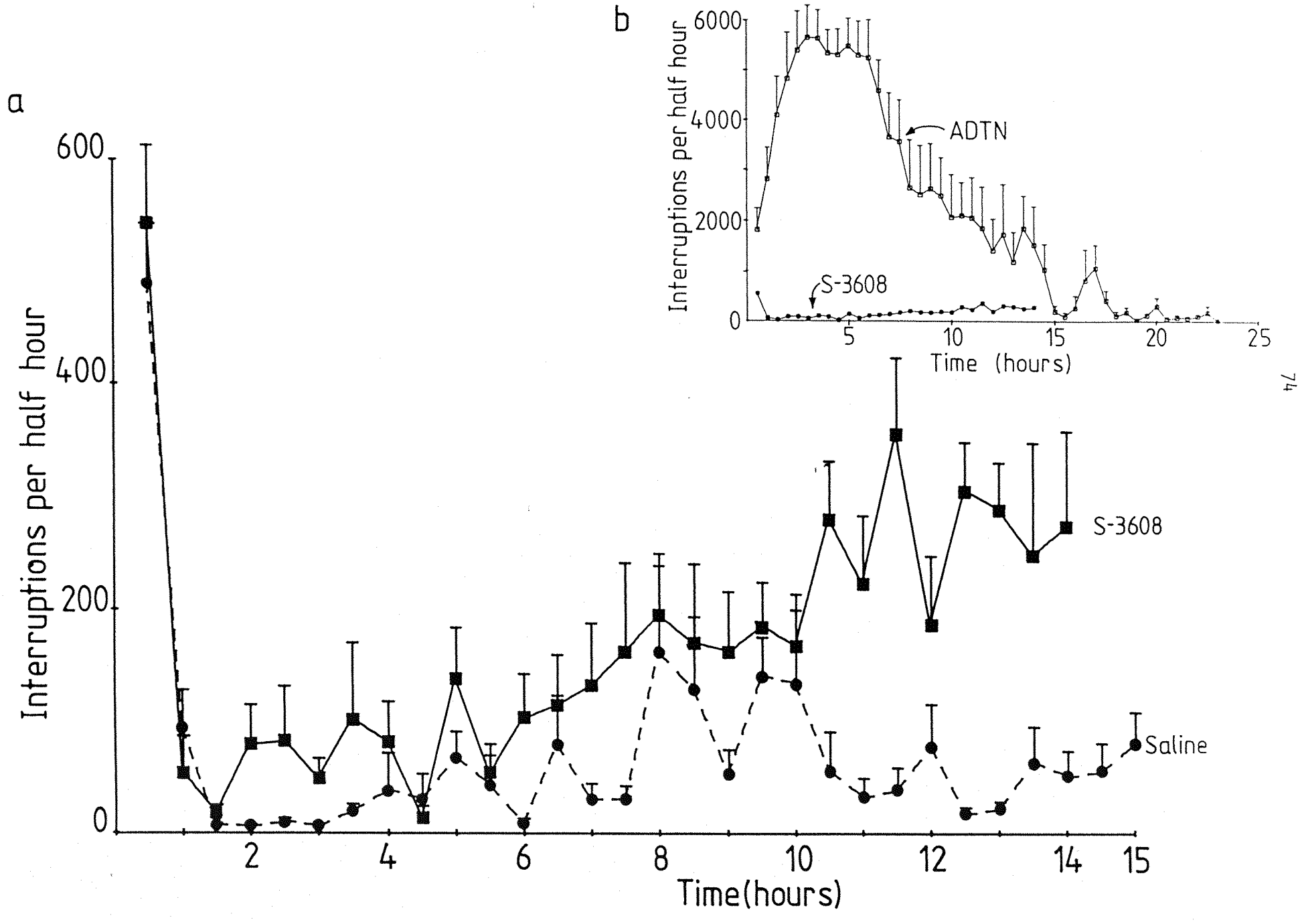
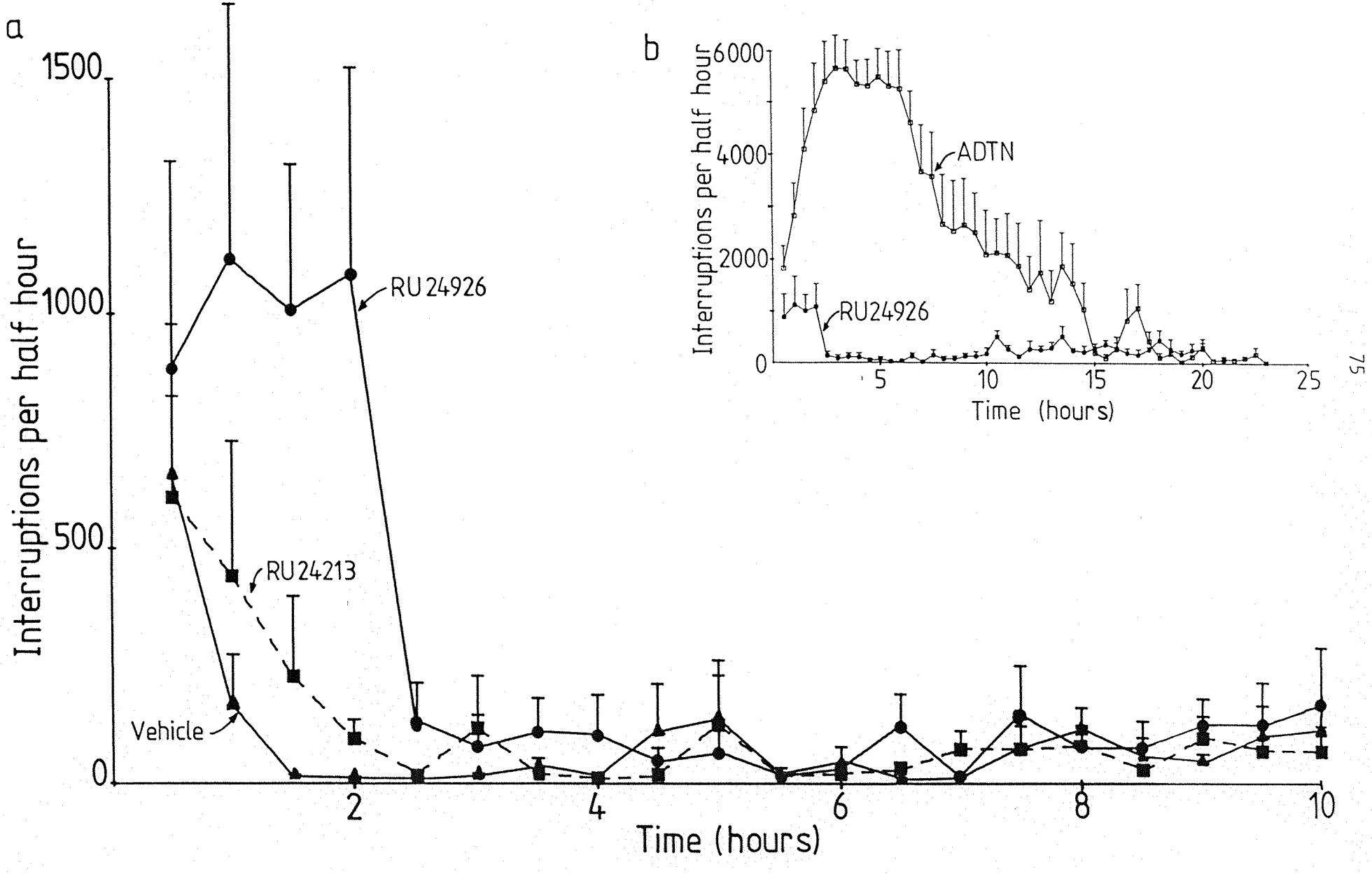

a
b
Interruptions per half hour
600
400
200
0
2
4
6
8
10
12
14
15
Time(hours)
S-3608
Saline
6000
4000
2000
0
5
10
15
20
25
Time (hours)
ADTN
S-3608

Figure 3.13 Graphs of the locomotor activity induced in rats following the bilateral microinjections directly into the nucleus accumbens.

(a) The locomotor activity induced by the bilateral microinjections of RU24213 (200 nmol/side; n = 6; ■– –■), RU24926 (150 nmol/side; n = 6; ●——●), compared with the microinjection of the vehicle used to dissolve these drugs (▲——▲; n = 4).

(b) The locomotor activity induced by bilateral injections of ADTN (100 nmol/side; n = 10; □——□), compared with the microinjection of RU24926 (150 nmol/side; ●——●).


a
1500
1000
500
0
Interruptions per half hour
RU24926
RU24213
Vehicle
2
4
6
8
10
Time (hours)
b
6000
4000
2000
0
Interruptions per half hour
ADTN
RU24926
5
10
15
20
25
Time (hours)

tested, when the response is compared with the locomotor activity produced by bilateral microinjections of vehicle alone. In addition, figure 3.13a shows that a bilateral microinjection of RU24926, injected directly into the nucleus accumbens at a dose of 150 nmol/side (n=6), produced a significant stimulation of locomotor activity. This stimulation of locomotor activity lasted for two hours; after this time the activity of the rats rapidly returned to the control values. Figure 3.13b shows that this stimulation was weak when compared with the stimulation of locomotor activity produced by microinjections of ADTN (100 nmol/side).

The lack of potency of both these compounds was unexpected as both were potent and long lasting dopamine agonists when tested on the SNC neuronal activity and in other model systems (Euvrard et al., 1980).

3.3 Antagonist studies

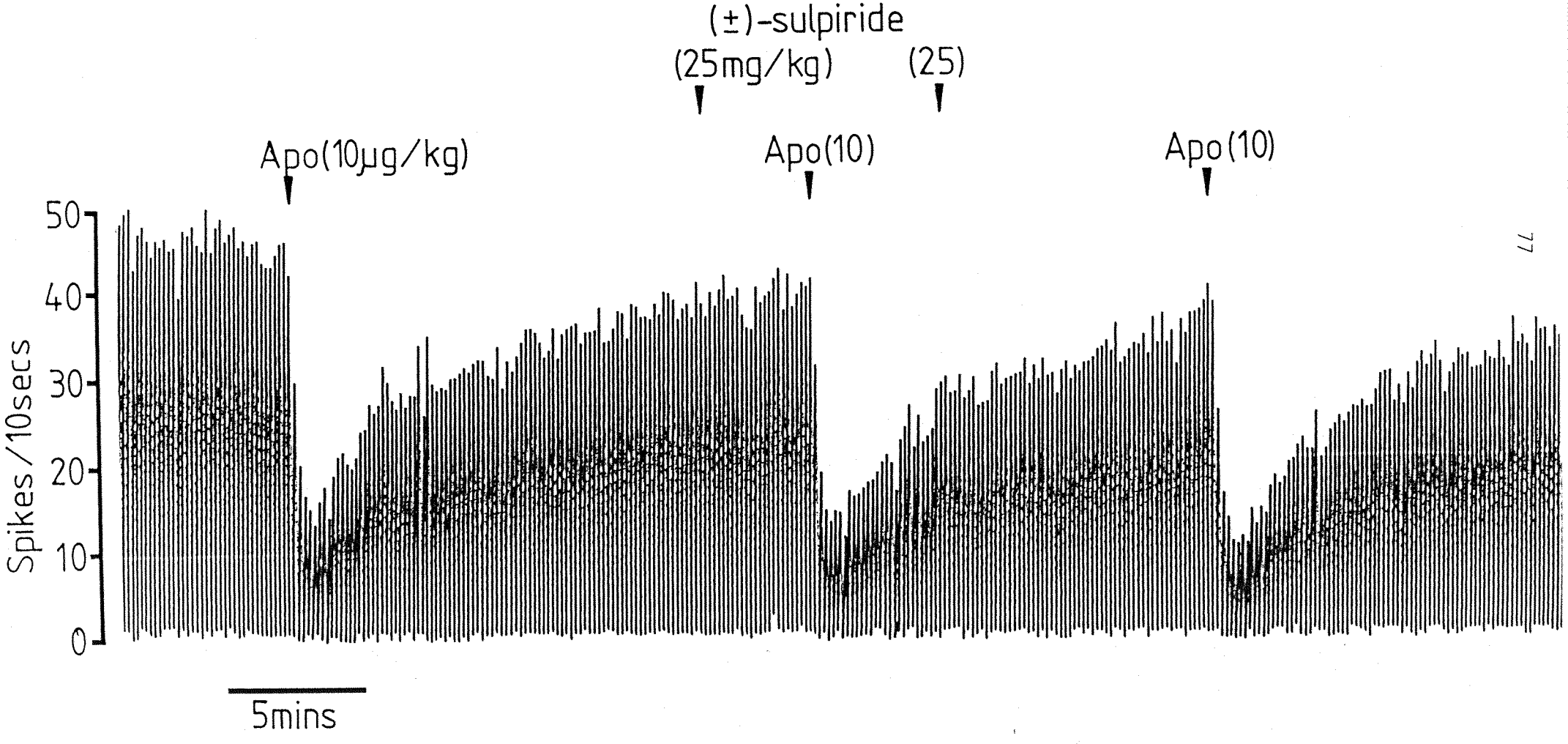
3.3.1 The effect of intravenous sulpiride on apomorphine responses in the SNC

The substituted benzamide, sulpiride, is a potent dopamine antagonist, but, when given periperally, it does not act as a dopamine antagonist on behavioural models of dopamine receptors (see section 1.7). This is proposed to be due to its poor lipid solubility (Honda et al., 1977; Woodruff, Poat and Freedman, 1980) and, consequently, its inability to cross the blood brain barrier.

(±)-Sulpiride was tested against the inhibitory responses of apomorphine (10 µg/kg) on four cells at a cumulative dose of 50 mg/kg without any effect on the apomorphine response. Figure 3.14 shows that the inhibitory responses of apomorphine on neuronal firing rate were unaffected by two doses of sulpiride (25 mg/kg). Thus these results demonstrate that, at a dose of 50 mg/kg, insufficient sulpiride is able to cross the blood brain barrier and antagonise the inhibitory responses of apomorphine. However, it has been reported that similar high doses can antagonist dopamine agonist responses on dopamine synthesis and locomotor activity (Argiolas, Mereu, Serra, Melis, Fadda and Gessa, 1982) as well as responses to apomorphine on SNC neurones (Mereu, Casu and Gessa, 1983). These results are in conflict with the present observations.

Figure 3.14

A continuous ratemeter recording of the firing rate of an SNC cell. Apomorphine (10 µg/kg, i.v.) inhibits the firing of the cell which slowly reverses. Two doses of (±)-sulpiride (25 mg/kg, i.v.) fails to antagonise the inhibitory effect of subsequent doses of apomorphine (figures in brackets indicate the doses given).


(±)-sulpiride
(25mg/kg)
(25)
Apo(10µg/kg)
Apo(10)
Apo(10)
Spikes/10secs
50
40
30
20
10
0
5mins

3.3.2 Antagonism of apomorphine by intravenous zetidoline

Zetidoline (1-(3-chloro phenyl)-3-[2-(3,3-dimethyl-1-azetidinyl) ethyl] imidazolidin-2-one HCl) is a recently discovered compound with properties which are indicative that it acts as an antipsychotic agent in both behavioural and biochemical test systems (Barone, Corsico, Diena, Restelli, Glasser and Rodenghi, 1982), but zetidoline is not active on the dopamine sensitive adenylate cyclase and is thus similar to sulpiride. The effect of this potential dopamine antagonist was thus tested on the SNC cells responses of drugs.

Figure 3.15a shows that apomorphine, given intravenously at 1-10 µg/kg, caused dose-dependent depressions of SNC neuronal firing rate. This was associated with an increase in spike amplitude, as shown in figure 3.15b. Zetidoline (100 µg/kg), given intravenously, rapidly reversed the depression of the firing rate (within 20 seconds), induced by apomorphine, on all 12 cells that were tested. Figure 3.15 shows that following administration of zetidoline the firing rate returned to its pre-drug baseline rate. In seven of the cells tested with zetidoline, a transient increase in the firing rate (above the control firing rate) was observed, before the rate returned to the pre-drug levels. Apomorphine, at doses of 1-10 µg/kg given after zetidoline, had no effect on the firing rate. Doses of apomorphine, 20 µg/kg and greater, did produce depressions of the firing rate; this is indicative of competitive antagonism. In one cell the antagonism of the dopamine receptor following zetidoline administration was monitored for 2 hours with no appreciable return of the response to apomorphine (10 µg/kg) for 1½ hours. Responses to apomorphine gradually began to return after this time and were approximately 20% of their original level after 2 hours.

Thus the results demonstrate that zetidoline is a potent and reasonably long acting dopamine antagonist. They also show that zetidoline, unlike sulpiride, can rapidly cross the blood brain barrier.

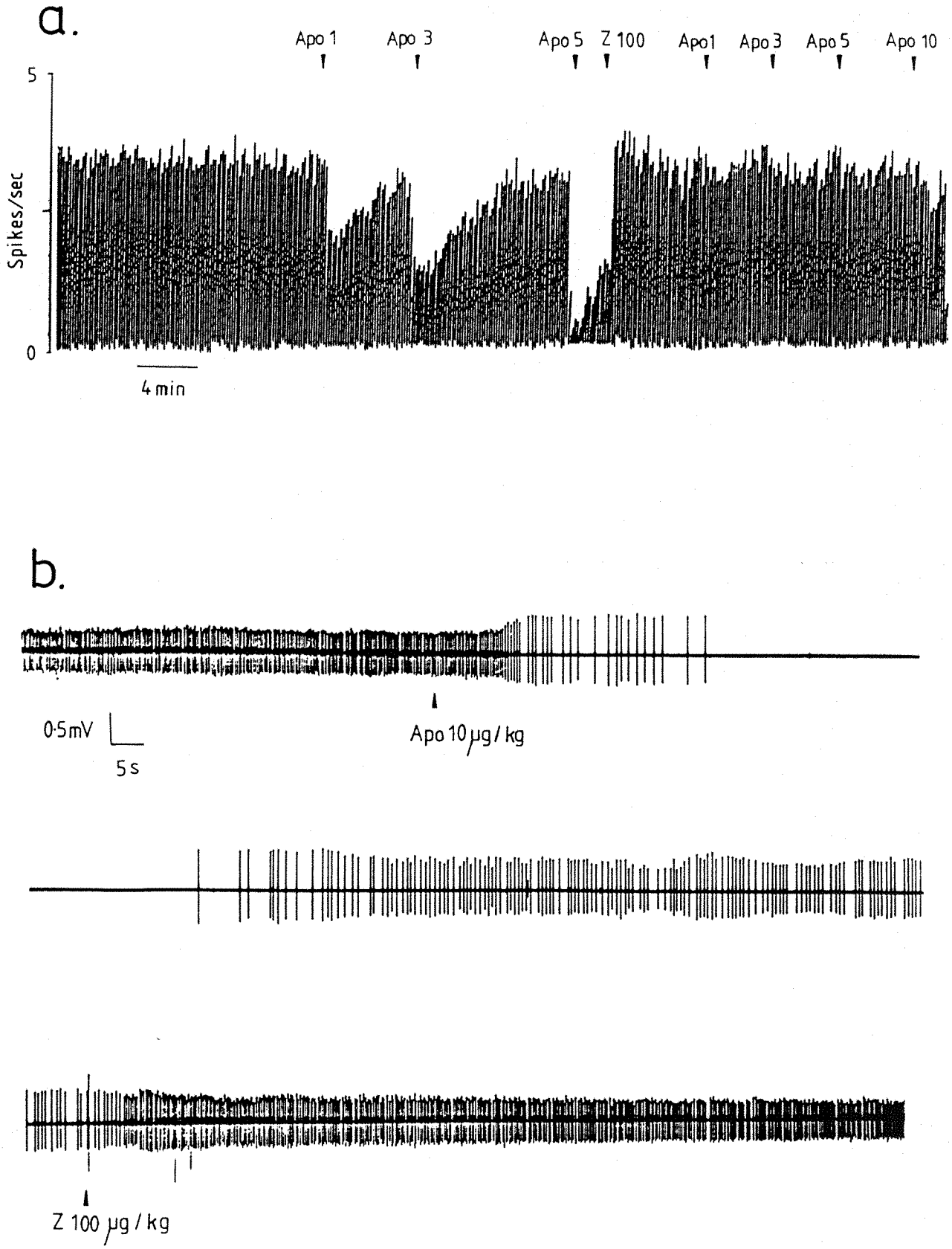
3.3.3 The effect of iontophoretically applied zetidoline on SNC responses

Having shown that intravenously administered zetidoline would antagonist apomorphine induced inhibitions of SNC cell firing, the effect of zetidoline applied directly onto the SNC cells was tested.

