TYPICAL AND ATYPICAL NEUROLEPTIC DRUG EFFECTS ON DOPAMINE AND OTHER NEUROTRANSMITTER FUNCTIONS IN RODENTS

A thesis submitted to the University of London
for the degree of Doctor of Philosophy

by

Jeffrey W. Dalley

Department of Pharmacology,
University College London
Gower Street
London WC1E 6BT

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ABSTRACT

The antischizophrenic effect of neuroleptic drugs probably arises from blockade of central dopamine receptors which also leads to extrapyramidal side effects (EPSEs). The aim of the work described in this thesis was to elucidate how some drugs, the so-called atypical neuroleptics, alleviate the symptoms of schizophrenia without producing marked EPSEs.

An intracerebral microdialysis system was developed and used to monitor the acute effects of neuroleptic drugs on dopamine release and metabolism in the caudate putamen (CP), nucleus accumbens (NAc) and medial prefrontal cortex (MPFC) of halothane-anaesthetised rats. Dopamine and its metabolites DOPAC and HVA were estimated in brain dialysates by HPLC with electrochemical detection. In other studies extracellular recording techniques were used to assess the effects of neuroleptics and dopamine agonists on the spontaneous activity of neurons in the CP and MPFC.

Both haloperidol (typical neuroleptic) and clozapine (atypical) elevated the efflux of dopamine metabolites in the CP, NAc and MPFC although only clozapine significantly facilitated dopamine efflux and this was restricted to the MPFC. Neither neuroleptic showed any consistent effects on neuronal activity in the CP, however, unlike haloperidol, but similar to the dopamine agonist apomorphine, clozapine both stimulated and inhibited neuronal activity in the MPFC. The ability of these agents to antagonise the effects of apomorphine was also evaluated. Apomorphine produced a dose-dependent inhibition of neuronal activity and efflux of dopamine and metabolites in both the CP and the MPFC. Although fewer cells in the MPFC showed any response to apomorphine compared with those in the CP, those that did, were markedly more sensitive to the inhibitory effects of this agent. The effects of apomorphine were reversed by haloperidol but only partially reduced by clozapine. Since clozapine did not alter the time course of apomorphine appearance in CP dialysates and haloperidol only reduced the appearance at one dose level, it was concluded that the antagonism of apomorphine's actions in the CP and MPFC were directly mediated.

The differential effects of haloperidol and clozapine on dopamine function in the CP and MPFC are discussed in respect of their typical and atypical profiles. The weak dopamine (apomorphine) antagonism shown by clozapine in the CP would account for the low

incidence of EPSEs seen with this compound although its relatively weak action in the MPFC implies that its antischizophrenic action must stem, at least in part, from other actions. The ability of clozapine to enhance the efflux (release?) of dopamine in the MPFC could explain its efficacy against the negative symptoms of schizophrenia. A possible mechanism to account for the ability of clozapine to alleviate the positive aspects of this disorder (despite weak dopamine receptor antagonism) is also discussed.

In other experiments designed to reveal the nature of clozapine's atypical action its effects on 5-HT_3 mediated release of dopamine in the NAc as well as on the contraction of guinea-pig ileum *in-vitro* were studied. It was also compared with other atypical and typical neuroleptics on muscarinic, α-adrenergic and tachykinin receptors in the guinea-pig ileum and rat vas-deferens. The effects of typical and atypical neuroleptics in all these preparations is discussed in an attempt to explain their differential clinical effects.

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PUBLICATIONS

The following publications have arisen from the work described in this thesis:

1) Dalley J.W. & Webster R.A. (1990) Activity of typical and atypical neuroleptics at $5\text{-}HT_3$ and other peripheral non-dopaminergic receptors. *J. Psychopharmacology* **4**, 285.

2) Dalley J.W. & Webster R.A. (1991) Comparative effects of neuroleptics and apomorphine on dopamine release and metabolism in the rat caudate nucleus and medial prefrontal cortex. *Br. J. Pharmacol.* **105**, 2P.

3) Dalley J.W. & Webster R.A. (1991) Neuroleptic effects on apomorphine-induced inhibition of striatal neuronal firing and passage across the rat blood brain barrier. *Br. J. Pharmacol.* **105**, 151P.

ABBREVIATIONS and UNITS

APO	Apomorphine
CLN	Clonidine
CLZ	Clozapine
cm	Centimetre
CNS	Central nervous system
COMT	Catechol-O-methyl-transferase
CP	Caudate putamen
CPZ	Chlorpromazine
CSF	Cerebrospinal fluid
DA	Dopamine
DIW	De-ionised water
DOPA	Dihydroxy-phenylalanine
DOPAC	3,4-Dihydroxyphenyl-acetic acid
ECG	Electrocardiogram
EEG	Electroencephalogram
EPSE	Extrapyramidal side effect
EPSP	Excitatory postsynaptic potential
g	Gram
GABA	γ-Aminobutyric acid
HAL	Haloperidol
5-HIAA	5-Hydroxyindole-3-acetic acid
HPLC	High pressure liquid chromatography
5-HT	5-hydroxytryptamine
HVA	Homovanillic acid
Hz	Hertz
i.d.	Internal diameter
i.p.	Intraperitoneal
IPSP	Inhibitory postsynaptic potential
i.v.	Intravenous
kg	Kilogram
M	Molar
MAO	Monoamine oxidase
MET	Metoclopramide
mg	Milligram
min	Minute
mm	Millimetre
mM	Millimolar
MPFC	Medial prefrontal cortex
ms	Millisecond
n	number of experiments
Na	Nanoampere
NAc	Nucleus accumbens
NMDA	N-methyl-D-aspartate
o.d.	Outer diameter
pmol	Picomole
REM	Remoxipride
s	Second
s.c.	Subcutaneous
SEM	Standard error of mean
SP	Substance P
SUL	Sulpiride
THIO	Thioridazine
TTX	Tetrodotoxin
μl	Microlitre
μm	Micrometre
μM	Micromolar
V	Volts

CHAPTER 1

GENERAL INTRODUCTION

1.1 SCHIZOPHRENIA

1.1.1 Clinical features

" Soon after I was placed in my room all of my senses became perverted. None of my food had its usual flavour. This soon led to that common delusion that some of it contained poison. Familiar materials had acquired a different feel - in the dark, the bed sheets at times seemed like silk - I believed detectives had provided these silken sheets for some hostile purpose of their own. My sense of sight was subjected to many weird and uncanny effects. Imaginary breezes struck my face - they seemed to come from cracks in the wall and ceiling and annoyed me exceedingly "

This personal account (taken from Stevens, 1973) vividly describes some common features of schizophrenia, particularly, the delusional (paranoid) and sensory deceptive aspects of this disorder. It was Kraepelin (1899), who first delineated the concept of *dementia praecox* (as schizophrenia was then known), and recognised that whilst the cause of this disorder may in some way relate to cerebral dysfunction, it does not impair the intellectual processes of memory, consciousness and orientation (see Johnstone, Crow, Frith, Stevens, Kreel & Husband, 1978). In its original description, *dementia praecox* was categorised into hebephrenic (marked disorganisation of speech and behaviour, inappropriate affect [*emotion and mood*] and delusional content [*a false belief despite contradiction by social reality*]), catatonic and paranoid sub-types, and it was Bleuler (1911), who later modified this classification, and re-defined this disorder as schizophrenia (see Wistedt, 1981). The traditional Kraepelinian-Bleulerian divisions, described above, forms the basis of the DSM-III (*Diagnostic and Statistical Manual*) system which is widely employed in the diagnosis of schizophrenia (see Andreasen & Olsen, 1982).

Schizophrenia can only be defined in terms of its symptomology since the pathophysiology and etiology of this disorder remains unknown. The diagnosis depends largely on the presence of some of a variable number of symptoms which can conveniently be divided into positive and negative categories (see Table 1.1). The positive symptoms of schizophrenia which include such features as hallucinations (auditory and tactile), delusions (persecutory, paranoia, grandeur and omnipotence [*the conviction that one's thoughts control the outside world*]), formal thought disorder (illogicality, incoherence and dissociative thinking) and repeated instances of bizarre and disorganised behaviour, are characteristic of the acute schizophrenic syndrome (see Ashton, 1987; Crow, 1980;

Andreasen & Olsen, 1982; Carpenter & Buchanan, 1989). In contrast, the negative symptoms of schizophrenia which encompass such features as muteness, alogia (poverty of speech), affective flattening, apathy, attentional impairment, social withdrawal, diminished emotional range and decreased libido (see Crow, 1980; Carpenter, Heinrichs & Wagman, 1988) typically predominate in chronic schizophrenic patients (Andreasen & Olsen, 1982). Unlike positive symptoms, negative symptoms are not exclusive to schizophrenia. Indeed, they can also occur in endogenous depressive states and as a consequence of institutionalization (see Angst, Stassen & Woggen, 1989). Negative symptoms tend to be the most persistent features of schizophrenia whereas positive symptoms tend to fluctuate dramatically over time (Crow, 1980). As well, negative symptoms, but not positive symptoms, are normally associated with intellectual (cognitive) impairment (Johnstone, Frith, Kreel, Crow & Husband, 1976; Crow, 1980; Taylor, 1984), gross structural brain damage (Frangos & Athanassenas, 1982; Nasrallah, Jacoby, McCalley-Whitters & Kuperman, 1982) and exhibit poor response to neuroleptic drugs (Crow, 1980; Carpenter, Heinrichs & Wagman, 1988).

	Positive	Negative
1) clinical presentation	- hallucinations *auditory* *tactile* *olfactory* - delusions *persecutory - paranoid* *religious* *grandiose* - thought disorder *incoherence* *illogicality* - bizarre & disorganised behaviour	- affective flattening - apathy - attentional & cognitive impairment - mutism alogia - anhedonia - social withdrawal - decreased libido
2) symptoms onset	acute/reversible	insidious/enduring
3) cognition	normal	diminished
4) gross brain pathology	absent (?)	present
5) response to neuroleptics	good	poor
6) central dopamine function	raised (?)	reduced (?)

Table 1.1 Characteristic features of positive and negative symptoms in schizophrenia.

1.1.2 Etiology

Although schizophrenia is most commonly diagnosed in late adolescence (Loranger, 1984; Torrey, 1989) and is associated with structural brain pathology such as enlarged ventricles (especially negative symptoms) and has a strong genetic basis (Owen & Cross, 1989) there is evidence that neuroleptics (dopamine receptor antagonists) can ameliorate the positive symptoms of this disorder (Crow; 1980; Carpenter, Heinrichs & Wagman, 1988).

A popular etiological theory of schizophrenia relates to the presence of a fixed brain "lesion", incurred in early life, which subsequently disrupts, or is made apparent by, the normal maturation process of one or more developing brain systems (Weinberger, 1987). This "lesion" could presumably, develop from numerous, and unrelated causes, such as perinatal trauma, hereditary predisposition, infection (virus?; see Crow, 1978; Crow, Johnstone, Owens, Ferrier, MacMillian & Parry, 1979), toxin exposure or a primary metabolic disorder. The site and extent of this brain pathology could, conceivably, determine the manifestation and severity of schizophrenic symptoms. Weinberger (1987) argues that the primary lesion in schizophrenia may be located in the dorsolateral aspects of the prefrontal cortex. In this argument, he proposes that the diagnostic symptoms of schizophrenia only become apparent during the maturation process of dopaminergic systems innervating a "lesioned" prefrontal cortex. The observations that the prefrontal cortex only reaches full functional maturity late in life (Goldman & Alexander, 1977), which coincides with the time of maximal dopaminergic activity in this area (Weinberger, 1987), perhaps accounts for the late adolescent onset of schizophrenia (see above).

In an attempt to explain the effectiveness of neuroleptic drugs in schizophrenia, which by blocking dopamine receptors, would be expected to further compromise dopamine function in the dorsolateral prefrontal cortex, Weinberger (1987) argues that a functionally underactive dopamine system in the prefrontal cortex (caused by brain pathology), may render dopamine function in certain limbic structures overactive, a view for which there is experimental support from lesion studies in the rat (Pycock, Kerwin & Carter, 1980). This, he argues, may account for the development of both positive (caused by a hyperactive dopamine function in the mesolimbic system) and negative (caused by hypoactive dopamine function in the mesocortical system) symptoms in schizophrenia. The fact that neuroleptic drugs predominantly alleviate the positive symptoms of

schizophrenia would be consistent with this theory (see above). Whilst there is no direct evidence that mesolimbic dopamine hyperactivity exists in schizophrenia, post-mortem neurochemical studies have found increased numbers of limbic dopamine receptors (Seeman *et al.*, 1984) and elevated tissue levels of dopamine in the nucleus accumbens (Bird, Spokes & Iverson, 1979) and left-sided amygdala (Reynolds, 1983). The cause of the asymmetrical distribution of dopamine in the amygdala is not clear but this may relate to the dominance of the left cerebral hemisphere (see Roberts, 1990).

Although abnormal dopamine function, in some, as yet ill-defined brain areas, may contribute to the final expression of symptoms in schizophrenia, it is possible that the primary dysfunction in schizophrenia may lie with other transmitter systems involved in the regulation of dopamine systems. Thus, schizophrenia may be a condition of multiple etiology expressed through a common defect in cerebral dopamine function since neuroleptic drugs, which all share potent antagonist activity at dopamine receptors, can alleviate the symptoms of this disorder. This assumption may help explain why drugs such as lysergic acid diethylamide (LSD), phencyclidine (PCP) and yohimbine which act by blocking serotoninergic, NMDA and alpha-2 adrenergic receptors respectively, are all psychotomimetic drugs and can exacerbate schizophrenic symptoms (Allen & Young, 1978; Crow, 1980; Wachtel & Turski, 1990; Snyder, 1980; Holmberg & Gershon, 1961).

1.2 DOPAMINE AND SCHIZOPHRENIA

1.2.1 The dopamine hypothesis of schizophrenia

The idea that some aspects of schizophrenia may be related to an excessive dopaminergic function is the most enduring, and arguably, the most intensively researched hypothesis of schizophrenia. This hypothesis has arisen from several lines of evidence:

1) Neuroleptic drugs, which are beneficial in alleviating the symptoms of schizophrenia (Johnstone, Frith, Price, Crow & Carney, 1978), have potent antagonistic actions at central dopamine receptors (Creese, Burt & Snyder, 1976). Moreover, the therapeutic potency of neuroleptics correlates well with their affinity for dopamine D_2 receptors (Sokoloff, Martres & Schwartz, 1980; Richelson & Nelson, 1984).

2) Drugs which can release dopamine such as amphetamine (see Hornykiewicz, 1982), as well as L-dopa (see Ashton, 1987) and directly acting dopamine agonists (Davies, Sant & Ellison, 1985) all exacerbate pre-existing schizophrenic symptoms. Indeed, amphetamine, can induce in normal individuals, a state closely resembling paranoid schizophrenia (see Robinson & Becker, 1986).

3) Increased dopamine receptor binding sites have been detected in the schizophrenic brain (but see below), specifically in the caudate putamen (Wong *et al.*, 1986) and nucleus accumbens (Seeman, Ulpian, Bergeron, Riederer, Jellinger, Gabriel, Reynolds & Tourtellotte, 1984). Elevated levels of dopamine have also been found in several limbic forebrain structures of post-mortem schizophrenic brains (Bird, Spokes & Iverson, 1979; Reynolds, 1983).

Despite the above observations, that implicate a hyperactive dopamine influence in schizophrenia, the dopamine hypothesis of schizophrenia suffers from a number of drawbacks. Quite apart from the obvious limitation that no clear neuroanatomical site of dopaminergic dysfunction has been defined, the dopamine hypothesis of schizophrenia cannot account for the fact that an irreducible time delay of several weeks is required before the therapeutic effects of neuroleptic drugs become apparent. This suggests that dopamine receptor blockade, which presumably occurs immediately, is not directly responsible for the anti-schizophrenic effects of neuroleptics and that secondary processes, with a much longer time course, must be involved.

A second drawback of the dopamine hypothesis is the fact that neuroleptic drugs do not alleviate all the symptoms of schizophrenia (Crow, 1980). Significant drug effects are usually only apparent against the positive symptoms of this disorder (hallucinations, delusions and thought disorder). Moreover, in chronic schizophrenia, where negative symptoms predominate, some have argued that dopamine function may be relatively underactive (Karoum, Karson, Bigelow, Lawson & Wyatt, 1987; Ashton, 1987; Weinberger, 1987; Chouinard & Jones, 1978; Gerlach & Luhdorf, 1975). Since positive and negative symptoms appear to co-exist in both acute and chronic schizophrenia (Andreasen, 1982; Angst, Stassen & Woggon, 1989) it is apparent that a simple dopamine hyperactivity hypothesis of schizophrenia is incompatible with their co-existence.

Another criticism of the dopamine hypothesis relates to the significance of the increased numbers of dopamine receptors found in the caudate putamen and nucleus accumbens of schizophrenic brains. In a recent PET (positron emission tomography) study (using [^{11}C]-methyl spiperone as the radioligand), dopamine D_2 receptors were reported to be more numerous in the basal ganglia of neuroleptic-naive schizophrenics compared with non-schizophrenics (Wong *et al.*, 1986). This finding, however, was not replicated in a more recent PET study where [^{11}C]-raclopride was employed as the radioligand (Farde, Wiesel, Hall, Hallidin, Stone-Elander & Sedvall, 1987). Although numerous postmortem studies of schizophrenic brains have revealed elevated numbers of dopamine D_2 receptors in the basal ganglia (Lee, Seeman, Tourtellotte, Farley & Hornykeiwicz, 1978; Seeman, Ulpian, Bergeron, Riederer, Jellinger, Gabriel, Reynolds & Tourtellotte, 1984; Hess, Bracha, Kleinman & Creese, 1987), it is unclear whether the dopamine D_2 receptor increase reported in these studies is etiologic to schizophrenia, or simply iatrogenic, since an up-regulation of dopamine receptors also occurs in response to neuroleptics (MacKenzie & Zigmond, 1985). The relevance of an up-regulation of dopamine receptors in the basal ganglia to this disorder as a whole is not clear since a dysfunction of the nigrostriatal system is probably more associated with disturbances of motor behaviour (see section 1.3.3). Moreover, it is difficult to reconcile an increased dopamine transmission in schizophrenia with a resulting up-regulation of dopamine receptors. Presumably therefore, if dopamine receptors increase in number as a result of the schizophrenic process itself (which on balance appears to be unlikely) this must occur through postsynaptic mechanisms rather than through any change in the release of dopamine.

Finally, and as a further criticism of the dopamine hypothesis of schizophrenia, few post-mortem neurochemical studies have consistently revealed an increase in the turnover of dopamine, in either the brain, or in the CSF of schizophrenics (see Owen & Cross, 1989). This, the authors believe, may relate to the instability of dopamine and dopamine metabolites in post-mortem tissue. In CSF samples, the expected increase in dopamine turnover may be obscured if this increase only occurs in relatively discrete brain regions (e.g. the amygdala?, see Reynolds, 1983).

Given the above considerations, it is clear that the dopamine hypothesis of schizophrenia, is not, without its problems. Nevertheless, it would seem, that whatever the underlying cause of schizophrenia, that blockade of dopamine (D-2) receptors can slowly ameliorate

the positive symptoms of this disorder. Indeed, it is reported that fluphenazine, a neuroleptic with a particularly high affinity for the dopamine D-2 receptor (Seeman & Grigoriadis, 1987), slowly (over several weeks), decreases the level of HVA in the plasma of schizophrenics and, in close temporal agreement, alleviates the symptoms of schizophrenia in these patients (Pickar, Labarca, Linnoila, Roy, Hommer, Everett & Paul, 1984). In summary therefore, even though the dopamine hypothesis of schizophrenia suffers from a number of limitations, it may still provide a useful theoretical framework within which the mode of action of neuroleptic drugs can be elucidated. It is therefore necessary to consider the central functions of dopamine in some detail.

1.2.2 Neuroanatomical pathways of dopamine

Dahlstrom and Fuxe (1964) first described the distribution of catecholamine-containing cells and classified them, primarily, on the basis of topographical and morphological criteria, into twelve groups, designated A1-12. Dopamine containing cells in the ventral mesencephalon can be divided into four systems: (1) those located in the substantia nigra (A9), where most of the cells are aggregated in the pars compacta and pars lateralis with only a few cells located in the pars reticulata; (2) those located in the ventromedial mesencephalic tegmentum (A10) where cells originate from the Tsai's ventral tegmentum area, the paranigral nucleus, the caudal lineary nucleus and the nucleus parabrachialis pigmentosus; (3) those extending caudally and dorsally from the substantia nigra into the ventrolateral midbrain tegmentum, lying caudal and ventrolateral to the nucleus ruber (A8); (4) those located in the hypothalamic arcuate and periventricular nuclei (A12).

The axons from A9, particularly those of the pars compacta, form the nigrostriatal pathway (Fig.1.1). These axons, together with some axons from A8, ascend rostrally to innervate predominantly the caudate nucleus and the putamen, as well as the central amygdaloid nucleus. In this thesis the caudate nucleus and putamen will be collectively referred to as either the striatum or the caudate putamen, since in rats, unlike in more highly evolved species, the caudate nucleus and putamen appear to be structurally homogeneous (i.e. they are not separated by an internal capsule). The nigrostriatal pathway gives rise to an extensive terminal plexus that is distributed throughout the entire caudate putamen. About 20 per cent of all terminals in this area are dopaminergic, making it one of the most enriched dopamine-containing regions in the brain. The levels

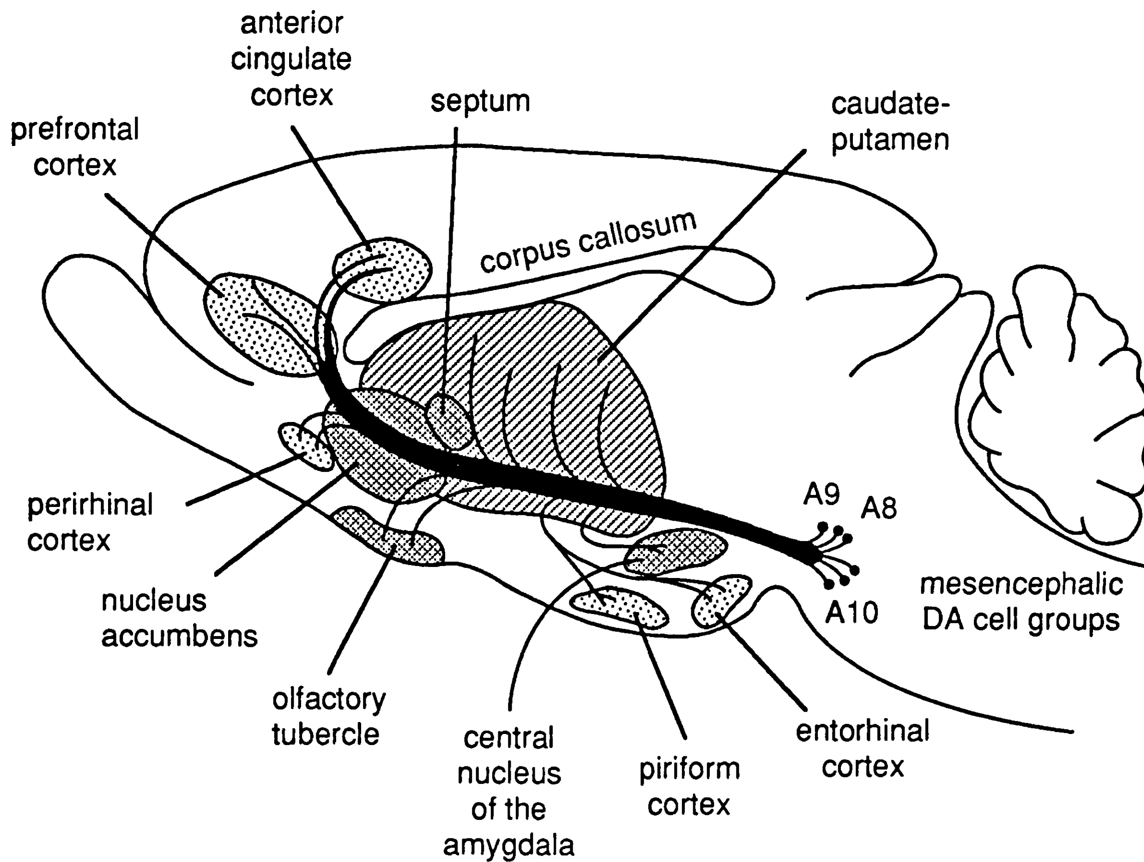


Figure 1.1 Schematic diagram illustrating the neuroanatomy of the nigrostriatal, mesolimbic and mesocortical dopaminergic systems in the rat brain (see section 1.2.2) (taken from Wolf & Roth, 1990).

of dopamine in the caudate putamen are however, not homogenous, with the highest levels found in dorso-rostral regions, and the lowest content found in the ventro-caudal part of this structure (Widmann & Sperk, 1986).

The axons from A10 form the mesolimbic and the mesocortical dopamine systems (see Fig.1.1). These systems, which run within the medial forebrain bundle, in a position immediately ventromedial to and closely associated with the nigrostriatal pathway, innervate the nucleus accumbens, olfactory tubercle, the medial part of the lateral septal nucleus as well as parts of the cortex (mesocortical system) such as the medial prefrontal and perirhinal cortex. The dopamine innervation to the anterior cingulate cortex is also derived from A10 but with some axons from A9.

The medial prefrontal cortex (an area studied in this thesis), which is also known as the pregenual part of the antero-medial cortex (Lindvall, Bjorklund & Divac, 1978; Palkovits, Zaborszky, Brownstein, Fekete, Herman & Kanyicska, 1979), lies rostral to the genu (of the corpus callosum) and medial to the forceps minor of the corpus callosum (see Paxinos & Watson, 1982). The greatest dopamine innervation of this area has been shown to occur in layers V and VI (Berger, Thierry, Tassin & Moyne, 1976), whereas the more superficial layers (I-III) of this area, receive only a light dopamine innervation and a prominent noradrenergic input (Hokfelt, Fuxe, Johansson & Ljungdahl, 1974). The low tissue concentration of dopamine in the medial prefrontal cortex (97-111 ng/g, see Bannon, Wolf & Roth, 1983; Galloway, Wolf & Roth, 1986) compared with that of the caudate putamen (10,560-12,880 ng/g, see Widman & Sperk, 1986; Bannon, Wolf & Roth, 1983) reflects the relative density of dopamine innervation of these two areas.

1.2.3 Pharmacology of dopamine synapses

Dopamine is synthesized from the amino acid precursor tyrosine in neuronal perikarya and nerve terminals (Figs.1.2 & 1.3). Tyrosine is hydroxylated to dihydroxy-phenylalanine (L-dopa) by the enzyme tyrosine hydroxylase, which is located in the cytoplasm and on cell membranes. Tyrosine hydroxylase contains iron and utilises tetrahydrobioterin as its cofactor. The conversion of L-dopa to dopamine is catalyzed by the enzyme L-amino acid decarboxylase (sometimes called dopa decarboxylase) which utilises pyridoxal phosphate as its cofactor. Dopamine is stored in the nerve terminal, evidently, in several releasable

and non-releasable storage sites, either in vesicles or free in the cytoplasm (Arbuthnott, Fairbrother & Butcher, 1990), and is released into the synaptic cleft following depolarisation in a calcium-dependent manner. Once in the synaptic cleft, dopamine diffuses to, and interacts with its receptors (pre- and postsynaptically located). The most important mechanism whereby dopamine is removed from the synaptic cleft, and its influence on dopamine receptors terminated, is by re-uptake.

In essence, two enzymes are required in the metabolism of dopamine (see Fig.1.2). Monoamine oxidase (MAO_A and MAO_B) is located in neuronal and non-neuronal tissues, mainly attached to mitochondrial membranes (MAO_A?), as well as extraneuronally (MAO_B?) (Garrett & Soares-Da-Silva, 1990) and catalyses the deamination of dopamine and 3-methoxytyramine (3-MT). Catechol-O-methyltransferase (COMT) is located mainly extracellularly and catalyses the transfer of methyl groups from S-adenosyl-methionine to the meta-hydroxy group of catecholamines (i.e., O-methylation) (Webster, 1989). The major metabolites of dopamine formed as a consequence of MAO and COMT activity are 3,4-dihydroxy-phenylacetic acid (DOPAC) and 3-MT respectively. It has been argued that since dopamine re-uptake blockers (e.g. nomifensine) do not consistently reduce the extracellular levels of DOPAC, as one would expect if these drugs reduced the metabolism of newly released and taken-up dopamine, then this metabolite must be formed mainly from the intraneuronal deamination of freshly synthesized, but unreleased dopamine (Zetterstrom, Sharp, Collin & Ungerstedt, 1988), perhaps by the action of MAO_A (Garrett & Soares-Da-Silva, 1990). A final metabolite of dopamine is homovanillic acid (HVA) which is formed from the O-methylation of DOPAC, as well as from the deamination of 3-MT.

The metabolism of dopamine can be selectively modified by some drugs (see Fig.1.3). Whilst it is possible to inhibit the activity of COMT using pyrogallol, this compound is considered toxic and of no real use in clinical practice (see Webster, 1989). In contrast, modulation of MAO activity can be readily achieved by many non-toxic and therapeutically useful compounds. This enzyme exists in two forms, MAO_A and MAO_B, although MAO_B shows the highest substrate specificity for dopamine. The activity of MAO_B can be selectively inhibited by selegiline, which when used in conjunction with L-dopa, reduces "end-of-dose" akinesia in Parkinson's disease, and unlike other non-selective MAO inhibitors (iproniazid, pargyline and tranylcypromine), which find use as

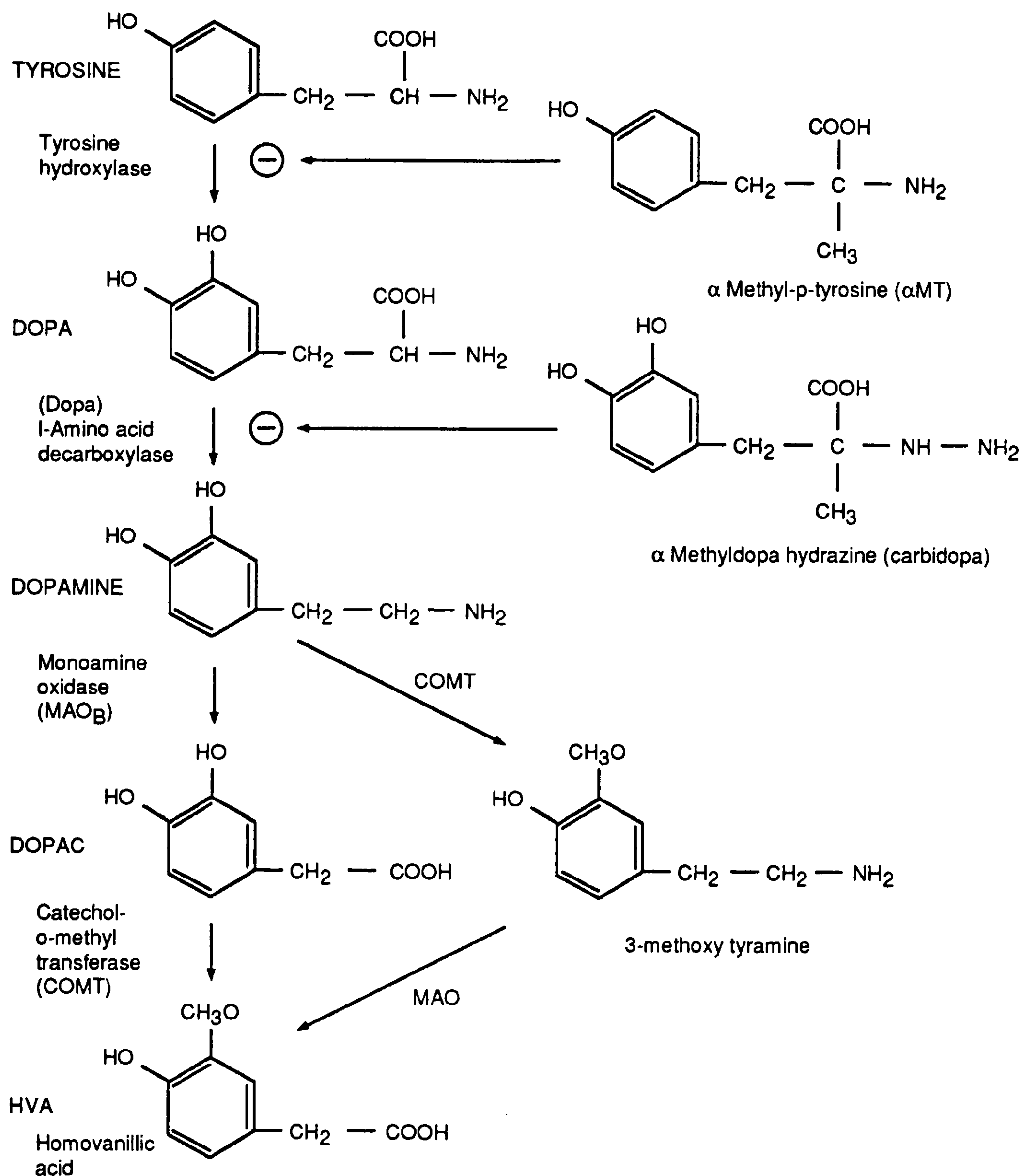
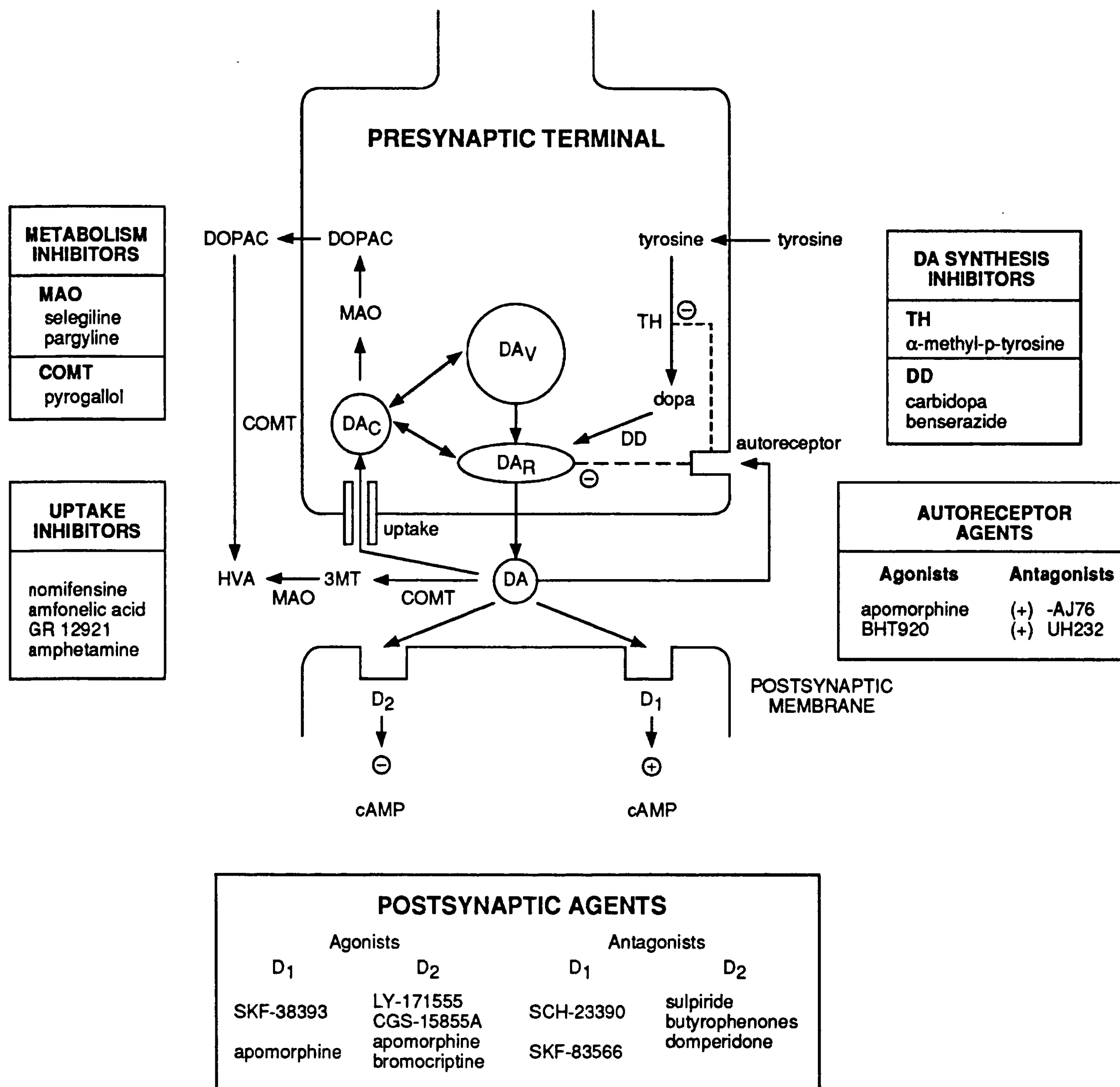


Figure 1.2 The synthesis and metabolism of dopamine. The sites at which α-methyl-p-tyrosine and carbidopa act to reduce the synthesis of dopamine are also shown (see also section 1.2.3).



POSTSYNAPTIC AGENTS			
Agonists		Antagonists	
D_1	D_2	D_1	D_2
SKF-38393	LY-171555 CGS-15855A	SCH-23390	sulpiride butyrophenones
apomorphine	apomorphine bromocriptine	SKF-83566	domperidone

Figure 1.3 Schematic diagram of a dopaminergic synapse (modified from Arbuthnott, Fairbrother & Butcher, 1990) showing the sites at which drugs may act to modify the transmission of dopamine (see also 1.2.3). Abbreviations: DA, dopamine; TH, tyrosine hydroxylase; DD, dopa decarboxylase; DA_R, releasable dopamine; DA_V, vesicular long-term dopamine store; DA_C, cytoplasmic dopamine; MAO, monoamine oxidase; DOPAC, 3,4-dihydroxy-phenylacetic acid; COMT, catechol-O-methyl-transferase; HVA, homovanillic acid; 3MT, 3-methoxy-tyramine; cAMP, cyclic adenosine 3',5'-monophosphate.

antidepressants, selegiline does not cause severe hypertensive effects to certain tyramine-containing foods. The activity of MAO_A can be inhibited by high doses of amphetamine (see Zetterstrom, Sharp & Ungerstedt, 1986a) as well as clorgyline (Webster, 1989).

A number of agents are thought to act by directly releasing dopamine from dopaminergic nerve terminals and dendrites (Imperato & Di Chiara, 1985; Wolf & Roth, 1990), and it is likely for some compounds, at least in the striatum, that this action may be directed at different intraneuronal stores of dopamine. Thus, amphetamine is thought to release dopamine, in a calcium-independent, carrier-dependent manner (Arbuthnott, Fairbrother & Butcher, 1990; Hurd & Ungerstedt, 1989a), from a newly-synthesized, reserpine-resistant pool of dopamine (Niddam, Arbilla, Scatton, Dennis & Langer, 1985), whereas methylphenidate and tyramine are believed to selectively release dopamine from long-term vesicular stores only (Zetterstrom, Sharp, Collin & Ungerstedt, 1988; Arbuthnott, Fairbrother & Butcher, 1990). The depolarising influence of high potassium may release dopamine from both of these nerve terminal stores. Amphetamine and methylphenidate also enhance the synaptic availability of dopamine by blocking the re-uptake of dopamine into the nerve terminal although this can be more selectively achieved using nomifensine and GR 12921 (Hurd & Ungerstedt, 1989b). The release of dopamine induced by amphetamine can be inhibited by nomifensine (Hurd & Ungerstedt, 1989a) suggesting that carrier-mediated up-take across the presynaptic membrane is an important pre-requisite in the action of this compound.

Until recently it was assumed that most, if not all, of the effects of dopamine in the CNS, could be accounted for by the existence of two, G-protein regulated, dopamine receptor subtypes (D_1 and D_2) (Stoof & Kebabian, 1984). It has become clear, however, that this rather limited classification can not account for all the actions of dopamine, nor the effects of drugs which can modify dopamine function (Andersen, Gingrich, Bates, Dearry, Falardeau, Senogles & Caron, 1990). More recently, other dopamine receptors have been cloned (D_3, D_4, and D_5; see Sibley & Monsma, 1992), though it is apparent that their signal transduction pathways (but not their affinity for dopamine and related compounds) are broadly similar to those of dopamine D_1 and D_2 receptors. Dopamine D_1 receptors, which are by far the most abundant subtype of dopamine receptor in the CNS (Boyson, McGonigie & Molinoff, 1986), and which are selectively labelled by [^{3}H]-SCH 23390 (Hyttel, 1983), activate the enzyme adenylate cyclase and increase the intracellular level

of cyclic AMP (Creese, Sibley, Hamblin & Leff, 1983). In some brain areas (e.g. the amygdala), it is clear that dopamine D_1 receptors may not always be linked to this enzyme (Anderson, Gingrich, Bates, Dearry, Falardeau, Senogles & Caron, 1990). In contrast to dopamine D_1 receptors, dopamine D_2 receptors are either not linked to adenylate cyclase, or exert an inhibitory influence on this enzyme (Creese, Sibley, Hamblin & Leff, 1983). Dopamine D_2 receptors may also regulate additional second messenger systems including the inhibition of phosphatidylinositol turnover, opening of potassium channels and inhibition of calcium channel activity (Vallar & Meldolesi, 1989; Lacey, Mercuri & North, 1987). The dopamine D_2 receptor is found presynaptically as well as postsynaptically (the dopamine D_1 receptor is only located postsynaptically) and functionally, the presynaptic D_2 dopamine receptor (i.e. autoreceptor) inhibits the synthesis and release of dopamine (Wolf & Roth, 1990). There is evidence that the function of D_1 and D_2 dopamine receptors are mutually regulated (Saller & Salama, 1986; Strange, 1991). Thus, in some dopamine receptor-mediated electrophysiological and behavioural effects, both dopamine receptor subtypes (acting synergistically to one another), need to be simultaneously stimulated (Wachtel, Hu, Galloway & White, 1989; Waddington, 1989). This functional interaction appears to be mediated on the same postsynaptic neuron since this effect can be demonstrated using dissociated cells (Bertorello, Hopfield, Aperia & Greengard, 1990), and moreover, both dopamine receptor subtypes have been shown to co-exist on the same striatal neuron (Ohno, Sasa & Takaori, 1987). Although the exact signal transduction pathway underlying the functional regulation of dopamine receptor subtypes is, at present, unclear, this may relate to the reported synergistic effects of dopamine D_1 and D_2 receptor stimulation on the inhibition of the Na^+/K^+ pump (responsible for maintaining the disparate distribution of Na^+ and K^+ across neuronal membranes) (Bertorello, Hopfield, Aperia & Greengard, 1990) and the potentiation of arachidonic acid release (Piomelli *et al.*, 1991).

Drugs may also act by blocking (see section 1.3) and stimulating dopamine receptors (see Fig.1.3). Dopamine D_1 receptor agonists such as SKF-38393, SKF-82526 (fenoldopam) and dihydronomifensine (as well as apomorphine), although showing no effects on prolactin secretion, all stimulate adenylate cyclase activity and cause vasodilatation of renal blood vessels (Stoof & Kebabian, 1984). Dopamine D-1 receptors are also thought to participate in the control of some rodent behaviours such as grooming, sniffing, and locomotion (Boyar & Altar, 1987; Molloy & Waddington, 1987), and as well, these receptors may be

involved in the regulation of dopamine metabolism (Boyar & Altar, 1987; Coward, Imperato, Urwyler & White, 1989) and control of postsynaptic (caudate putamen, globus pallidus) neuronal activity (Carlson, Bergstrom & Walters, 1986; Ohno, Sasa & Takaori, 1987; Calabresi, Mercuri, Stanzione, Stefani & Bernardi, 1987; Hara, Sasa & Takaori, 1989).

The consequences of dopamine D_2 receptor stimulation, by agonists such as LY-171555 (quinpirole), CGS-15855A, LY-141865 and apomorphine, are either to decrease or have no effect on the formation of intracellular cyclic AMP (Creese, Sibley, Hamblin & Leff, 1983; Stoof & Kebabian, 1984). In other functional studies, these agents inhibit both the secretion of prolactin (Stoof & Kebabian, 1984) and the central neuronal release of acetylcholine (Baud, Arbilla & Langer, 1985), glutamate (Kornhuber & Kornhuber, 1986), dopamine (Boyar & Altar, 1987) and noradrenaline (Rossetti, Pani, Portas & Gessa, 1989). Dopamine D_2 receptors also mediate the inhibitory effects of dopamine receptor stimulation on the firing of both dopamine (Lacey, Mercuri & North, 1987) and dopamine-innervated (White & Wang, 1986) neurons. Selective dopamine D_2 receptor agonists also reduce dopamine synthesis (Wolf & Roth, 1990) and metabolism (Boyar & Altar, 1987; Coward, Imperato, Urwyler & White, 1989) and can elicit in rodents, a number of distinctive behaviours such as stereotypy (Seeman, 1981; Dreher & Jackson, 1989). An important role for dopamine D_2 receptors is in the presynaptic (autoreceptor) control of dopamine function (see below). Thus, the stimulation of these receptors in the somatodendritic region (substantia nigra and ventral tegmental area) slows the firing rate of dopamine neurons, whilst the stimulation of autoreceptors on dopamine nerve terminals results in the inhibition of the synthesis (via an action on tyrosine hydroxylase) and the release of dopamine (Wolf & Roth, 1990).

Recently, a number of selective dopamine autoreceptor agents have been developed (Abbott, 1990). Although the signal transduction pathways of pre- and postsynaptic dopamine D_2 receptors appear to be similar (inhibition or no effects on adenylate cyclase activity), it is apparent that presynaptic dopamine receptors are 5-10 times more sensitive to the effects of dopamine and other dopamine agonists than postsynaptic dopamine receptors in biochemical and electrophysiological models (Zetterstrom & Ungerstedt, 1984; Wolf & Roth, 1990; White & Wang, 1986). It is thought that autoreceptor agonists (e.g. BHT920 and 3-PPP) may be useful antipsychotic agents devoid of extrapyramidal side effects (see section 1.3.4), since these drugs can inhibit the motor activity of animals over

a wide range of doses (presumably through a reduction of dopamine function), without stimulating motor activity at higher doses like classical dopamine agonists, such as apomorphine (at higher doses apomorphine also stimulates postsynaptic dopamine receptors; see Zetterstrom & Ungerstedt, 1984). This approach assumes of course that dopamine autoreceptors are also present in cortical and limbic pathways, which at least in the cortex (particularly synthesis-regulating autoreceptors), may not be a valid assumption (Bannon, Reinhard, Bunney & Roth, 1982; Chiodo, Bannon, Grace, Roth & Bunney, 1984; Cubeddu, Hoffman & Talmaciu, 1990). Moreover, by reducing dopamine function in the caudate putamen, selective dopamine autoreceptor agonists would, in essence, act like dopamine receptor antagonists and induce an up-regulation of postsynaptic dopamine receptors with the possibility perhaps of producing adverse motor effects. Interest has also been expressed in the development of preferential dopamine autoreceptor antagonists which also have some antagonistic action at postsynaptic dopamine D_2 receptors (e.g [+]-AJ76 and [+]-UH232 (Abbott, 1990; Sokoloff, Giros, Martres, Bouthenet & Schwartz, 1990; see also Fig.1.3). These agents have been reported to have a normalising effect on dopaminergic activity (i.e., they restore dopamine function to baseline activity despite this being either raised or reduced), which is presumably mediated, by restoring the balance of pre- and postsynaptic mechanisms. The implication therefore, is that this class of drugs, unlike typical neuroleptics, may be useful against both the positive and negative features of schizophrenia.

1.3 TYPICAL AND ATYPICAL NEUROLEPTIC DRUGS

1.3.1 Clinical and molecular classification

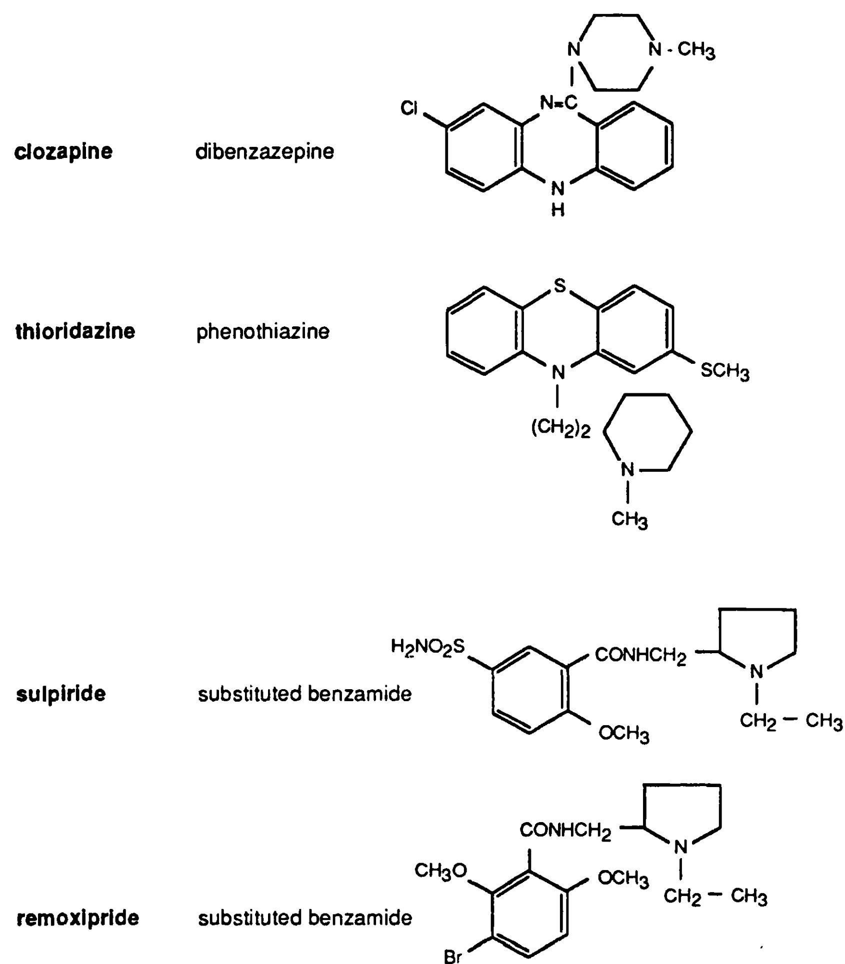
Neuroleptic drugs are effective in alleviating the florid or positive symptoms of schizophrenia (Johnstone, Frith, Price, Crow & Carney, 1978; Neborsky, Janowsky, Munson & Depry, 1981) but they are less effective against the negative (poor prognosis) symptoms of this disorder (Carpenter, Heinrichs & Wagman, 1988; Kane, Honigfeld, Singer & Meltzer, 1988). Although the term neuroleptic is widely used, particularly in Europe, it is rather a vague expression literally defined as a "clasping of the neuron". Despite its popularity of usage, this term more correctly refers to the extrapyramidal effects of these agents (see section 1.3.4) rather than their antischizophrenic effects. Thus, terms such as "antipsychotic" or "antischizophrenic" which give some indication of the

beneficial clinical effects of these agents should be preferred. In this thesis however, the term neuroleptic is used (perhaps incorrectly) to describe drugs which alleviate the psychomotor behaviour of schizophrenics with, or without, the tendency of inducing extrapyramidal side effects. Although neuroleptics are often categorised as either typical or atypical, there is no absolute definition of these terms. This dichotomous clinical classification is based on the ability of some compounds (i.e., atypical neuroleptics such as clozapine, thioridazine, sulpiride and remoxipride), but not others (i.e., typical neuroleptics such as haloperidol, thiothixene, fluphenazine, chlorpromazine and flupenthixol), to control schizophrenia without, or with less tendency of, producing extrapyramidal side effects (Gerlach, Thorsen & Fog, 1974; Mielke, Gallant & Kessler, 1977; Gerlach & Simmelsgaard, 1978; Lindstrom, Besev, Stening & Widerlov, 1985; Andersson, Haggstrom, Nilsson & Widerlov, 1988).

Neuroleptic drugs can also be classified according to their molecular structure (some of which are shown in Fig.1.4). The first or "prototypic" neuroleptic to be introduced was the aliphatic phenothiazine, chlorpromazine. Other phenothiazine derivatives were subsequently developed such as the piperazine derivatives, fluphenazine, trifluperazine and perphenazine, as well as the piperidine derivatives, thioridazine and pericyazine. The phenothiazine neuroleptics vary enormously in their tendency to induce extrapyramidal (anti-dopaminergic), hypotensive (anti-adrenergic) and "atropine-like" (dry mouth, constipation and blurring of vision) side effects. Thus, the piperazine compounds are more potent than the aliphatic compounds on dopamine receptors, and have relatively less α-adrenoceptor blocking activity, while the piperidine group are intermediate in potency and possess more anti-muscarinic activity than the aliphatic derivatives. The thioxanthene neuroleptics, chlorprothixene, clopenthixol and flupenthixol, are closely related to the aliphatic phenothiazines, both chemically and pharmacologically. The butyrophenone neuroleptics, haloperidol, benperidol and droperidol, differ from the phenothiazine and thioxanthine neuroleptic derivatives, in not possessing a tricyclic structure, but they do resemble the piperazine phenothiazines in their high tendency to induce extrapyramidal side effects.

The first synthesized atypical neuroleptic, clozapine, varies from other related tricyclic neuroleptics, in being a piperazine-substituted dibenzazepine. The substituted benzamides, sulpiride (2-methoxysulfamoylethylpyrrolidine) and remoxipride (2,6-

A. ATYPICAL NEUROLEPTICS



B. TYPICAL NEUROLEPTICS

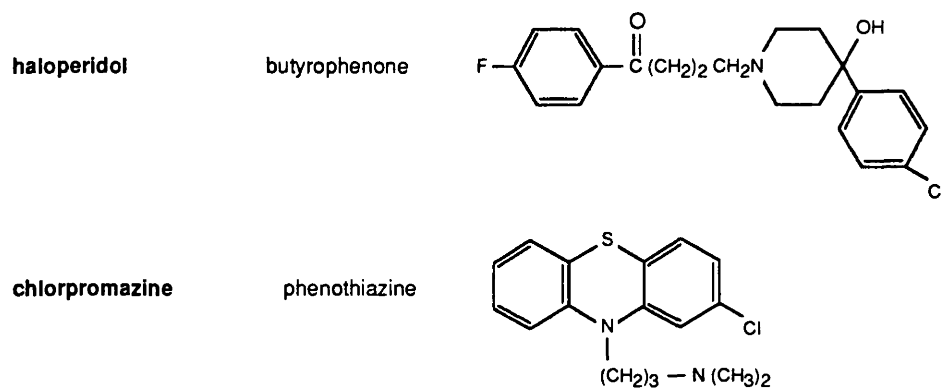


Figure 1.4 Molecular structures of some atypical and typical neuroleptic compounds.

dimethoxybromoethylpyrrolidine), which like clozapine are also atypical neuroleptics, are structurally related to procainamide and metoclopramide. Apart from the phenothiazine thioridazine, which, by the virtue of its strong anti-muscarinic activity which could offset its tendency to induce extrapyramidal effects (Millar & Hiley, 1974 but see also Ljungberg & Ungerstedt, 1979), it is apparent that typical neuroleptics are characterised by either a phenothiazine, thioxanthene or a butyrophenone structure, whereas atypical neuroleptics, are structural derivatives of either dibenzazepines or benzamides. It is not clear, however, what structural features are unique to either typical or atypical neuroleptics since these classes of neuroleptics represent a very diverse range of compounds.

1.3.2 Mode of action

Although neuroleptic drugs interact with a number of central neurotransmitter systems, it is probably their ability to act as dopamine receptor antagonists which, at least in part, underlies their therapeutic effects (but see below). This view has emerged since there is an excellent correlation between the average daily dose of neuroleptics required to control schizophrenia and the affinity of these agents for striatal dopamine receptors (Seeman, 1981). The most striking correlation, and one which has been repetitively demonstrated (Seeman, Lee, Chau-Wong & Wong, 1976; Sokoloff, Martres & Schwartz, 1980; Richelson & Nelson, 1984; Hall, Sallemark & Jerning, 1986; Sokoloff, Giros, Martres, Bouthenet & Schwartz, 1990), is that between the clinical potency of neuroleptics and their affinity for dopamine D_2 receptors. It is important to emphasis, however, that receptor affinity data does not reveal whether neuroleptics are acting as agonists, partial agonists or as antagonists at dopamine receptors. Nonetheless, it has become abundantly clear that neuroleptics block the effects mediated by dopamine receptor stimulation in behavioural (Creese, Burt & Snyder, 1976; Ungerstedt & Ljungberg, 1977; Bianchi, Landi & Garattini, 1986), biochemical (Bacopoulos & Roth, 1981) and electrophysiological (Chiodo & Bunney, 1983b; Mereu, Collu, Ongini, Biggio & Gessa, 1985; Grace & Bunney, 1985; Martin, Cox & Waszczak, 1990) studies. The significance of the correlation between dopamine D_2 receptor affinity and anti-schizophrenic potency is not without its interpretive difficulties. It could be argued that such a correlation is merely co-incidental and only reveals the tendency of these drugs to induce extrapyramidal effects. Nevertheless, it is assumed in this thesis that this action contributes, at least to some extent, to the effectiveness of neuroleptic drugs in schizophrenia.

The neuronal site where neuroleptics block dopamine (D_2) receptors to elicit their actions is not certain since these receptors are located pre- and postsynaptically (see section 1.2.3) and both of them are blocked by neuroleptics (Seeman, 1977; Starke, Spath, Lang & Adelung, 1983; Spampinato, Girault, Danguir, Savaki, Glowinski & Besson, 1986). When administered acutely, neuroleptics enhance the release (Imperato & Di Chiara, 1985; Moghaddum & Bunney, 1990) and turnover of dopamine (Nicolaou, 1980; Zetterstrom, Sharp & Ungerstedt, 1985; Karoum & Egan, 1992), as well as increase the firing of dopaminergic neurons (Bunney, Walters, Roth & Aghajanian, 1973; Mereu, Fanni & Gessa, 1984). These effects have been attributed to the blockade of presynaptic dopamine receptors on dopamine neurone cell bodies and axon nerve terminals, and postsynaptically, affecting compensatory cholinergic and GABAergic feedback pathways (Bunney, Walters, Roth & Aghajanian, 1973; Zetterstrom, Sharp & Ungerstedt, 1985; Ashton, 1987; Stamford, Kruk & Millar, 1988; Commissiong, Slimovitch & Toffano, 1990) to also increase dopamine neurone firing. Whether such effects could balance or even overcome the effects of dopamine receptor antagonism is uncertain but they are probably not directly involved in their antischizophrenic effect for this is only apparent after chronic administration (about 2-3 weeks). More recent evidence has revealed that the antagonism of dopamine receptors, and particularly dopamine D_2 receptors, by chronically administered neuroleptics, gradually, over several weeks, inactivates the firing of A9 and A10 dopaminergic neurons. This effect provides an excellent temporal correlation with the therapeutic lag-time of neuroleptics (and the emergence of extrapyramidal side effects), and singularly, restores the credibility of excessive dopaminergic activity in schizophrenia, or at least, the antagonism of dopamine transmission as the key to neuroleptic drug action.