Figure 3.15

(a) Ratemeter recording of an SNC neurone showing the inhibitory effect of apomorphine (1, 3 and 5 µg/kg, i.v.). Zetidoline (100 µg/kg, i.v.) reverses the inhibition by apomorphine. Subsequent doses of apomorphine at 1, 3, 5 and 10 µg/kg, i.v. has no effect on the neuronal firing rate.

(b) Continuous spike record of an SNC neurone showing the inhibition caused by apomorphine (10 µg/kg, i.v.), which caused an associated increase in the spike amplitude during the inhibition. Zetidoline (100 µg/kg) caused a reversal of the apomorphine-induced inhibition and a return of the spike amplitude to its predrug levels.


a.
Apo 1
Apo 3
Apo 5
Z 100
Apo1
Apo 3
Apo 5
Apo 10
5
Spikes/sec
0
4 min
b.
0·5mV
5 s
Apo 10 µg / kg
Z 100 µg / kg

In addition, zetidoline was tested for its selectivity against a number of putative transmitters in the substantia nigra. The results of these experiments are summarised in table 3.1.

Cells initially identified as being the SNC dopamine containing neurones by their characteristics described in section 2.1 were tested for their sensitivity to iontophoretically applied dopamine (30-120 nA) before proceeding further. Using the above criteria, 68 identified cells were tested with dopamine, and 65 cells were inhibited by dopamine which was dependent on the ejection current (see figure 3.16). These inhibitions were associated with an increase in the spike amplitude, as shown in figure 3.17. One other cell gave a transient excitation followed by an inhibition. The remaining two cells were unaffected by dopamine iontophoresed at high currents for 2 minutes.

Of the cells inhibited by dopamine, 25 cells were tested against zetidoline, applied iontophoretically (40-70 nA) for a period of 10-15 minutes. Figure 3.18 shows that the inhibitory responses to dopamine were blocked on 23 of the cells tested. The antagonistic effect of zetidoline occurred within 2-5 minutes of the start of the iontophoretic ejection. Antagonism was reversible, the responses returning 5-10 minutes after iontophoretic ejection of zetidoline was terminated. On the two other cells tested with zetidoline, a partial block of the dopamine responses was obtained, but these cells were lost before full antagonism was achieved. In 14 cells the iontophoretic application of zetidoline caused an elevation of the basal firing rate as can be seen in figure 3.18. This is mainly due to an increase in the bursting activity of the cells which decreased after zetidoline iontophoresis was terminated. This decrease in the neuronal bursting activity corresponded to the return of the dopamine responses.

GABA (30-100 nA; 30-60 seconds) was tested on 26 cells. The firing rates of 22 of these cells were inhibited (see figure 3.18) and in a dose related manner.

These inhibitory responses were rapid in onset and recovery as can be seen from figure 3.19. Figure 3.18 shows the GABA responses tested during the application of zetidoline. All 11 cells on which zetidoline was tested against GABA were unaffected.

Table 3.1

Summary of the results obtained by the iontophoretic application of putative transmitters on the SNC neurones and the effect of zetidoline on these responses.

Drug	Number of cells			Antagonised by zetidoline
	-	0	+	
Dopamine	65	3	0	$^{23}/_{25}$*
Noradrenaline	15	1	0	$^{7}/_{9}$*
GABA	22	4	0	$^{0}/_{11}$
Glutamate	2	4	21	$^{0}/_{4}$
Glycine	13	1	0	$^{0}/_{7}$
Taurine	1	7	0	N.T.

*The other two cells were partially antagonised before the cells were lost.

- = inhibition of firing
0 = no effect on firing
+ = excitation of firing
N.T. = not tested

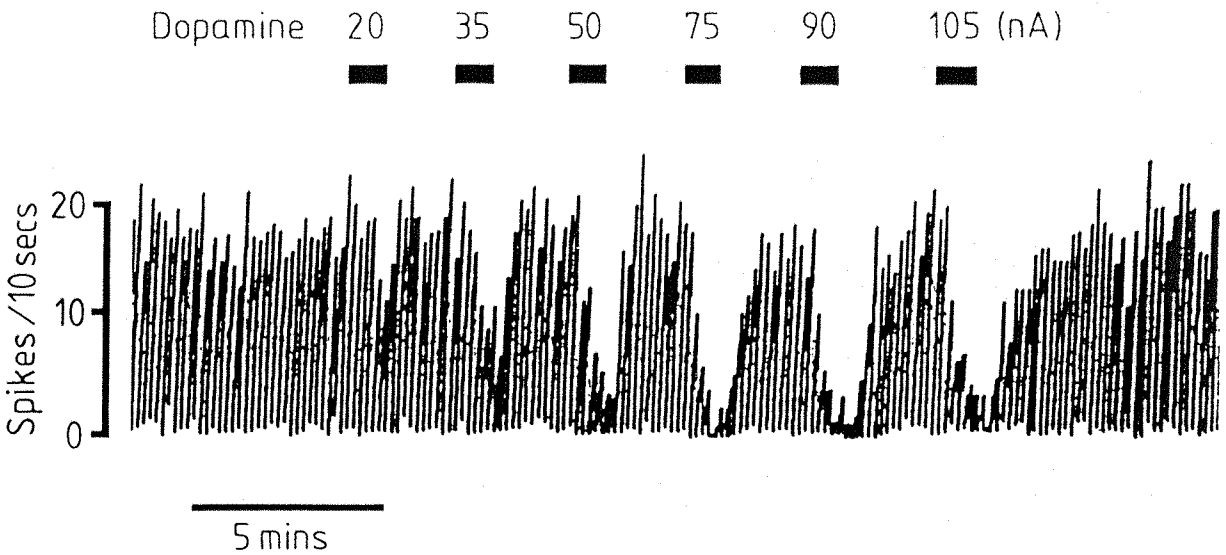


Figure 3.16

Ratemeter recording of a SNC neurone showing the dose related inhibition of firing rate produced by iontophoretic pulses of dopamine.

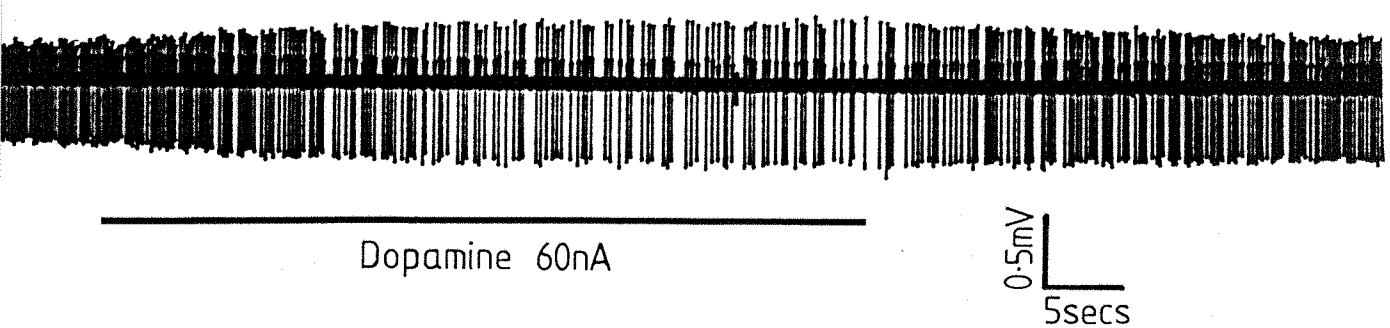


Figure 3.17

Spike record of a SNC neurone showing the inhibition of firing rate and associated increase in spike amplitude produced by an iontophoretic pulse of dopamine.

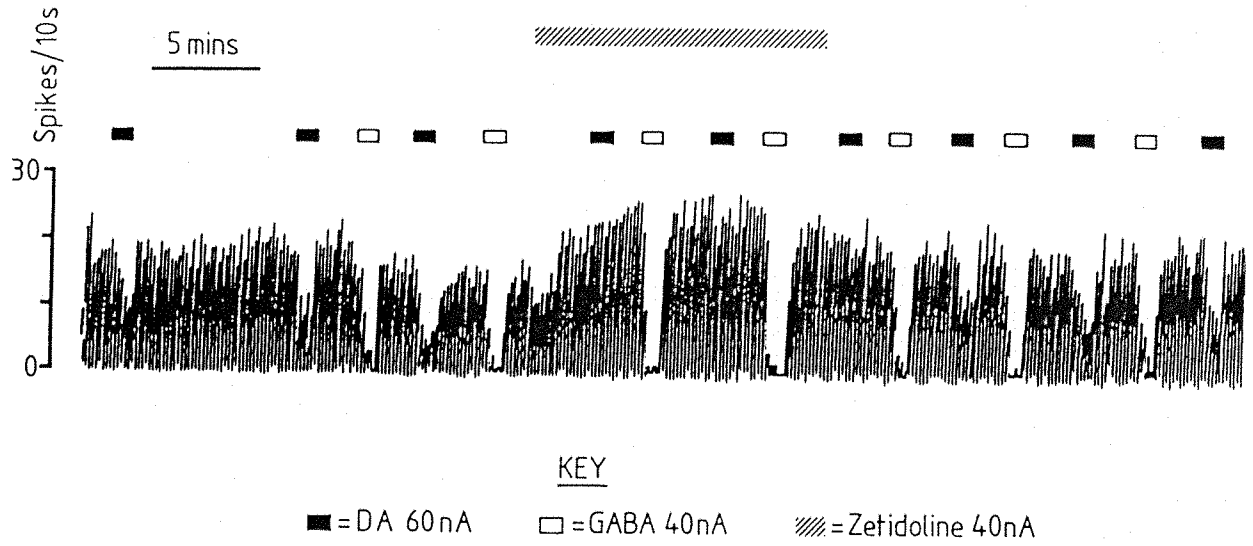


Figure 3.18

Ratemeter recording of a SNC neurone inhibited by iontophoretic pulses of dopamine (60 nA) and GABA (40 nA). Zetidoline applied iontophoretically continuously (40 nA), reversibly antagonised the inhibitory action of dopamine without affecting the responses to GABA. Note, during the application of zetidoline, the firing rate increased slightly over the base-line levels.

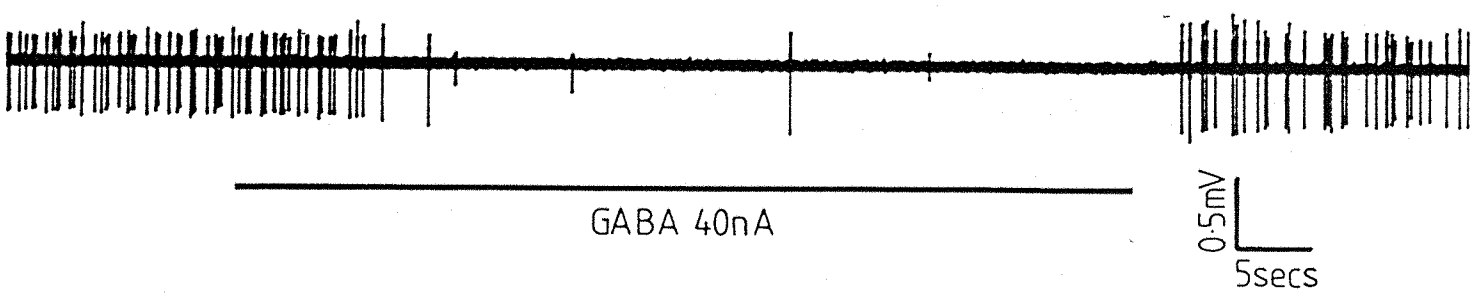


Figure 3.19

Spike record of a SNC neurone showing the inhibition of firing produced by an iontophoretic pulse of GABA 40 nA.

Noradrenaline (40-100 nA) caused an inhibition of neuronal firing on 15 of the 16 cells tested. Inhibitions were dose dependent and were approximately equipotent with dopamine, although dopamine was generally more potent than NA. This observation is in agreement with those of Aghajanian and Bunney (1977). Zetidoline antagonised the inhibitory responses of NA on 7 of the 9 cells tested; the two remaining cells were partially blocked but were lost before full antagonism of the NA response had occurred. Figure 3.20 shows that both DA and NA responses were reversibly antagonised by zetidoline (and sulpiride, see below).

Glycine, an inhibitory amino acid (60-100 nA), inhibited the neuronal firing of 13 of the 14 cells tested. As can be seen in figures 3.21 and 3.22a, the inhibitory responses were rapid in onset and recovery. Zetidoline, applied iontophoretically, did not block the inhibitory responses produced by glycine (as can be seen in figure 3.21) on all 4 of the cells tested. Another inhibitory amino acid, taurine, was iontophoretically applied (50-150 nA) to 8 cells. On seven cells, this was without effect, as shown in figure 3.21; the one remaining cell was inhibited.

Glutamic acid, an excitatory amino acid, was applied iontophoretically (50-100 nA; 15-60 seconds) to 27 cells. On 21 cells an excitation of the neuronal firing rate was observed, as can be seen from figure 3.21. This was generally slow in onset but rapidly returned to the baseline firing rate when ejection was terminated, sometimes with a rebound inhibition afterwards. As figure 3.22b shows, these cells show a decrease in the spike amplitude associated with the excitation. In some cases this becomes indistinguishable from the background noise. These observations are in agreement with those of Collingridge and Davies (1979). Two cells were inhibited by glutamate without an excitation occurring. This may be due to an excessive overdepolarization of the cell membrane rather than a true inhibitory response.

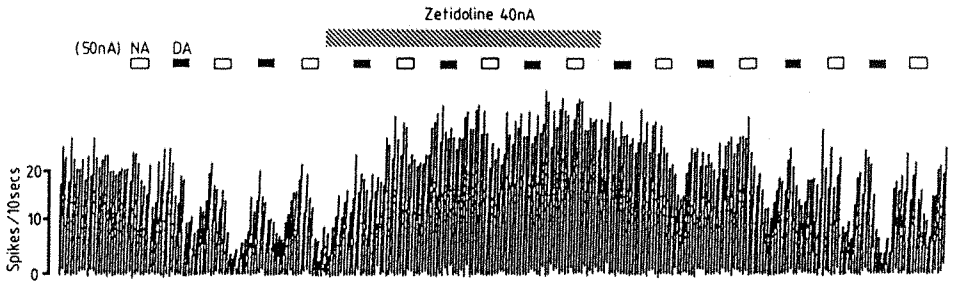
Zetidoline was tested against the excitatory responses of glutamate on 4 cells without any effect on these responses, as can been in figure 3.21.

Figure 3.20

Ratemeter trace of a SNC neurone inhibited by iontophoretic pulses of noradrenaline (50 nA) and dopamine (50 nA). Zetidoline (40 nA) applied iontophoretically, reversably antagonised the inhibitory effects of noradrenaline and dopamine. During the application of zetidoline the firing rate increased over the baseline levels seen before drug application.

Figure 3.21

Ratemeter trace of an SNC neurone inhibited by iontophoretic doses of dopamine (60 nA) and glycine (60 nA), but excited by glutamate (90 nA) while taurine (140 nA) had no effect on the firing rate.
Zetidoline (40 nA) did not block the responses produced by glutamate and glycine.


Zetidoline 40nA
(50nA) NA
DA
Spikes/10secs
20
10
0

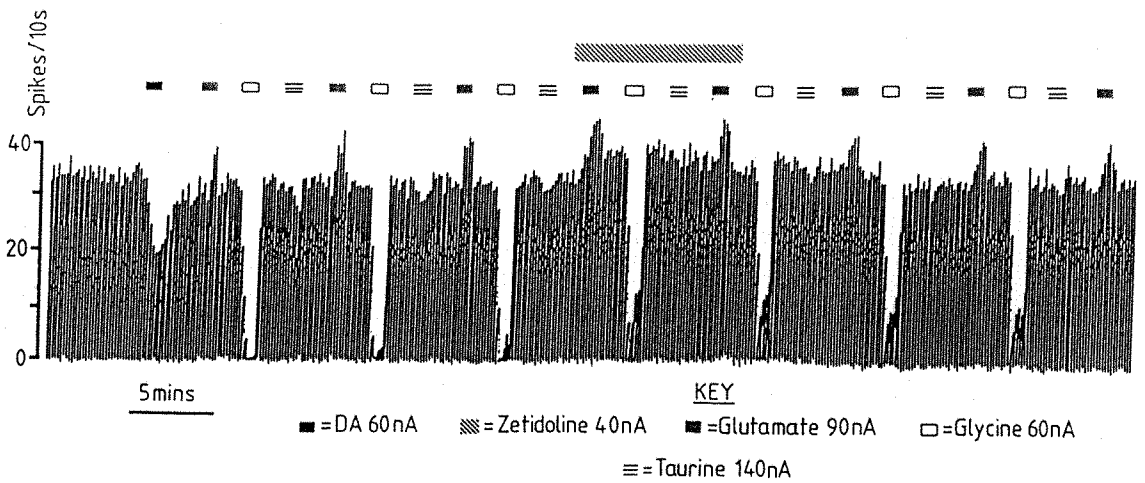

Spikes/10s
40
20
0
5mins
KEY
=DA 60nA
=Zetidoline 40nA
=Glutamate 90nA
=Glycine 60nA
=Taurine 140nA

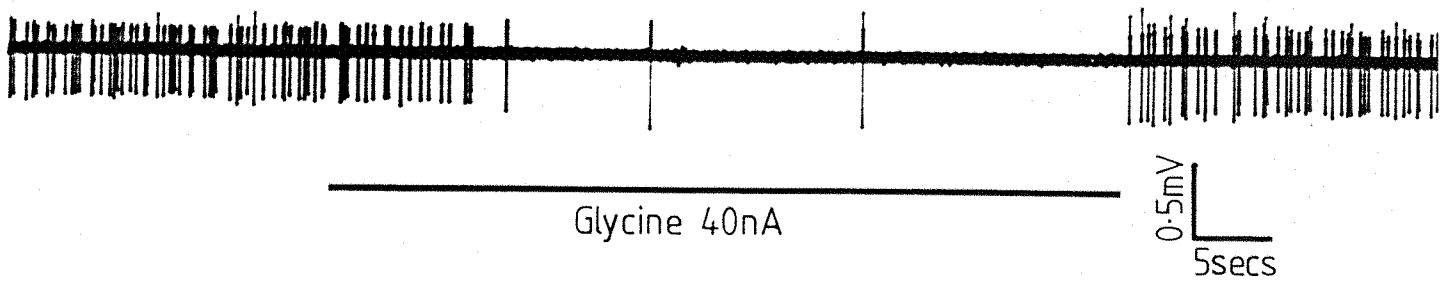
Figure 3.22

Spike records of the neuronal activity of SNC neurones:

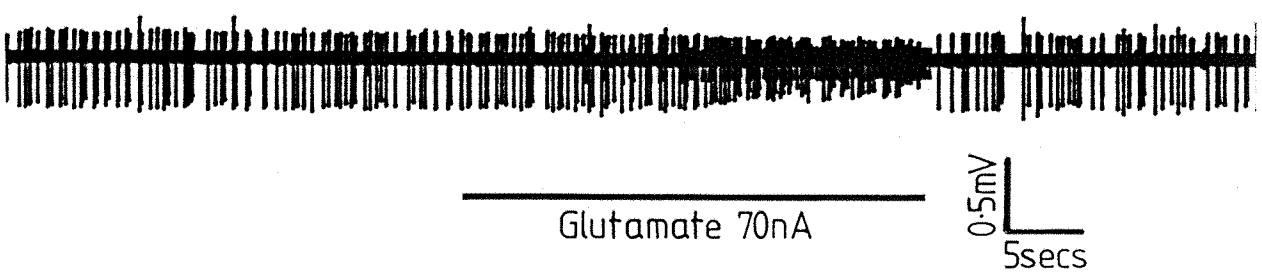
(a) inhibited by glycine (40 nA) showing an increase in the spike amplitude.