Neuroleptics, in fact, produce very different effects on dopaminergic mechanisms after their chronic administration. It is well recognised that in the nigrostriatal and the mesolimbic dopamine systems (Nicolaou, 1980; Scatton, 1977; Lerner, Nose, Gordon & Lovenberg, 1977; Guidotti, Gale, Toffano & Vargas, 1978; Meller, Friedhoff & Friedman, 1980), but not in the mesocortical dopamine system (Bacopoulos, Bustos, Redmond, Baulu & Roth, 1978; Matsumoto, Uchimura, Hirano, Kim, Yakoo, Shimomura, Inoue & Oomagari, 1983), tolerance develops to the facilitatory effects of neuroleptics on dopamine release and turnover. This tolerance corresponds in the striatum with an up-regulation of postsynaptic dopamine (D_2) receptors (Yarbrough, 1975; Sayers, Burki, Ruch & Asper,

1975; Creese & Snyder, 1980; Severson, Robinson & Simpson, 1984; Rupniak, Mann, Hall, Fleminger, Kilpatrick, Jenner & Marsden, 1984; MacKenzie & Zigmond, 1985; Liskowsky & Potter, 1987), similar to the effects of depleting the dopamine innervation to this area using 6-hydroxydopamine (Feltz & De Champlain, 1972), as well as the inactivation of dopamine neuronal firing (White & Wang, 1983a; Chiodo & Bunney, 1983a; see also below). It has been argued that the development of tolerance (or indeed lack of tolerance such as in the cortex) and the up-regulation of dopamine receptors in some areas may relate to the emergence of the clinical effects of neuroleptics (both therapeutic and adverse) (see sections 1.3.3 & 1.3.4). It should be noted that only dopamine D_2 receptors (not dopamine D_1 receptors), up-regulate in response to chronic neuroleptic treatment (MacKenzie & Zigmond, 1985) and that dopamine D_1 mechanisms appear not to be involved in the inactivation of dopamine neuronal firing (Esposito & Bunney, 1989). It is perhaps worth mentioning though, that in rodents, chronic treatment with sulpiride, an agent virtually devoid of dopamine D_1 activity, has been reported to elevate the activity of striatal adenylate cyclase (Rupniak, Mann, Hall, Fleminger, Kilpatrick, Jenner & Marsden, 1984). This unexpected effect may relate to the functional interrelationship which exists between dopamine D_1 and dopamine D_2 receptors (see section 1.2.3).

A convincing time-related model of neuroleptic drug action is provided by recording the effects of these agents on the activity of A9 and A10 dopamine neurons (White & Wang, 1983a; White & Wang, 1983b; Chiodo & Bunney, 1983a; Skarsfeidt, 1988). Unlike their acute effects (see above), chronically administered neuroleptics, produce an almost complete inhibition of dopamine neuronal firing in rodents. This effect is thought to be due to an over-depolarisation of these neurons since their activity can be reinstated, by either, the application of GABA (Chiodo & Bunney, 1983a), intravenous injection of apomorphine (White & Wang, 1983a), or the intracellular injection of a hyperpolarising current (see Ashton, 1987). All neuroleptic drugs were found to inactivate cells in A10, the origin of the mesolimbic and the mesocortical pathways, however, only typical neuroleptics inactivated cells in A9, the origin of the nigrostriatal pathway. Moreover, drugs devoid of antipsychotic activity such as metoclopramide and promethazine did not alter the firing of A10 cells, although metoclopramide, which can produce extrapyramidal side effects, decreased the firing of A9 cells. The inactivation of dopaminergic neurons developed after about 1 week of neuroleptic treatment, although the firing of some cells, particularly those thought to project to the prefrontal and cingulate cortex, was not

altered. The mechanism underlying the differential effects of typical and atypical neuroleptics on dopamine cell activity appears to involve intact feedback pathways (White & Wang, 1983a) and may relate to the anti-adrenergic and the anti-cholinergic activity of some atypical neuroleptics (Chiodo & Bunney, 1985). These results strengthen the evidence from other sources that the therapeutic effects of neuroleptics are, eventually, due to dopamine receptor blockade in the mesolimbic system (see 1.3.3).

The results from neurochemical studies of dopamine function are not entirely consistent with the idea that dopamine neurons are inactivated by chronic treatment with neuroleptics. Thus, following the chronic administration of clozapine, the release and turnover of dopamine are not, as would be expected, decreased in the nucleus accumbens (Ichikawa & Meltzer, 1990; Invernizzi, Morali, Pozzi & Samanin, 1990; but see also Blaha & Lane, 1987). Furthermore, chronic treatment with haloperidol, unlike the effects of gammabutyrolactone (an agent which inhibits the firing of dopamine neurons), does not increase the tissue concentration of dopamine in the caudate putamen and other dopamine-innervated structures (Anden, Grenhoff & Svensson, 1988). These neurochemical discrepancies may arise, since the basal release of dopamine, at least in the caudate putamen, is maintained at near normal levels, even after the extensive (but not complete), destruction of the nigrostriatal pathway (Robinson & Whishaw, 1988; Zigmond, Abercrombie, Berger, Grace & Stricker, 1990; Bean & Roth, 1991). Thus, despite a reduction in the activity of dopamine neurons induced by the chronic administration of neuroleptics, the synaptic function of dopamine may still be conserved.

Apart from dopamine, neuroleptics also affect the function of many other transmitter systems (e.g. cholinergic, adrenergic, serotoninergic, GABAergic, enkephalinergic). Although non-dopaminergic mechanisms are generally not thought to play a major causative role in schizophrenia (Ashton, 1987), they may, nonetheless, be important in modifying the tendency of neuroleptic drugs to induce extrapyramidal side effects.

1.3.3 Site of action

As dopamine D_2 receptors are located in the nigrostriatal, mesolimbic and the mesocortical dopaminergic systems (Boyson, McGonigie & Molinoff, 1986), the question arises as to which of these sites is responsible for mediating the clinical effects of neuroleptics. It is well recognised that dopamine dysfunction in the nigrostriatal system results in Parkinson's disease in which motor activity is disrupted. The features of Parkinson's disease (akinesia, rigidity and tremor) are virtually indistinguishable from some of the extrapyramidal effects produced by typical neuroleptics (see section 1.3.4). The evidence that only typical neuroleptics, but not atypical neuroleptics, inactivate A9 dopamine neurons, following their chronic administration (see above), again indicates that the extrapyramidal effects of neuroleptics are attributable to a dysfunction of the nigrostriatal dopamine system. If this hypothesis were correct then the anti-schizophrenic effect of neuroleptics may arise from dopamine receptor blockade within either the mesolimbic and/or the mesocortical systems. This would then imply that the atypical neuroleptics only affect dopamine function in these areas and conserve the functional integrity of the nigrostriatal dopamine system. There is evidence to support this claim. Thus, only typical neuroleptics are reported to antagonise the stereotypic behaviours induced in rats by dopamine agonists (Iverson & Koob, 1977; Robertson & MacDonald, 1985), an effect thought to result from dopamine receptor stimulation in the nigrostriatal system, whereas both typical and atypical neuroleptics, antagonise the hyperactivity induced in rats by dopamine agonists (Ljungberg & Ungerstedt, 1979; O'Connor & Brown, 1982), an effect thought to result from a facilitation of mesolimbic dopamine function (Fuxe, Ogren, Hall, Agnati, Andersson, Kohler & Schwarcz, 1980). The question remains, however, of how drugs that are basically dopamine antagonists could have such different effects in two dopamine innervated areas.

It has been argued that since neither neurochemical tolerance (Scatton, Glowinski & Julou, 1976; Matsumoto, Uchimura, Hirano, Kim, Yakoo, Shimorura, Nakahara, Inoue & Oomagari, 1983) nor any changes in the regulation of dopamine D_2 receptors (Bacopoulos, 1981; Liskowsky & Potter, 1987) have been reported to occur in the mesocortical system, following the chronic administration of neuroleptics, unlike that reported in either the nigrostriatal or the mesolimbic systems (see above), that the therapeutic effects of neuroleptics are mediated within the mesocortical system (i.e., dopamine receptor

blockade continues to occur in this system). Indeed, others have shown that after the chronic administration of neuroleptics, such dopamine-mediated functions as postsynaptic neuronal inhibition and behavioural activation to applied dopamine agonists, continue, either unabated, or with enhanced sensitivity in the nigrostriatal and the mesolimbic system (Yarbrough, 1975; Zarzecki, Blake & Somjen, 1977; Fleminger, Hall, Jenner, Kelly, Kilpatrick, Marsden & Rupniak, 1982). These effects are, however, postsynaptically-mediated, and do not reveal the level of presynaptic dopaminergic activity, which, presumably may be reduced in these dopamine systems since an up-regulation of dopamine receptors is known to occur under these conditions (see above). Thus, whether pre- or postsynaptically mediated, it is still plausible that neuroleptics elicit their therapeutic effects in schizophrenia by reducing raised mesolimbic dopamine function.

1.3.4 Extrapyramidal side effects

Neuroleptic drugs can give rise to a wide range of unwanted side effects (e.g. hypotension, dry mouth, constipation, blurred vision, skin reactions), many of which, apart from the blood dyscrasias caused by clozapine, only produce slight discomfort to the patient. However, the most prevalent, and arguably, the most troublesome side effects associated with the use of typical neuroleptics are those which are thought to arise from a dysfunction of the extrapyramidal motor system (nigrostriatal system). The earliest extrapyramidal side effects observed are acute dystonic reactions which are particularly likely to occur in males and adolescents and are a feature of the butyrophenones and piperazine phenothiazines. Acute muscle dystonias include retrocollis (where the head is drawn directly backwards), torticollis (a twisting of the neck), tongue protrusion, dysarthria (disturbance of speech), facial grimacing, opisthotonus and scoliosis (spinal curvature). A few doses, or even only a single dose of a neuroleptic, may be sufficient to produce these effects. The mechanism whereby neuroleptics can cause local spasm in particular groups of muscles is unknown, but since these effects respond to anti-muscarinic drugs (e.g. procyclidine), it may relate to an overactivity of striatal cholinergic function, secondary to dopamine receptor blockade.

As well as acute dystonias, patients receiving neuroleptics may also develop akathisia. This effect, which is also thought to arise within the nigrostriatal system, is described as an uncontrollable physical and psychological restlessness, which is characterised by

fidgeting and pacing. It usually occurs within 2 to 10 weeks of neuroleptic treatment and is only partially alleviated by anticholinergics. Since benzodiazepines are sometimes effective in reducing the intensity of akathisia (Ashton, 1987), this effect may, in part, be mediated by GABAergic mechanisms.

The most common extrapyramidal side effects seen with typical neuroleptics resemble those of Parkinson's disease. The condition usually supervenes within a few weeks and is often associated with the emergence of a clinical improvement in schizophrenia. The most common disturbance is akinesia and this tends to be associated with muscle weakness, particularly in those muscles involved in repetitive actions such as walking. Akinesia also affects the control of fine muscular movements. Thus, handwriting is sometimes severely affected. In more severe cases of neuroleptic-induced Parkinsonism, rigidity, coarse tremor, stooped posture, and excessive salivation may also be present. The Parkinsonian effects of neuroleptics, whilst being most common in woman and the elderly, sometimes disappear or improve after several months of continued treatment. The mechanism underlying neuroleptic-induced Parkinsonism is not altogether clear, but since anticholinergic drugs are effective, it is probably due to a relative excess of cholinergic function in the basal ganglia, secondary to the antagonism of dopamine receptors in the nigrostriatal system. The reason why tolerance seems to develop to these extrapyramidal effects is less certain since this does not appear to be a feature of the antipsychotic effects of these agents (Palmstierna & Wistedt, 1987).

The high risk of developing tardive dyskinesia, a condition which is generally considered to be irreversible (Gerlach & Casey, 1988; but see also Klawans, Goetz & Perlik, 1980), is a constraint to the long-term use of typical neuroleptics (about 10-20% of neuroleptic-treated patients; Ashton, 1987). This condition, which develops after months or years of neuroleptic administration, consists of involuntary repetitive movements that typically affect oral, lingual, buccal, facial, trunk and limb muscles. These often bizarre movements can manifest as lateral movements of the jaw, sucking, lip-smacking and tongue darting ("fly-catching"). The cause of tardive dyskinesia is, at best, poorly understood, as this condition also occurs in neuroleptic-naive chronic schizophrenics (Owens, Johnstone & Frith, 1982; Waddington & Youssef, 1986). The incidence of tardive dyskinesia is highest in woman and the elderly (Klawins, Goetz, Perlik, 1980; Kane & Smith, 1982; Gerlach & Casey, 1988), but surprisingly, previous neuroleptic-induced dystonia, parkinsonism, or

akathisia have been inconsistently associated with the risk of developing tardive dyskinesia (Ashton, 1987; Gerlach & Casey, 1988). This disorder is thought to arise from the development of dopamine D_2 receptor supersensitivity and raised dopamine function in the nigrostriatal system (Ashton, 1987; Klawans, Goetz & Perlik, 1980; Gerlach & Casey, 1988), although no consistent differences have been reported in the density of dopamine D_2 receptors in post-mortem schizophrenic brains of patients with, or without, tardive dyskinesia (Ashton, 1987). Thus, the fact that tardive dyskinesia is not a feature seen in all patients treated with (typical) neuroleptics, despite presumably showing striatal dopamine receptor supersensitivity, argues against a raised nigrostriatal dopamine function in this disorder. Nevertheless, tardive dyskinesia can be ameliorated, to some extent, by neuroleptics, cholinergics (physostigmine, lecithin) and dopamine-depleting drugs like tetrabenazine, whilst it is aggravated by dopamine receptor agonists and anticholinergic agents (Ashton, 1987; Gerlach & Casey, 1988). The practice of treating tardive dyskinesia with progressively larger doses of neuroleptics is considered shortsighted, because although this approach can achieve some relief, this can only be a temporary measure, since the underlying pathophysiology of this disorder is likely to be aggravated (Klawans, Goetz, Perlik, 1980).

Tardive dyskinesia is reported to be closely associated with negative symptoms of schizophrenia (Csernansky, Kaplan, Holman & Hollister, 1983; Barnes & Braude, 1985; Waddington & Youssef, 1986; Waddington, Youssef, Dolphin & Kinsella, 1987). It may be no coincidence therefore, that some atypicals neuroleptics have been reported to alleviate the negative symptoms of schizophrenia (Kane, Honigfeld, Singer & Meltzer, 1988), as well as either protecting against the development of tardive dyskinesia, or reducing its severity (Sayers, Burki & Asper, 1975; Ashton, 1987; Andersson, Haggstrom, Nilsson, Widerlov, 1988). These findings suggest that negative symptoms and tardive dyskinesia may share a common pathophysiology. To reconcile this possibility with the view that these conditions are thought to result from reduced (negative symptoms) and raised (tardive dyskinesia) dopamine function, it could be argued that these clinical conditions arise from dopamine dysfunction in distinct dopaminergic systems and that these systems may perhaps be inversely regulated. This possibility is examined in more detail in chapter 9.

1.4 AIMS AND OUTLINE OF STUDIES

It will be clear from the preceding sections that a disturbance of dopamine function may, at least to some extent, underlie the pathophysiology of schizophrenia as well as the tendency of neuroleptic drugs to induce extrapyramidal side effects. This thesis attempts to establish how the atypical neuroleptics (and in particular clozapine) alleviate the symptoms of schizophrenia without causing marked neurological side effects by comparing their actions with those of a typical neuroleptic (haloperidol) on dopamine transmission in the rat nigrostriatal, mesolimbic and mesocortical dopamine systems.

To this end, an *in-vivo* intracerebral microdialysis system was developed and employed to determine the extracellular levels of endogenous dopamine and dopamine metabolites in these areas, together with the activity of neurons in the caudate putamen and the medial prefrontal cortex, monitored by conventional extracellular recording. In chapter 2 these procedures are described in detail, as are the *in-vitro* methods employed to examine the activity of neuroleptics at non-dopaminergic receptors (this data is given in chapter 4). The results from experiments designed to validate the *in-vivo* techniques adopted in this thesis are presented in chapter 3. Subsequent chapters describe the effects of haloperidol and clozapine, either alone, or combined with apomorphine, on the release and turnover of dopamine in the caudate putamen and the medial prefrontal cortex (chapter 5) as well as on the activity of neurons in these areas (chapter 6). Intracerebral microdialysis was also employed to evaluate the effects of 5-HT_3 receptor-acting agents on dopamine function in the caudate putamen and the nucleus accumbens (chapter 8). The results of all the *in vivo* studies are discussed in the light of known facts and the *in vitro* analysis is an attempt to explain the distinction between typical and atypical neuroleptics.

CHAPTER 2

METHODS

2.1 INTRODUCTION

From the outset it was recognised that a wide variety of techniques could be employed to study the actions of neuroleptic drugs. Ideally, such techniques should accommodate the ability of neuroleptic drugs to act as antagonists at central dopamine receptors and be sensitive enough to discriminate between typical and atypical neuroleptic compounds.

Since the ability of neuroleptics to antagonise dopamine receptors in either the mesocortical or the nigrostriatal systems may underlie their anti-schizophrenic and parkinson-like extrapyramidal effects respectively (see chapter 1), it was decided to evaluate the actions of neuroleptic drugs on dopamine function in both the rostral caudate putamen and the medial prefrontal cortex. The rostral caudate putamen was chosen, in preference to the caudal region of this structure, since much higher levels of dopamine are found there (Widmann & Sperk, 1986; Beal & Martin, 1985). It was thus anticipated that the chances of locating neurons sensitive to apomorphine in later recording experiments (see section 2.4) would be greatly improved by working in this area. The following section outlines those considerations that led to the adoption of the specific *in vivo* procedures used in the present work.

Several techniques are available which can continuously monitor the release of chemicals in cerebral neurotransmission *in-vivo*. These range from *in-situ* techniques such as voltammetry (Stamford, Kruk & Millar, 1988; Maidment & Marsden, 1985) which can directly measure the oxidation of monoamines in the brain interstitial space, to techniques such as the cortical cup (see Benveniste, 1989), push-pull cannula (Yaksh & Yamamura, 1974) and intracerebral microdialysis (Tossman & Ungerstedt, 1981) which collect substances from the brain interstitial space for subsequent analysis *ex-situ*. Clearly, as suggested by its name, the cortical-cup technique is unsuitable as a tool to monitor the chemical transmission of dopamine in subcortical regions. The push-pull cannula technique was also ruled out since, in comparison to voltammetry and microdialysis, it suffers from a number of drawbacks. These include on-going trauma arising from the direct contact of perfusion fluid with neuronal elements, cannula blockage, the potential for enzymatic degradation of collected substances and the necessity to deproteinise samples prior to analysis. Although *in vivo* voltammetry can measure changes in the brain interstitial concentration with a time resolution of less than one second (Benveniste, 1989)

and can be used to record in more discrete brain areas compared to microdialysis, it can suffer from poor selectivity (Benveniste, 1989). By comparison, microdialysis has some disadvantages (poorer time resolution and larger probe dimensions), but is perhaps the most flexible method available and, providing a sensitive and reliable assay can be developed, appears to be ideally suited to monitor the events of cerebral dopamine release and metabolism. The interpretation of dialysis results is hampered, however, by its inability to provide an absolute estimation of synaptic neurochemical concentrations.

In essence, the technique of brain dialysis involves the perfusion of a small (200-800μm o.d) dialysis fibre implanted in discrete areas of the brain, and the collection and analysis of the perfusate. Low molecular weight compounds that are in a higher concentration in the extracellular fluid diffuse across a porous membrane into a physiological salt solution which is pumped through the dialysis probe at a low and constant flow rate. Existing dialysis probes fall into three basic designs: transcerebral, U-shaped and concentric (see Di Chiara, 1990). Since transcerebral dialysis probes perfuse both cerebral hemispheres, unlike intracerebral dialysis probes (concentric and U-shaped), much higher levels of neurochemicals are recovered in the perfusate. As the extracellular level of dopamine is very low, even in the striatum (20-50nM), this was recognised as a useful means to enhance the basal levels of this transmitter. In some preliminary experiments transcerebral probes were developed which were essentially of the same design as described by Imperato & Di Chiara (1984). Despite the high recovery of transmitters encountered using these probes they proved both laborious and traumatic to implant and could not reliably be inserted into deep cerebral structures such as the nucleus accumbens. This approach was subsequently abandoned in preference for a rigid concentric-designed probe.

A variety of options are available to record neuronal activity, from dissociated neurons in culture, to intact neurons *in situ*. A popular approach employed to monitor dopaminergic activity *in vivo* is to record the spontaneous activity of putative dopamine neurons in either the substantia nigra (Bunney, Walters, Roth & Aghajanian, 1973; Carlson, Bergstrom & Walters, 1986; Esposito & Bunney, 1989; Bergstrom, Carlson, Bromley, Jackson & Walters, 1986) or the ventral tegmental area (Grenhoff, Aston-Jones & Svensson, 1986; Trulson, Trulson & Arasteh, 1987). Whilst this approach can demonstrate the ability of neuroleptic drugs to block presynaptic dopamine receptors (Carlson, Bergstrom & Walters, 1986) since an intact striatonigral feedback pathway is not

required for a dopamine agonist such as apomorphine to inhibit the activity of these neurons (Baring, Walters & Eng, 1980), it will not directly reveal the effects of neuroleptics on postsynaptic dopamine receptors. This is an important consideration since the ability of neuroleptic drugs to block postsynaptic dopamine receptors is believed to contribute to both the increase in dopaminergic neuronal firing and the elevation of dopamine turnover seen after the acute administration of these agents (Ashton, 1987). Thus it was decided to monitor the activity of neurons in the caudate putamen and the medial prefrontal cortex to give a direct measure of dopamine transmission to compare with studies on its release using microdialysis.

Recording the spontaneous activity of neurons in the caudate putamen, in both anaesthetised and immobilized animals, is hampered by low neuronal activity and the predominance of silent cells (see Skirboll & Bunney, 1979). For this reason, most studies of striatal activity is carried out on cells activated by the microiontophoretic application of an excitatory substance such as glutamic acid (Ohno, Sasa & Takaori, 1987; Chiodo & Berger, 1986; Hu & Wang, 1988). Unfortunately, the use of an excitatory substance to enhance neuronal activity may bias results. Although the same could be said for only recording from cells which are spontaneously active. Whilst it is not impossible to find spontaneously active striatal neurons (Siggins, Hoffer & Ungerstedt, 1974; O'Donnell, Murer & Pazo, 1989; Ohno, Sasa & Takaori, 1987) they are not common and so it was decided to try various strategies in order to enhance the level of activity in this area.

Although microiontophoresis appears to be the most favoured method employed to apply excitatory substances to neurons, the results can be difficult to interpret (Bloom, 1974). This limitation together with the observations that many neuroleptics are insoluble at the high concentrations needed for iontophoretic studies and can only block the inhibitory effects of locally applied dopamine on striatal neuronal firing when applied iontophoretically (Siggins, Hoffer & Ungerstedt, 1974) but not parenterally, despite high doses (Johnson, Hoffer & Freedman, 1986), led me to explore other possibilities. One strategy tried was to use intracerebral dialysis to deliver locally both directly acting (glutamic acid and aspartic acid), as well as indirectly acting (picrotoxin, bicuculline and leptazol), excitatory agents to the recording site. Despite a range of doses employed, none of the agents listed above, with the possible exception of leptazol, produced any stimulatory effects on the discharge of striatal neurons. Indeed, paradoxically, they

produced predominantly inhibitory effects. This may have resulted from either the activation of intrinsic inhibitory neurons or, as reported recently for glutamate, which was added to the dialysis perfusion medium (1mM), through the release of dopamine in the striatum (Shimizu, Duan, Hori & Oomura, 1990). Some success, however, was obtained using this technique to administer various dopamine receptor agonists (dopamine and CGS 15855A) as well as drugs which are thought to act by releasing dopamine (amphetamine and 2-methyl-5HT). These agents all produced a concentration-dependent decrease in the activity of striatal neurons (see chapter 3). Although this preliminary data was promising, the technique as a whole lacked flexibility and with the apparent interpretive difficulties of the results, was considered to be of no real use in the study of neuroleptic drug action.

It is clearly desirable to avoid the use of general anaesthetics since they are known to impair the normal functioning of central neuronal mechanisms (McGivern & Scholfield, 1990). In recent years the technique of microdialysis has been sufficiently refined to enable its use in conscious freely moving animals (Imperato & Di Chiara, 1985; Zetterstrom, Sharp & Ungerstedt, 1985). This possibility was not exploited in the present studies, however, principally due to the widely reported stimulatory effects of stress on dopamine utilisation in the mesocortical system (Bannon & Roth, 1983; Thierry, Tassin, Blanc & Glowinski, 1976). Given that stress is a likely consequence of microdialysis employed in awake freely moving animals it was considered appropriate to use a general anaesthetic agent which can provide a very standardised set of experimental conditions. Although chloral hydrate has been widely used in the study of central dopamine neurotransmission it may not be the best choice since it has been reported to produce a considerable dampening effect on dopaminergic transmission in rodents (Zhang, Tilson, Stachowiak & Hong, 1989; Ford & Marsden, 1986) as well as altering the responsiveness of dopamine neurons to both dopamine receptor agonists (Kelland, Freeman & Chiodo, 1989) and antagonists (Zhang, Tilson, Stachowiak & Hong, 1989). In this regard halothane may be a better agent since although it impairs the ability of neuroleptic drugs to enhance both the firing of dopamine neurons (Mereu, Fanni & Gessa, 1984) and to facilitate the release of dopamine (Spampinato, Girault, Danguir, Savaki, Glowinski & Besson, 1986), it produces a less marked depressant effects on dopaminergic transmission than chloral hydrate (Ford & Marsden, 1986) and is recognised, in practical terms, to be a more convenient general anaesthetic to use in rodents (Ford & Marsden, 1986).

2.2 GENERAL PROCEDURES *IN VITRO*

2.2.1 Isolated guinea-pig ileum

This preparation was used to examine the activity of typical (haloperidol, chlorpromazine) and atypical (clozapine, thioridazine, sulpiride and remoxipride) neuroleptic compounds at 5-HT_3, muscarinic and tachykinin (substance P) receptors.

Figure 2.1 illustrates the possible mechanisms through which stimulation of these receptors are thought to elicit a contractile response in this preparation (from Buchheit, Engel, Mutschler & Richardson, 1985). Thus 5-HT can contract this tissue, either directly, by stimulating 5-HT receptors located on the longitudinal smooth muscle (D-receptors of Gaddum & Picarelli, 1957), or indirectly, through the release of both substance P (Chahl, 1983) and acetylcholine (Kilbinger, Kruel, Pfeuffer-Friederich & Wessler, 1982) via the stimulation of neuronally located 5-HT_3 receptors (M-receptors of Gaddum & Picarelli, 1957). The contractile-induced response of this tissue to substance P (SP), like 5-HT, is both directly mediated (Buchheit, Engel, Mutschler & Richardson, 1985), and indirectly mediated via the release of acetylcholine (Chahl, 1983).

It was anticipated that the activity of various neuroleptic compounds at these receptors (listed above) could be specifically evaluated by incorporating selective receptor antagonists in the bathing medium to block confounding receptor interactions. Thus when assessing these compounds for 5-HT_3 and tachykinin receptor activity, atropine and methysergide were used to antagonise the direct contractile effects of acetylcholine and 5-HT respectively. The bathing medium also contained diphenhydramine (histamine H_1 receptor antagonist). Atropine was omitted from the bathing medium when assessing neuroleptic activity at muscarinic receptors.

Procedures

Male Dunkin-Hartley guinea-pigs (250-350g) bred in the Joint Animal House, University College London, were killed by cervical dislocation and a 15cm length of whole ileal tissue was removed, 2cm from the ileo-caecal junction, and washed internally with Tyrodes solution (see Appendix II). Segments approximately 2cm long were placed in 5ml organ baths containing Tyrodes solution, aerated with 95% O_2 and 5% CO_2 at 37°C, and

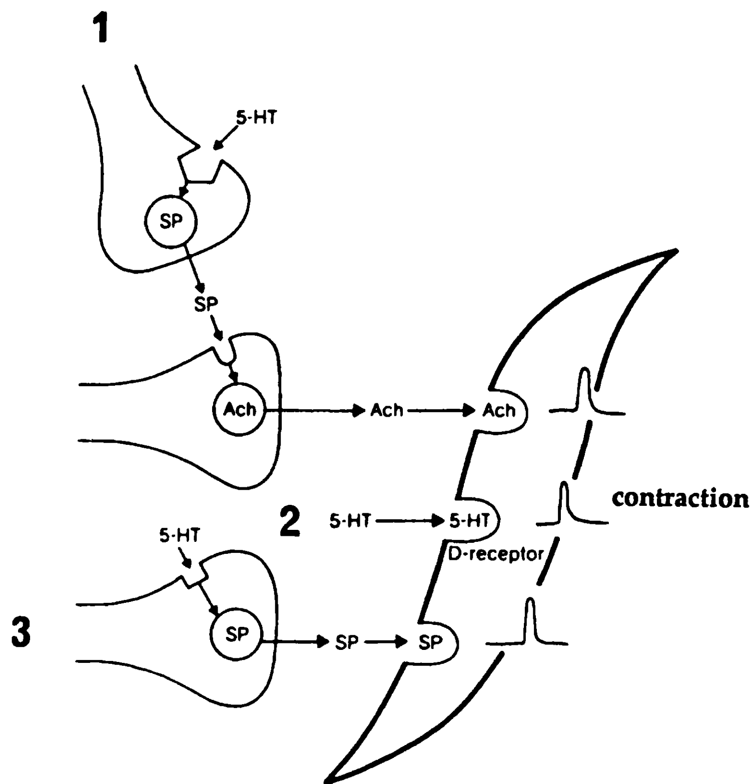


Figure 2.1 Schematic diagram showing the possible mechanisms underlying the contractile effect of 5-HT on the isolated guinea-pig ileum (adapted from Buchheit *et al.*, 1985). 5-HT is thought to induce its effects in this preparation either directly (2), by stimulating 5-HT receptors located on the smooth muscle (D-receptors), or indirectly (1 & 3), through the release of substance P (SP) and acetylcholine (ACh) secondary to the stimulation of neuronally located 5-HT receptors (M-receptors). In the present study the activity of neuroleptics and other compounds at neuronal 5-HT_3 receptors (3) were evaluated by adding atropine and methysergide to the bathing medium to block mechanisms 1 and 2 respectively.

suspended under a tension of 500mg for isotonic recording. The bathing medium also contained 100nM atropine, 100nM methysergide and 100nM diphenhydramine except when acetylcholine was used as the agonist, in which case, atropine was omitted. Agonists (2-methyl-5-HT, acetylcholine and substance P for $5\text{-}HT_3$, muscarinic and tachykinin receptors respectively) were applied directly to the bath and the contractions recorded on flatbed chart recorders (Servoscribe 1s RE 541). Non-cumulative concentration-response curves were constructed for each agonist with a 15 minute dosing cycle to prevent tissue desensitisation (with two bath wash-outs). For bath concentrations of 2-methyl-5-HT above 10μM, a dosing interval of 30 minutes was employed to prevent desensitisation and the bath was replenished with fresh bathing medium every 10 minutes. Measurements were made at the highest point of contraction and agonists were washed out as soon as the maximum response was observed. Neuroleptics and selective receptor antagonists (see chapter 4) were added, in increasing concentrations, to the reservoir containing Tyrodes, and were allowed to equilibrate with the ileum for 30 minutes before the concentration-response curves were repeated. Preliminary experiments revealed that each ileal segment could be used to record at least 4 concentration-response curves before a significant loss in the contractile response was observed. EC_{50} values (agonist concentration required to produce a 50% maximum contractile response) for each agonist, either alone, or in the presence of each antagonist, were determined graphically and pA_2 values were calculated from a Schild plot (see section 2.2.3).

2.2.2 Isolated rat vas-deferens

This preparation was used to quantitate the activity of typical and atypical neuroleptic drugs as antagonists at both α_1 and α_2 adrenoceptors. The activity of these compounds at α_1 adrenoceptors was assessed against the contractile effects of adrenaline whereas α_2 adrenoceptor activity was evaluated against the effects of clonidine on the field stimulated vas-deferens. Adrenaline was the preferred agonist at α_1 adrenoceptors, since unlike noradrenaline which is less potent in this preparation, it is not taken up by sympathetic nerve terminals. Propranolol (100nM) was added to bathing medium in order to counter any possible β-mediated effects of adrenaline.

Procedures

Male Sprague-Dawley rats (250-300g) bred in the Joint Animal House, University College London, were killed by cervical dislocation and both vas-deferens were removed and placed in an aerated (95% O_2 and 5% CO_2) Krebs-Henseleit solution (see Appendix II) where the muscles were stripped of superficial fascia and blood vessels. Experiments were conducted on 3cm lengths of prostatic vas-deferens tissue under a resting tension of 500mg which were mounted on perspex holders in 20ml organ baths containing Krebs-Henseleit solution maintained at 37°C. Adrenaline was added directly to the bath and the resulting isotonic contractions were displayed on a flatbed chart recorder (Servoscribe 1s RE 541). Non-cumulative concentration-response curves were constructed for adrenaline using a dosing cycle of 15 minutes and a tissue contact time of 1 minute. Thereafter, neuroleptics and selective receptor antagonists, were added to the bathing medium, in increasing concentrations, and were allowed to equilibrate with the tissue for 30 minutes before concentration-response curves were repeated. It was possible to record at least 4 concentration-response curves in each tissue before any significant reduction in response was observed. EC_{50} values (concentration of adrenaline required to produce a 50% maximum contractile response) were determined graphically and pA_2 values for each compound tested were calculated from a Schild plot of the data (see section 2.2.3).

Electrical field stimulation of the isolated vas-deferens was used to assess the α_2 adrenoceptor activity of neuroleptic drugs. The vas-deferens was placed between parallel platinum electrodes on a perspex holder (500mg tension) in 20ml organ baths which contained Krebs Henseleit solution (see Appendix II) maintained at 37°C. Following a one hour equilibration period electrical field stimulation was applied to the tissue using a Grass S48 stimulator (Grass Medical Instruments, U.S.A.) and the resulting isometric (Dynamometer, UF1 force transducer) contraction was recorded on a flatbed chart recorder (Servoscribe 1s RE 541). The stimulation parameters used were: train rate 1 per 33 seconds; train duration 500ms; pulse duration 2ms; pulse frequency 1Hz; pulse amplitude 60 volts. When a steady response to stimulation was obtained, clonidine was directly added to the bath in a cumulative manner, until at least a 90% inhibition of the field stimulated contraction was observed. Cumulative concentration-response curves were constructed for clonidine, either alone, or against increasing concentrations of neuroleptic compounds (listed above) which were added to the bathing medium. IC_{50} values (concentration of clonidine required to produce a 50% inhibition of the field

stimulated contractile response) were determined graphically and pA_2 values were calculated from a Schild plot of the data (see below).

2.2.3 Estimation of pA_2 values by Schild analysis

The pA_x scale (Schild, 1947) provides an empirical measure of drug antagonism. In its original form, the pA_x estimate was defined as:-

" the negative logarithm to base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose (x) of an active drug to that of a single dose "

The most widely employed multiple (or dose ratio) is 2. The pA_2 value can be calculated using the Schild equation (given below) which is based on the assumption that drug molecules (agonists and antagonists) compete for a common receptor site such that at equilibrium, the number of drug-receptor associations and dissociations are equal (Schild, 1949).

$$x-1 = K_B [B]$$

where: x is the dose ratio; K_B is the affinity constant for the combination of the antagonist with the receptor; [B] is the molar concentration of the antagonist.

The Schild equation predicts that a plot of logarithm (x-1) versus logarithm [B] should be linear with slope unity if the antagonism is competitive in nature. There is some confusion, however, whether the abscissa intercept, when the dose ratio is equal to 2, estimates the pA_2 or the log K_B (Jenkinson, 1991). These values are only identical when the slope of the Schild plot is exactly unity, which in practice is most unlikely. It could be argued that if the slope of the "best fit" line is not statistically different from unity then the antagonism is competitive and the abscissa intercept (x=2) estimates the pA_2. In the present work this was the method employed to estimate the pA_2 value. This was achieved by measuring dose ratios over as wide a range of antagonist (neuroleptic) concentrations as practicable.

2.3 GENERAL PROCEDURES *IN VIVO*

2.3.1 Animals and anaesthesia

Male Sprague-Dawley rats (250-300g) obtained either from the Joint Animal House, University College London, or from Charles Rivers, Kent, U.K., were used in these *in vivo* experiments. The animals were anaesthetised with halothane (Flurothane, ICI). The induction of anaesthesia was achieved by placing the rat in a covered perspex box which was supplied with 3% halothane in a carrier gas (95% O_2 and 5% CO_2) delivered at a flow rate of 500 ml/min (Fluotec 3' dispenser, Cyprane Ltd, U.K). The halothane-carrier gas mixture was delivered to the "induction" box via one arm of a small polythene Y-piece (Portex, 700/140/000, 2mm i.d., 4mm o.d.) and waste gases were vented to the outside through the other arm of the Y-piece. Following the induction of anaesthesia (3-5 minutes), the animal was placed with its dorsal surface on a heating blanket (Harvard homeothermic blanket, Harvard Apparatus Ltd., Kent, U.K.) and halothane (2%) was delivered at a flow rate of 500 ml/min via a face mask (excess halothane was drawn off via a connection to a tap-mounted vacuum suction pump). Surgery was not attempted until the animal was adequately anaesthetised which was assessed by general muscle flaggidity and the loss of both corneal and limb-withdrawal reflexes. A small flap of skin was reflected from the ventral neck region directly below the larynx and the longitudinal muscles surrounding the trachea were teased apart. A short length (2cm) of polythene tubing (Portex, PP220 100FT, 2.1mm o.d.), sealed within the Y-piece assembly described above, and cut obliquely at one end, was inserted into the trachea and tied in place. During maintenance anaesthesia the flow rate of the carrier gas was adjusted to 350 ml/min and the level of halothane reduced to 1.0-1.5%. For some experiments a 25 gauge needle was inserted into the peritoneum to allow i.p. drug administration (to avoid unnecessary artifacts during recording experiments) and a femoral vein was cannulated to allow intravenous drug administration. This cannula (Portex, 3FG, 0.75mm o.d.) was filled with normal saline and connected to a two-way stopcock (Baxter, K75B).

2.3.2 Stereotaxic procedures

The animal was placed in a stereotaxic frame (Kopf Small Animal Stereotaxic Model 900) with the head fixed in position with an ear bar in each external auditory meatus and the

snout secured within an upper incisor bar/nose-clamp assembly. The skin overlying the dorsal cranium was reflected away from the midline and any connective tissue was scraped away from the bone until the positions of lambda and bregma were apparent (see Fig.2.2). Any persistent bleeding either from the cranial surface or from the margins of the reflected skin was stopped by cauterisation. The position of the head was adjusted so that lambda and bregma were aligned in the same horizontal plane. This was normally achieved when the incisor bar was lowered approximately 3mm below the horizontal zero (interaural line). Stereotaxic brain coordinates were derived from the atlas of Paxinos & Watson (1982). Based on these coordinates (relative to bregma), holes were trephined (2.0mm o.d.) through the dorsal cranium (on either side of the midline), vertically above either the rostral caudate putamen (rostral 1.0-1.5mm; lateral 2.5-3.0mm; ventral 7.0mm), the nucleus accumbens (rostral 1.7-2.2mm; lateral 1.0-1.5mm; ventral 8.0mm) or the medial prefrontal cortex (rostral 2.5-3.0mm; lateral 0.5-1.0mm; ventral 5.5mm). The dura-mater was split using a 25 gauge needle and care was taken to avoid disrupting the cerebral blood vessels.

2.3.3 EEG, ECG, heart rate and temperature monitoring

The EEG was occasionally monitored to assess the condition of the animal. This was achieved by securing two screw-electrodes (0.75mm o.d) in the dorsal cranium such that their tips rested on the surface of the underlying parietal cortex. The signal from the screw-electrodes was amplified (Devices), viewed on an oscilloscope (Telequipment D63) and displayed on a pen chart recorder (Washington, Kent, U.K.) (see Fig.2.3).

The ECG was periodically recorded to assess both the cardio-respiratory function of the animal during the experiment and the effects of some drugs on the ECG and heart rate. This was achieved by placing two needle (23 gauge) electrodes beneath the skin of the chest wall. The signal from the electrodes was amplified (Devices) and fed to an audio-monitor (Devices Audio Unit 4010) which enabled concurrent monitoring of the heart beat and the respiration rate. The output from the audio-monitor was fed to an oscilloscope (Telequipment D63) and displayed on a pen chart recorder (Washington 400 MD/2, Kent, U.K.). The core body temperature of the animal was maintained at 37°C using a homeothermic heating blanket (Harvard Apparatus Ltd, U.K.) in conjunction with a rectal probe.

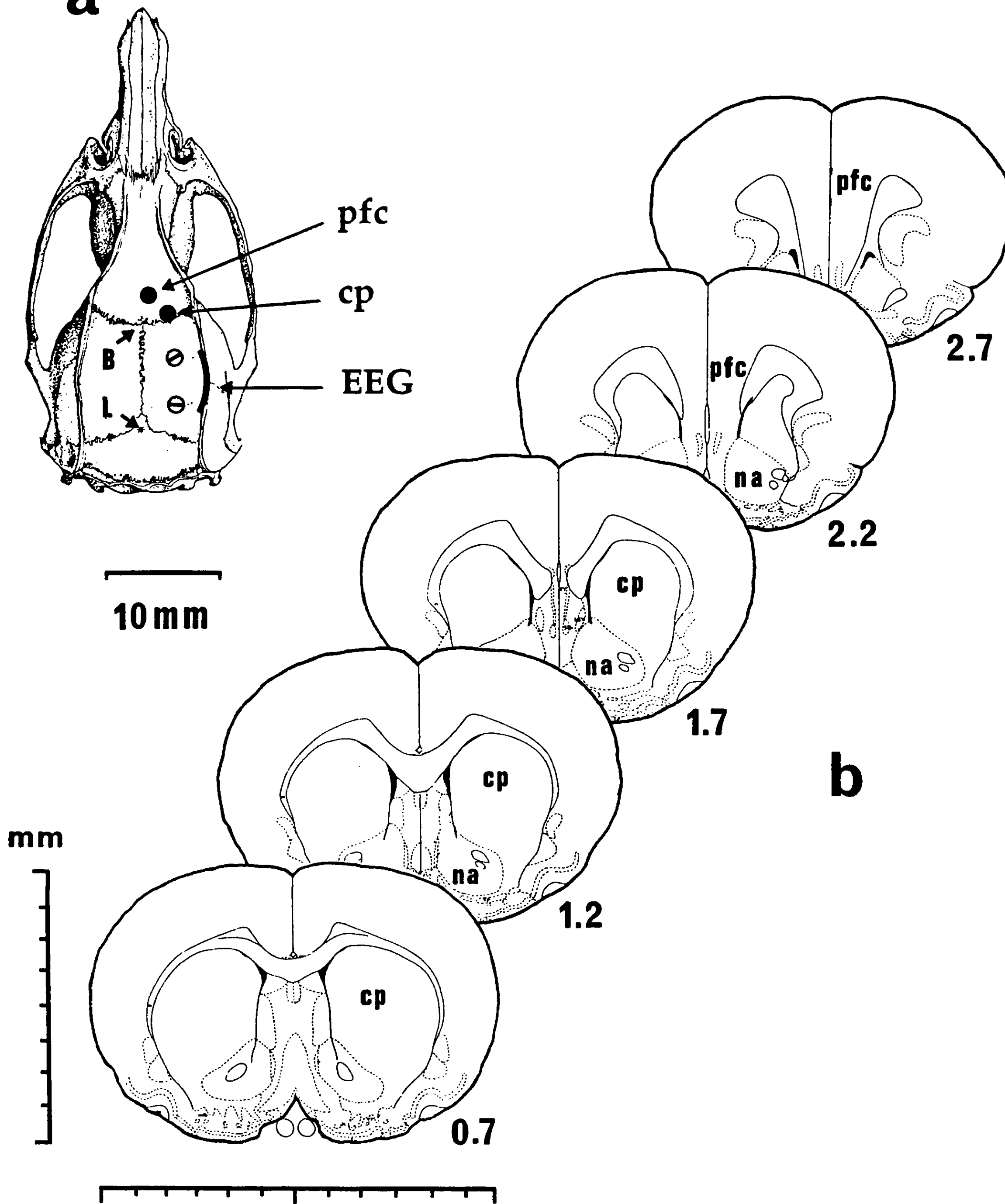


Figure 2.2 Dorsal view of the rat skull showing the positions of the drill sites overlying the caudate putamen (cp) and medial prefrontal cortex (pfc) as well as bregma (B), lambda (L) and EEG screw electrodes located on the surface of the parietal cortex (a). Coronal sections of the rat brain showing the locations of the rostral caudate putamen, nucleus accumbens (na) and medial prefrontal cortex are given in (b). The figures to the right of each section are rostral stereotaxic coordinates (mm) relative to bregma (adapted from Paxinos & Watson, 1982).

2.4 MONITORING OF SPONTANEOUSLY ACTIVE NEURONS IN THE ROSTRAL CAUDATE PUTAMEN AND MEDIAL PREFRONTAL CORTEX

The spontaneous activity of neurons in the rostral caudate putaman and medial prefrontal cortex was monitored using standard extracellular recording techniques. Recordings were generally made from single neurons, but periodically and especially in the caudate putamen, as many as three active neurons were encountered at any given recording site. Either glass-coated tungsten microelectrodes (platinum tipped; length 20-25μm; impedance 5-10MΩ) made by Mr A. Ainsworth, Department of Anatomy, University College London (see Merril & Ainsworth, 1972), or carbon-fibre microelectrodes (Graffex, PD Systems, Surrey, U.K.) were employed. The glass-coated electrodes were adapted to fit tightly within the lumen of a 25 gauge needle. The needle-electrode assembly was mounted vertically on a perspex block attached to a micromanipulator on the stereotaxic frame. The carbon-fibre microelectrodes were glued inside a 1ml syringe barrel which was positioned vertically on the micromanipulator. The electrode assembly was connected to the headstage (Neurolog NL100, Digitimer Ltd., Welwyn Garden City, U.K.) by a short length of unscreened cable. A 19 gauge needle was inserted beneath the skin of the dorsal cervical region of the animal which was grounded to the stereotaxic frame by an unscreened cable. To reduce electrical interference from ambient radiation the stereotaxic frame and headstage were placed inside an earthed Faraday cage. Differential recording (see Fig.2.3) was performed between channel A (connected to the recording electrode) and channel B (grounded by a short length of unscreened cable to the stereotaxic frame) of the headstage (NL100) and following a 5k amplification (NL104), the signal was filtered with a bandpass of 1000Hz (-3dB), 2.5kHz (-3dB) (NL125). The amplified and filtered signal was fed to a gated amplitude discriminator (Digitimer D130) (set to give a standard impulse whenever the spike amplitude exceeded a set threshold) and viewed on an oscilloscope (Tektronix 5A18N). If the action potential was predominantly negative-going (see chapter 3), a more reliable spike discrimination was achieved by inverting the signal at the amplifier (NL104). The absolute noise level of the glass-coated tungsten and carbon-fibre microelectrodes *in situ* were estimated to be 15-20μV and 25-40μV respectively. The cumulative spike count was fed to a flatbed chart recorder (Servoscribe 1s, RE 541) in the form of voltage ramps which was reset every ten seconds and the cumulative spike count was numerically recorded on an alpha-numeric printer (IPP 144-40, ITT Instruments) connected to the spike processor. The output from the spike

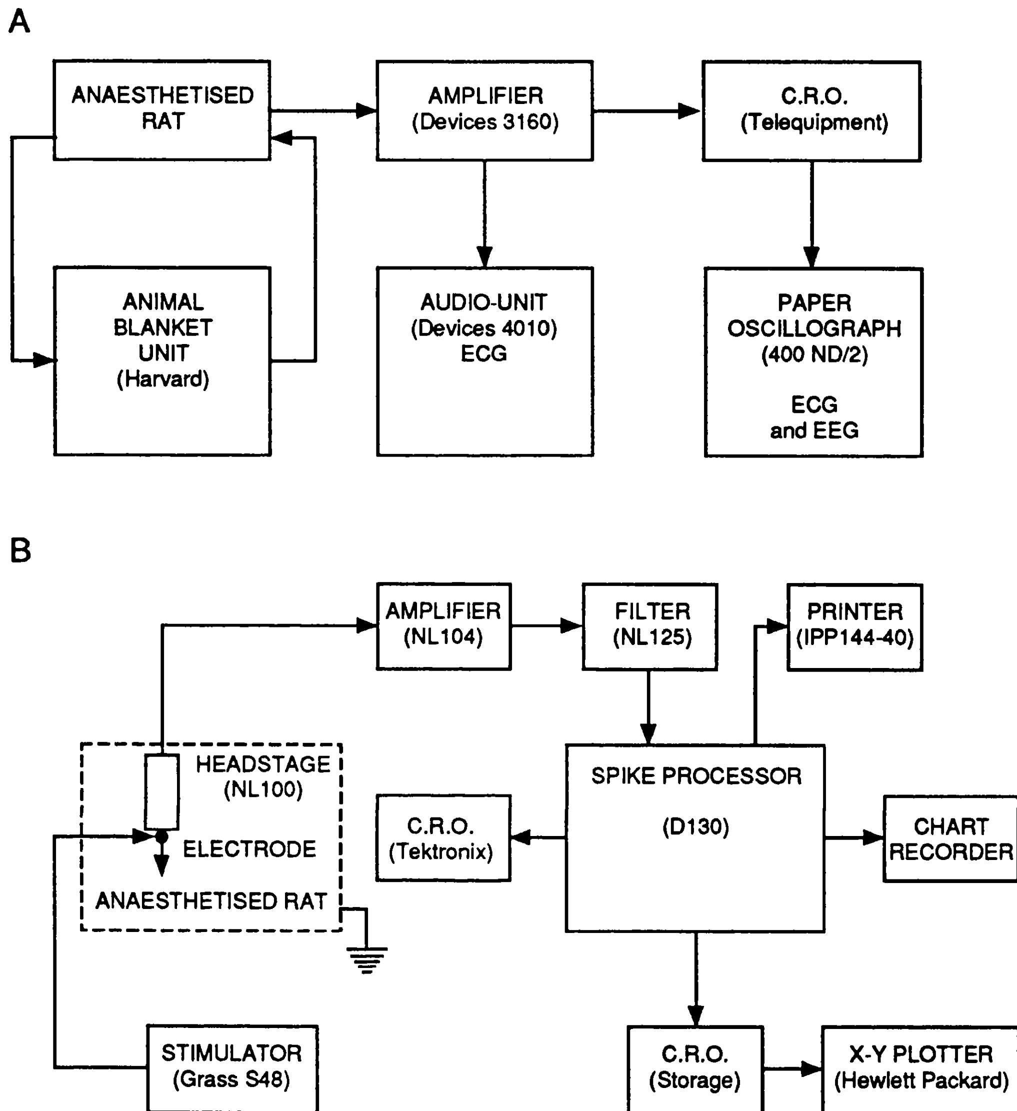


Figure 2.3 Schematic representation of the apparatus used for the recording and analysis of the ECG, EEG and spontaneous activity of neurons in the rostral caudate putamen and medial prefrontal cortex (C.R.O; oscilloscope).

processor was also fed to a Racal tape recorder (Racal Recorders Ltd; Hythe, U.K.) and subsequently displayed on a storage oscilloscope (OX7520, ITT Instruments). Photographs of stored images (multiple or single action potentials) were taken with an oscilloscope camera (Shackman 7000) using polaroid 667 film. Alternatively, stored waveforms were displayed on a flatbed X-Y plotter (Hewlett Packard, 7475A). The spike counting apparatus described above was periodically calibrated with a square pulse signal (Grass S48 stimulator, Grass Medical Instruments, U.S.A.) fed simultaneously to the spike processor and an oscilloscope (Tektronix 5A18N). A typical calibration graph with voltage ramp histograms are given in Fig.2.4.

2.4.1 Attempts to facilitate activity using microdialysis

In some preliminary experiments attempts were made to enhance the level of activity in the caudate putamen using microdialysis to deliver excitatory substances to the recording site (see section 2.1). This was achieved by implanting a concentric-designed microdialysis probe (Fig.2.5) into the caudate putamen, perfused at 5.0 µl/min with artificial CSF (see Appendix III: composition I), and positioning a microelectrode proximal (<500 µm) to the dialysis membrane surface. Recording experiments were commenced three hours after surgery. Baseline firing was monitored for 10-15 minutes before pulsing (see below) various substances (bicuculline, picrotoxin, leptazol, glutamic acid, aspartic acid) through the dialysis probe. In other experiments, directly acting (dopamine, CGS 15855A) and indirectly acting (amphetamine and 2-methyl-5HT) dopamine receptor agonists were locally applied in the caudate putamen and their effects on neuronal firing evaluated.

2.4.2 Effects of systemic apomorphine and neuroleptics on neuronal activity

In these experiments the effects of the non-selective dopamine receptor agonist apomorphine hydrochloride (25-500µg/kg i.v.), as well as the effects of the neuroleptics haloperidol (0.5mg/kg, i.p.) and clozapine (20mg/kg, i.p.), were evaluated on the spontaneous discharge of neurons in the caudate putamen and medial prefrontal cortex. In studies on the caudate putamen, apomorphine was tested either alone in increasing doses administered every 40 minutes, or, 40 minutes after the administration of either haloperidol or clozapine. In other experiments, the effects of haloperidol and clozapine (doses listed above), given alone, were tested on the spontaneous firing of neurons in the

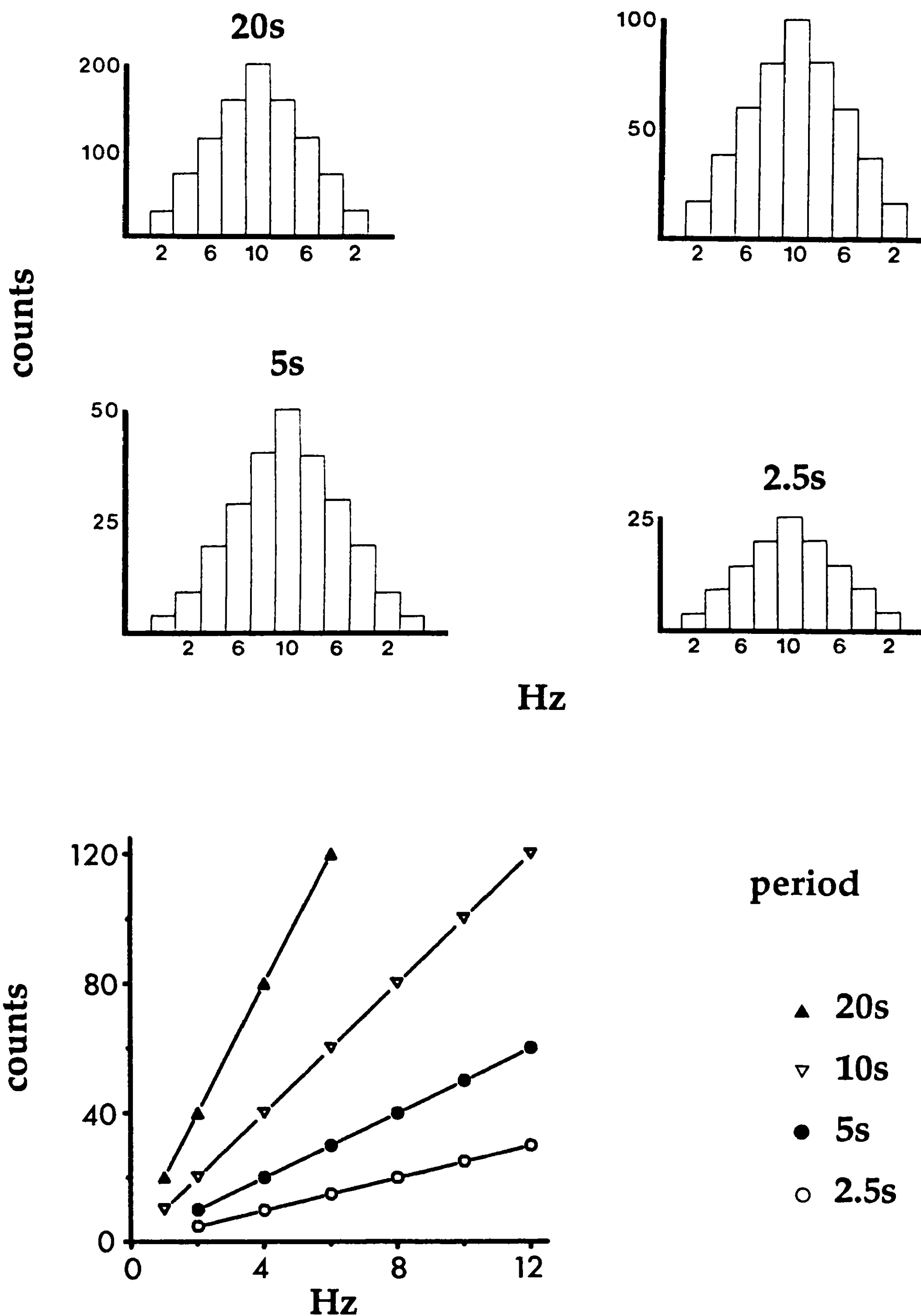


Figure 2.4 Calibration of the spike counting apparatus (Digitimer D130). A square pulse signal (1V amplitude and 1ms duration), generated by a Grass stimulator (S48), was fed concurrently, at 2, 4, 6, 8 and 10 Hz in ascending and descending order, to the D130 ('store' mode) and an oscilloscope. The cumulative pulse counts over 2.5, 5.0, 10 and 20 second periods are displayed as voltage ramp histograms (upper graphs) and plotted for linearity in the lower graph ($r^2 > 0.999$ for all counting durations).

caudate putamen and the medial prefrontal cortex over about 2 hours. Since in the cortex, apomorphine produced a variety of effects on neuronal activity in the cortex (inhibition, facilitation and no change), compared with only inhibition in the caudate putamen, it was necessary to study the effects of apomorphine before and after either haloperidol or clozapine on the same neuron in this area. The cumulative spike counts over 10 seconds were recorded numerically and displayed as cumulative rate histograms. The spike counts were averaged over five minute time intervals and the response to apomorphine was measured as the maximum percentage change in the frequency of firing (over 5-10 minutes after drug administration) from basal (control) neuronal activity recorded for 10-15 minutes before drug administration.

The doses of haloperidol and clozapine employed in these studies were carefully chosen on the basis of relative clinical potency. The daily doses of these agents which are normally required to treat schizophrenia are reported to be 5-20 mg/day (haloperidol) (see Van Putten, Marder, Mintz & Poland, 1989; Rama Roa, Bailey, Bishop & Coppen, 1981) and 200-600 mg/day (clozapine) (see Liebermann, Johns, Cooper, Pollack & Kane, 1989; Kane, Honigfeld, Singer & Meltzer, 1988; Clozaril prescribing information, Sandoz Pharmaceuticals, U.K.). These figures suggest that clozapine is about 40 fold less potent than haloperidol. Given this information and since in rodents, 0.5mg/kg is a widely employed dose of haloperidol (Imperato & Di Chiara, 1985; Skirboll & Bunney, 1979; Stamford, Kruk & Millar, 1988; Zetterstrom, Sharp & Ungerstedt, 1985; Bacopoulos & Roth, 1981; Hernandez & Hoebel, 1989), a dose of 20mg/kg of clozapine was employed in the present work.

2.5 INTRACEREBRAL MICRODIALYSIS

A concentric designed microdialysis probe was employed in these experiments (see Fig.2.5). In essence the probe consists of a hollow fibre dialysis membrane (300-340μm o.d.) pulled over two glass micropipettes. The perfusion fluid (see Appendix III) is pumped at a slow and constant rate (2.0-5.0μl/min) through one pipette and the dialysate is collected through the adjacent pipette. To accommodate the smaller dorso-ventral dimensions of the nucleus accumbens compared to either the caudate putamen or the medial prefrontal cortex the conducting membrane length of the accumbal dialysis probes was considerably shortened (see Fig.2.5). Other variations of the basic probe design included the use of two different hollow fibre dialysis membranes (acrylic copolymer and cellulose).

2.5.1 Microdialysis probe construction

Glass micropipettes (borosilicate 2.0mm o.d., Jencons Scientific Ltd, U.K.) were pulled under gravity using a glass pipette puller (Levick). The glass pipettes were secured in a 3D micropositioner and broken back to an outer tip diameter of 90μm using a small heated glass bead (Corning 7570) under a standard binocular microscope with an eye-piece graticule. The tapered tips (about 15mm in length) of some pipettes (see Fig.2.5) were bent at an oblique angle (about 45°) by heating the tips within a coil of platinum wire connected to a 6V step-down transformer. Thereafter, the pipette tips were separated from the pipette shaft using a diamond-head glass cutter and glued (Epoxy-adhesive, RS Components Ltd, U.K.) onto long 25 gauge needles (bevels removed). The pipettes (one straight and one bent) were mounted and glued onto a 26 gauge aluminum template (see Fig.2.5) under a stereo-dissecting microscope. Lengths (2mm for accumbal probes; 5mm for striatal and cortical probes) of hollow fibre dialysis membrane (vitafiber, o.d. 0.34mm, Amicon, U.K.; cuprophan, o.d. 0.30 mm, Medicell, U.K.) were pulled over the glass pipettes and sealed with a small quantity of glue.