(b) excited by glutamate (70 nA) showing a decrease in the spike amplitude during the excitation.

a.



b.



3.3.4 The effect of iontophoretically applied sulpiride on neuronal responses in the SNC

As zetidoline antagonises the effects of NA as well as DA on the SNC neurones, sulpiride was used in a comparison with zetidoline to check that these NA responses were due to an agonist action on the dopamine receptor and not mediated through adrenoceptors. Sulpiride has a similar pharmacological profile to zetidoline and is a selective dopamine receptor antagonist (O'Connor and Brown, 1982). The results of this study are summarised in table 3.2.

Figure 3.23 shows that sulpiride, applied iontophoretically (50-60 nA), antagonised the inhibitory responses of iontophoretic dopamine (50-80 nA); this was observed on all seven of the cells tested. Two of these cells had previously had their DA responses antagonised by zetidoline. In addition, four of these seven cells were inhibited by NA and all these inhibitory responses to NA were antagonised by (±)-sulpiride. One of these cells had previously been antagonised by zetidoline. Thus, the DA and NA responses are antagonised by the dopamine antagonist, sulpiride, suggesting that the NA responses are mediated via DA receptors.

3.3.5 The effects of iontophoretically applied sulpiride and zetidoline on cerebellar neuronal responses

The cerebellum receives noradrenergic afferents from the locus coeruleus, which terminate predominantly on the Purkinje cells (Bloom, Hoffer and Siggins, 1971). Noradrenaline, released from the nerve endings or applied iontophoretically, inhibited the spontaneous firing rate. This effect was mediated through β-adrenoceptors, since iontophoretically applied isoprenaline inhibited the cell firing rate. In addition β-adrenoceptor antagonists blocked the inhibitory actions of NA on the firing rate (Hoffer, Siggins and Bloom, 1971; Yeh and Woodward, 1983). The cerebellum contains no detectable dopamine receptors, as measured by binding assays (Burt, Creese and Snyder, 1976; Woodruff and Freedman, 1981). Thus the cerebellum was used for a comparison of the antagonistic effects of sulpiride and zetidoline on adrenoceptor mediated NA and DA responses and for a comparison of the effects of these drugs on the SNC neurones. The results of this study are summarised in table 3.3.

Table 3.2 Summary of the results obtained from the iontophoretic application of sulpiride onto the SNC neuronal responses to iontophoretic dopamine and noradrenaline.

Drug	Number of cells			Antagonised by	
	-	0	+	(±)-sulpiride	Sulpiride and zetidoline
Dopamine	7	0	0	$^{7}/_{7}$	$^{2}/_{2}$
Noradrenaline	4	0	0	$^{4}/_{4}$	$^{1}/_{1}$

- = inhibition of firing rate
0 = no effect
+ = excitation of firing rate

Table 3.3 Summary of the results obtained by the iontophoretic application of sulpiride on the neuronal responses of the cerebellar neurones.

Drug	Number of cells				Antagonised by sulpiride
	-	0	+	±	
Dopamine	13	5	0	1	$^{0}/_{5}$
Noradrenaline	19	3	0	0	$^{0}/_{7}$
Glutamate	1?	2	6	0	N.T.
Zetidoline	10	0	0	0	N.T.
Propanolol	5	0	0	0	N.T.

Current ejection had no effect on 4 cells tested.

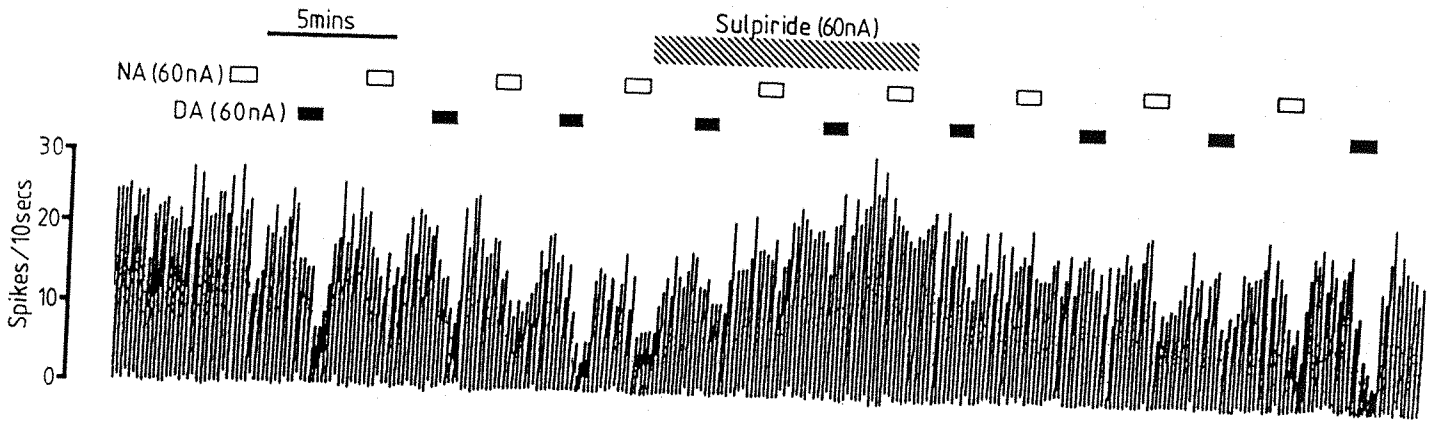
+ = excitation of firing rate
0 = no effect
- = inhibition of firing rate
± = biphasic effect on firing rate
? = result possibly due to rapid overdepolarisation of the cell
N.T. = not tested.

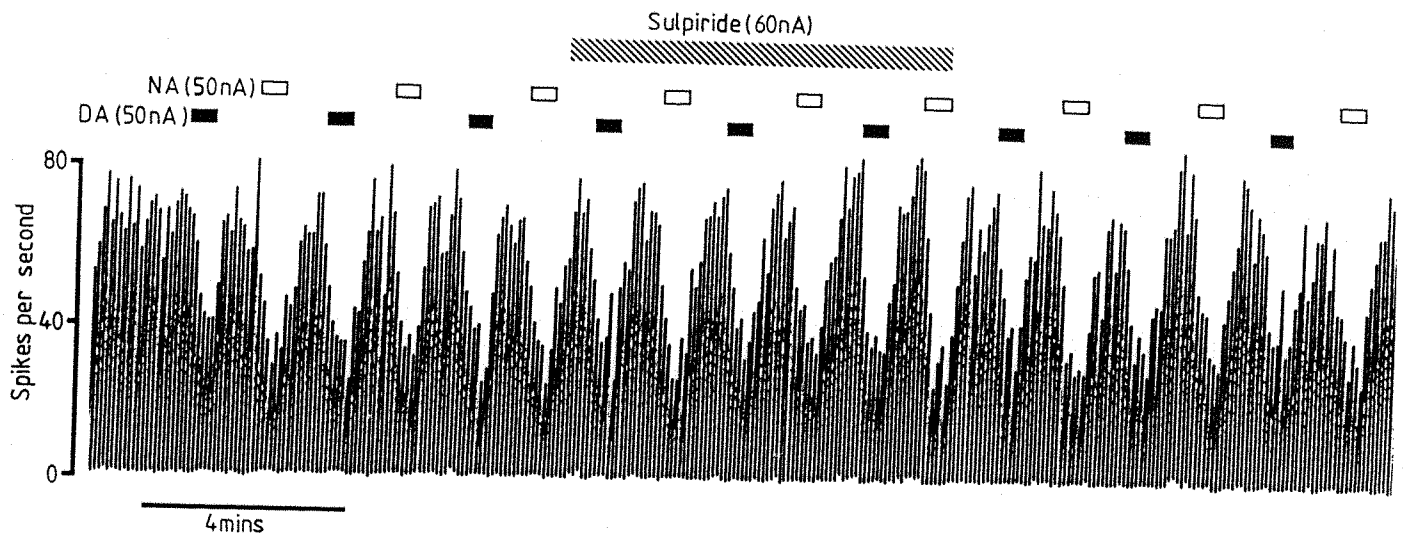
Figure 3.23

Ratemeter recording of the neuronal activity of a SNC neurone inhibited by iontophoretic pulses of dopamine (60 nA) and noradrenaline (60 nA). Sulpiride (60 nA) antagonised the inhibitory responses of dopamine and noradrenaline which began to return 10 minutes after sulpiride application was terminated.

Figure 3.24

Ratemeter trace of the neuronal activity of a cerebellar neurone inhibited by iontophoretic pulses of noradrenaline (50 nA) and dopamine (50 nA). Iontophoretic sulpiride (60 nA) has no effect on the inhibitory responses produced by dopamine and noradrenaline (the vertical lines represent the spikes counted in each 5 second period).


5mins
Sulpiride (60nA)
NA (60nA)
DA (60nA)
30
20
10
0
Spikes/10secs


Sulpiride (60nA)
NA (50nA)
DA (50nA)
80
40
0
Spikes per second
4mins

Iontophoretic responses to NA and DA were consistently achieved. Noradrenaline (30-100 nA) caused inhibition of the spontaneous firing rate in 19 of 22 cells tested (see figure 3.24). Dopamine (30-100 nA) caused inhibitions of the firing rate in 13 of the 19 cells tested. Noradrenaline was slightly more potent than dopamine. The dopamine response in one cell was biphasic, showing an initial excitation followed by a longer lasting inhibition of the firing rate.

Glutamic acid (20-100 nA) resulted in excitation of the firing rate in 6 of the 9 cells tested. One cell produced consistent inhibitions of the firing rate.

Figure 3.24 shows that iontophoretically applied (±)-sulpiride (40-60 nA) failed to antagonise any of the NA or DA responses on 7 and 5 cells respectively. When zetidoline (10-100 nA) was applied iontophoretically to 10 cells in the cerebellum, all the cells were inhibited (see figure 3.25). This effect appeared to be dose-dependent as the degree of depression was related to the length of time and amount of current used to eject the zetidoline. The inhibition was slowly reversable on terminations of the ejection current. Similarly, propanolol applied iontophoretically (20-80 nA), inhibited the firing rate in a dose dependent manner. The inhibition by propanolol reversed slowly on termination of the ejection current, as shown in figure 3.26. On three cells inhibited by zetidoline, glutamate was excitatory. Figure 3.25 shows that during zetidoline application, these excitatory responses were blocked. This effect could be the consequence of local anaesthetic properties of zetidoline. Ejection of current (100 nA for 5 min) on four of these cells had no effect on the firing rate while on one other cell an increased firing rate of approximately 10% occurred.

3.3.6 The effect of zetidoline on neuronal responses in vitro

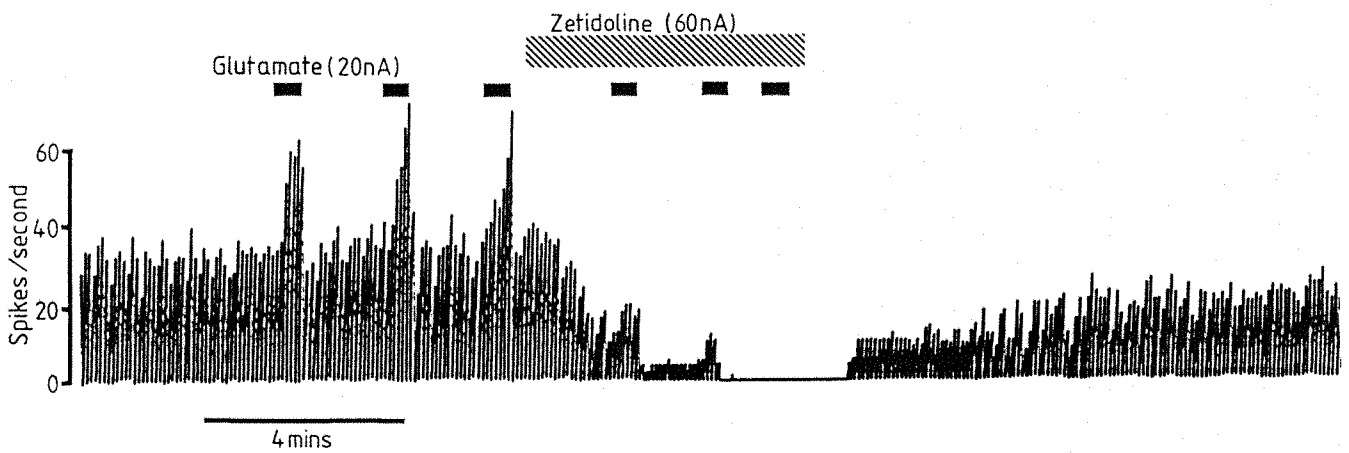
The recording of neuronal activity from brain slices from a variety of areas is a relatively new technique. This has been extended recently to include the substantia nigra (Pinnock, 1983a,b). Having shown that zetidoline antagonises the effects of dopamine and apomorphine in vivo, this technique was used to quantify the antagonistic activity of zetidoline on the dopamine autoreceptors of the substantia nigra.

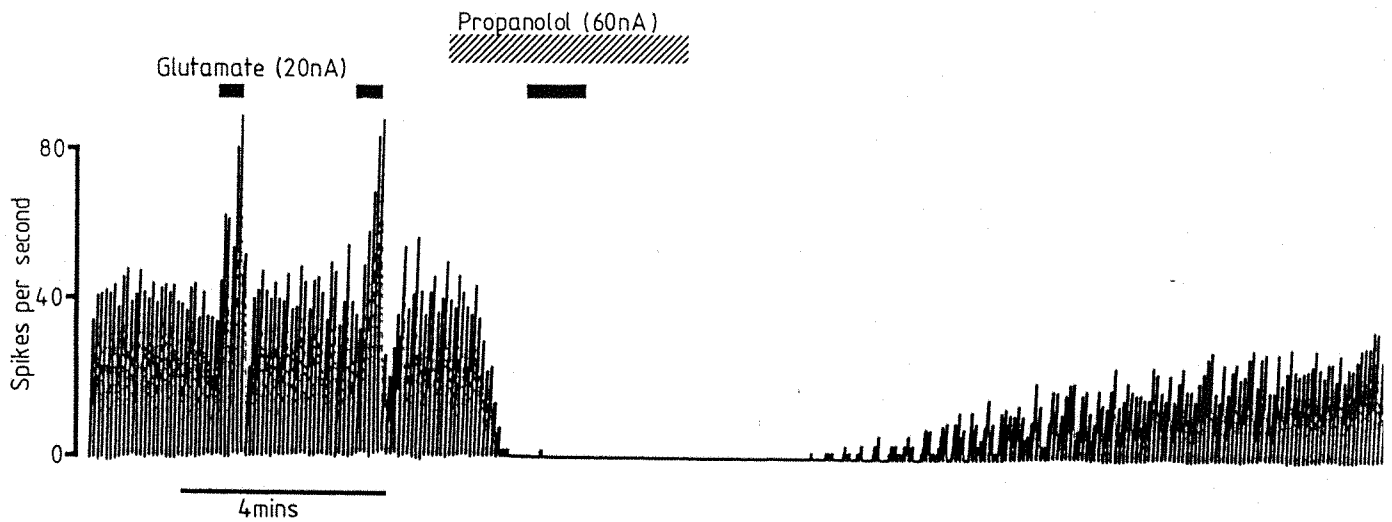
Figure 3.25

A ratemeter recording of a cerebellar neurone which is excited by the iontophoretic application of glutamate (20 nA). Zetidoline applied iontophoretically directly depresses the neuronal firing rate and abolishes the responses to glutamate. On termination of the iontophoretic application of zetidoline the firing of the cell slowly returns (each vertical line represents the spikes counted per 5 second period).

Figure 3.26

A ratemeter recording of a cerebellar neurone excited by iontophoretically applied glutamate (20 nA). Iontophoretically-applied propanolol (60 nA) which abolished the response to glutamate. When the application of propanolol is ceased the neuronal firing rate slowly returns.


Zetidoline (60nA)
Glutamate (20nA)
60
40
20
0
Spikes/second
4 mins


Propanolol (60nA)
Glutamate (20nA)
80
40
0
Spikes per second
4mins

In the brain slice containing part of the substantia nigra, cells were identified by their characteristic extracellular action potentials (see figure 3.1f) and firing rate. The firing rate is very regular with none of the bursting activity associated with the recordings of the in vivo preparation. The identified cells were tested for their sensitivity to dopamine. Dose-dependent responses were obtained by applying DA to the bath perfusate in known concentrations over the range of 3-100 μM on five cells in different nigral slices. Figure 3.27 shows the responses to DA at several concentrations of zetidoline applied to the bathing c.s.f. The threshold dose for an inhibitory response was 3 μM; doses less than this had no effect on the firing rate. The calculated ED_{50} for dopamine on these cells was 28.8 ± 1.6 μM. The cell firing rate was quickly depressed and allowed to reach a plateau level to determine the level of inhibition for each dose (see figure 3.27). Artificial c.s.f. containing known concentrations of zetidoline were allowed to perfuse over the slice for 2-3 minutes before dose-response curves were again obtained to dopamine in the concentration range, 3 μM to 3 mM (see figure 3.27). Zetidoline was used at five different concentrations over the range of 100 nM to 10 μM. Zetidoline itself had no effect on the firing rate in the slice but caused parallel shifts in the dose response curves to the right of the control, as can be seen in figure 3.28. The antagonism of the response by zetidoline (1 μM) was monitored after returning to the normal artificial c.s.f. perfusate. The responses to DA had not returned significantly after 2 hours. The response to GABA (30 and 100 μM) was tested in one cell following the perfusion of zetidoline (10 μM) through the bath. GABA was inhibitory on the firing rate, the effect being concentration dependent. Ascorbate (which was used to dissolve the drugs) at a concentration of 1 mM in the bathing c.s.f. caused a slight reduction (less than 10%) in the firing rate.

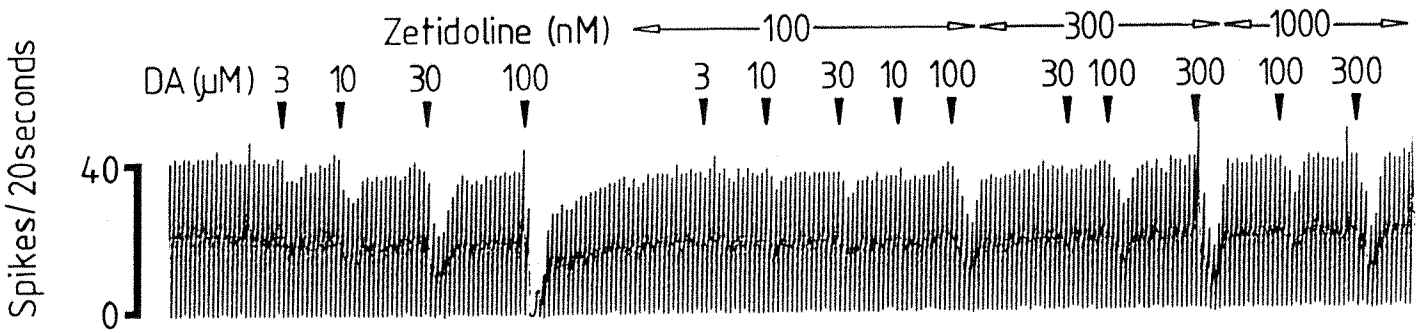
From the dose-response curves, the agonist dose-ratios for each concentration of agonist was obtained. The negative log of antagonist concentration was plotted against the log of the dose-ratio-1, as shown in figure 3.29. Regression analysis of these results gave a slope of 0.84 with a correlation coefficient of 0.997. The intercept on the abscissa gave a pA_2 value of 7.02 with a pA difference (pA difference = pA_2-pA_{10}) of 1.14. The results are compatible with

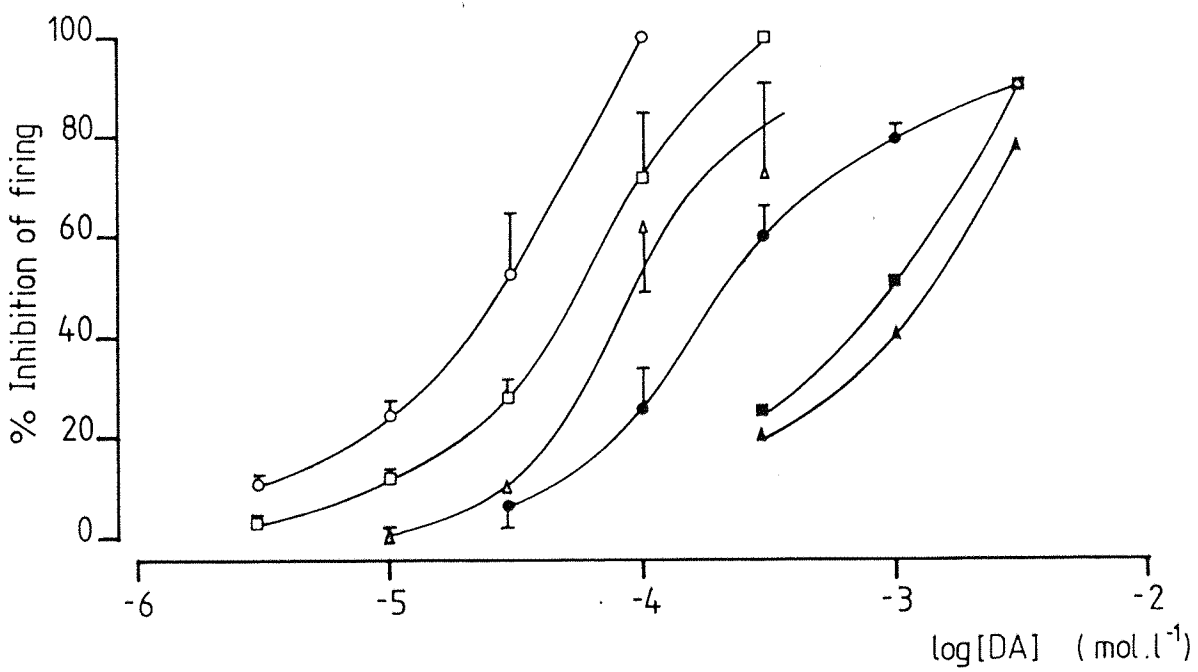
Figure 3.27

A ratemeter trace of the neuronal activity of a SNC neurone recorded in vitro from a brain slice. Dopamine added to the perfusing artificial cerebrospinal fluid at concentrations of 3, 10, 30 and 100 μM, causes a dose dependent inhibition of the neuronal firing rate. Zetidoline applied continuously to the
at concentrations of 100, 300 and 1000 nM, antagonised the responses produced by dopamine.

Figure 3.28

Log dose-response curve for dopamine at inhibiting the firing rate of SNC neurones in brain slices. Zetidoline at five different concentrations caused a parallel shift to the right of the dose-response curves. Each point is the mean ± S.E.M.; ○—○, dopamine alone (n=5); □—□, dopamine + zetidoline, 100 nM (n=5); △—△, dopamine + zetidoline, 300 nM (n=5); ●—●, dopamine + zetidoline, 1 μM (n=5); ■—■, dopamine + zetidoline, 3 μM (n=1); ▲—▲, dopamine + zetidoline, 10 μM (n=1).


Zetidoline (nM)
100
300
1000
DA (μM) 3 10 30 100 3 10 30 10 100 30 100 300 100 300
40
0
Spikes/20seconds


100
80
60
40
20
0
% Inhibition of firing
-6
-5
-4
-3
-2
log[DA] (mol.l^{-1})

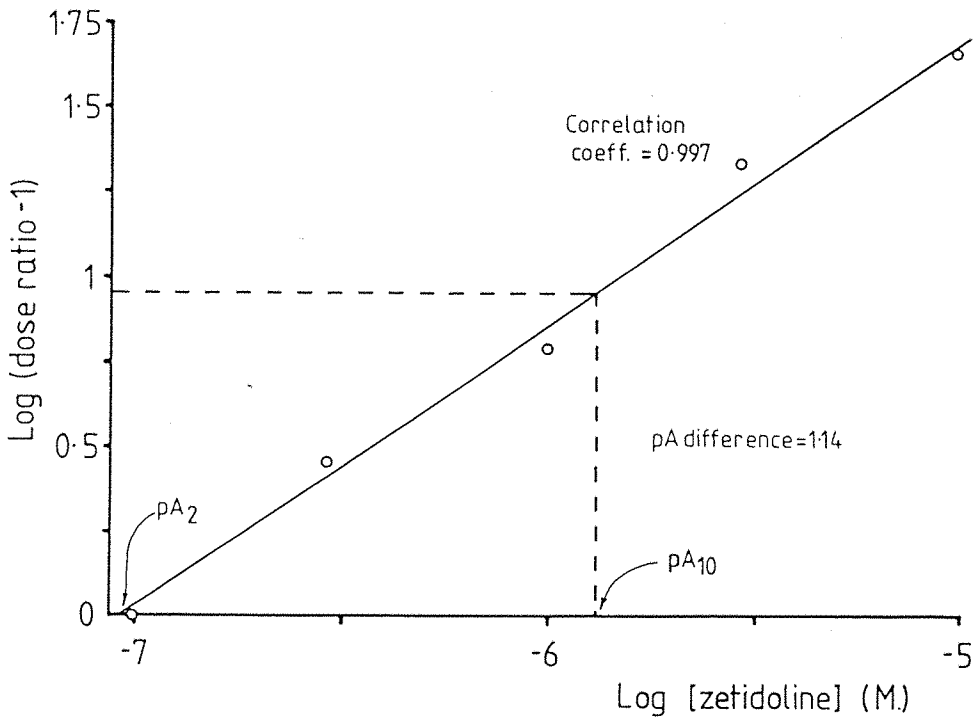


Figure 3.29

Schild plot taken from the dose response curves to dopamine on the neuronal firing rate of the SNC cells recorded in vitro, in the presence of varying concentrations of zetidoline. Regression analysis gives the line with a correlation coefficient of 0.997. The intercept on the abscissa gives a pA_2 value of 7.02 and the slope gives a pA difference of 1.14.

zetidoline being a competitive antagonist of the dopamine autoreceptor in the substantia nigra.

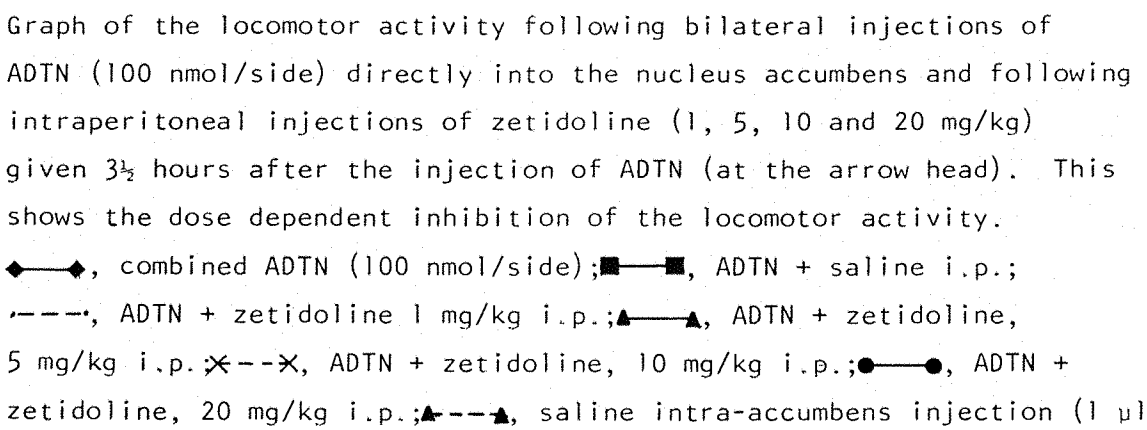
3.3.7 The effect of zetidoline on ADTN induced locomotor activity

The locomotor activity induced by intracerebral injections of dopamine agonists directly into the nucleus accumbens has been used as a model for the study of postsynaptic dopamine receptors. This increase in the locomotor activity can be antagonised by a range of neuroleptics (Woodruff, 1978; Elkhawad and Woodruff, 1975).

In this series of experiments the animals were tested for their responses to ADTN (100 nmol/side) when injected bilaterally into the nucleus accumbens. As can be seen in figure 3.31, the intense hyperactivity induced by ADTN in these animals lasted for over 12 hours. These animals were tested with an injection of the antagonist $3\frac{1}{2}$ hours after the start of the experiment and once the locomotor activity induced by ADTN had begun.

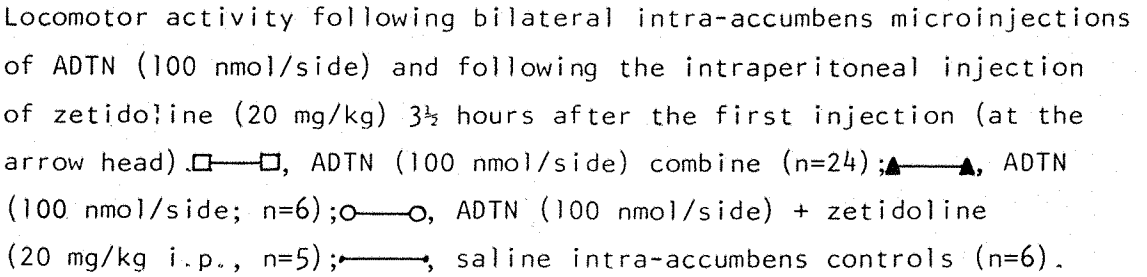
Zetidoline was given intraperitoneally at doses between 0.5-20 mg/kg. A dose-dependent inhibition of the locomotor activity was achieved (see figure 3.30), but only at the high doses (10 mg/kg or greater) was locomotor activity significantly reduced. Figure 3.31 shows the antagonism of the ADTN induced locomotor activity by 20 mg/kg of zetidoline. This is an extremely high dose when compared with other lipid soluble antagonists such as fluphenazine (Woodruff, 1978), and much larger than expected from the results in the previously described electrophysiological studies. One explanation for the lack of activity could be poor access, by this drug, into the nucleus accumbens. In order to test this possibility, zetidoline was given bilaterally, directly into the nucleus accumbens via the permanently implanted cannulae. Injections of zetidoline in this manner at doses of between 3-200 nmol-side had little effect on the ADTN induced locomotor activity. Only at the highest dose given (200 nmol/side) was any attenuation of the locomotor observed (as shown in figure 3.32). In a separate series of experiments, sulpiride was an extremely potent antagonist of the ADTN induced locomotor activity. Figure 3.33 shows that direct bilateral intra-accumbens microinjection of (±)-sulpiride (5 nmol/side) completely inhibited the stimulation of locomotor activity by ADTN. The locomotor activity measured following the sulpiride treatment was less than the

Figure 3.30

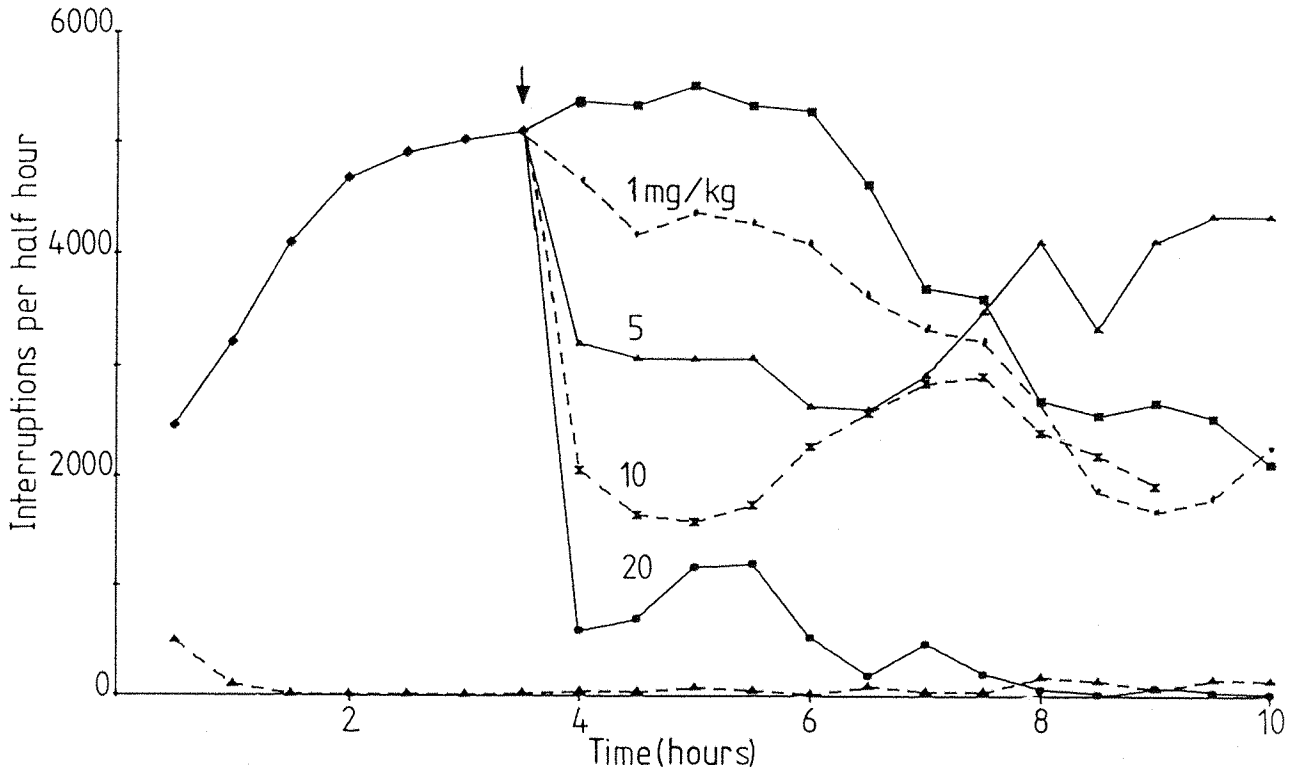


Graph of the locomotor activity following bilateral injections of ADTN (100 nmol/side) directly into the nucleus accumbens and following intraperitoneal injections of zetidoline (1, 5, 10 and 20 mg/kg) given 3½ hours after the injection of ADTN (at the arrow head). This shows the dose dependent inhibition of the locomotor activity. ◆——◆, combined ADTN (100 nmol/side); ■——■, ADTN + saline i.p.; ·– – –·, ADTN + zetidoline 1 mg/kg i.p.; ▲——▲, ADTN + zetidoline, 5 mg/kg i.p.; ✕– –✕, ADTN + zetidoline, 10 mg/kg i.p.; ●——●, ADTN + zetidoline, 20 mg/kg i.p.; ▲– –▲, saline intra-accumbens injection (1 µl).

Figure 3.31



Locomotor activity following bilateral intra-accumbens microinjections of ADTN (100 nmol/side) and following the intraperitoneal injection of zetidoline (20 mg/kg) 3½ hours after the first injection (at the arrow head). □——□, ADTN (100 nmol/side) combine (n=24); ▲——▲, ADTN (100 nmol/side; n=6); ○——○, ADTN (100 nmol/side) + zetidoline (20 mg/kg i.p., n=5); ·——·, saline intra-accumbens controls (n=6).