2.5.2 General microdialysis procedures

The probes were tested for patency and any air-bubbles removed by increasing the flow rate to 10μl/min and applying suction (20ml syringe) to the outlet port. Before using the

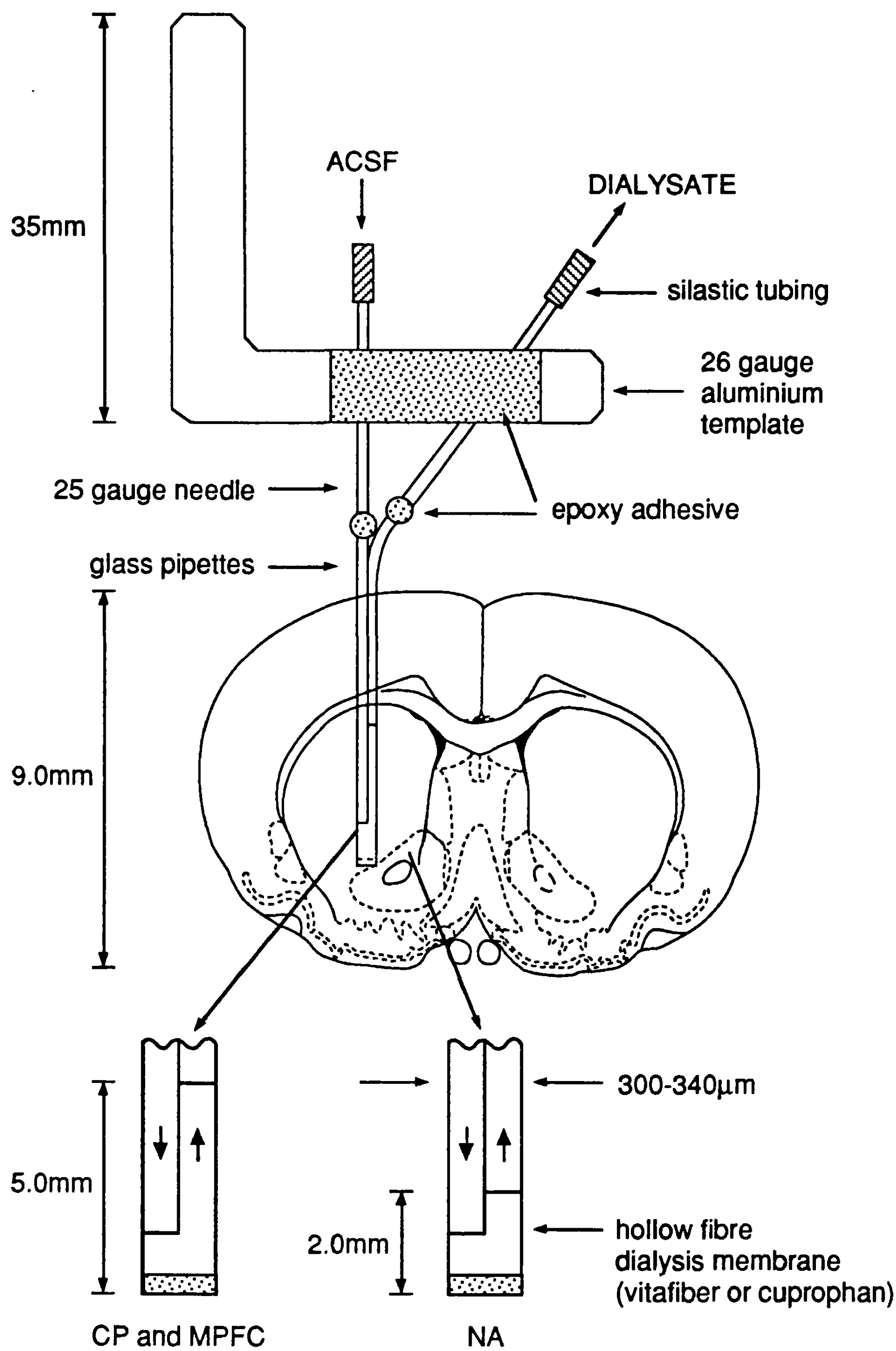


Figure 2.5 Schematic diagram (not to scale) showing the construction and location of a microdialysis probe within the caudate putamen of the rat brain (0.7mm forward of bregma). The tip of the dialysis probe has been enlarged in the lower diagrams to reveal the dimensions and construction of probes used in either the caudate putamen (CP) and medial prefrontal cortex (MPFC) or the nucleus accumbens (NA).

dialysis probes *in vivo*, the probes were calibrated *in vitro* for catecholamine recovery (see below). Microdialysis probes were slowly lowered (over 3-4 minutes) into either the caudate putamen, nucleus accumbens or medial prefrontal cortex and perfused at low flow rates (202U/AA cassette pump, Watson-Marlow Ltd., U.K.) with artificial CSF. Various drug solutions (in artificial CSF) were perfused through the dialysis probes using a 3-way switching valve (made by Mr F. Ballhatchet, Department of Pharmacology, University College London). Apart from a few studies (see below), intracerebral dialysis experiments were commenced 3 hours after probe insertion to give a necessary recovery period. The dialysates were collected every 20 minutes into pre-weighed acidified (0.1M $HClO_4$) tubes, "on-ice", and analysed for their monoamine content (see section 2.7), either on the same day, or within 2 days of the experiment (samples stored at -70°C). The sample tubes were weighed immediately after the collection of the dialysate to ensure the desired flow rate was maintained. The efflux levels of neurochemicals were either expressed in terms of absolute quantities appearing in the dialysate (pmoles/20 minutes) or more generally as a percentage of the basal efflux of each substance (2-3 hours after implanting the dialysis probe).

2.5.3 Calculation of absolute and relative recoveries

Prior to using the microdialysis probe *in vivo*, each probe was calibrated for catecholamine recovery *in vitro*. Probes were immersed in standard solutions of dopamine, DOPAC, HVA and 5-HIAA (all 1μM in artificial CSF containing 8.5mg/L l-ascorbic acid and maintained at 37°C). After a 30 minute equilibration period, the performance of the probes was assessed by measuring the absolute and relative recovery of these substances. The absolute recovery was calculated as the amount of substance in the outflow solution per unit time (pmoles/20 minutes). The relative recovery was calculated as the ratio between the concentration of a substance in the outflow solution and the concentration of the same substance surrounding the probe (expressed as a percentage).

2.5.4 Estimation of potassium efflux during microdialysis

In preliminary studies it was considered necessary to assess the extent of neuronal damage during microdialysis. Although direct neuronal trauma occurs during probe

implantation (see Benveniste, 1989) it is not known what damage results from dialysis experiments conducted over many hours. One approach employed to clarify this uncertainty was to measure the efflux of potassium in dialysates of the caudate putamen during microdialysis. Local insult to CNS neurons is known to elicit a spreading depression (Leao, 1944) which is characterised by a large rise in the extracellular level of potassium with corresponding falls in the extracellular levels of sodium, calcium and chloride (Hansen & Zeuthen, 1981). In the present study microdialysis probes were implanted in the rostral caudate putamen and perfused with potassium-free artificial CSF (tonicity of the perfusion medium was maintained with additional NaCl). Potassium was estimated in the brain dialysates using flame photometry (Corning 400 flame photometer, Corning Ltd, U.K.). Dialysates were collected every 10 minutes and diluted with artificial CSF to provide sufficient sample volume for analysis (0.5ml). The instrument was zeroed during aspiration of artificial CSF and the linear range of the assay was determined to be 0.01-2.0mM.

2.5.5 Calcium and activity-dependency of dopamine release

To test whether the dopamine content appearing in brain dialysates of the caudate putamen was neurogenic in origin, either calcium was removed from the perfusion medium (tonicity maintained with additional NaCl), or in separate studies, tetrodotoxin (1µmol/l) was added to the perfusion medium. These studies were commenced at least three hours after the implantation of the dialysis probe.

2.5.6 Administration of drugs by microdialysis

Substances were locally administered to various brain areas using microdialysis (see chapters 3 & 8). A 3-way switching valve (see above) was employed to perfuse these agents through the dialysis probe. Apart from 2-methyl-5-HT, where a small quantity of l-ascorbic acid (8.5mg/L) was added to the perfusion medium, all drugs were dissolved directly in artificial CSF. In some studies *in vitro*, the release of dopamine and 2-methyl-5-HT from dialysis probes was measured. The medium (artificial CSF) in which the probes were immersed (1.0-1.5 ml) was analysed for dopamine and 2-methyl-5-HT content following their perfusion. Various flow-rates and perfusion concentrations were employed in these studies.

2.6 VERIFICATION OF ELECTRODE AND DIALYSIS PROBE PLACEMENT

Periodically at the end of representative experiments, the brain was removed, sectioned, and microscopically examined to establish the position of the recording electrode or the microdialysis probe. A small electrolytic lesion was produced at the electrode tip by the passage of a d.c current (20-50μA for 45 seconds; monitored by an Avometer). This was achieved by applying a constant voltage (10-25V) across the electrode assembly using a Grass S48 stimulator.

The animal was deeply anaesthetised with halothane (3%), the external jugular veins cut, and the left ventricle slowly perfused with normal saline (40ml). In order to remove the brain the occipital muscles were cut from the occipital bone to expose the foramen magnum. The cranium overlying the hindbrain was cut away (as far forward as lambda) taking care not to damage the underlying cortex. The bone covering the olfactory bulbs was crushed with a large pair of forceps allowing the remaining cranial bone to be teased away from the dura mater. Using a small spatula, and by cutting the optic and trigeminal nerves, the brain was removed from the cranial vault and placed in 10% formol-saline.

The brain was sectioned after at least 4 days of formaldehyde fixation. A single vertical cut was made through the midbrain (McIlwain tissue chopper) and the remaining forebrain tissue block washed in running water for 24 hours. The tissue was infiltrated with 10% gelatin and 1% glycerol at 40°C for about 12 hours before being re-immersed in formol-saline (10%). The cut surface of the tissue was placed on the stage of a freezing microtome (Leitz) and sections 50-100μm thick were cut in the coronal plane. Following a rinse in water, the sections were dehydrated and defatted in absolute ethanol (1 hour), before being stained with cresyl-fast violet (diluted 1:1). The sections were then rinsed in water, dehydrated in ethanol for 1 minute and immersed in beechwood creosote to flatten and clear the sections. The sections were mounted on glass slides, blotted with filter paper (Whatman), and rinsed in either xylene or histoclear to remove the beechwood creosote. A cover-slip was placed over the sections and fixed in place with DPX (standard histological mounting agent). All sections were examined under a low-power binocular microscope (Zeiss) for the presence of electrolytic lesions and localised tissue damage and photographed (Ecktachrome 64T film) using a 35mm camera (Zeiss MC63) mounted vertically over the illuminated stage of the microscope.

2.7 DETECTION AND ESTIMATION OF CATECHOLAMINES AND INDOLAMINES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

The estimation of catecholamines and indolamines in brain dialysates was carried out using reverse-phase chromatography with electrochemical detection (see Fig.2.6).

2.7.1 Basic apparatus

The mobile phase (see below) was delivered at 1.5 ml/min through an in-line degasser (Erma, ERC 3510) and 0.2µm filter assembly (Whatman, cat.no:-6725 5002) by an isocratic pump (SA 6410C, Severn Analytical) connected to a pulse damper (Severn Analytical). A conditioning guard cell (5020, ESA) with an in-line filter assembly (2µm graphite, ESA) was connected between the pump and sample injection station and set to an oxidising potential of 500mV (Coulochem Model 5100A, ESA). The injection of samples (dialysates and standards) onto the analytical column (Spherisorb 5ODS, 25cm x 4.6mm i.d., HPLC Technology: ambient laboratory temperature maintained at 20-22°C) via a low-volume pre-column (Upchurch, C-130-B, 2mm i.d. x 2cm, 0.5µm & 2.0µm frits, Perisorb RP-18 30-40µm pellicular) was controlled by a Kontron autosampler (either a Kontron MSI 660 with 20-50µl sample loop or a Kontron 465, 1µl-80µl injection volume) fitted with an ethylene glycol (20%) cooling system. Amines were oxidised and detected at a high sensitivity analytical cell (5011, ESA) connected with an in-line filter assembly (0.2µm graphite, ESA) at working potentals of 20mV (D1) and 300mV (D2) (Coulochem Model 5100A, ESA) and the results analysed on an integrator (Spectra-Physics, SP4400). The low oxidising potential set at D1 (20mV) was employed to reduce the "noise" contribution from each sample and so enhance the signal-to-noise ratio. The mobile phase was continuously recirculated through the system for at least 1 week or until the background current from D2 became unacceptably high (> 0.1µA).

2.7.2 Mobile phase

A citric acid-phosphate buffer mobile phase was employed to produce optimal separation of catecholamines (l-dopa, dopamine, DOPAC and HVA) and indolamines (5-HIAA and 2-methyl-5-HT). The mobile phase contained acetonitrile (2%) as well as (mmol/l): citric acid (60); di-sodium hydrogen phosphate (40); sodium heptane sulphonate (1.0); di-

sodium ethylenediamine-tetra-acetic acid (0.054). This was adjusted to pH 2.60 with perchloric acid and filtered (Ultipor-N66 0.1μm, Pall). All materials used in the preparation of the mobile phase were of the highest grade obtainable (AnalaR and AristaR).

2.7.3 Assay sensitivity, calibration and sample analysis

Since the extracellular concentration of dopamine is very low in some brain areas (nucleus accumbens and medial prefrontal cortex), and since the recovery of dopamine using dialysis is at best only 10%, it was important to optimise the assay sufficiently to detect at least 20 to 50 fmoles of dopamine. In practice this was achieved using the system in an oxidative-screen mode where an oxidising potential was applied to both the guard cell and the analytical cell (see above). Other options were tried such as applying equal but opposite working potentials (oxidising and reducing) to the two analytical electrodes (differential mode) and combining their absolute current outputs. Although an increased signal was obtained using this approach the detection limit was not significantly enhanced since the absolute noise was also increased. Using a detector gain of between 2000 and 5000 it was possible, using the oxidative-screen mode, to routinally achieve a detection limit of about 25 fmoles on column (exceptionally 10 fmoles). A lower gain (500) was employed when striatal dialysates were analysed to avoid saturation of the detector output during the elution of DOPAC and HVA which were present in considerably greater quantities than dopamine. The detection limits of DOPAC, HVA and 5-HIAA were routinally about 50-100 fmoles on column. The observation that 2-methyl-5-HT could be analysed in the same chromatograph as other monoamines was exploited in some experiments (see chapter 8). It did, however, have an inconveniently long elution time of 35-40 minutes. This potential problem was avoided by injecting samples about every 20 minutes so that 2-methyl-5-HT eluted between DOPAC and 5-HIAA in the next sample.

Individual monoamine stock solutions (10mM) were prepared from crystalline material (see Appendix I) dissolved in 0.1M perchloric acid and stored at -70°C for up to 1 week. Working standard solutions containing mixtures of the monoamines were prepared by diluting the stock solution in artificial CSF (containing 0.1M perchloric acid) to give a final injection amount (pmoles on column) of: dopamine (0.5-1); DOPAC (5-10); 5-HIAA

(5-10); HVA (5-10). In a few experiments l-dopa was also analysed (see chapter 8) which was calibrated with 5-10 pmole standard solutions. The system was calibrated (peak area) after the response to repeated injections of standards was stable (see Fig.2.7 and Fig.2.8).

Prior stability studies revealed that the monoamines in the brain dialysates were stable over a period sufficient to enable their analysis to be automated. Dialysates were routinally run in batches of 20-30 and were analysed either on the same day, or within 2 days of the experiment (samples stored at -70°C).

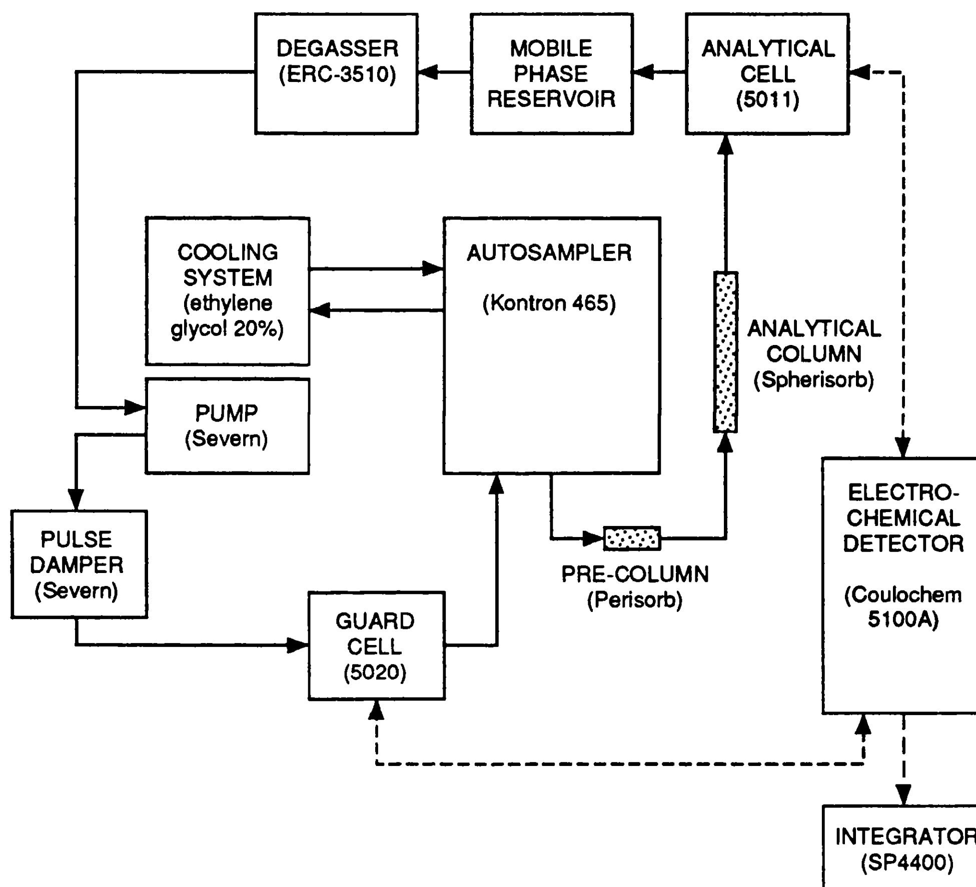


Figure 2.6 Schematic representation of the apparatus used in the separation and detection of monoamines. The solid and dashed lines indicate flow and electrical connections respectively.

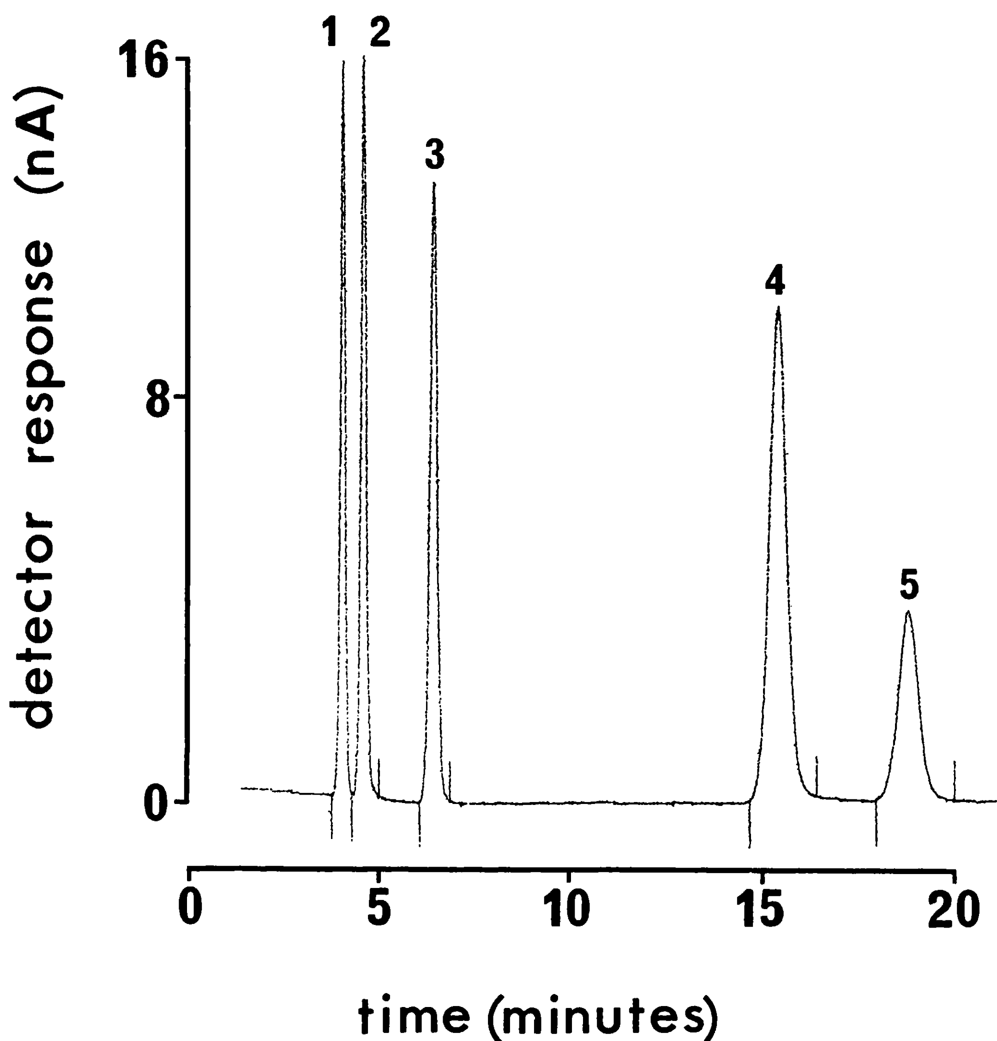


Figure 2.7 Chromatogram of a standard solution of the following monoamines (pmoles): 1, l-dopa (10); 2, dopamine (10); 3, DOPAC (10); 4, 5-HIAA (20); 5, HVA (10). Separation was achieved on a Spherisorb analytical column (5ODS, 25cm x 4.6 mm i.d.) using the mobile phase described in section 2.7.2 pumped at 1.5 ml/min. The gain used on the electrochemical detector was 500 with applied potentials of 20mV (D1) and 300mV (D2).

2.8 DETECTION AND ESTIMATION OF APOMORPHINE IN DIALYSATES OF THE RAT CAUDATE PUTAMEN USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

In some experiments (see chapter 7) apomorphine was estimated in striatal dialysates following its systemic administration. This was achieved using a high pressure liquid chromatography assay coupled with electrochemical detection.

2.8.1 Basic apparatus

The mobile phase (see below) was delivered at 2.0 ml/min through an in-line 0.2µm filter assembly (Whatman, cat.no:- 6725 5002) by an isocratic pump (Kontron 420). Samples (dialysates and standards) were injected (Rheodyne 7125, 50µl sample loop) onto the analytical column (Spherisorb S5CN-3677, 25cm x 4.6mm i.d., Anachem) and detected at a potential of 800mV (LCA15, EDT Research Ltd., 30nA fsd). Sample chromatographs were either displayed on a flatbed chart recorder (Servoscribe 1S, RE 541) or on an integrator (Spectra-Physics SP4400).

2.8.2 Mobile phase

The composition of mobile phase which achieved an optimal separation of apomorphine from the solvent front contained 11% acetonitrile, 0.15M KH_2PO_4 and 0.3mM disodium ethylenediamine-tetra-acetic acid, adjusted to pH 3.0 with phosphoric acid (diluted 1:5 with distilled deionised water). The mobile phase was filtered (Ultipor-N66, 0.1µm, Pall) prior to use.

2.8.3 Assay calibration and sample analysis

Working standard solutions of apomorphine (0.25-10 pmoles) were freshly prepared each day in artificial CSF (acidified with 0.1M HCl). Standards were injected repeatedly until a stable response was obtained. Under the conditions employed apomorphine eluted between three and four minutes and the detection limit of the assay was estimated to be approximately 0.25 pmoles. In order to facilitate the detection of apomorphine in brain dialysates it was necessary to inject the entire volume of sample collected (65µl).

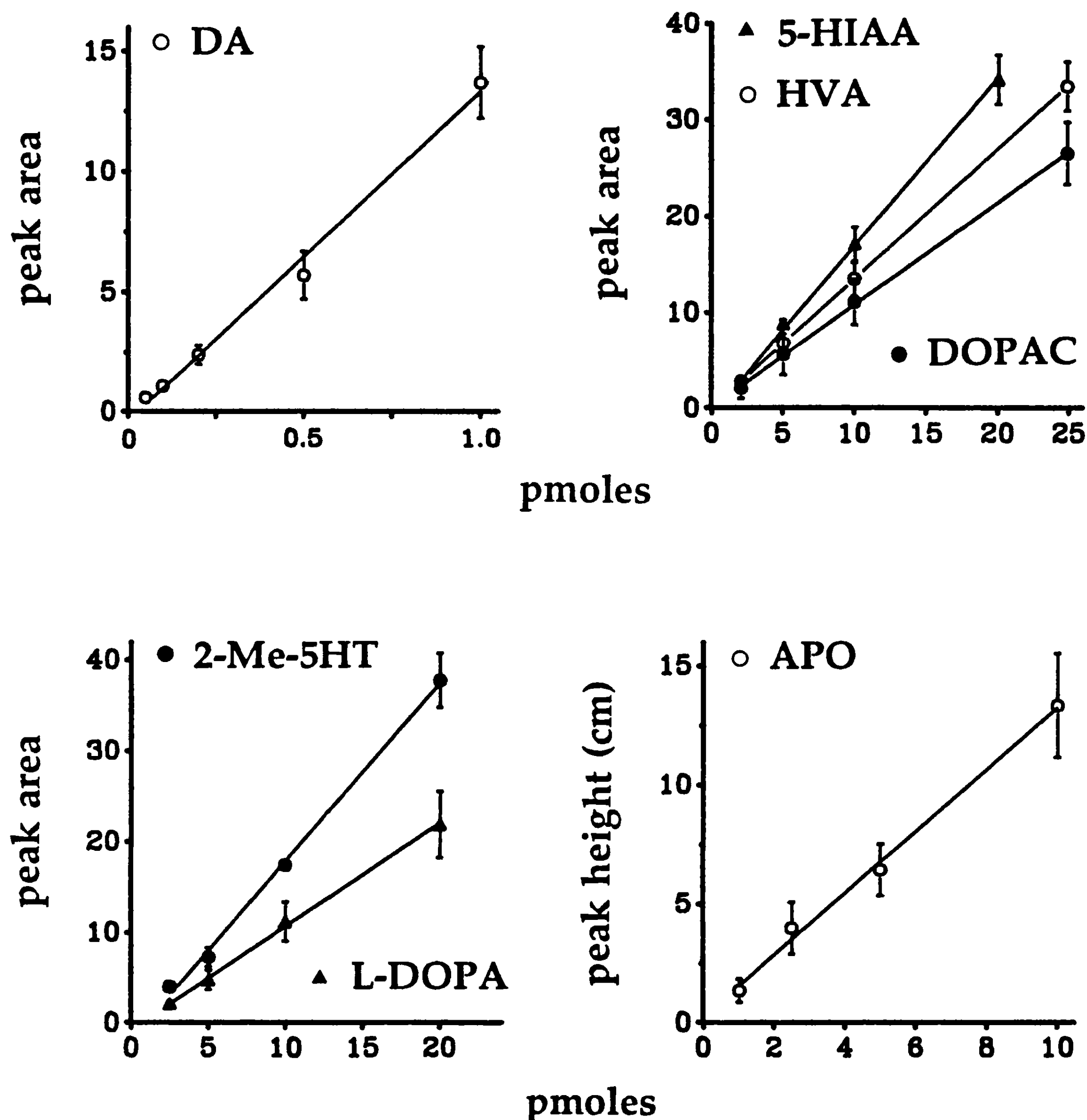


Figure 2.8 Calibration curves showing linearity of detector output (see Table 2.1) in response to standard solutions (pmoles on column) of monoamines (see section 2.7.3) and apomorphine (APO) (see section 2.8.3). The response (± s.e.mean, n=4-5) was measured in terms of either peak area (arbitrary units) for the monoamines or peak height (cm) for apomorphine (50mV fsd). A detector gain of 5000 was employed in the detection of dopamine (DA) (500 for the remaining monoamines).

compound	Regression equation*	Coefficient of determination (r^2)
dopamine	y = 1.37x - 0.37	0.9964
DOPAC	y = 1.05x + 0.22	0.9998
HVA	y = 1.34x - 0.01	0.9999
5-HIAA	y = 1.74x - 0.51	0.9994
L-Dopa	y = 1.14x - 0.66	0.9993
2-Me-5-HT	y = 1.96x - 1.74	0.9988
apomorphine	y = 1.30x + 0.29	0.9976

Table 2.1 Relationship between the concentration of monoamines and apomorphine with peak area and peak height respectively (see Fig.2.8). * regression equation in the form y = mx + c, where y is the peak area or peak height, m is the slope, x is the concentration (pmoles/50µl) and c is the y intercept.

CHAPTER 3

DEVELOPMENT AND VALIDATION OF IN-VIVO TECHNIQUES: INTRACEREBRAL MICRODIALYSIS AND EXTRACELLULAR NEURONAL RECORDING

3.1 INTRODUCTION

In chapter 2 the considerations which led to the adoption of the specific techniques used in this thesis were outlined. This chapter presents the results of preliminary experiments designed to validate the techniques of intracerebral microdialysis and extracellular neuron recording. In some of these studies attempts were made to combine these procedures in order to enhance the activity of neurons in the rostral caudate putamen. Although the results from these experiments were encouraging the procedure as a whole lacked flexibility and was not employed in the subsequent studies detailed in this thesis.