6000
4000
2000
0
Interruptions per half hour
1mg/kg
5
10
20
2
4
6
8
10
Time (hours)

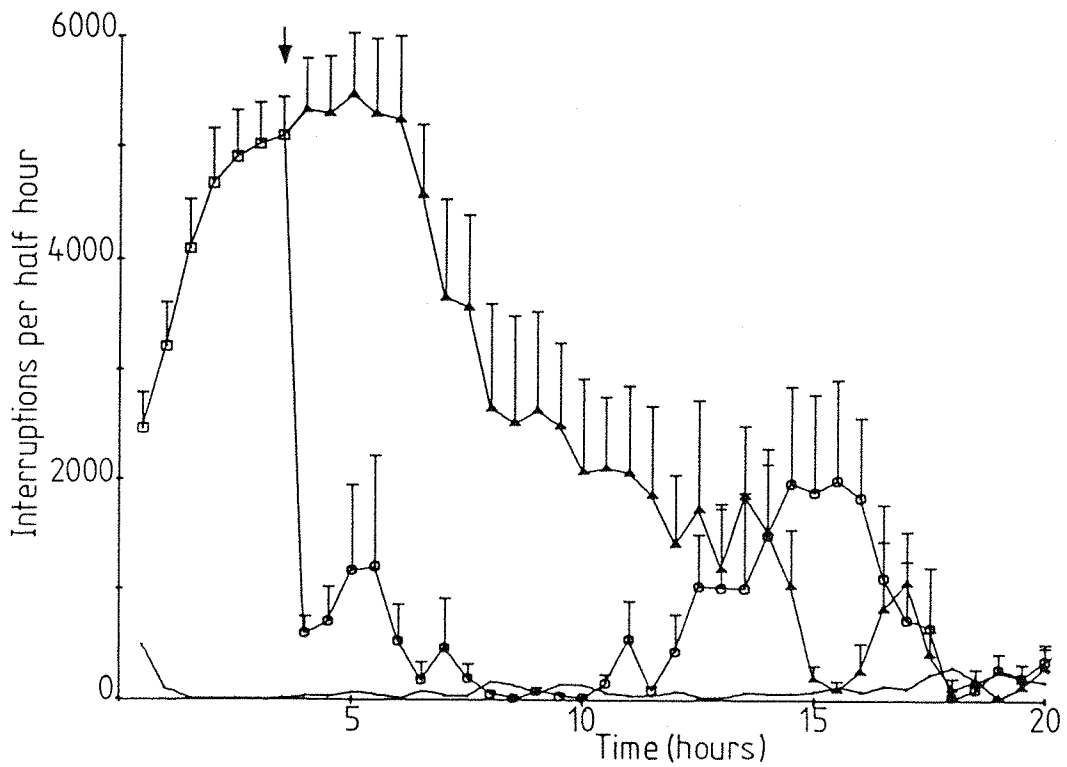

6000
4000
2000
0
Interruptions per half hour
5
10
15
20
Time (hours)

Figure 3.32

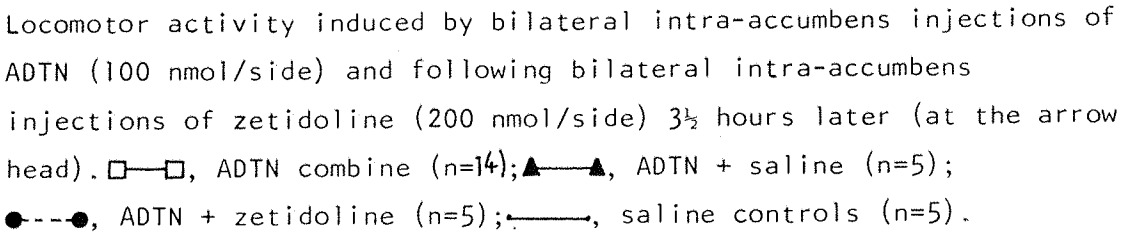
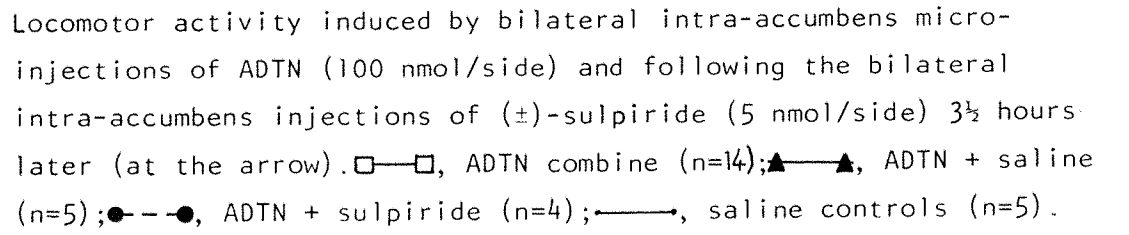
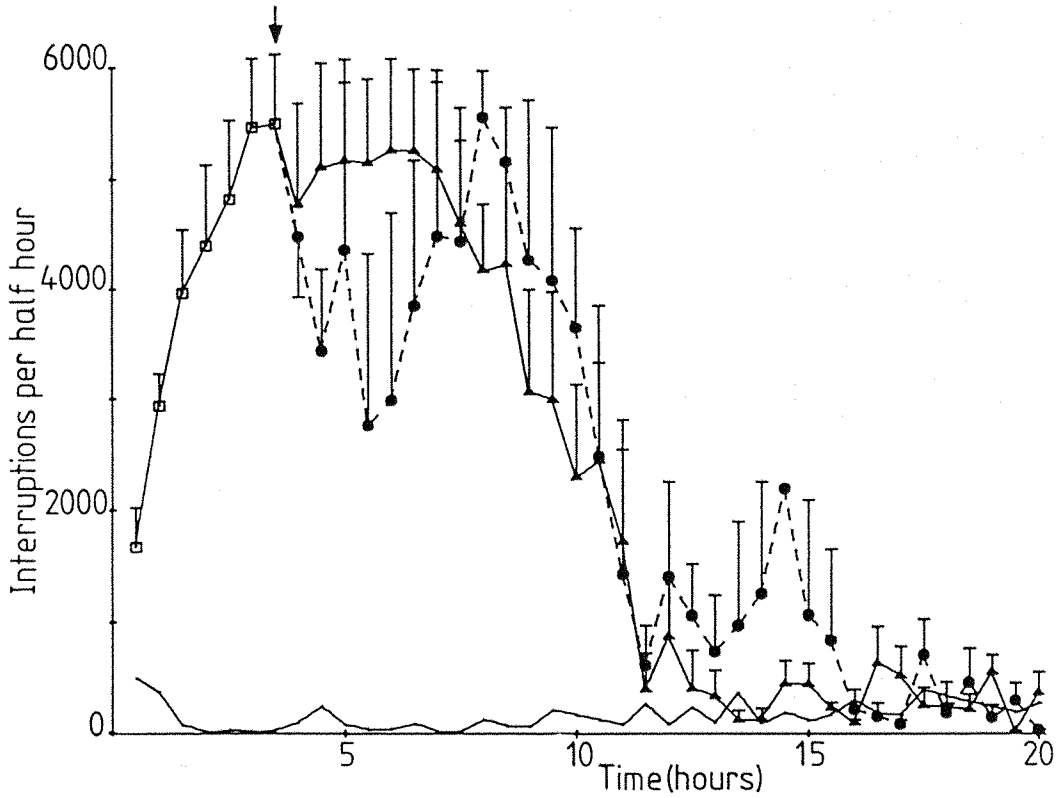
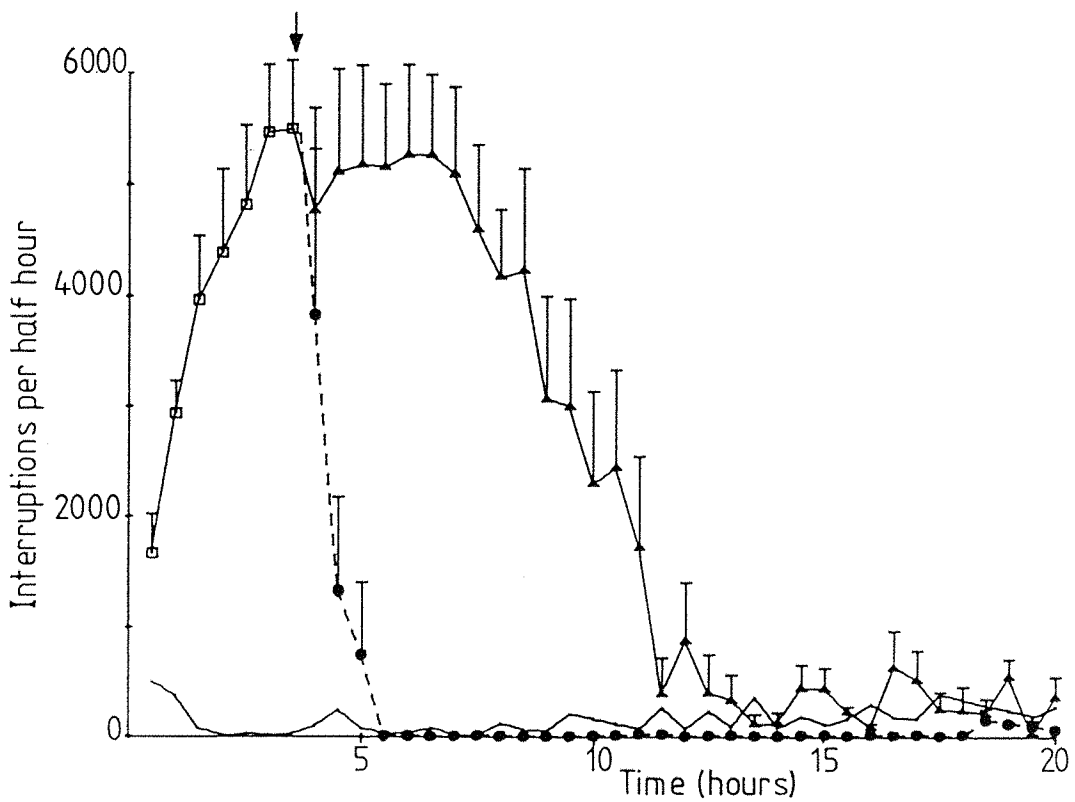


Figure 3.33




6000
4000
2000
0
Interruptions per half hour
5
10
15
20
Time (hours)


6000
4000
2000
0
Interruptions per half hour
5
10
15
20
Time (hours)

activity measured after a bilateral injection of vehicle alone. This may indicate that the dose of sulpiride used may be causing a sedative effect in the animals.

Following a successful experiment, the positions of the cannulae were checked. Figure 3.34 shows the positioning of the cannulae from a successful experimental animal.

The results obtained by direct intra cerebral injections show that the lack of potency at attenuating the locomotor activity is not the result of poor access into the nucleus accumbens.

Thus, this study has examined the effects of some novel dopamine agonist and antagonists. The study has demonstrated the potential of the electrophysiological technique, on the SNC neurones, as a pharmacological tool in vivo as well as in vitro. Drugs given intravenously can be shown to cross the blood brain barrier and have effect on neuronal activity within the CNS. Subsequently, in the case of zetidoline, the effects were shown to be the result of a direct action on the SNC neurones by microiontophoresis. Again the activity of zetidoline as a dopamine receptor antagonist was tested in vitro using a brain slice preparation. In addition, the effect of some of these drugs were assessed in a behavioural test for postsynaptic dopamine receptor activity. This has highlighted discrepancies between the activity of these drugs at the cellular level and their activity on the behavioural test.

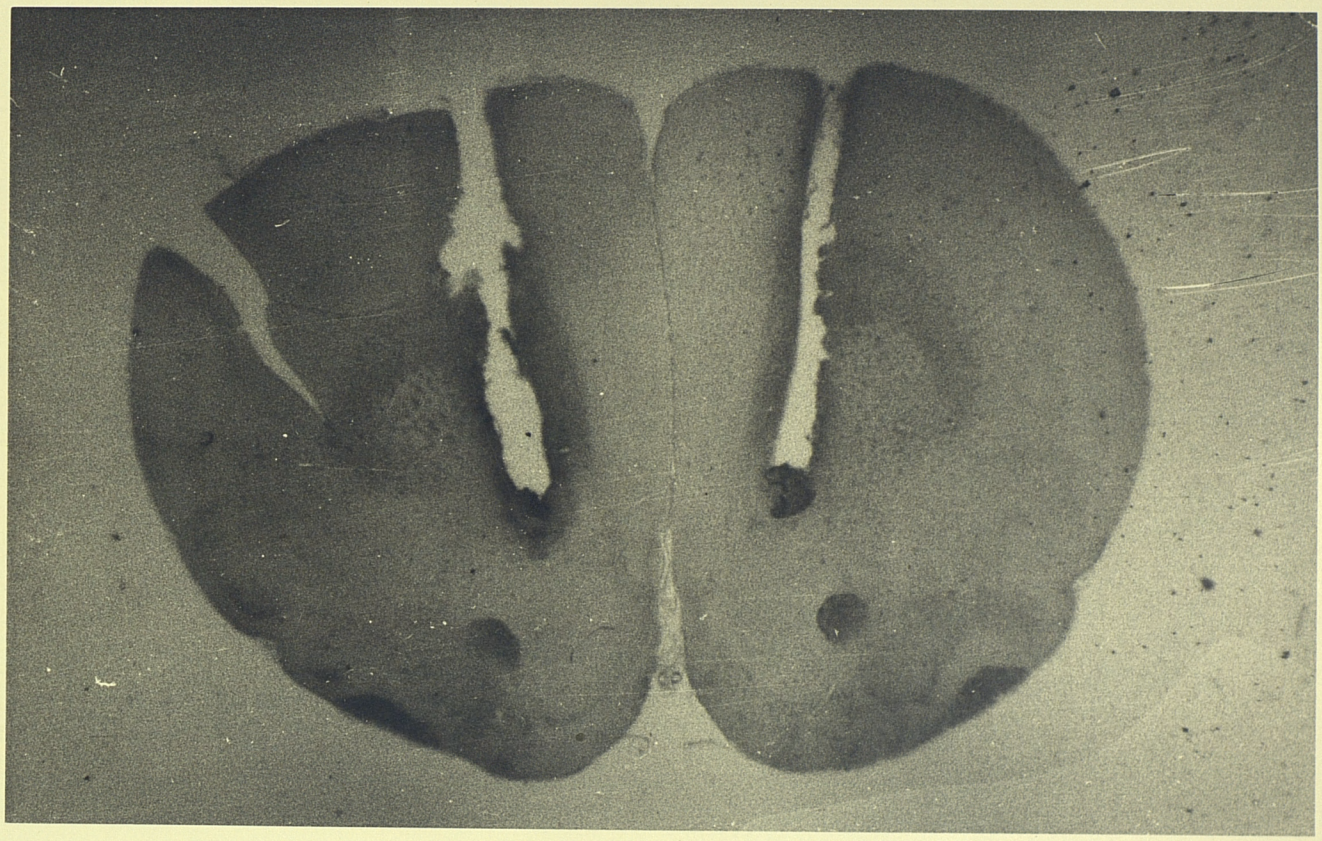


Figure 3.34

A coronal section through the brain of a rat cannulated with guide cannulae, which terminate above the nucleus accumbens, for the microinjections of drugs directly into the nucleus accumbens.

Chapter 4

4. Discussion

In recent years, compounds with dopamine receptor activity have been developed in an attempt to produce new and selective DA receptor agonists and antagonists for clinical use in the treatment of Parkinson's disease and schizophrenia. Some of these drugs have proven to have novel pharmacological profiles which has led to the suggestion that they may act on specific dopamine receptor subtypes (as explained in section 1.7). The assessment of the actions of new drugs needs to be performed in as wide a range of model systems as possible in order for discrepancies in the expected activity and for the detection of possible side effects in the clinical situation. The assessment of dopamine receptor drugs has been poorly studied in electrophysiological preparations, possibly because of technical difficulties. The present study set out to assess some novel drugs in an electrophysiological system on a restricted population of neurones in the SNC, which are known to be sensitive to dopamine; in addition to test these drugs on the locomotor activity of rats.

4.1 Identification and stimulation of the SNC cells

The cells recorded in this study were shown to have similar electrophysiological characteristics to the previously reported observations of Bunney et al. (1973), Guyenet and Aghajanian (1978) and Grace and Bunney (1980). The cells have been identified as the DA containing cells whose axons form the nigrostriatal pathway. Approximately 20% of the cells recorded were identified as nigrostriatal neurones following stimulation of the caudate nucleus. Antidromic activation of dopaminergic neurones was established by the criteria proposed by Lipski (1981). These criteria are constant latency; one spike per stimulus; the ability to follow high frequency stimulations without spike failure and collision of antidromic with orthodromic potentials. A conduction velocity of the antidromic potential, calculated from the latent period, of 0.45 m/sec is close to the previously reported value of 0.54 m/sec (Grace and Bunney, 1980). This is typical

of small diameter unmyelinated fibres, which are the type believed to constitute the nigrostriatal pathway (Hokfelt and Ungerstedt, 1973). However, in some cells, the antidromic latency varied slightly to a constant stimulus intensity. Similar findings have been reported by Collingridge, James and MacLeod (1980) following caudate stimulation. In addition, changes in antidromic latencies have been noted during rapid stimulation in noradrenergic neurones (Aston-Jones, Segal and Bloom, 1980). These variations in antidromic latency have been attributed to changes in the excitability following the conduction of previous spontaneous orthodromic action potentials or an alteration of the ion concentrations along the unmyelinated nerve fibre. These fibres have a relatively small volume to surface area ratio and so may be more susceptible to the changes in ion concentrations during repetitive stimulations. Alternatively, the nigrostriatal pathway may be activated in different parts of their highly branched and twisted axons. This slight variation in the antidromic latency has been suggested to account for the failure of earlier studies to antidromically identify SNC cells. Although not all of the cells were identified as nigrostriatal projection neurones, the study was assumed to be on a restricted population of neurones in the SNC that give projections to the striatum.

The SNC cells are sensitive to dopamine and dopamine mimetic drugs such as apomorphine. In this study, intravenously administered apomorphine inhibited the neuronal firing rate of the SNC cells in low doses (1-20 µg/kg). Previous studies have calculated an ED_{50} for apomorphine on the SNC cell firing rate of 9.3 µg/kg, i.v. (Guyenet and Aghajanian, 1978). The doses used in this study produced inhibitions of SNC cell firing rate which are comparable with the previous reports. These inhibitory effects were antagonised by fluphenazine. Fluphenazine has been shown in pharmacological tests to cross the blood-brain barrier and to be a selective antagonist at dopamine receptors (Fjalland and Boeck, 1978; Andrews and Woodruff, 1979). Thus the responses to apomorphine on the SNC cells were confirmed to be due to dopamine receptor stimulation.

4.2 The effect of novel DA receptor agonists on dopaminergic systems

Apomorphine was the only dopamine receptor agonist known for many years; consequentially it has been used widely in many behavioural and

biochemical models of DA receptors. When given peripherally it rapidly crosses the blood-brain barrier to act on the CNS. Low doses of apomorphine (that is, less than 1 mg/kg) were shown to cause sedation in mice, an effect which was antagonised by non-sedative doses of neuroleptics while doses of 1 mg/kg or larger causes hyperactivity (Carlsson, 1975). The synthesis of DA in the striatum after axotomy or GBL pretreatment is inhibited by low doses of apomorphine and blocked by neuroleptics (Anden, Corrodi, Fuxe and Ungerstedt, 1971; Walters et al., 1975). Both effects are considered to be due to presynatic DA receptor activity. In particular, the effect by DA agonists on DA synthesis is due to dopamine autoreceptors activity in the nerve endings of the nigrostriatal pathway. Thus, the responses to low doses of apomorphine on the neuronal firing rate of the SNC cells in this study are due to DA autoreceptor stimulation. These inhibitory responses to apomorphine were used to test the sensitivity of the SNC cells prior to further intravenous drug administration and to allow a comparison to be made between agonist response on the DA autoreceptors of single neurones.

Piribedil (or ET495) was first proposed as a dopamine receptor stimulating agent by Corrodi, Fuxe and Ungerstedt (1971). They showed that peripheral administration produced increases in the turnover of dopamine, but had no effects on the metabolism of noradrenaline. In addition, contralateral rotations were elicited in animals with 6 OHDA lesions of the substantia nigra, similar to those produced by apomorphine. Piribedil was found to be a longer lasting agonist but was less potent than apomorphine in that study. However, the contraversive turning produced by piribedil was prevented by α-methyl paratyrosine pretreatment, suggesting the involvement of a presynaptic receptor response.

In this study intravenously administered piribedil had an inhibitory effect on the SNC cell firing rate. These inhibitions were rapid in onset and relatively short in action, a dose of piribedil lasting 5 minutes (for complete recovery) whereas an equipotent dose of apomorphine would last 30 minutes. In addition, piribedil has approximately a third of the potency of apomorphine. These results are in agreement with the report by Walters et al. (1975). In their study they applied piribedil and apomorphine intraperitoneally and used doses capable of producing postsynaptic receptor activation. The doses used in this study were small in order to stimulate only the DA autoreceptors, particularly on the SNC cells. Despite these differences the results of both studies are

similar. Piribedil was less potent than apomorphine, both compounds having a rapid but slower onset of action following intraperitoneal administration, the response taking 1-2 minutes to have maximal effect. In this study, the inhibitory responses to piribedil on the SNC cell firing rate were antagonised by low doses of fluphenazine. Similarly, the inhibitory effects of piribedil were antagonised by low doses of haloperidol (Walters et al., 1975).