The most widely used method to assess the performance of microdialysis probes is to measure their ability to recover substances of interest *in-vitro*. This procedure was routinely employed to ensure an adequate recovery of dopamine, DOPAC and HVA. The effects of perfusion flow rate as well as the nature of the dialysis membrane employed (acrylic or cellulose) on the recovery of these catecholamines are summarised in this chapter. Apart from monitoring the extracellular levels of substances, a further application of microdialysis, and one which is used often in this thesis, is the administration of substances to discrete brain areas. In this chapter this approach was used and calibrated *in-vitro* by estimating the amount of dopamine released from dialysis probes following its inclusion in the perfusion medium (see also chapter 8). In subsequent experiments this procedure was used to test the effects of various agents on the firing of striatal neurons.

In this chapter a novel method for assessing the extent of neuronal trauma incurred during the acute phase of cerebral dialysis is described. Potassium is predominantly an intracellular ion and so trauma to central neurons is known to be associated with an enhanced extracellular level of potassium (see section 2.5.4). It was therefore assumed that by measuring its efflux in striatal dialysates it might be possible to provide an indication not only of the time required for striatal neurons to recover from the effects of surgery (i.e., for the potassium gradient across neuronal membranes to be restored) but by when dialysis could then be reliably performed. The evidence to support the claim that ionic mechanisms may be disrupted, at least during and immediately after dialysis probe positioning, stems from the observations that neurotransmitter release is only partially dependent upon neuronal activity during acute dialysis experiments (see section 3.4).

One of the most important factors to consider when employing invasive brain techniques is to be certain that the intended area of study is indeed being examined. To this end, histological studies were undertaken to assess the position of recording electrode tips and dialysis probe tracks. In other experiments described in this chapter the levels of dopamine, DOPAC and HVA appearing in dialysates of the caudate putamen and prefrontal cortex were estimated and monitored over several hours. The dependency of dopamine efflux into striatal dialysates on calcium and neuronal activity, as well as the effects of high potassium, were also assessed. To enable a comparison with the results of other dialysis studies to be made it was decided to test the effects of pargyline, nomifensine and amphetamine (following their systemic administration) on dopamine release and turnover in the striatum. In other studies amphetamine was also added to the perfusion medium in order to determine whether its actions on striatal dopamine function were markedly different when administered locally to dopamine nerve terminals.

This chapter also aims to characterise the spontaneous activity of neurons in the caudate putamen and the medial prefrontal cortex. By recording the discharge pattern and individual shape of action potentials it should be possible to identify the types of potentials present as well as characterise the firing pattern of spontaneously active neurons in these areas.

3.2 INTRACEREBRAL MICRODIALYSIS

3.2.1 *In-vitro* recovery of catecholamines

Figure 3.1 shows the effect of perfusion flow rate as well as the effect of dialysis membrane type on the relative and absolute recovery of dopamine, DOPAC and HVA from standard 5μM solutions (see section 2.5.3). The relative recovery, whilst being approximately equal for dopamine, DOPAC and HVA, increased markedly as the flow rate was decreased (see also Johnson & Justice, 1983). In contrast, the absolute recovery of these monoamines (vitafiber membrane) showed a tendency to increase as the flow rate was increased. The cellulose hollow-fibre membrane used (cuprophan), except in its recovery of HVA, was significantly superior to the acrylic co-polymer membrane employed (vitafiber). Based on these results it was decided to use a perfusion rate of 5 μl/min (where for the vitafiber membrane the relative recoveries of DA, DOPAC and

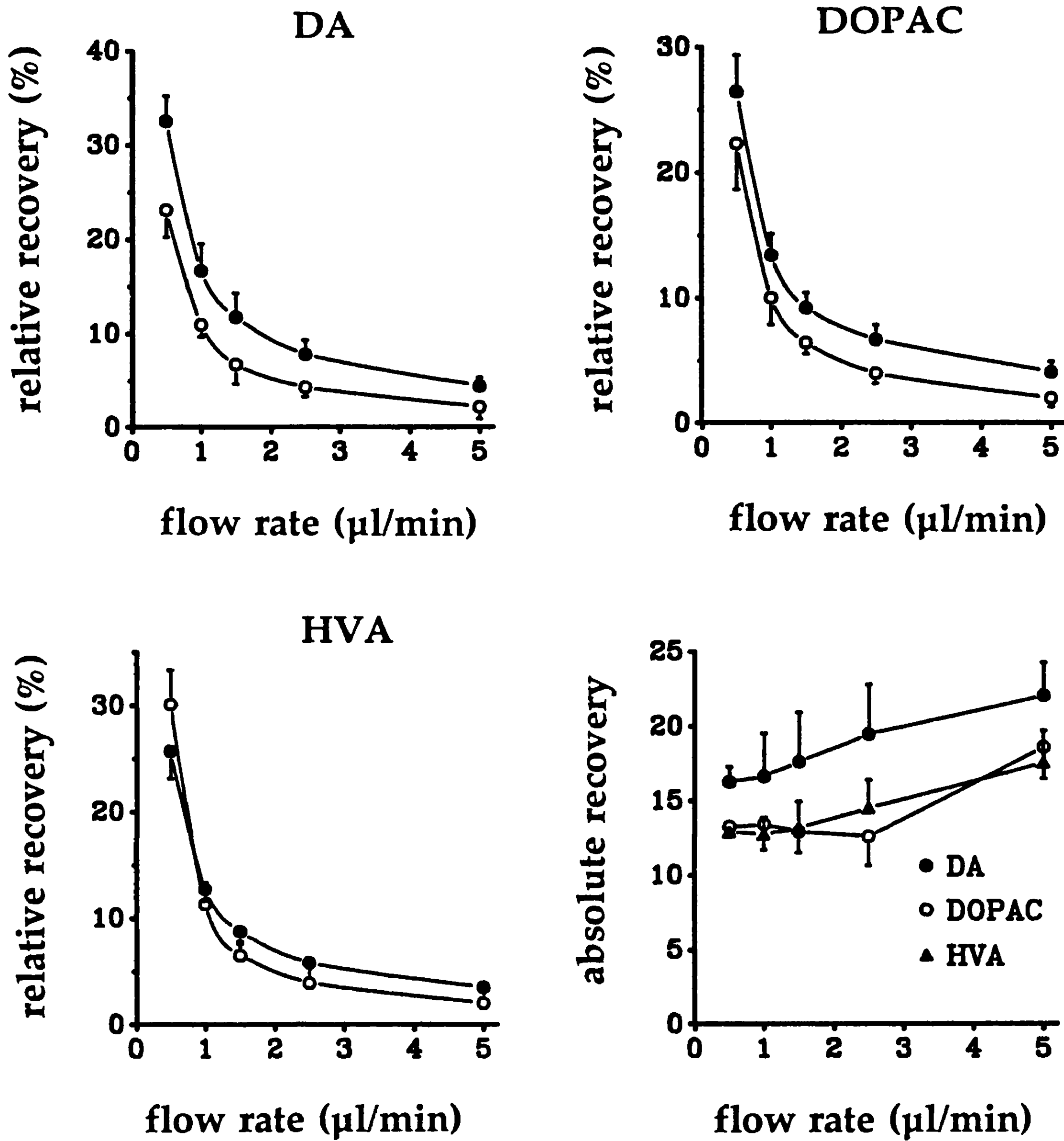


Figure 3.1 Effect of perfusion flow rate on the *in vitro* recovery of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by concentric dialysis probes constructed with either vitafiber (○) or cuprophan (●) hollow-fibre membranes. The effect of flow rate on the absolute recovery (pmoles/20 mins) of DA, DOPAC and HVA by vitafiber dialysis probes is also shown. Error bars represent s.e.means (*n*=5).

HVA were estimated to be 2.17, 2.01 and 2.04 per cent) in subsequent *in-vivo* studies. Despite the fact that the relative recovery was considerably higher at lower flow rates, it was decided nonetheless, to adopt a higher flow rate since the absolute recovery would be higher. Thus, by analysing the entire volume of dialysate collected it should be possible to obtain higher levels of monoamines without, as is necessary for lower flow rates, the need to dilute the dialysate for analytical purposes (the minimum injection volume required for reliable HPLC analysis was approximately 50µl, although in practice, at least 80µl was injected). The vitafiber hollow-fibre membrane was used both in the remaining studies of this chapter as well as in chapter 5 (unfortunately the cuprophan membrane only become available toward the latter stages of this thesis). In other studies (although the data is not shown), the absolute recoveries (pmoles/20 minutes) of DA, DOPAC and HVA were directly related to their concentration across the range 100nM to 10µM. The recovery data presented in Fig.3.1 will be used to estimate the approximate extracellular concentration of these catecholamines in the caudate putamen and prefrontal cortex (see section 3.4).

3.2.2 *In-vitro* calibration of dopamine release by dialysis

The release of dopamine *in-vitro* following its inclusion (in progressively increasing concentrations) in the perfusate of vitafiber dialysis probes is shown in Fig.3.2. The total transfer of dopamine from the probes into the surrounding fluid was found to be directly related to its concentration in the perfusion medium. At a flow rate of 5 µl/min, and by keeping the length of tubing connecting the 3-way switch and the dialysis probe to a minimum, the peak release of dopamine following a 5 min pulse occurred between 2.5 and 5 minutes. In other studies designed to optimise the administration of drugs by dialysis the effects of flow rate and pulse time were examined. Not surprisingly, dopamine release (for a given flow rate) increased as the pulse time was increased. The release of dopamine also increased as the flow rate increased although this was by no means a linear relationship. A flow rate of 5 µl/min and a pulse time of 5 mins were chosen on the basis of consistency and to avoid prolonged inhibitory effects of dopaminergic agents in subsequent recording studies. The concentration-dependency of drug administration by dialysis is confirmed in chapter 8 where the effects of 2-methyl-5-HT (a selective 5-HT_3 agonist) are examined on dopamine function in the nucleus accumbens and caudate putamen following its administration by cerebral dialysis.

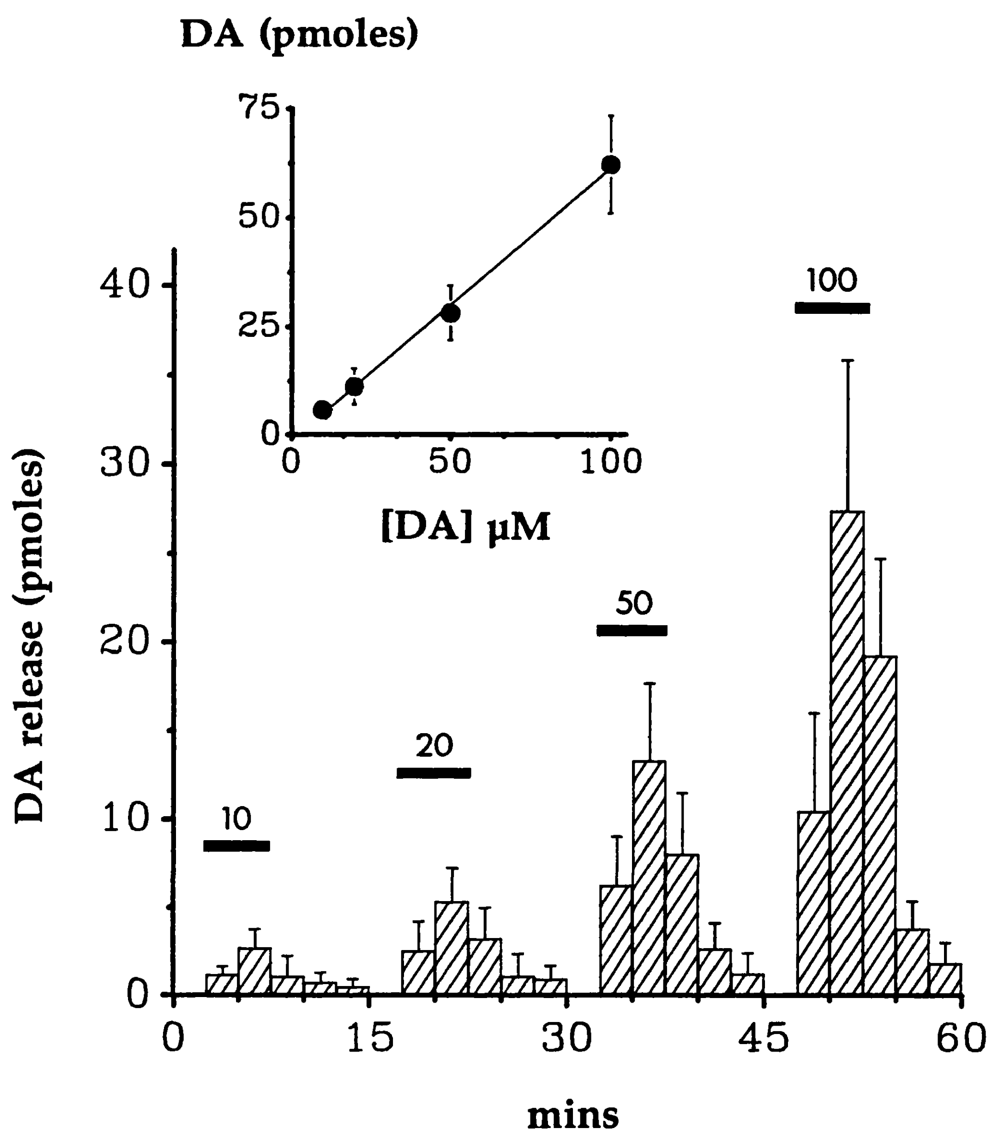


Figure 3.2 Release of dopamine from vitafiber dialysis probes immersed in artificial CSF (1 ml). Dopamine was added to the perfusion medium (flow rate 5 µl/min) in varying concentrations (µM) and pulsed for 5 mins. The surrounding medium was replenished every 2.5 minutes and analysed for its dopamine content. The smaller insert graph gives the total amount (pmoles) of dopamine released for each concentration of dopamine employed (r^2 = 0.9876). Error bars represent s.e.means (*n*=5). Horizontal bars show the period of dopamine administration.

3.2.3 Potassium efflux as a measure of neuronal trauma

The efflux of potassium in dialysates of the rostral caudate putamen (in potassium-free but isotonic artificial CSF of composition I; see appendix III) was appreciably higher during the first hour of dialysis compared with that estimated 3 to 4 hours after probe implantation (Fig.3.3). The efflux concentration of potassium ($[K^+]_e$), not surprisingly, was higher when a lower flow rate was employed. The relative recovery of potassium determined *in-vitro* ranged from 10% to 2.5% for flow rates of 2 and 10 µl/min respectively (see also Benveniste, Hansen & Ottosen, 1989). Thus, there is no evidence from these results to suggest that higher flow rates are associated with more neuronal damage (see section 3.4) since this 4-fold difference in relative recovery is consistent with the variation observed in the steady-state levels (at 3.5 hours post-probe implantation) estimated for potassium (± s.e.mean, n=5) of 0.199 ± 0.056 mM and 0.057 ± 0.009 mM for 2 and 10 µl/min respectively. The results indicate that dialysis experiments should not be started until at least 1 to 1.5 hours have elapsed after probe positioning. In practice, experiments were commenced 3 hours following surgery. The analysis of potassium by flame-photometry was impeded by the presence of other electrolytes in artificial CSF (Fig.3.3B). This spectral interference was mainly associated with sodium and calcium ions. Fortunately, this problem could be avoided by diluting the dialysates at least 1 in 25 in DIW prior to analysis (diluted potassium-free CSF was used to "zero" the instrument).

3.2.4 Evaluation of dopamine function by cerebral dialysis

In this section the ability of microdialysis to reflect the molecular events of dopamine neurotransmission was evaluated by testing procedures and drugs that should modify dopamine efflux and metabolism in specified ways. The catecholamines, dopamine, DOPAC and HVA, as well as the 5-HT metabolite 5-HIAA, which were present in striatal and cortical dialysates were analysed as described in section 2.7. In Fig.3.4 representative chromatograms showing the separation of these monoamines are given. The extracellular levels of dopamine, DOPAC and HVA were appreciably higher in the rostral caudate putamen than in the medial prefrontal cortex (see also Fig.3.6). In contrast, the levels of 5-HIAA in the prefrontal cortex were either higher, or more typically, quite similar to the levels estimated in the caudate putamen. In practice, the gain on the electrochemical detector was set at 2000 to 5000 in order to maximise the dopamine signal in cortical

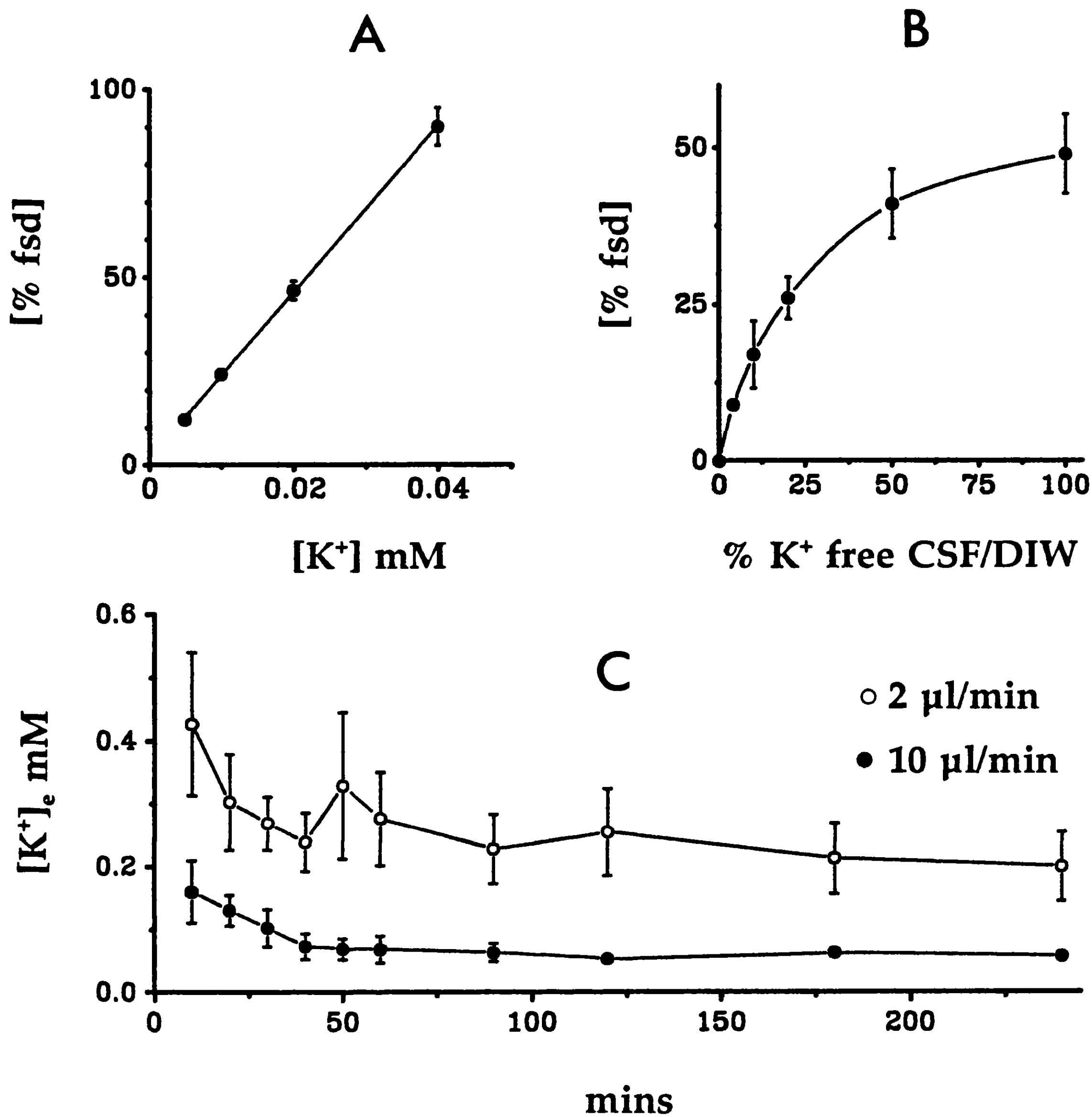


Figure 3.3 Estimation of potassium efflux concentration (mM) in dialysates of the rat caudate putamen (C). The assay calibration curve is given in (A) (r^2 = 0.995) and the interference of potassium-free CSF on the flame photometric signal is shown in (B). Dialysis probes were perfused with potassium-free CSF (containing extra NaCl) at either 2 or 10 µl/min and dialysates were diluted at least 1:25 in DIW prior to analysis. Error bars represent s.e.means (*n*=5).

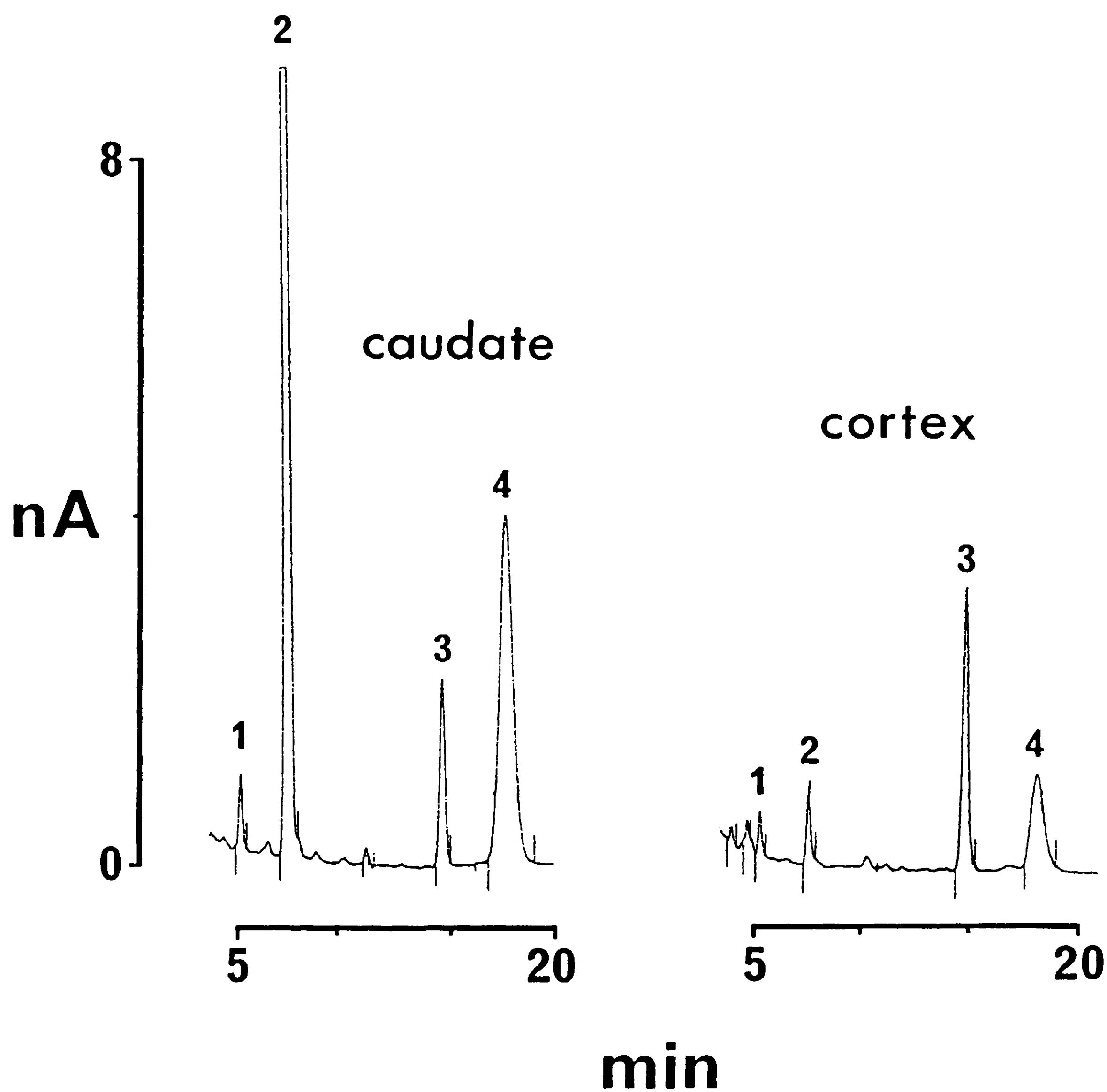


Figure 3.4 Representative chromatograms of dialysates of the caudate putamen and the medial prefrontal cortex (see also Fig.2.8). A perfusion flow rate of 5 µl/min was employed and dialysates were collected every 20 minutes into 5.0 µl 6M $HClO_4$. The eluted peaks are: (1) dopamine; (2) DOPAC; (3) 5-HIAA; (4) HVA. The detector gain was 1000 and the applied potentials used were 20mV (D1) and 300mV (D2).

dialysates but was reduced to 500 to avoid detector saturation of the DOPAC signal when analysing striatal samples (as is apparent in Fig.3.4).

In Fig.3.5 the effects of high potassium (30 and 60 mM), calcium-free CSF and TTX (10 μmol/l) on the efflux of dopamine in striatal dialysates are shown. The depolarising influence of potassium caused a significant and dose-related increase in the efflux of dopamine with (although not shown) a corresponding fall in the levels of DOPAC and HVA. The exclusion of calcium from the perfusion medium resulted in a significant decline in the basal efflux of dopamine (by about 40-45%), which, with the reintroduction of calcium, quickly returned to basal levels. There was, however, no discernible change observed in the efflux of either DOPAC or HVA during the perfusion of calcium-free CSF. The inclusion of the sodium-channel blocker tetrodotoxin in the perfusate induced a rapid and significant reduction in the efflux of dopamine (by about 60%) which returned only slowly to basal levels following its removal. TTX perfusion was also associated with a significant decline in DOPAC and HVA efflux (by about 40-50%).

Fig.3.6 shows the time course of the basal efflux of dopamine, DOPAC and HVA detected in dialysates of the caudate putamen and the medial prefrontal cortex (see Table 5.1 for the actual amounts detected). The output of dopamine from both brain areas, similar to the efflux of potassium in striatal dialysates (see Fig.3.3), showed a tendency to decrease over the first 2 to 3 hours. The basal efflux of DOPAC and HVA, whilst being about 150 to 250 fold that of dopamine in the caudate putamen but only 25 to 45 times higher than dopamine in the prefrontal cortex, tended to increase during the first 2 to 3 hours after probe implantation, whereupon, at least in the caudate putamen, it stabilised and remained constant over about 6 hours. Rather surprisingly, the ratio of DOPAC to HVA efflux, while greater than unity in dialysates of the caudate putamen, was reversed in dialysates of the prefrontal cortex. This finding is discussed in some detail in section 5.4.1. The basal efflux values of catecholamines detected in dialysates of the nucleus accumbens are listed in section 8.2.2.

The non-selective MAO inhibitor pargyline (50 mg/kg i.p.) resulted, as shown in Fig.3.7, in a steady increase in the efflux of dopamine in striatal dialysates to a peak of about 5 times the basal efflux within 1 hour. The high levels of dopamine efflux fell only gradually with significantly elevated levels detected over 2 hours. The efflux of DOPAC

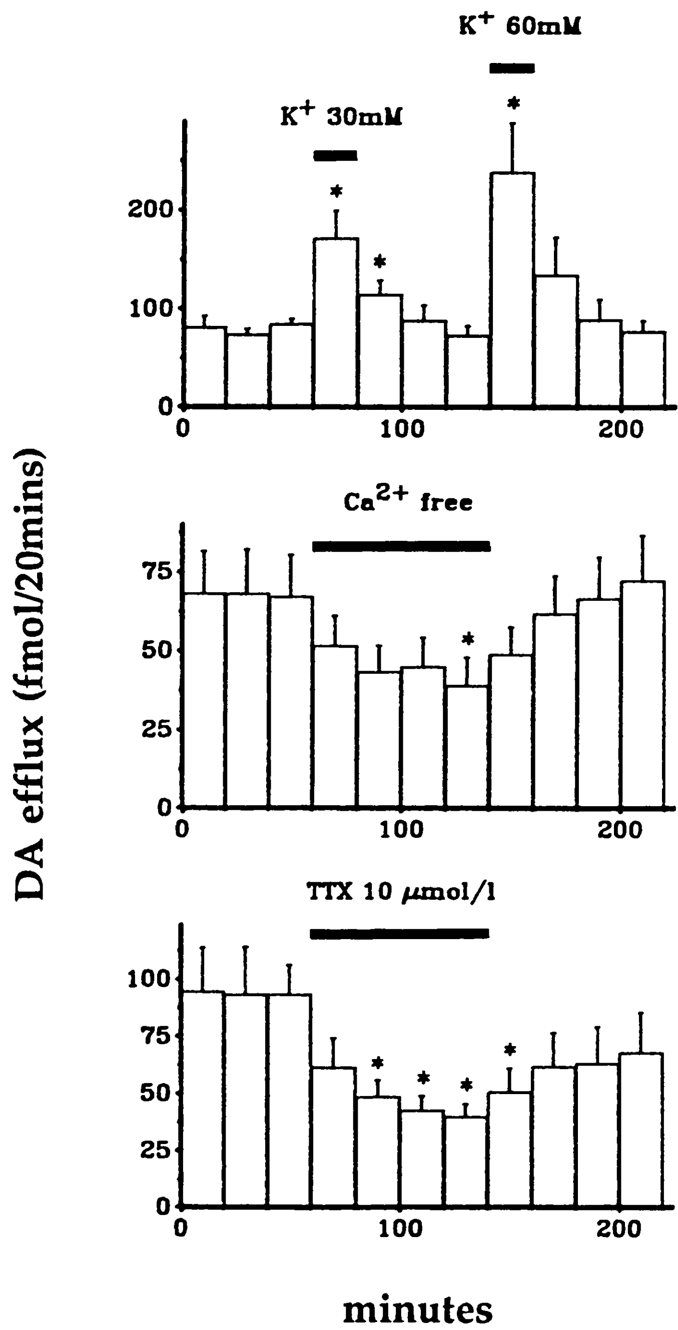


Figure 3.5 Effects of potassium, calcium-free artificial CSF and tetrodotoxin (TTX) on dopamine efflux in dialysates of the rat caudate putamen (presence shown by bars). Error bars represent s.e.means (n=4-5) and significance was assessed using a Student's t-test (* $p<0.05$).