However, these results do not agree with the findings of Corrodi et al. (1971), who found piribedil to be longer acting than apomorphine. Piribedil is extensively metabolised in the rat (Jenner, Taylor and Cambell, 1973); its chief metabolite (S584) contains a catechol group, and was suggested as the active compound in vivo (the metabolism of piribedil is shown in figure 4.1). Other evidence in favour of this suggestion has been found. The metabolite S584 when injected directly into the striatum demonstrated dopamine receptor stimulatory activity (Costall and Naylor, 1974), but piribedil and its other metabolites were inactive. Furthermore, S584, but not piribedil, had a stimulatory effect on the striatal dopamine stimulated adenylate cyclase (Miller and Iversen, 1974). However, piribedil became active after a preincubation with the striatal homogenates before cAMP levels were measured (Makman, Mishra and Brown, 1975). Creese and Iversen (1974) showed that both piribedil and S584 produced stereotyped behaviours similar to apomorphine but had different time courses of action, again in support of the metabolite as the active molecule, but the results of the present study and those of Walters et al. (1975) suggest that piribedil is a directly acting dopamine receptor stimulating agent. The rapidity with which piribedil acts on the SNC firing rate following intravenous injection would suggest that the metabolism was virtually instantaneous. The duration of the piribedil response is relatively short, lasting only 5-10 minutes, and correlates well with the kinetics of piribedil metabolism in the plasma and brain. The concentrations of piribedil rapidly reach a peak after injection and quickly decline in an exponential manner, which is reflected in the recovery rate of the neuronal activity of the SNC cells. Additionally the levels of S584 measured in the brain following parenthral administration are small compared with the levels after an injection of S584 (Consolo, Fanelli, Garattini, Ghezzi, Jori, Ladinsky, Marc and Samanin, 1975).

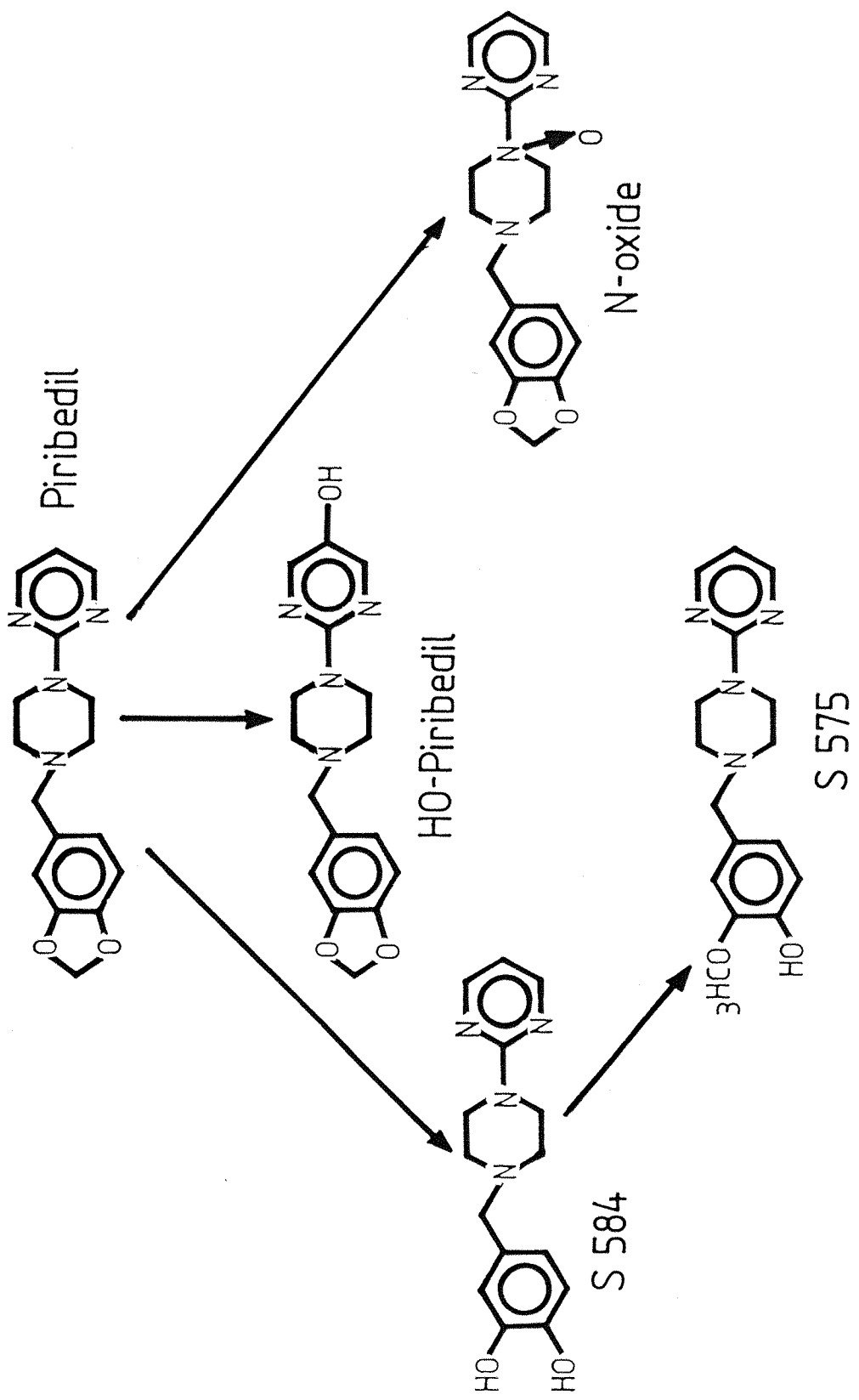


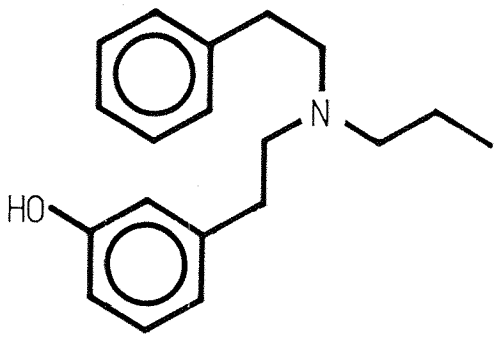
Figure 4.1 Diagram showing the routes of metabolism for piribedil

Subsequent to the proposal that piribedil might act through a metabolite, a new compound was synthesised in which the methylenedioxy group of piribedil was replaced by a moiety that could not be metabolised to a catechol. The compound, S-3608, was introduced as a dopamine agonist with this methylenedioxy group; the structure is shown in figure 4.2 (Poignant, Gressier, Petitjean, Regnier and Canevari, 1975). Although this initial study demonstrated that the properties of this compound were indicative of a dopamine agonist, producing stereotyped behaviours and contralateral rotations in rats with 6 OHDA lesions of the SNC, little is known about its pharmacology.

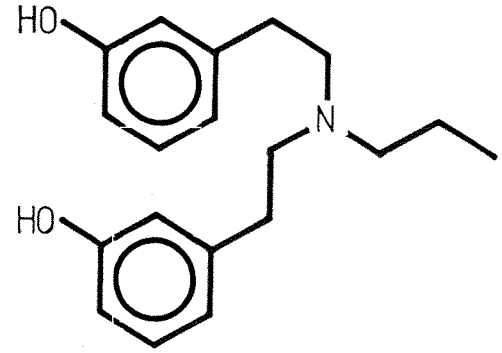
In this study, S-3608 was administered intravenously and inhibited the neuronal firing rate of the SNC cells, being approximately equipotent with piribedil. This showed that, like piribedil, S-3608 was able to cross the blood brain barrier, in agreement with the previous report where peripheral administration produced the behavioural changes. All cells that responded to S-3608 were responsive to apomorphine and vice versa. These inhibitory effects on SNC cell firing rate were antagonised by fluphenazine and haloperidol confirming that these responses were mediated by dopamine receptors. A comparison of the effects of piribedil with S-3608 show that the inhibitions produced were almost identical in onset and length of action. In addition, both compounds were equipotent at producing inhibitions of neuronal activity. Thus, on the firing rate of the SNC cells, S-3608 and piribedil are short acting agonists compared with apomorphine.

As there is no evidence to suggest S-3608 might act through a metabolite and both S-3608 and piribedil have very similar actions, it may be concluded that both compounds are acting directly on the DA receptor on the SNC cells and not via a metabolite.

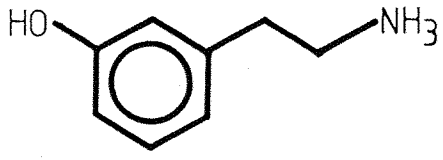
The dopaminergic system in the nucleus accumbens plays an important role in the control of locomotor activity in the rat. Dopamine and dopamine agonists injected directly into the nucleus accumbens produces hyperactivity in rats. Dopamine is quickly metabolised following injection and requires pretreatment with a monoamine oxidase inhibitor to elicit a strong and long lasting stimulation of hyperactivity (Costall and Naylor, 1976). ADTN has been routinely used to stimulate hyperactivity in rats following direct injections into the nucleus accumbens, because ADTN is not metabolised by MAO and therefore does not



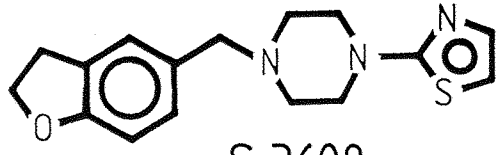
RU 24213



RU 24926



m-tyramine



S 3608

Figure 4.2 The structures of the dopamine agonists RU24213, RU24926 and S-3608

require the monoamine oxidase inhibitor pretreatment (Elkhawad and Woodruff, 1975; Andrews and Woodruff, 1978).

In this study, ADTN injected directly into the nucleus accumbens, induced an intense and long lasting stimulation of locomotor activity of the rats, similar to that seen in other studies. This hyperactivity was used as a test to check the success of the cannulation procedure and as a control response for the comparison with other DA agonist responses. The injection of S-3608 directly into the nucleus accumbens failed to produce any locomotor activity even when compared with the control injections of saline. This was unexpected as piribedil had been shown to produce hyperactivity in rats (Butterworth et al., 1975), and S-3608 seemed to have a similar pharmacological profile to piribedil, but this response by piribedil on motor activity was produced by high doses injected intraperitoneally, far in excess of the doses required to produce inhibitions of SNC cell firing in this study. Thus, the doses used in the present study, although high, may not be enough to elicit hyperactivity. Alternatively, the locomotor response to piribedil was not produced by activation of dopamine receptors in the nucleus accumbens but elsewhere. The discrepancy in the lengths of action between piribedil and apomorphine on the SNC cell firing seen in this study and on the behavioural responses seen by Corrodi et al. (1971) may be a result of the dopamine agonist activity of the metabolite of piribedil. S584 has agonist activity causing stereotyped behaviours and rotational activity and is active at stimulating the dopamine sensitive adenylate cyclase, whereas piribedil has no activity on this model (Miller and Iversen, 1974). Under the Kebabian and Calne classification, S584 would be classified as a D-1 receptor agonist and piribedil and S-3608 as D-2 receptor agonists. The DA autoreceptors in the SNC are considered D-2-like receptors because sulpiride antagonises the responses to dopamine (Pinnock et al., 1979; Woodruff and Pinnock, 1981; and the present study).

Against [^{3}H]-sulpiride binding to rat striatal membranes, both S-3608 and piribedil were potent displacers of binding, piribedil being four times more potent than S-3608 but four times more potent than apomorphine (as table 4.1 shows; Woodruff and Freedman, 1983). Similar results were obtained against [^{3}H]-spiperone binding to rat striatal and nucleus accumbens membranes (Hall, Jenner and Marsden, 1983). Thus,

it might be expected that S-3608 and piribedil inhibit the neuronal firing rate of the SNC cells, but not by S584 or other D-1 agonists. The effect of S584 on the SNC cell firing rate has not been tested and would be of interest to do so.

If the behavioural responses previously reported were elicited through D-1 receptors in the striatum, then this might account for the differences in the duration of action of piribedil and apomorphine on the SNC and the behavioural responses. In addition the lack of effect of S-3608 on the nucleus accumbens-induced locomotor activity model could be due to a lack of activity on D-1 receptors. However, since the locomotor activity induced by direct intra-accumbens injections of ADTN can be blocked by sulpiride (Andrews and Woodruff, 1978), D-2 receptor must be involved in the locomotor response. Therefore, the explanation that D-1 receptors are involved in the locomotor activity response, to account for S-3608's weak activity in this model, is inadequate. An alternative explanation might be that different responses are produced from pre and postsynaptic receptor activation. Piribedil and S-3608 demonstrated in this study that the presynaptic DA receptor is responsive to low doses of dopamine agonists, whereas much higher doses may be required for postsynaptic receptor activity. Thus, higher doses than used in this study may be required to produce locomotor activity.

Two other recently proposed dopamine agonists, RU24213 and RU24926, are based on N-diphenyl ethylamine (Euvrard, Ferland, DiPaolo, Beaulieau, Labrie, Oberlander, Raynaud and Boissier, 1980). These compounds have a structural similarity with meta-tyramine (the structures of these compounds are shown in figure 4.2). It is interesting to note that the structures of RU24213 and RU24926 have only one hydroxyl group on the phenolic ring, but structure activity studies of the DA receptor have shown a requirement for two hydroxyl groups in the 3 and 4 positions of the phenolic ring (Woodruff and Walker, 1969; Goldberg et al., 1968; Miller et al., 1974). On sulpiride binding both RU24213 and RU24926 were potent displacers of binding (as table 4.1 shows; Woodruff and Freedman, 1983) being more potent than S-3608 and piribedil.

This study showed that both RU24213 and RU24926 had an inhibitory action on the firing rate of the SNC cells. Compared with the action of apomorphine, both RU24213 and RU24926 were less potent but of a similar potency to piribedil and S-3608 and much longer acting than all of these

drugs. If the potency of the agonists found in this study (albeit qualitative) is compared with the ability to displace binding in the receptor binding studies (see table 4.1), then a discrepancy is detected. In the binding studies, apomorphine and RU24926 are equipotent at displacing sulpiride binding, but apomorphine is 3, 6 and 24 times more potent than RU24213, piribedil and S-3608 respectively at displacing sulpiride binding. Yet, apart from apomorphine, RU24213, RU24926, piribedil and S-3608 were all approximately equipotent at inhibiting the cell firing rate of the SNC cells. This discrepancy may be explained by the fact that binding can only measure the ability of a drug to displace a ligand from the recognition site of the receptor, whereas an agonist has the ability to initiate the sequence of events which causes the physiological response (intrinsic activity). This is a disadvantage of the binding technique. An additional explanation which may account for the different affinities seen in the binding data is that the pharmacodynamics of the drugs might alter their measured affinity for the receptor. Thus, the long acting drugs, RU24213 and RU24926, may have an enhanced affinity when measured by receptor binding, compared with the physiological measurement where the length of action is observed but is not used in the assessment of the pharmacological efficacy. The drugs in the in vivo preparation have to cross the blood brain barrier to have effect, but do not in receptor binding studies. In this study, the drugs were able to cross the blood brain barrier and have effect on the SNC cells firing rate.

Recovery from the inhibition of cell firing by a single dose of either RU24213 or RU24926 had two phases, this effect being more pronounced than with apomorphine inhibitions. The two phase recovery for apomorphine has been suggested as an effect on pre and postsynaptic dopamine receptors (Walters et al., 1975). Evidence suggests that the dopamine autoreceptor modifying the neuronal activity of the SNC cells are more sensitive to iontophoretic dopamine or intravenous apomorphine than the postsynaptic dopamine receptors (Skirboll et al., 1979). Thus, when the DA agonists are given they may act initially on both auto and postsynaptic DA receptors, so that both the nigrostriatal and striatonigral pathways are inhibited. With the metabolism of these compounds, the concentration of the agonists at the receptors decreases. As the postsynaptic DA receptors are less sensitive to agonists,

Table 4.1 Displacement of [^{3}H]-sulpiride binding to rat striatal membranes.

Antagonists	IC_{50} (nM)
Spiroperidol	0.5*
Domperidone	0.8
Fluphenazine	0.9
(+)-Butaclamol	1.6*
Haloperidol	4.5
Zetidoline	6.0*
Cis-flupenthixol	8.9*
Chlorpromazine	9.0
(-)-sulpiride	11.0

Agonists	IC_{50} (nM)
Bromocryptine	4.5
(±) ADTN	28.0
Apomorphine	32.0
RU24926	32.0
RU24213	110.0
Piribedil	180.0
Dopamine	580.0
S-3608	780.0
Noradrenaline	3000.0

Taken from: Woodruff and Freedman (1983)

*Holden-Dye et al. (1983)

inhibitions mediated by the postsynaptic receptor ceases before that of the DA autoreceptor. This would allow for an initial rapid recovery of the firing rate as a result of the release of the striatonigral pathway from the inhibitory effects of the dopamine agonists in the striatum and a slower recovery from the inhibitory effects of the agonists on the autoreceptors in the SNC.

The tachyphylaxis seen with subsequent doses of RU24213 and RU24926, described in the results, might reflect desensitisation of the dopamine autoreceptor on the SNC cells. A tachyphylactic effect was observed on the SNC neuronal firing rate following large doses of apomorphine and piribedil given intraperitoneally (Walters et al., 1975). A second dose failed to produce the expected degree of inhibition. Long-lasting desensitisation was also seen following a short iontophoretic pulse of ADTN on these cells (Woodruff and Pinnock, 1981). This may be the result of slow dissociation of the agonist from the receptor site without stimulation of the receptor effector mechanism, acting like a partial agonist. Another explanation for this effect could be that these drugs have an indirect action causing the release of DA and that the reduced responses were due to a depletion of the stores of DA released. However, RU24213 produces contralateral turning in 6 OHDA unilaterally lesioned rats, and this was reduced by AMPT pretreatment (Gershanik, Heikkila and Duvoisin, 1983), but this compound does not act indirectly, otherwise it would induce ipsilateral rotation. Furthermore, the responses to apomorphine were also reduced following the applications of either RU24213 or RU24926.

The action of both RU24213 and RU24926 lasted for over an hour following a single dose (50 µg/kg) which was far longer than equipotent dose of apomorphine. The length of action of these two agonists may reflect a low rate of metabolism at the nerve terminals. Another possibility is that both molecules being lipophylic become incorporated into the membrane and are slowly released into the extracellular fluid. This prolonged action of the two agonists is in agreement with the study of Euvrard et al. (1980). They showed that both agonists produced a long lasting inhibition of plasma PRL concentration in the rat and decreased striatal turnover, as well as long lasting effects on stereotyped behaviours in rats and emesis in dogs.

When tested on the locomotor activity model by direct intra-nucleus accumbens injections, RU24213 caused no locomotor activity at the doses given, compared with the control responses; RU24926 did cause a weak stimulation of locomotor activity requiring a high dose to elicit this activity. The locomotor response produced by RU24926 is of similar duration to that produced by apomorphine but was approximately five times less potent (Andrews, 1981). This DA agonist appears to be very weak on the locomotor activity model.

Both RU24213 and RU24926, at a dose of 100 µM, had no effect on the basal or dopamine stimulated adenylate cyclase activity (Euvrard et al., 1980), and would thus be classified as D-2 agonists under the Kebabian and Calne (1979) classification. A correlation between the activity on adenylate cyclase and on the ability to cause vasodilation of the perfused rat kidney has been made (Schmidt, Imbs, Giesen and Schwartz, 1982). In that study, SKF38393 is a proposed selective D-1 agonist and causes locomotor activity following intra-accumbens injections, but since this activity induced by SKF38393 is antagonised by (-)-sulpiride (Freedman et al., 1979), the locomotor activity response cannot be classified in terms of the D-1 or D-2 receptor stimulation. A possible explanation could be that these agonists have low potency on postsynapic receptors but are potent on the dopamine autoreceptor. In the GBL pretreated rat, striatal L-DOPA accumulates which can be inhibited by dopamine agonists. This forms the basis for a model for presynaptic dopamine receptors. In this model, RU24926 inhibited L-DOPA accumulation having less activity than apomorphine, but was more potent than 3-(3-hydroxyphenyl)-N-n-propyl piperidine (3-PPP) (Hambrich and Pflueger, 1982). 3-PPP is a proposed selective presynaptic DA agonist (Hjorth, Carlsson, Wilkström, Lindberg, Sanchez, Hacksell, Arvidsson, Svensson and Nilsson, 1981). 3-PPP has only one hydroxyl on its phenolic group (similar to RU24926 and RU24213), yet other selective presynaptic DA agonists have been proposed such as TL-99, EMD-23448 and RDS-127, which show no structural similarity as shown in figure 4.3. Perhaps the structure-activity requirement for presynaptic DA receptor activity are less stringent than for the postsynaptic DA receptor. These so-called selective presynaptic agonists inhibit the neuronal activity of the SNC cells in vivo (Bunney, Meltzer, Kauer and Chiodo, 1983; Arneric, Long, Williams, Goodale, Mott, Lakoski and Gebhart, 1983;

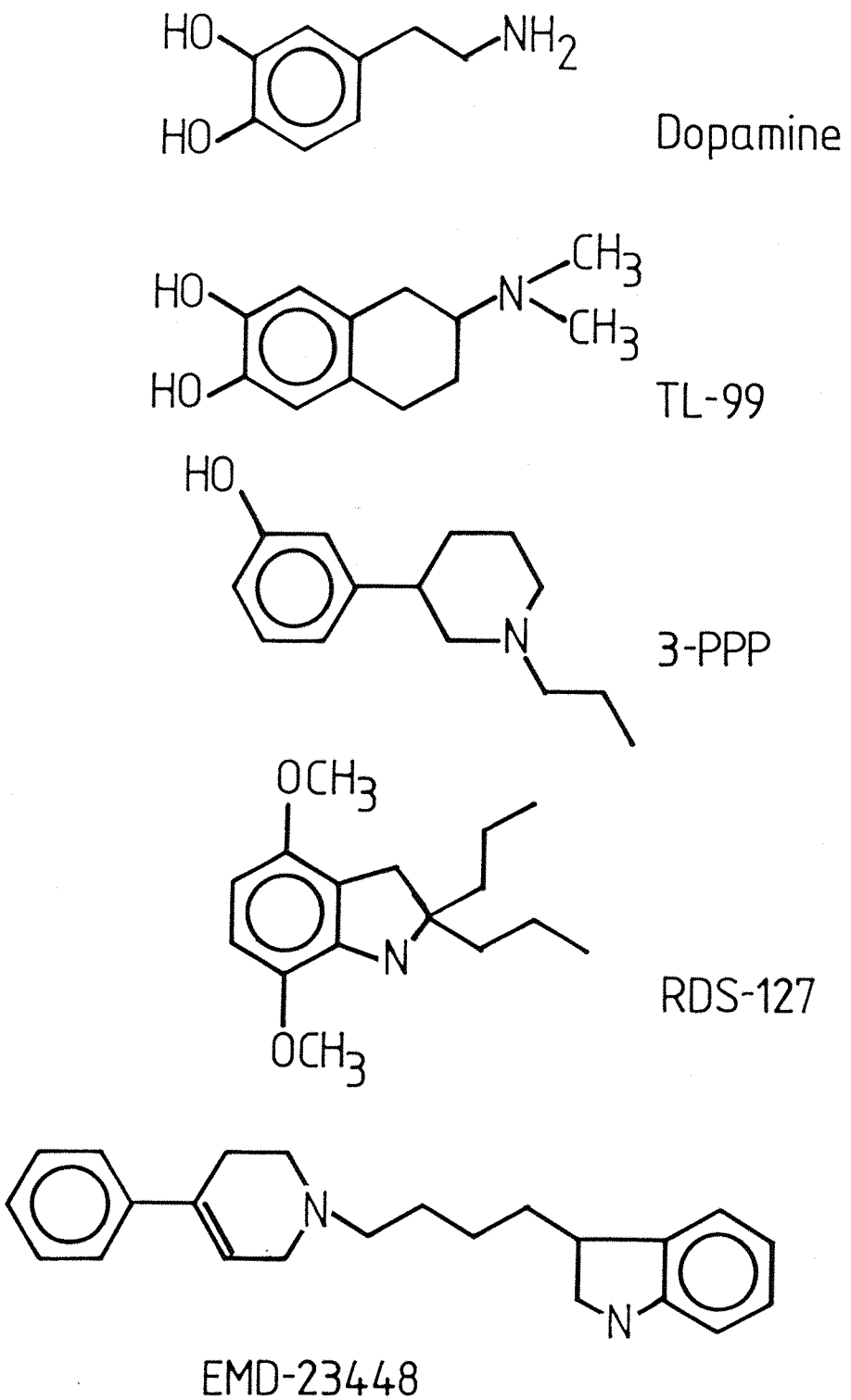


Figure 4.3

The structures of the putative selective presynaptic dopamine receptor agonists.

Chiodo and Bunney, 1983). In vitro studies on the substantia nigra brain slice have shown that these compounds inhibit the neuronal firing rate of the SNC cells. A series of epipotent molar ratios for these agonists have been calculated (see table 4.2; Pinnock, 1983b, 1984). However, it is difficult to assess from these studies whether or not the DA autoreceptors on the SNC cells have a different structural requirement from the postsynapic dopamine receptor. More studies are needed with a wider range of compounds tested. The differential sensitivity of the postsynaptic and presynaptic dopamine receptors detected by Skirboll et al. (1979) could reflect a tighter coupling between the receptor recognition site and the effector mechanism than the postsynaptic dopamine receptor, but may still have the same structural requirements for activity. Alternatively, there may be a difference in the accessibility of the receptors to exogenously administered drugs.

The selectivity of some of these proposed selective presynaptic dopamine receptor agonists is questionable. TL-99 has been shown to stimulate the postsynaptic DA receptor (Watling and Williams, 1982) as well as having some α_2-adrenoceptor activity (Horn, de Vries, Dijkstra and Mulder, 1982). 3-PPP has been resolved into the (+) and (-)-3-PPP enantiomers. Both enantiomers have dopamine receptor activity. It is suggested that (+)-3-PPP is a DA agonist at both autoreceptors and postsynaptic receptor. In contrast (-)-3-PPP is an agonist at the DA autoreceptor and an antagonist at the postsynaptic receptor (Hjorth, Carlsson, Clark, Svenson, Wilkstrom, Sanchez, Lindberg, Hacksell, Arvidsson, Johansson and Nilsson, 1983).

In this study, a slight difference in potency was detected in the locomotor activity responses to RU24213 and RU24926 and in other behavioural and biochemical tests. The more potent of the two compounds was RU24926, as can be seen by its ability to displace ^{3}H-sulpiride binding in table 4.1. There is, however, only a small difference in the structures: RU24213 lacks a hydroxyl group as can be seen fromthe structures in figure 4.2. Because of the structural difference in RU24213, where one of the phenylethylamine side chains bears a closer resemblance to the DA molecule than the other, this side chain must have a higher affinity for the DA receptor than the other side chain without the hydroxyl group, whereas both side chains in RU24926 have hydroxyl groups and therefore have equal affinity for the DA receptor.

Table 4.2 The structure-activity relationship for dopamine agonists on the neuronal activity of the SNC cells in the in vitro nigral slice preparation.

Drug	Equipotent molar ratio
TL-99	$2.4 \pm 0.96 \times 10^{-4}$ *
RDS-127	$2.66 \pm 1.7 \times 10^{-3}$ *
Apomorphine	$3.2 \pm 1.8 \times 10^{-3}$
ADTN	$1.0 \pm 0.25 \times 10^{-2}$
DA	1
NA	1.07 ± 0.11
3-PPP	1.1 ± 0.41 *
d-amphetamine	1.3 ± 0.4
Iso-ADTN	1.5 ± 0.24
p-tyramine	4.7 ± 1.4
m-tyramine	8.2 ± 1.7
d,l-octopamine	19.6 ± 3.2
d,l-norphenephrine	20.1 ± 2.7
Nomifensine	32 ± 7.2

Taken from: Pinnock (1983)
*Pinnock (1984)

Thus, RU24926 has, overall, a higher affinity for the DA receptor because both side chains may act on the DA receptor site, which is reflected in its agonist potency.

From their pharmacological profile, both RU24213 and RU24926 have potential clinically as anti-hyperprolactinemia and Parkinsonian drugs. They are long acting agonists on nigral, pituitary and striatal neurones, but show low activity in the nucleus accumbens and would therefore tend not to exacerbate or induce psychotic behaviour in man.

ADTN is one of the most potent DA agonists known on physiological responses. One drawback to its use clinically is its inability to penetrate through the blood brain barrier in sufficient quantities to have central effects. The dibenzoyl ester of ADTN (DBADTN) was synthesised as a lipid soluble prodrug to overcome this problem.

In this study, large doses of DBADTN were given peripherally but had no effect on the firing rate of the SNC cells. At the doses given intravenously some of the animals died, which was to be expected from other studies (A.S. Horn, personal communication), but the absence of any detectable agonist activity on this system was unexpected. This lack of activity might reflect a slow rate of metabolism of DBADTN to the proposed active molecule ADTN within the CNS. Large doses of DBADTN given peripherally produced decreases in the striatal HVA levels. Decreases were detectable one hour after peripheral injection of DBADTN, but were not maximal until 4 hours. These decreases in striatal HVA levels were antagonised by haloperidol (Horn et al., 1978). Over this time scale the single unit recording technique is not suitable to measure a drug's agonist activity. This is because it cannot be guaranteed that a neurone will be recorded for that amount of time in order to observe the effects on the neuronal firing rate. Even so, cells were monitored continuously for long periods of time without showing any inhibition of the neuronal firing rate. Interestingly, DBADTN injected directly into the nucleus accumbens, does cause locomotor activity similar to that induced by ADTN, but the onset of this response is delayed by approximately two hours when compared with the ADTN response (S.K. Long, personal communication). Such a delay would be expected while the prodrug is metabolised to its active form and sufficient amounts accumulate to elicit the behavioural response.

Two explanations may account for the lack of activity detected in this study. The enzymes required to debenzoylate DBADTN to the active molecule may not be present in the substantia nigra, whereas they may be present in other brain areas such as the nucleus accumbens and striatum. However, no enzyme has been suggested to perform the metabolism of DBADTN, but presumably the metabolism is by non-specific esterases within the CNS. After peripheral injections of large doses of DBADTN, ADTN was detected in the brain and accumulated in dopaminergic regions (Horn et al., 1980; Westerink, Dijkstra, Feenstra, Grol, Horn, Rollema and Wrix, 1980), but when DBADTN is compared with its isomer, DBisoADTN, in their ability to inhibit DA turnover in the striatum, DBisoADTN had half the activity of DBADTN, which is greater than might be expected from other studies where ADTN is 100 times more potent that isoADTN (Westerink et al., 1980). Despite the detection of ADTN in the brain following peripheral injection of DBADTN, the levels of ADTN obtained were low compared with the levels achieved after intercranial injections of ADTN (Horn et al., 1980). If only a small amount of the prodrug crossed the blood brain barrier, this might account for the low levels of ADTN detected. Furthermore, the locomotor activity seen after intra-accumbens injection suggests that if sufficient amounts of the prodrug are present inside the CNS then the DA receptor mediated responses are elicited.

Alternatively the actions of DBADTN may not be due to direct activation of DA receptors. In 6 OHDA unilaterally lesioned rats, DBADTN failed to produce rotational activity when administered peripherally even after 4 hours. However, sedation was produced in these animals (Horn, Kelly, Westerink and Dijkstra, 1979). Although the sedation was blocked by sulpiride it was not antagonised by the classical neuroleptics, haloperidol or cis-flupenthixol, yet the α_2-adrenoceptor antagonists, yohimbine and piperoxane (but not the α_1-adrenoceptor antagonist, prazosin) were able to block the sedative effect of DBADTN (Summers, de Vries, Dijkstra and Horn, 1981). As this sedation may be produced by the α_2-adrenoceptor agonist, clonidine, some of the actions of DBADTN might be mediated via α_2-adrenoceptors and thus may not be selective for DA receptors.

4.3 The effects of sulpiride and zetidoline on central dopaminergic systems

Sulpiride is a potent dopamine antagonist on many test systems, but does not antagonise the stimulation of the adenylate cyclase by DA and DA agonists. When given peripherally, sulpiride has no activity on central dopaminergic functions. Sulpiride has a low lipid solubility as measured by its oil/water partition coefficient and is unable to penetrate the blood brain barrier as a result. The poor lipid solubility of sulpiride has been suggested to account for its inability to antagonise the dopamine sensitive adenylate cyclase (Woodruff, Freedman and Poat, 1980).

In this study, the peripheral administration of (±)-sulpiride had no effects on the inhibitory actions of apomorphine even when extremely high doses (50 mg/kg) were given intravenously. Yet, at a dose of 10 mg/kg, (-)-sulpiride was reported to antagonise the hypomotility and inhibition of SNC neuronal firing induced by norpropylapomorphine and apomorphine (Argiolas, Mereu, Serra, Melis, Fadda and Gessa, 1982; Mereu, Casu and Gessa, 1983). Although, in their study, these workers used the active stereoisomer of sulpiride, the racemic mixture used in the present study should have provided sufficient quantities of the active (-)-sulpiride isomer at the doseages given. Unless the presence of the inactive enantiomer hinders the passage of the active (-)-sulpiride isomer through the blood brain barrier, then the conclusion to be made from this study is that insufficient amounts of (-)-sulpiride are able to penetrate into the brain at the doses given. Thus, the results of this study are in disagreement with those of Argiolas et al. (1982) and Mereu et al. (1983).

The direct application of sulpiride onto the SNC neurones by iontophoresis antagonised the inhibitory responses to iontophoretically applied dopamine. This confirms the previous study by Pinnock et al. (1979) where dopamine but not GABA responses were antagonised. The inhibitory responses produced by iontophoretically applied noradrenaline were approximately equipotent with dopamine, in agreement with the observations of Aghajanian and Bunney (1977). These responses were antagonised by the (±)-sulpiride. However, the inhibitory responses produced by NA are possibly due to agonist activity on dopamine receptors,

since neither isoprenaline nor clonidine (the adrenoceptor agonists) have any activity on the neuronal firing rate of SNC cells when applied iontophoretically. In addition the adrenoceptor antagonists, piperoxane and sotalol had no antagonist activity, whereas the dopamine receptor antagonist, trifluoperazine, potently blocked both NA and DA responses (Aghajanian and Bunney, 1977).

The development of new dopamine antagonists has sought to produce drugs with reduced side effects. Sulpiride has been used in clinical trials and has been reported to have a low incidence of extrapyramidal side effects and tardive dyskinesias (Lewis, Bond and Curry, 1983). These side effects have been associated with neurotransmitter inbalance in the basal ganglia, whereas the mesolimbic and mesocortical dopaminergic systems have been implicated in the therapeutic action of antipsychotics (Snyder, 1976). There is evidence to suggest that the atypical neuroleptics have preferential action on the mesolimbic system. The atypical neuroleptics have lower potency on striatal mediated behaviours (Costall and Naylor, 1975), but are more effective on dopamine turnover and dopamine receptor ligand binding in limbic areas (Bartholoni, 1976; Borison, Fields and Diamond, 1981). Because of the low incidence of side effects produced by sulpiride clinically new antagonists with similar properties to sulpiride are being sought.

Zetidoline is a new DA antagonist with similar properties to sulpiride, on biochemical and behavioural tests. Like sulpiride, it is unable to antagonise the dopamine stimulated adenylate cyclase (Barone et al., 1982). This study demonstrated that, when given intravenously, zetidoline antagonised the inhibitory actions of apomorphine on the SNC cell firing rate. The block of dopamine receptors following peripheral administration demonstrates that, unlike sulpiride, zetidoline was able to rapidly cross the blood brain barrier and have an effect on the dopamine autoreceptors on the SNC cell bodies. When applied iontophoretically, zetidoline antagonised the inhibitory responses produced by DA and NA but had no effect on the responses produced by the iontophoretic application of GABA, glutamate or glycine. The NA responses in the SNC were presumed to be due to activity on the DA receptor (as previously explained). However, further work must be done to confirm this assumption.

As zetidoline is effective as an antagonist of DA responses when applied iontophoretically onto the SNC cells, it is exerting a direct effect on the DA receptor and is not acting by an indirect mechanism, which is one explanation for the effect following the intravenous application of drugs. Thus, zetidoline will be a useful antagonist of central dopamine receptor models and will allow the peripheral administration of the antagonist instead of the intracerebral injections required with sulpiride.

The development of the in vitro brain slice technique has provided a means of quantifying the potency of agonists and antagonists on central neurones. The previous experiments on SNC neuronal firing were only a qualitative assessment of zetidoline's antagonistic activity. The present study showed that the dose dependent responses to dopamine on the neuronal activity of SNC cells in brain slices could be antagonised, producing parallel shifts in the dose response curves. From these experiments a pA_2 value of 7.02 was calculated, indicating that zetidoline is a potent antagonist of the DA autoreceptors on the SNC cell bodies. This compared well with the pA_2 values reported for three other dopamine antagonists on the responses of nigral cells to dopamine ((-)-sulpiride, pA_2 7.5; haloperidol, pA_2 8.4 and cis-flupenthixol, pA_2 6.9; Pinnock, 1984). However, in that study, the pA_2-pA_{10} differences were outside the limits considered to be compatible with competitive antagonism over the concentration range tested. In the present study a pA difference of 1.14 was calculated for zetidoline on the responses of the SNC cells to dopamine, which is within the limits compatible with competitive antagonism, but does not necessarily prove competitive antagonism. Divergence from the theoretical pA difference of 0.95 may be explained in three ways: that antagonism is not competitive or that there may be a multimolecular interaction between the drugs and the receptor. Alternatively, that equilibrium conditions were not reached during the experimental periods. The most likely explanation for the divergence of the pA difference from the expected theoretical value in this study is that equilibrium at the receptor site was not fully achieved, but other factors that may influence the equilibrium might include an effect by the antagonist on the uptake of neurotransmitters or an effect on other transmitter functions. The antagonistic effect of zetidoline in this preparation was long-acting

and was not easily washed out of the slice preparation on returning to the 'drug free' artificial c.s.f. Zetidoline was longer lasting than haloperidol in this respect (R.D. Pinnock, personal communication). This length of action could be a result of the lipid solubility of the drug. If zetidoline is highly lipid soluble it may become incorporated into the cell membrane in the slice and may then slowly leak back into the extracellular fluid. If sufficient amounts of zetidoline are incorporated into the membranes they may still exert an antagonistic effect for hours after the drug was removed from the perfusing c.s.f. It would be unlikely for zetidoline to occupy the receptor site for an extended period of time because the antagonism was competitive over the concentrations tested.