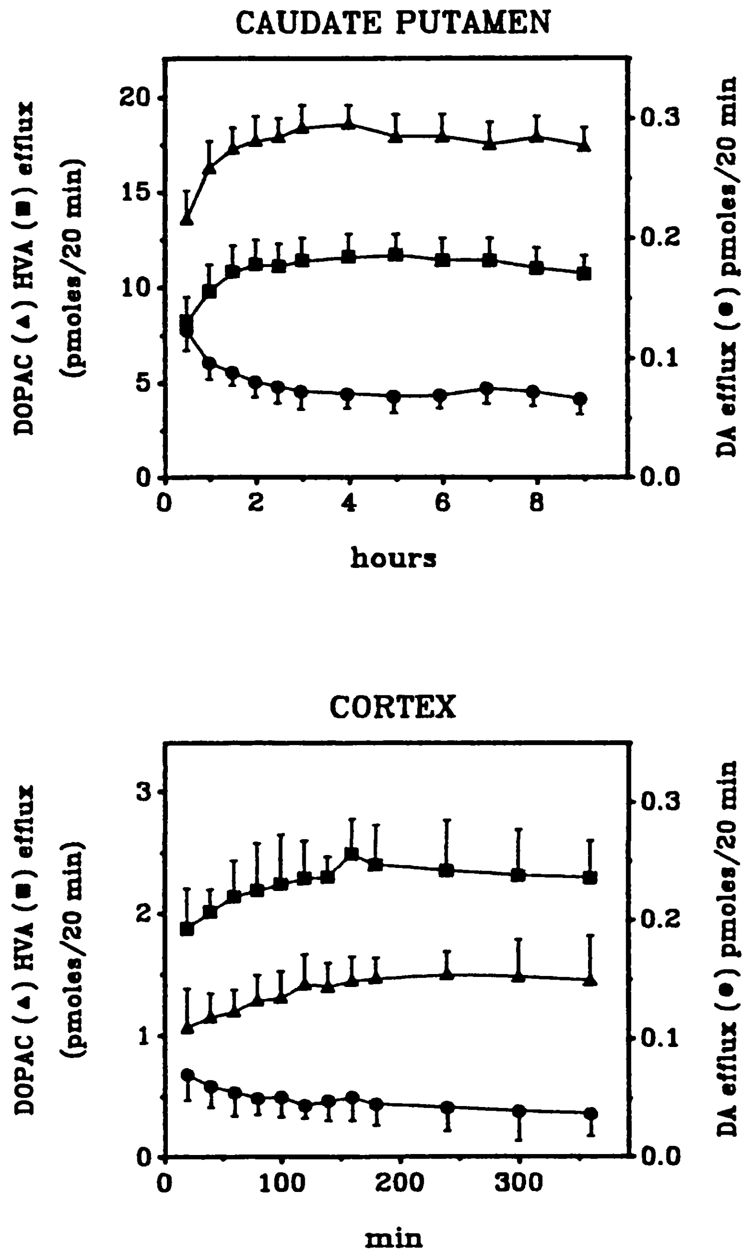


Figure 3.6 Time course of the basal efflux of dopamine (●), DOPAC (▲) and HVA (■) in dialysates of the caudate putamen and the medial prefrontal cortex. Error bars represent s.e.means (*n=5*).

declined sharply, falling to about 40% of basal values after 20 mins and to less than 5% after 60 mins. HVA efflux decreased after a delay of about 20 mins, and its fall roughly paralleled that of DOPAC.

The dopamine re-uptake inhibitor, nomifensine (10 mg/kg i.p.), also like pargyline, significantly elevated the efflux of dopamine (reaching a peak of about 2 times the basal efflux) but did not decrease the efflux of either DOPAC or HVA (Fig.3.7). In fact, nomifensine produced a gradual and significant increase in the extracellular level of HVA, without any effect on the efflux of DOPAC. When nomifensine was added to the perfusion medium (composition II; see appendix III) it also enhanced the efflux of dopamine in a concentration-dependent manner (see chapter 8). In some initial experiments, however, where buffered artificial CSF was employed (i.e., composition I; see appendix III), no significant effects of nomifensine could be detected when it was added to the perfusion medium although it was active when given systemically. This rather unusual effect might be due to a physical interaction between nomifensine and a component of composition I which could attenuate its passage across the dialysis membrane. Whatever the reason it was decided in subsequent experiments to always use composition II (composition I was only used in the experiments of Fig.3.3 and Fig.3.8).

D-amphetamine, as shown in Fig.3.8, caused a pronounced and significant rise in the efflux of dopamine, either when given systemically (about 8 fold), or by microdialysis, where the effect was concentration-related. The efflux of DOPAC and HVA decreased after the systemic administration of amphetamine (rapidly for DOPAC but less so for HVA) to about 10-20% of basal values after 90 mins but this effect was less marked, especially for HVA, following its local application by dialysis despite producing comparable peak increases in the efflux of dopamine.

Representative track lesions indicating the position of dialysis probes in the rostral caudate putamen, nucleus accumbens and medial prefrontal cortex are clearly visible in the stained transverse sections of the rat forebrain displayed in Fig. 3.9. Although it is likely that some bleeding occurs at the time of probe removal the presence of blood in the track was not a consistent finding. High-power magnification of the margins of track lesions revealed no gross abnormalities (i.e., no peculiar staining or evidence of abnormal neuronal morphology) even after prolonged (>10 hours) experiments.

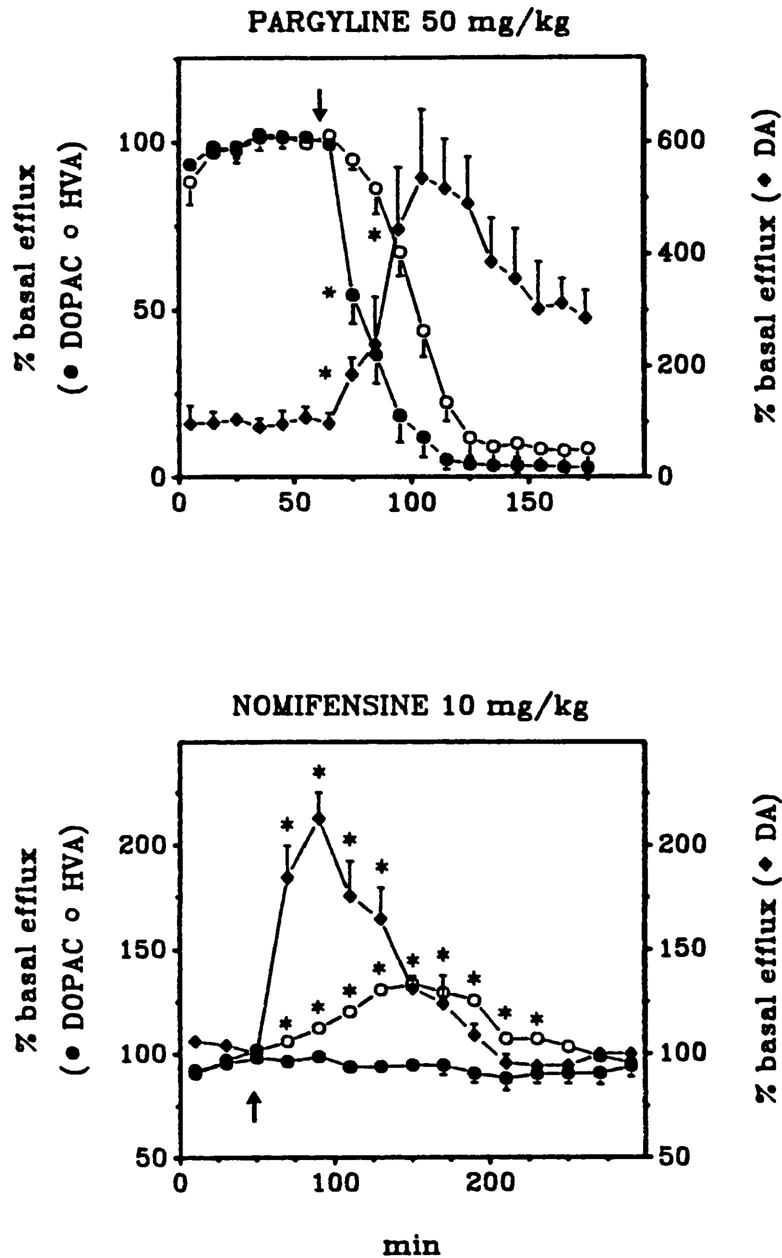


Figure 3.7 Effects of pargyline (50 mg/kg) and nomifensine (10 mg/kg) on the efflux of dopamine (◆), DOPAC (●) and HVA (○) in dialysates of the rat caudate putamen. These agents were administered by i.p. injection (1.0 ml/kg) as indicated by the arrows. Error bars represent s.e.means (n=4) and significance (versus basal efflux) was assessed using a Student's t-test (* $p<0.05$).

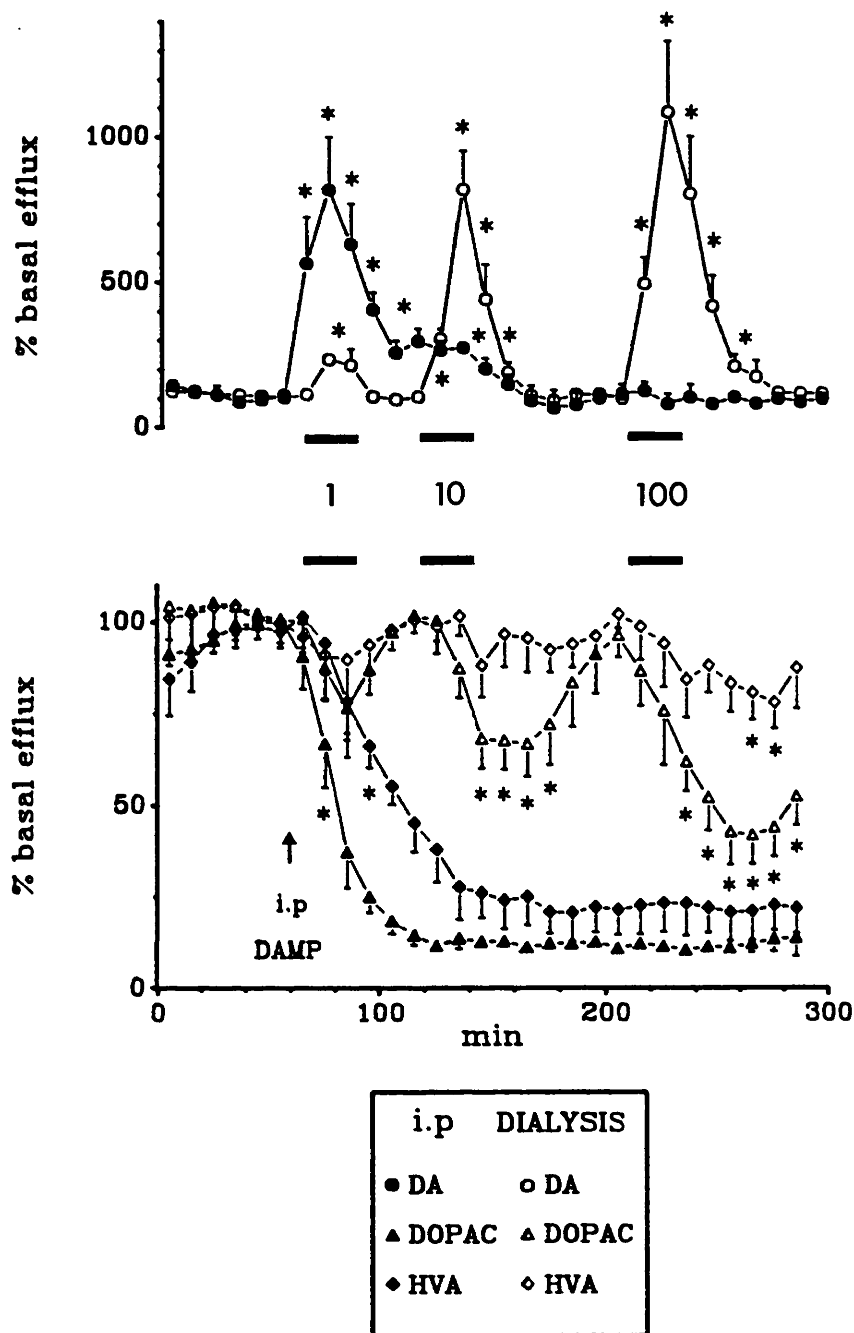
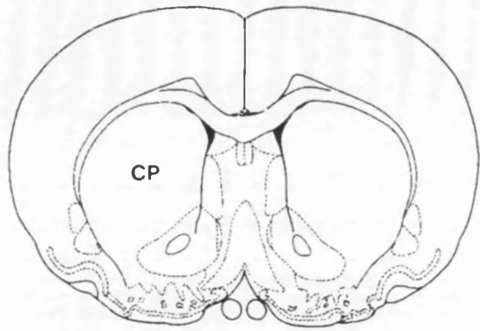
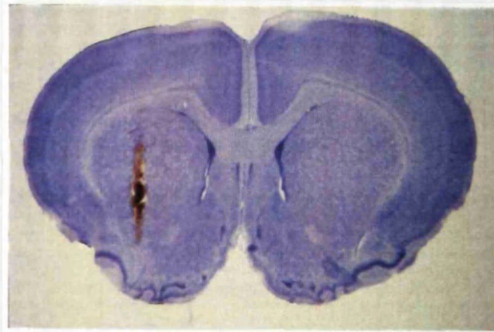
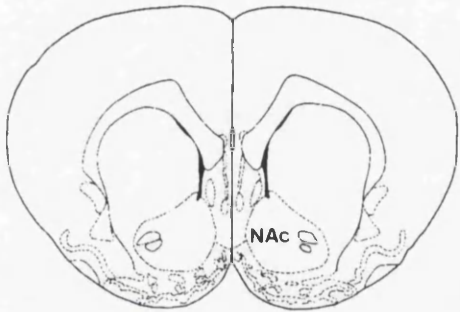
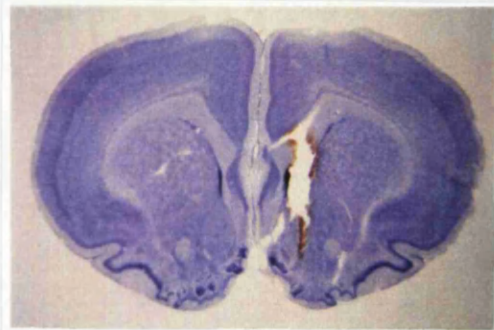


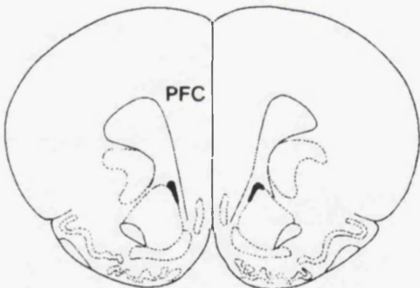
Figure 3.8 Comparison between the effects of D-amphetamine (DAMP) administered either by i.p. injection (2.0mg/kg) (shaded symbols) or by dialysis (open symbols) (presence shown by bars [µM]) on the efflux of dopamine, DOPAC and HVA in dialysates of the caudate putamen. Error bars represent s.e.means (n=4-5) and significance was assessed using a Student's t-test (* $p<0.05$).











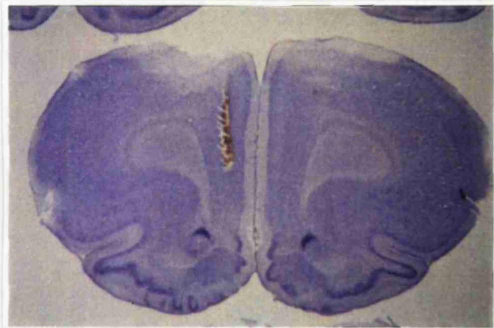


Figure 3.9 Transverse sections through the rat forebrain showing the position of the dialysis probe in the rostral caudate putamen (CP), nucleus accumbens (NAc) and medial prefrontal cortex (PFC). The diagrammatic sections (taken from the atlas of Paxinos & Watson, 1982) have been cut approximately 0.7mm (CP), 1.7mm (NAc) and 2.7mm (PFC) forward of bregma and correspond structurally within at least 0.5mm of each stained section.

3.3 EXTRACELLULAR NEURONAL RECORDING

3.3.1 Electrophysiological characterization

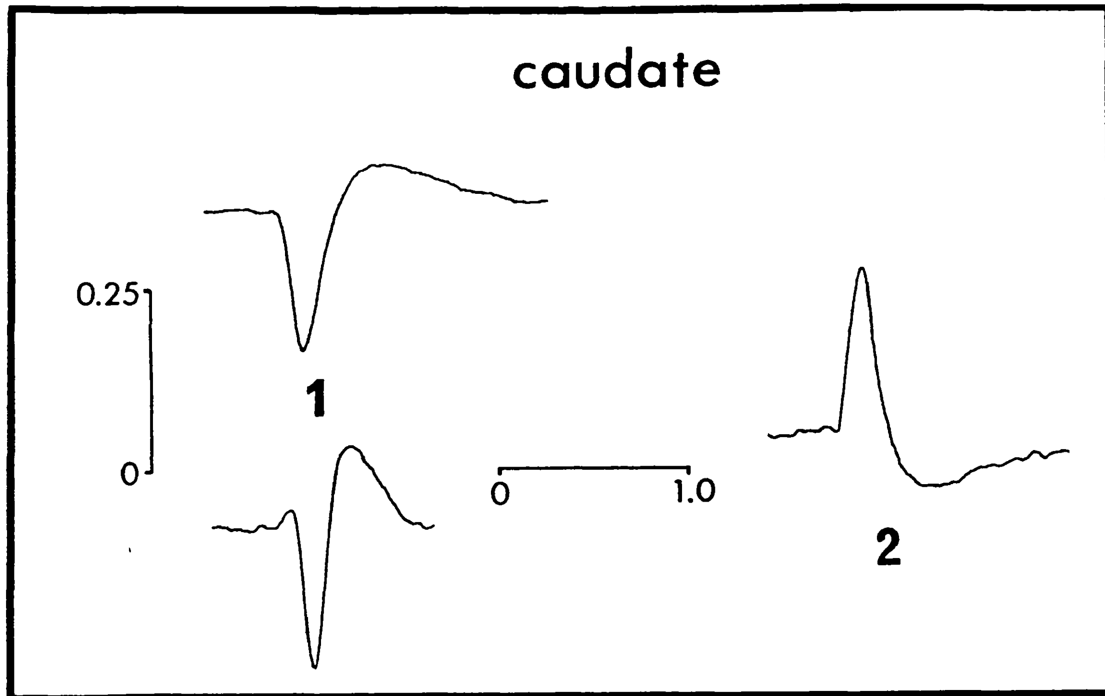
This section describes the nature and the pattern of the spontaneous activity recorded from individual cells in the rostral caudate putamen and the medial prefrontal cortex of halothane-anaesthetised rats.

Caudate putamen

Two types of spontaneously active neurons were found in the rostral caudate putamen: type I and type II (see Fig.3.10). Type I units, which were the most frequent type of neuron found in this area (80-85%), were biphasic and showed amplitudes of 80-350μV, with durations, generally of 0.65-0.85ms, but in some exception cases, as long as 1.4ms. Type I neurons exhibited two main patterns of spontaneous activity: (1) slow, fairly regular firing, and (2) burst firing. Burst sequences were most common (see also Wilson & Groves, 1981) and consisted of trains of 2-6 spikes over a short period separated by quite prolonged quiescent periods. Within each burst there was sometimes a progressive diminution of spike amplitude (this effect was most apparent during longer burst sequences). Most cells fired in a mixed fashion (i.e., bursts and single action potentials), although rarely, some cells fired entirely in burst sequences (see Fig.3.11). Type II units were much less commonly encountered, representing only 15-20% of active units, and showed spike amplitudes of 150-500μV with durations of 0.7-1.3msec. Type II potentials were biphasic and essentially a mirror image of type I potentials. Only very rarely could type II cells be recorded from since their activity was low and most often associated with the firing of more prevalent type I units.

The shape of type I and II potentials did not depend on the proximity of the electrode tip to the soma or the filtering parameters employed (see also Nisenbaum, Orr & Berger, 1988). For each neuron these two variables were adjusted to maximise the signal to noise ratio and enable consistent spike discrimination. The background noise (recorded after the death of the animal) was generally as low as 15-25μV.

The spontaneous activity of neurons in the caudate putamen was conspicuously confined to layers, between which, little or no activity was apparent. It was a noticeable feature,



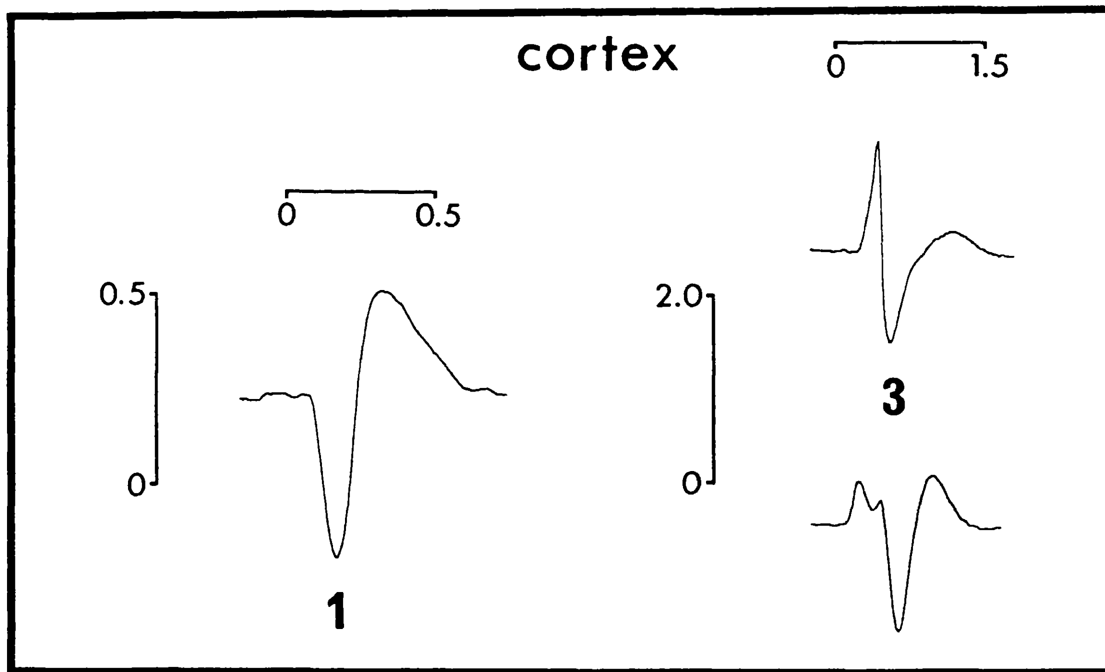


Figure 3.10 Oscilloscope traces showing the waveforms of representative action potentials recorded extracellularly in the caudate putamen and the medial prefrontal cortex. Type 1 and type 2 potentials are biphasic, the latter being essentially a mirror image (about the horizontal plane) of the former. In contrast, the larger type 3 potentials are triphasic, of long duration, and often display an initial-segment somatodendritic notch. The ordinate and abscissa scales are in units of mV and ms respectively.

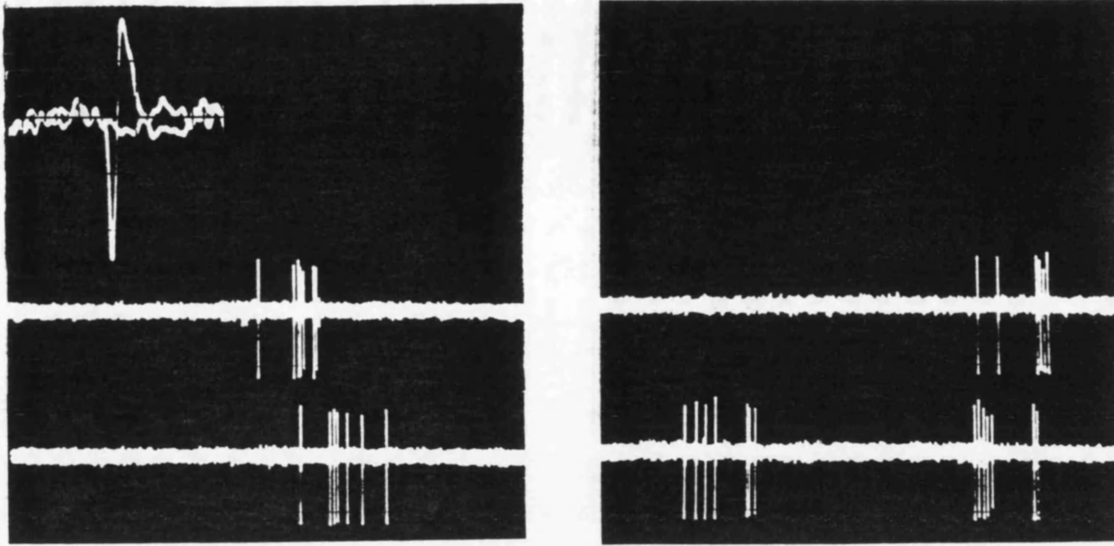
however, that the background noise increased on electrode descent through the "inactive" zones which normally ranged between 200µm and 500µm in depth. When activity was present, type I units were most frequently encountered and, more often than not, several of these units (of varying amplitudes), as well as slower firing Type II units, were present. In only very exceptional cases were type II units located individually. Overall, neurons fired at an average rate of 2.76 ± 0.25 Hz (mean ± s.e.m; range 0.5 to 6.2 Hz, *n=31*; Fig.3.12).

A transverse section through the rat brain at the level of the rostral striatum demonstrating the position of the recording electrode in a typical experiment is shown in Fig.3.13. Other histological examinations revealed that recording sites (from the present studies and those of chapter 6) were located throughout the full dorso-ventral extent of the rostral striatum.

Medial prefrontal cortex

Spontaneously active neurons in the medial prefrontal cortex were larger and generally fired faster than neurons in the caudate putamen (mean rate ± s.e.m, 5.03 ± 0.36 Hz, range; 0.7 to 11.9 Hz, *n=47*; Fig.3.12). The most frequent neurons found (60-65% of cells) were type I (see Fig.3.10) although these were invariably larger (400-1000µV) than their counterparts in the caudate putamen. These neurons exhibited regular as well as burst sequences, although, no one pattern predominated. The remaining cells recorded from were type III (see Fig.3.10). These showed triphasic potentials of long duration (1.3-1.6ms) with either a pronounced notch on the initial rising phase or, in some cases, a slight initial deflection with a smaller late positive phase (Fig.3.10). The shape of type III action potentials varied markedly with electrode movement. In most cases, as the electrode was advanced, the amplitude increased, typically to a maximum of about 1 to 2.5mV. Additional forward movement invariably destroyed the cell, either instantly, or after a brief period of rapid activity. Type III cells generally exhibited burst activity with a progressive within-burst decrease in spike amplitude, although single action potentials were usually dispersed between burst sequences. Type I and type III cells were uniformly distributed in the prefrontal cortex, so, unlike the caudate putamen, spontaneous activity was encountered throughout the entire ventral depth of this structure.

A



10s

B

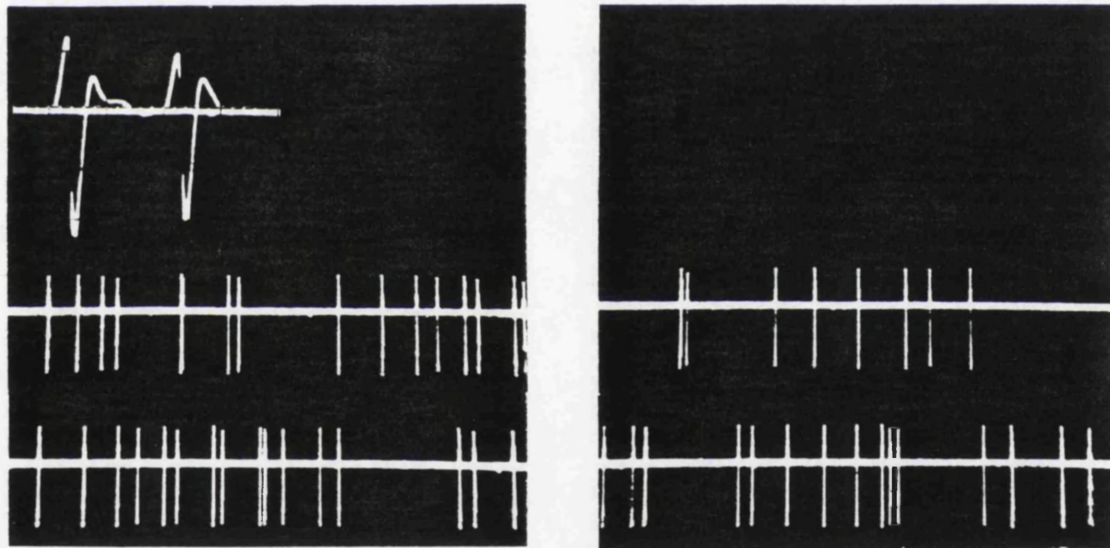


Figure 3.11 Oscilloscope recordings showing the spontaneous activity of single neurons in the caudate putamen (A) and medial prefrontal cortex (B). In each case the waveform of the extracellularly recorded action potential is shown at a fast sweep speed together with a longer record showing the pattern of firing (see section 3.3.1). The amplitude (mV)/duration (ms) of the action potential recorded for (A) and (B) are 0.25/0.97 and 1.85/1.43 respectively.