Recently, radioligand binding studies have shown that zetidoline is a potent displacer of [^{3}H]-sulpiride binding, as can be seen in table 4.1 (Holden-Dye, Poat, Senior and Woodruff, 1983). Some preliminary studies using [^{3}H]-zetidoline as a ligand on rat striatal membranes revealed a regional distribution of binding associated with dopaminergic areas in the brain. This binding was similar to the binding of sulpiride, being dependent on sodium ions and was displaced by neuroleptics with similar potency to other binding systems (Barone, Corsizo, Piazza, Rossetti and Condo, 1983a,b). Sensitivity to sodium ions may be due to a linkage with a GTP sensitive regulatory protein as suggested by Rodbell (1980). Thus, despite its inability to antagonise the adenylate cyclase, the binding may be to an adenylate cyclase linked receptor.

An interesting feature observed during the experiments on the nigral slices was that the neuronal firing of the SNC cells was very regular. In the in vivo preparation the firing rate was regular but with occasional slow bursting activity. Grace and Bunney (1983) have shown that there is a slow depolarisation occurring within these cells which may give a pacemaker role to these cells. In the slice preparation most of the inputs that synapse onto the SNC cells will be cut or damaged. This releases the second to second control of the cells' firing rate from these external influences, allowing the regular pacemaker-like activity of the SNC cells to become apparent. This may account for the slightly different pattern of firing seen between the in vivo and in vitro preparations and may explain why the in vitro nigral slice firing rate is so regular.

In order to test that the antagonism of the NA and DA responses by sulpiride and zetidoline were mediated via dopamine receptor and not by an effect on adrenoceptors, the cerebellum was chosen as a control area. The cerebellum has no dopaminergic innervation and contains no detectable dopamine receptors (Burt et al., 1976). The cerebellum received a large noradrenergic innervation from the locus coerulens which terminates mainly on Purkinje cells (Bloom et al., 1971). Thus, the antagonists could be tested on the adrenoceptor mediated responses of DA and NA.

This study showed that iontophoretically applied NA and DA produced inhibitory effects on the neuronal firing of the cerebellar cells. These responses were unaffected by the iontophoretic application of (±)-sulpiride, supporting the proposal that the action of sulpiride is selective for dopamine receptors. The inhibitory responses produced by NA and DA were presumed to be mediated via β-adrenoceptors located on the Purkinje cells, since the responses to NA and isoprenaline have been shown to be antagonised by propanolol (Hoffer et al., 1971). The iontophoretic application of zetidoline on cerebellar neurones caused a direct depression of the neuronal activity. This depression was slow in onset and recovery and interestingly decreased the excitatory responses to glutamate. Thus, a comparison of the effects of zetidoline on DA and NA responses with the SNC could not be made. The depressant action of zetidoline on the cerebellar neurones is possibly a result of a local anaesthetic action on these cells, as the excitatory responses produced by iontophoretically applied glutamate were also abolished. In the SNC, zetidoline had no effect on the responses to glutamate. Thus, the application of zetidoline may have impaired the action potential generating mechanism. The iontophoretic application of propanolol into the cerebellum had similar reversible depressant effects on these cells.

Propanolol and some other β-adrenoceptor antagonists have previously been shown to have anaesthetic properties on cerebellar Purkinje cells when applied by microiontophoresis (Hoffer et al., 1971). Nevertheless, other β-adrenoceptor antagonists, such as sotalol, do antagonise NA responses without causing the depression of neuronal firing themselves. The anaesthetic properties are possessed by a number of classical neuroleptics: chlorpromazine, fluphenazine and haloperidol are three examples. The ability of the neuroleptics to produce

anaesthesia has been correlated to the lipid solubility of these compounds (Seeman, 1966; Staiman and Seeman, 1974). Zetidoline is capable of rapidly crossing the blood brain barrier, as demonstrated in this study and must therefore be lipid soluble. Thus, it is not unexpected that zetidoline should have such an effect. In order to test the effects of zetidoline on the neuronal responses to NA and DA mediated through an adrenoceptor, an alternative area of the brain should be investigated.

The locomotor activity induced by ADTN provides a good model on which to test neuroleptic activity. All classes of neuroleptics have been shown to inhibit the locomotor activity (Woodruff and Andrews, 1979 ; Arnt, 1983). In the present study, zetidoline, given intraperitoneally, was very weak, requiring extremely high doses to antagonise completely the locomotor activity induced by ADTN. This was unexpected following the electrophysiological data where zetidoline was as potent as other neuroleptics at antagonising the inhibitory responses of apomorphine on the SNC cells. At the doses employed for intraperitoneal injections, the inhibition of locomotor activity may contain a cataleptic component. Costall and Naylor (1980) have pointed out that an ability to produce catalepsy may be reflected by a concomitant ability to inhibit induced hyperactivity and this may not be mediated by a direct antagonism of the dopamine agonist response. Zetidoline has been reported to be cataleptic in the rat (Barone et al., 1982), although little data was presented to support this observation. The range of neuroleptics tested against ADTN induced locomotor activity require much lower doses to antagonise the hyperactivity than zetidoline, with the exceptions of sulpiride, clozapine and metoclopramide. Sulpiride has low activity because of its poor lipid solubility. Clozapine is not very specific in its actions, having activity on muscarinic, β-adrenoceptors and serotonin receptors and thus will have multiple behavioural effects (Fjallund and Boeck, 1978; Peroutka and Snyder, 1980). Metoclopramide lacks potency on a variety of dopaminergic test systems. If the lack of activity of zetidoline was due to an inability to penetrate into the nucleus accumbens, then intracerebral injections of zetidoline would detect this. Nevertheless, the potency to produce an inhibitory effect on the ADTN induced locomotor activity following intracerebral injections, was again very weak in comparison with the effects of (±)-sulpiride in

this study and with the effects of fluphenazine, cis-flupenthixol and both enantiomers of sulpiride (Woodruff and Andrews, 1979). The lack of antagonistic activity here is therefore not due to an inability to enter the nucleus accumbens. The lack of potency in the nucleus accumbens may represent a differential ability of zetidoline on two receptor types with different locations within the brain. Yet, such an idea would not fit in with the D1/D2 classification because both sulpiride and cis-flupenthixol antagonise the induced hyperactivity. A pre and postsynaptic division might be apparent here, as zetidoline was shown in this study to be a potent antagonist of the dopamine autoreceptors in the SNC. The locomotor activity elicited by dopamine agonists is thought to be due to postsynaptic dopamine receptor stimulation. However, amphetamine induced stereotypy in rats and mice as well as apomorphine induced hypermotility in mice are all antagonised by zetidoline (Barone et al., 1982) and are thought to be due to postsynaptic dopamine receptors. Thus, the evidence does not support such a broad division of dopamine receptors.

If this lack of potency is to be substantiated, then it would be of interest to test the effect of iontophoretically applied zetidoline on the responses to dopamine in the nucleus accumbens. Furthermore, this compound should be tested against a wider range of dopamine receptor model systems in order to discover if zetidoline has weak activity on any other preparation which may aid the understanding of the DA receptor.

This study set out to investigate the actions of a number of DA agonists and antagonists on the neuronal activity of the SNC cells and on the locomotor activity model of dopamine receptors. The results obtained have shown that:-

The proposed dopamine agonist, S-3608, is able to rapidly cross the blood brain barrier and have an inhibitory effect on the SNC neuronal activity, being less potent than apomorphine but approximately equipotent with its structural analogue, piribedil. These responses were antagonised by the dopamine receptor antagonists, haloperidol and fluphenazine.

Two other model dopamine agonists, RU24213 and RU24926, were able to rapidly cross the blood brain barrier and inhibit the neuronal activity but were much longer acting. Both RU24213 and RU24926 caused tachyphylaxis to DA agonist responses following a single dose.

The dibenzoyl ester of ADTN, synthesised as a prodrug for ADTN, failed to produce any inhibition of the neuronal firing rate up to doses that were lethal to the rats.

In another model system, S-3608 and RU24213 had no activity at inducing the locomotor activity following intra-accumbens injections of these agonists, but RU24926 had a weak stimulatory effect on the locomotor activity when compared with the ADTN induced responses.

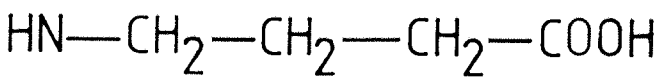
Intravenously applied (±)-sulpiride had no effect on the neuronal responses to apomorphine on the SNC neurones, but the new dopamine antagonist, zetidoline, given intravenously, rapidly inhibited the responses to apomorphine on the SNC cells. Thus, zetidoline is able to cross the blood brain barrier. When applied iontophoretically, both sulpiride and zetidoline antagonised the responses to DA and NA. Zetidoline did not affect the neuronal responses to glutamate, glycine and GABA. In the in vitro nigral slice preparation, zetidoline competitively antagonised the inhibitory responses to DA with a calculated pA_2 value of 7.02.

In the cerebellum, zetidoline applied iontophoretically directly depressed the neuronal activity, similar to propanolol. This is possibly due to a local anaesthetic effect on the cell membranes. Sulpiride was unable to antagonise the responses to iontophoretic NA and DA.

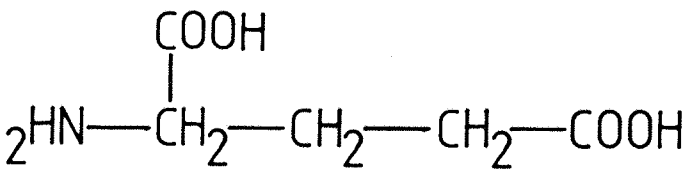
On the ADTN induced locomotor activity, zetidoline was a weak antagonist when applied either intraperitoneally or by direct intra-accumbens injections. However, (±)-sulpiride injected directly into the nucleus accumbens potently inhibited the ADTN induced locomotor activity.

STRUCTURES

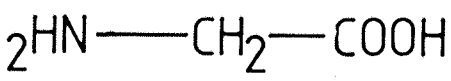
Amino acids



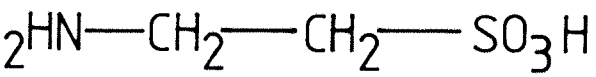
GABA.



Glutamate

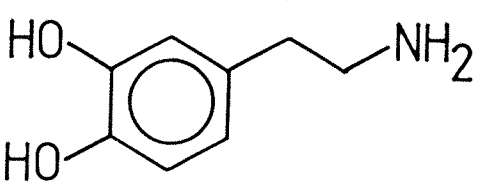


Glycine

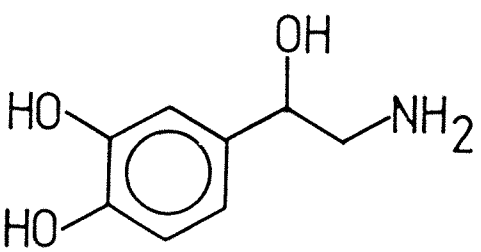


Taurine

Catecholamines

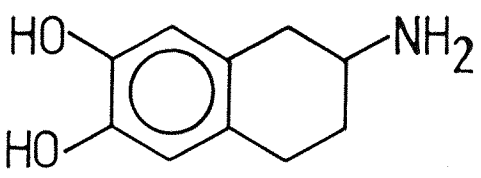


Dopamine

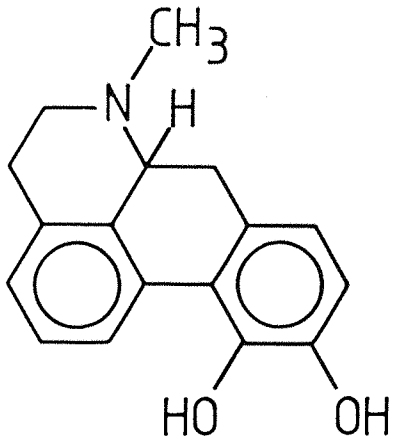


Noradrenaline

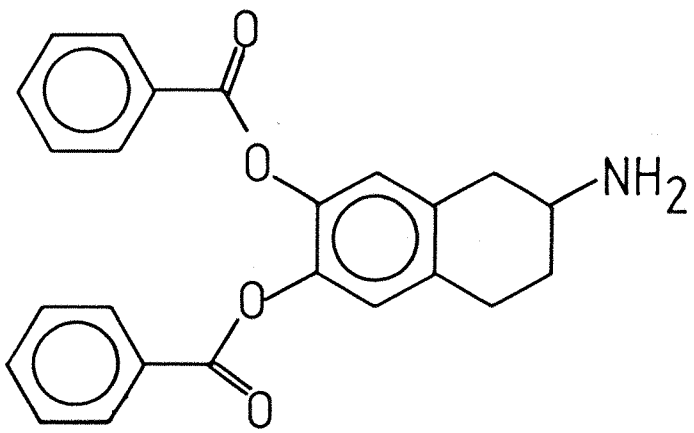
Agonists



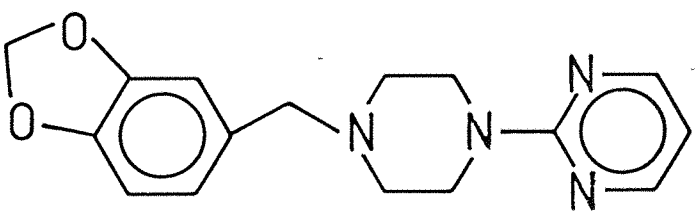
ADTN



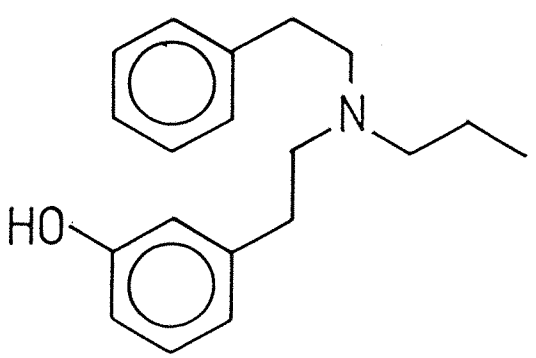
Apomorphine



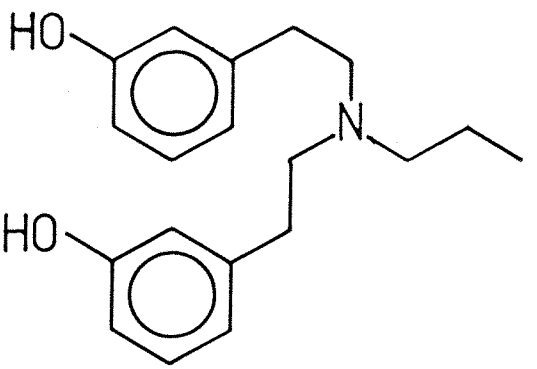
DBADTN



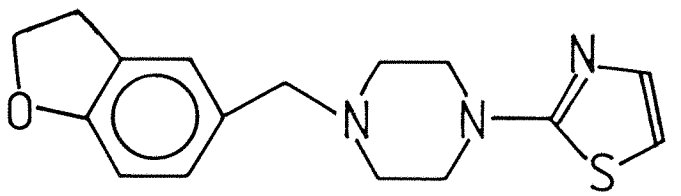
Piribedil



RU 24213

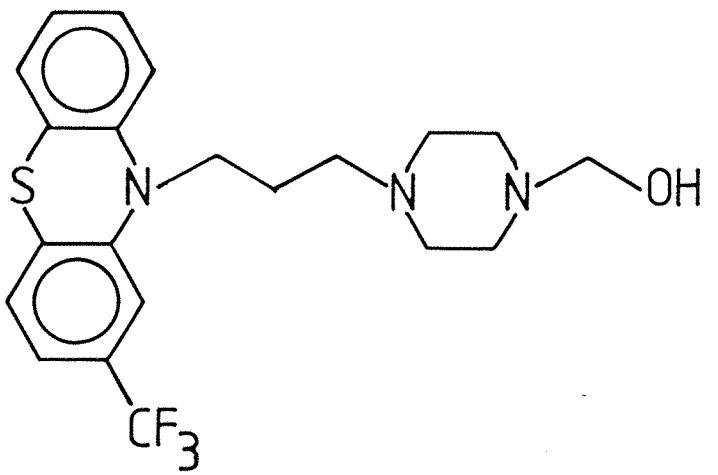


RU 24926

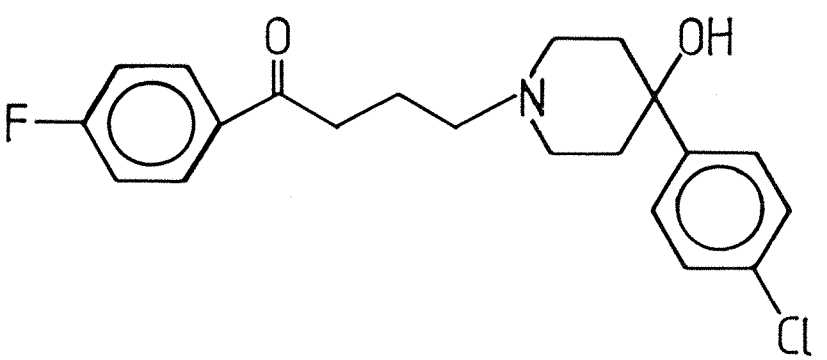


S 3608

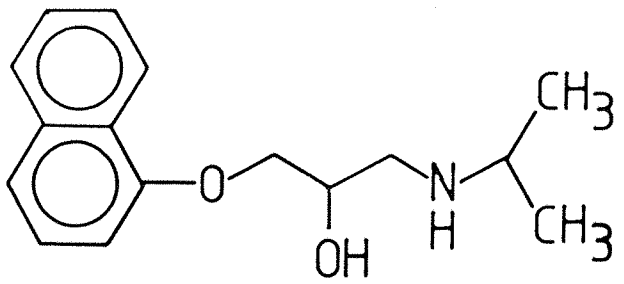
Antagonists



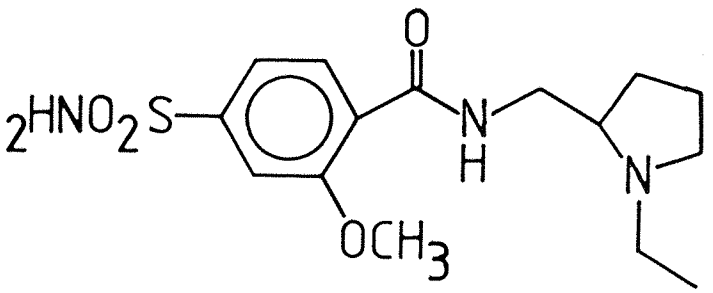
Fluphenazine



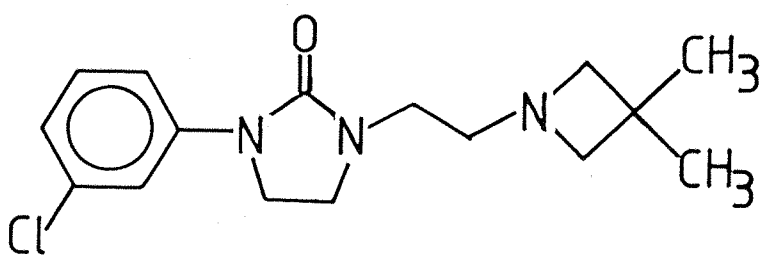
Haloperidol



Propranolol



Sulpiride



Zetidoline

Appendix I

Equation for calculating the amount of drug ejected from a microelectrode by microiontophoresis.

$$M = \frac{n.i.t}{Z.F}$$

M - amount of drug ejected in moles

n - transport number

i - ejecting current

t - time

F - Faraday's constant

Z - valency of ion (charged drug)

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