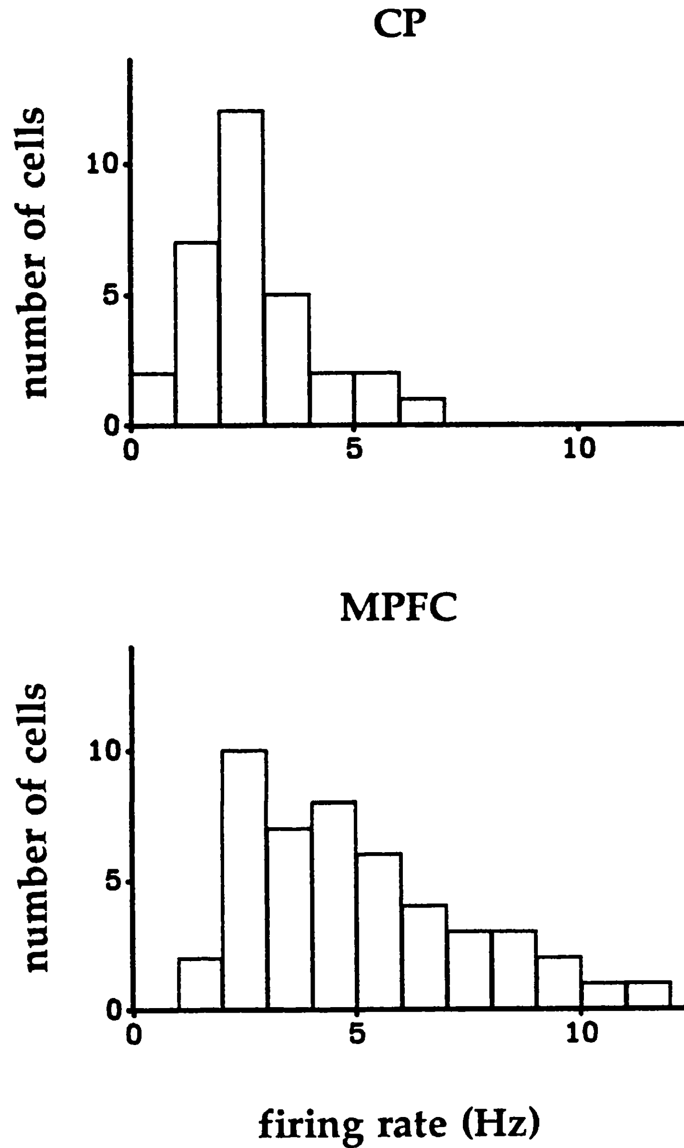
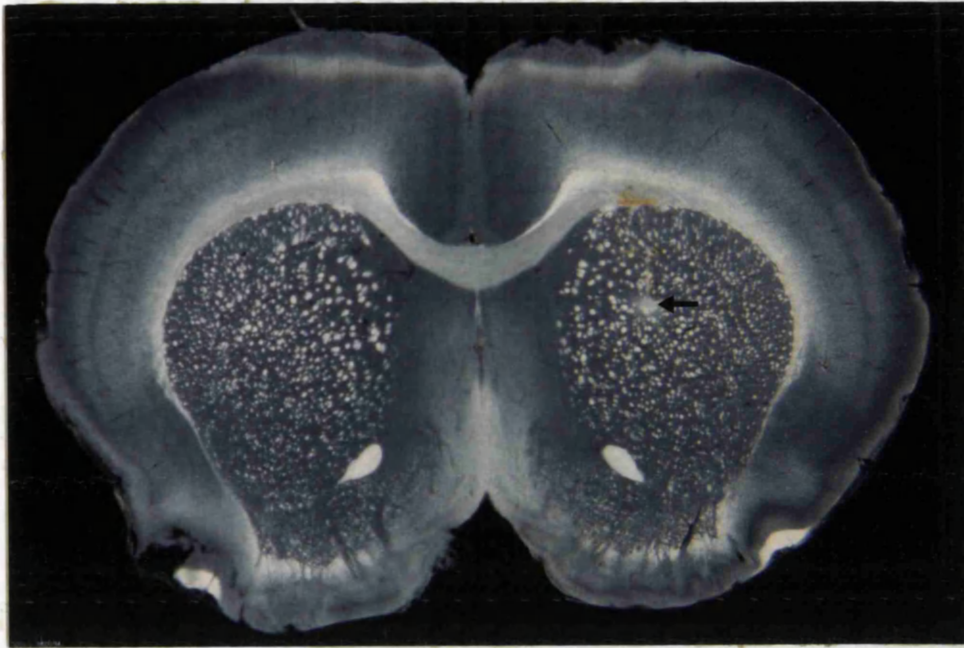


Figure 3.12 Histograms showing the frequency distribution of the firing rates of neurons in the caudate putamen (CP) and the medial prefrontal cortex (MPFC). The mean firing rates (± s.e.mean) were estimated to be 2.76 ± 0.25 Hz (CP) and 5.03 ± 0.36 Hz (MPFC).

A



B

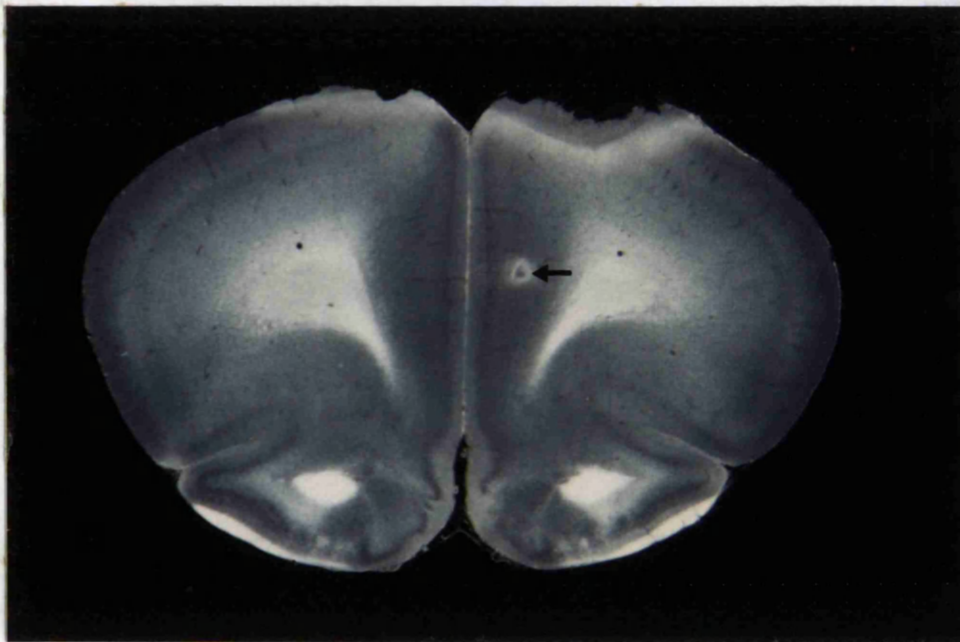


Figure 3.13 Representative transverse sections through the rat forebrain showing the position of the recording electrode tip (indicated by arrows) in the caudate putamen (A) and the medial prefrontal cortex (B).

Fig.3.13 demonstrates the position of the recording electrode tip within the medial prefrontal cortex. Histological examinations (see section 2.6) from representative experiments revealed that most neurons recorded from in this area were located proximal to the medial border of the forceps minor of the corpus callosum. This area lies rostral and slightly ventral to the genu of the corpus callosum (see Paxinos & Watson, 1982).

3.3.2 Pharmacological attempts to facilitate the activity of neurons in the caudate putamen using intracerebral dialysis

In some experiments efforts were made to enhance the level of striatal activity in order to facilitate the recording of extracellular action potentials. Fig.3.14 summarises the effects of some agents, applied by intracerebral dialysis as near as possible to the cell being studied. Rather surprisingly, glutamate and NMDA both produced concentration-dependent inhibitory effects on striatal firing (each compound $n=9$). Indeed, even with high concentrations of these agents (> 1mM) there was no sign of any transient facilitation of neuronal discharge. Glutamate was about 5 times more potent than NMDA in producing this inhibition (the response to glutamate was first apparent at about 10µM). Attempts to facilitate activity by disinhibition through perfusing GABA antagonists such as bicuculline, picrotoxin and leptazol, produced somewhat mixed results. In some cases, bicuculline enhanced the firing rate in a concentration-dependent manner, as it did when given systemically, but more generally, it produced inhibitory effects. In some experiments, particularly those in urethane-anaesthetised rats, leptazol dramatically stimulated neuronal activity in the striatum in nM concentrations. Unfortunately, this was not a consistent effect and it could not be demonstrated when using halothane. When perfused at considerably higher concentrations (50-200µM) such that GABAergic mechanisms were likely to be affected, leptazol, like picrotoxin (5-20µM), produced only inhibitory effects. Other approaches such as augmenting striatal cholinergic function by perfusing the reversible cholinesterase inhibitor physostigmine (5µM) resulted in an attenuation of neuronal discharge. Not surprisingly, GABA (10mM) and muscimol (1mM), were also inhibitory. The physiological significance of the rather unexpected results obtained using this technique are discussed in some detail in section 3.4.

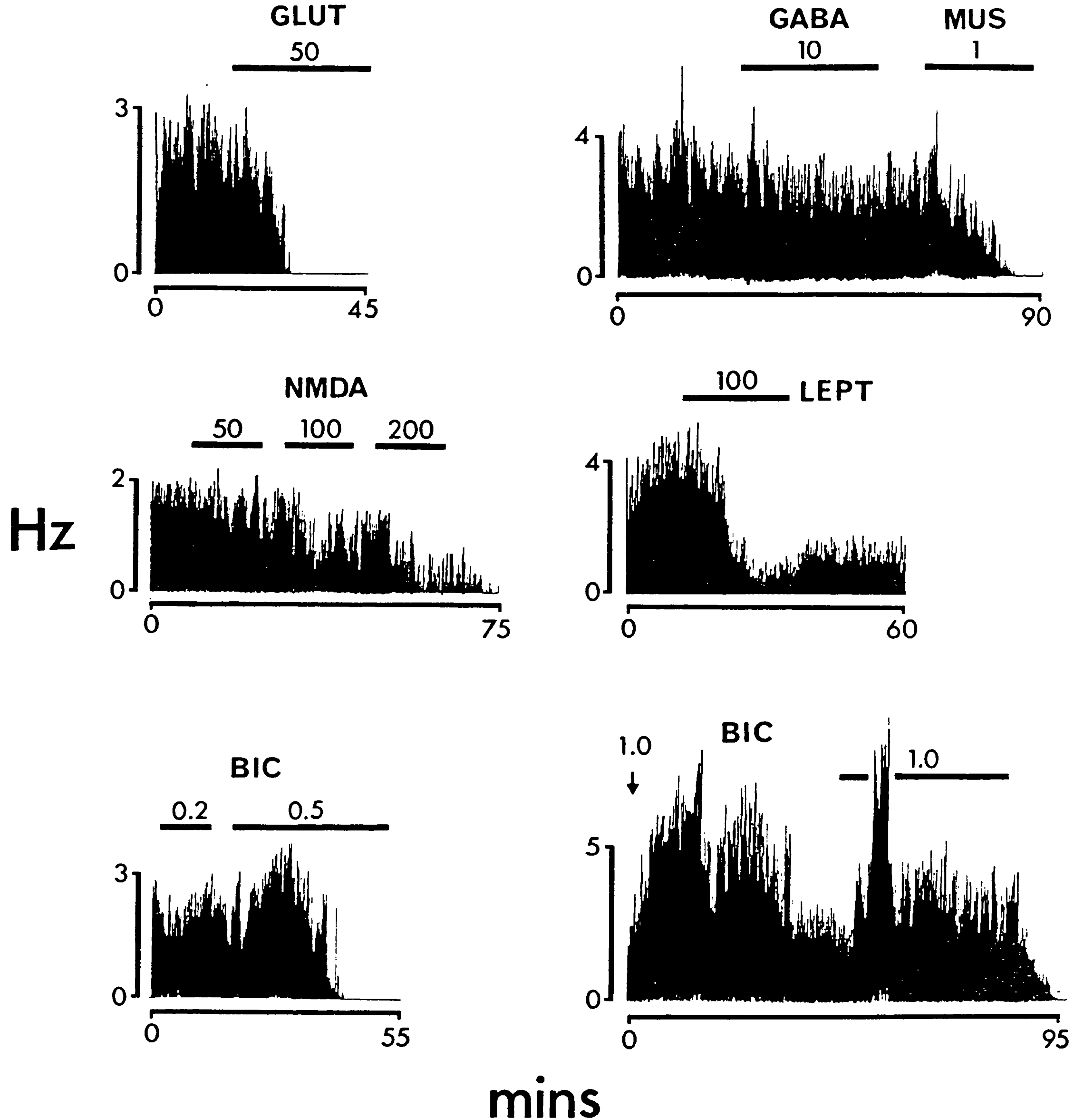


Figure 3.14 Attempts to facilitate the spontaneous activity of neurons in the caudate putamen using microdialysis. Recordings were made proximally to intra-striatal dialysis probes perfused at 5 µl/min with artificial CSF containing the following compounds (presence indicated by bars): GLUT (glutamate); GABA; MUS (muscimol); NMDA; LEPT (leptazol); BIC (bicuculline). The perfusion concentrations are in units of µM except those where GABA and MUS were used, in which case, the units are mM. In the lower right graph the effect of i.v. BIC (1.0 µmol/kg) is also shown.

3.3.3 Effects of directly and indirectly acting dopamine agonists applied by intracerebral microdialysis on striatal neuronal activity

In this series of experiments microdialysis was employed to administer dopaminergic agents close to striatal neurons in order to test their effects on spontaneous discharge. The representative rate histogram traces displayed in Fig.3.15 summarise the effects of dopamine, amphetamine, 2-methyl-5-HT and CGS-15855A on striatal activity. Each of these agents (apart from amphetamine which in 3 experiments was only tested at one concentration) produced a concentration-dependent inhibition of neuronal discharge (see Fig.3.16). Dopamine was more potent than 2-methyl-5-HT, which in turn, and rather surprisingly, was more potent than the selective dopamine D_2 agonist CGS-15855A. In all cases, where dopamine and 2-methyl-5-HT produced these effects, greater than 85% inhibition could be achieved. However, CGS-15855A, even at high concentrations (200μM), only attenuated neuronal discharge by about 40%. At low perfusion concentrations, dopamine (5μM) and CGS-15855A (2μM), albeit rather transiently, produced a stimulation of activity (without subsequent inhibition).

The effects of dopamine re-uptake inhibitors (nomifensine and benztropine) were also tested using this approach (mainly in an attempt to minimise the rapid termination of dopamine). Unfortunately, both of these agents, even at very low concentrations (<5μM), resulted in an inhibition of neuronal firing and no consistent potentiation of the effects of dopamine could be achieved. In other experiments the effects of dopamine on the firing of accumbal neurons was tested. Although preliminary results indicated that these neurons may be less sensitive to dopamine than those in the striatum this approach was subsequently abandoned since reliable access to the nucleus accumbens could not be achieved.

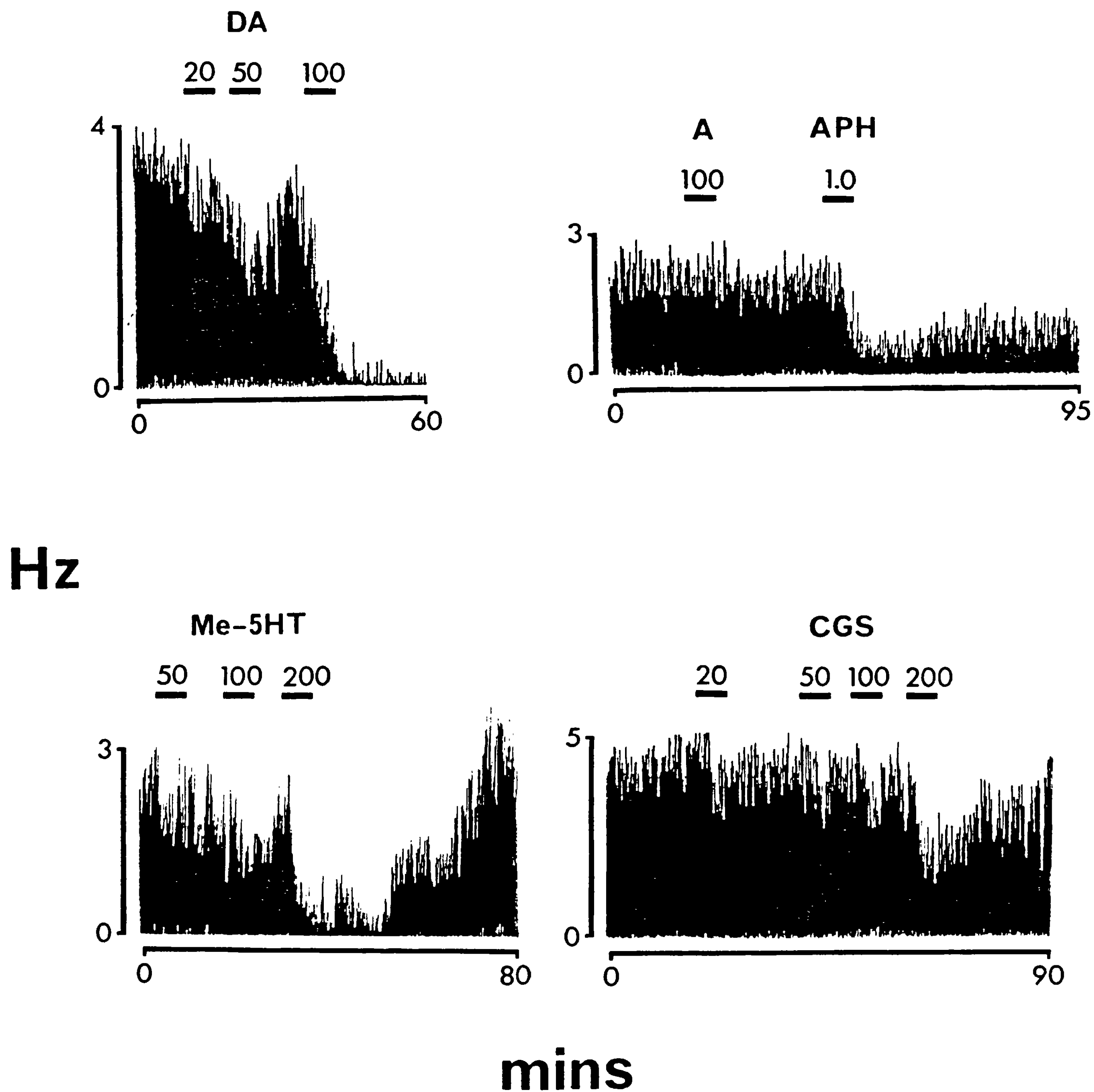


Figure 3.15 Representative rate recordings showing the effects of dopamine (DA), L-ascorbic acid (A), amphetamine (APH), 2-methyl-5-HT and CGS-15855A (CGS), administered by microdialysis on the spontaneous activity of neurons in the caudate putamen. The perfusion concentrations are in units of µM.

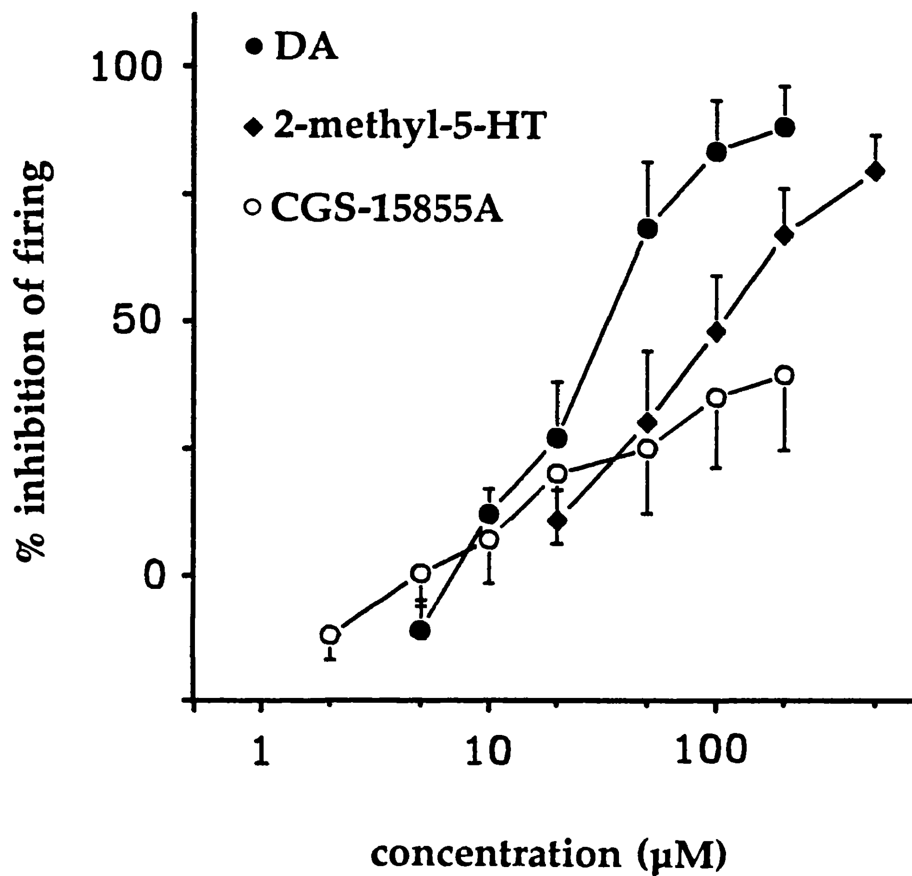


Figure 3.16 Dose-dependent effects of DA, 2-methyl-5-HT and CGS-15855A, administered by microdialysis (see Fig.3.15), on the spontaneous activity of neurons in the caudate putamen. Error bars represent s.e.means (*n*=5, DA, 2-methyl-5-HT; *n*=4, CGS-15855A).

3.4 DISCUSSION

3.4.1 Intracerebral microdialysis

The *in-vitro* recovery performance of the cellulose dialysis probes used in the present study compares well with other designs of intracerebral dialysis probes. Thus, from Fig.3.1, the recovery of dopamine, DOPAC and HVA, at a flow rate of 2 µl/min, is about 8-10%. Other workers (see Moghaddam & Bunney, 1990; Sharp, Zetterstrom & Ungerstedt, 1986; Butcher, Fairbrother, Kelly & Arbuthnott, 1988; Osborne, O'Connor, Drew & Ungerstedt, 1990; Santiago & Westerink, 1990) report catecholamine recoveries, at an equivalent flow rate, of 8-16%. The vitafiber membrane (apart from its recovery of HVA) did not perform so well, with a relative recovery of only about 5%. It is perhaps for this reason, as well as its inherent rigidity, that this membrane is mainly used in the construction of transcerebral dialysis probes (see Zetterstrom & Ungerstedt, 1984; Imperato & Di Chiara, 1984). There is some suggestion, however, that the vitafiber membrane performs considerably better *in-vivo* than what might be predicted from *in-vitro* tests. Thus, the basal efflux of DOPAC appearing in striatal dialysates (pmoles/20 min) for vitafiber transcerebral probes has been reported to be 43.3 (Zetterstrom & Ungerstedt, 1984) and 62.2 (Imperato & Di Chiara, 1984) and for vitafiber intracerebral probes to be 18.1 (see Table 5.1) similar to the efflux of DOPAC reported for cellulose intracerebral probes of 17 (Butcher, Fairbrother, Kelly & Arbuthnott, 1988) 23.2 (Sharp, Zetterstrom & Ungerstedt, 1986) and 30.6 (Fairbrother, Arbuthnott, Kelly & Butcher, 1990). Carnegie intracerebral dialysis probes (polycarbonate membrane) exhibit superior recovery with DOPAC efflux values reported in the striatum as high as 45 pmoles/20 min (Hurd & Ungerstedt, 1989b).

Some evidence was provided in this chapter that neuronal potassium conductance may be disrupted during the first hour of cerebral dialysis. Based on the *in-vitro* recovery of potassium (10% at 2 µl/min) the extracellular level of potassium estimated in the striatum would be about 2 mM. This is somewhat lower than that reported by other workers (2.5-2.7 mM; see Moghaddam & Bunney, 1989) though this discrepancy may be related to the omission of potassium from the perfusion medium. Thus, in the rat cortex, perfusion of calcium-free artificial CSF has been reported to reduce the extracellular concentration of this ion up to a distance of about 500µm from the dialysis probe (Benveniste, Hansen &

Ottosen, 1989). It is tempting to associate the raised extracellular level of potassium detected during the first hour of cerebral dialysis (about 4-5mM) with the equally high basal levels of dopamine detected during this time (see Fig.3.6). Unfortunately, potassium was not found to modify the basal efflux of striatal dopamine when perfused at 4.5mM (Moghaddum & Bunney, 1989). It could be argued, however, that since a concentration of 4.5mM in the perfusate would only raise the extracellular level of potassium by about 0.5-1.0mM, and with the excellent buffering capacity of glial cells for potassium (Nicholson, 1980), the effects of potassium on dopamine efflux are likely to be considerably attenuated.

The insertion of a cerebral dialysis probe, at least initially, is likely to disturb the immediate microenvironment of brain tissue. This trauma probably initiates, within the first few minutes, a propagating depression (Leao, 1944), characterised by an arrest of spontaneous and evoked activity, negative deflections of DC potentials, an increase of extracellular potassium concentration, and a decrease of extracellular calcium (Hansen & Zeuthen, 1981). Indeed, a rapid decline of extracellular calcium (over about 5 minutes) has been observed following the insertion of dialysis probes into cerebral tissue (Benveniste, Hansen & Ottosen, 1989). It is perhaps surprising though, given this transient fall in calcium levels, that the efflux of potassium remained high over about the first hour in the present study. This would suggest that neuronal trauma persists during this time although it does not appear, as originally expected, that higher flow rates are associated with increased trauma (as a result perhaps of increased hydrostatic pressure within the probe). The results of this study indicate that cerebral dialysis should not be commenced for at least 1-2 hours after probe insertion since voltage-dependent mechanisms are likely to be disturbed during this time (see below).

Neurotransmitter release, whether estimated by *in-vitro* or *in-vivo* techniques, should satisfy two criteria: it should depend on neuronal activity, and it should display calcium-dependency. It is likely that during the first few hours after implantating the probe, part of the dopamine efflux is derived from damaged nerve terminals which is unrelated to nerve impulse activity. It has been suggested, in the case of dopamine, that the amount of released transmitter derived from damaged nerve terminals, can be estimated by infusing the potent sodium-channel blocker tetrodotoxin (TTX) (Westerink & De Vries, 1988). In the present study TTX rapidly (but not completely) reduced striatal dopamine

efflux 3 hours after probe insertion. This finding is consistent with the reports of other studies which have also examined the effects of TTX after acute probe insertion (Santiago & Westerink, 1990; Osborne, O'Connor, Drew & Ungerstedt, 1990). TTX is reported to have more profound and more complete effects 24 hours after probe insertion (Westerink & De Vries, 1988) which suggests that impulse-mediated transmitter release mechanisms are virtually restored by this time.

The second criterion for determining a neurogenic origin of transmitters present in dialysates; i.e., a sensitivity to calcium supply, was also investigated in the present study. Calcium-free artificial CSF produced a readily reversible decline in the efflux of dopamine although this, consistent with another report (Santiago & Westerink, 1990), and like the effects of TTX, was not complete (about 45% inhibition). The residual calcium-insensitive overflow of dopamine is likely to be due, in part, to an incomplete recovery of voltage-dependent release mechanisms as well as the presence of endogenous calcium. Although it is possible to combine a calcium-free medium with either chelating agents (EGTA), calcium-channel blockers (verapamil) or inorganic ions (Mg, Cd), this approach is considered to be less specific and, in certain conditions, toxic (see Westerink, Hofsteede, Damsma & De Vries, 1988; Santiago & Westerink, 1990). The extracellular level of calcium in the striatum is reported to be about 1.2mM (Moghaddam & Bunney, 1989; Benveniste, Hansen & Ottosen, 1989) which is somewhat lower than what is normally employed in cerebral dialysis studies (2.0-2.4mM). Recently, Benwell, Balfour & Lucchi (1991) found that the calcium concentration of the initial perfusate is important in determining whether subsequent calcium-free CSF can lower the efflux of dopamine or not. Thus, dopamine efflux into accumbal dialysates was only dependent on calcium supply when a high perfusate calcium concentration (3.4 mM) was initially employed. Imperato & Di Chiara (1984) found that by omitting calcium from a perfusion medium which originally contained 3.4mM calcium striatal dopamine efflux was reduced to undetectable levels.

Perfusion of high potassium (30 and 60mM), resulted in a concentration-related facilitation of dopamine overflow in the striatum with a concomitant fall in the efflux of DOPAC and HVA, similar to the results of other dialysis studies (see Imperato & Di Chiara, 1984; Zetterstrom, Sharp, Collin & Ungerstedt, 1988; Fairbrother, Arbuthnott, Kelly & Butcher, 1990; Arbuthnott, Fairbrother & Butcher, 1990). These effects of potassium as well as the observation that nomifensine does not consistently alter the

extracellular levels of DOPAC (Fig.3.7; see also Zetterstrom, Sharp, Collin & Ungerstedt, 1988) are consistent with the idea that DOPAC is formed, to a large extent, by the deamination of newly synthesized but unreleased dopamine (i.e., by releasing dopamine potassium may deprive MAO of its substrate) (see also Soares-Da-Silva & Garrett, 1990).

The basal efflux of dopamine, DOPAC and HVA detected in dialysates of the caudate putamen and medial prefrontal cortex remained constant over several hours (see also Imperato & Di Chiara, 1984). The extracellular concentration of these substances can be estimated by extrapolating from their respective recoveries *in-vitro*. Obviously, this method depends on how well *in-vitro* conditions simulate the extracellular environment in the brain. This may not always be the case since the recovery of calcium is reported to be considerably higher in saline than in rat cerebral cortex (Benveniste, Hansen & Ottosen, 1989). Nevertheless, the determination of extracellular concentration using *in-vitro* data is a popular approach. Using this method (and the data given in Fig.3.1 and Table 5.1) catecholamine (dopamine, DOPAC, HVA) extracellular concentrations estimated in the caudate putamen and the medial prefrontal cortex would be approximately (33nM, 9µM, 5.5µM) and (22nM, 0.5µM, 1.0µM) respectively. These values, apart from the somewhat higher than expected levels of dopamine in the cortex (but see section 5.4.1), are consistent with those estimated in other studies (see Sharp, Zetterstrom & Ungerstedt, 1986; Benveniste, 1989).

Changes in the efflux of dopamine and its metabolites in the caudate putamen after pargyline, nomifensine and amphetamine were consistent with the results of other studies (Imperato & Di Chiara, 1984; Sharp, Zetterstrom & Ungerstedt, 1986; Hurd & Ungerstedt, 1989a; Pehek, Schechter & Yamamoto, 1990; Butcher, Fairbrother, Kelly & Arbuthnott, 1988) and the known actions of these agents on dopaminergic mechanisms as discussed in section 1.2.3.

Overall, these studies show that the microdialysis system used monitors changes in dopaminergic transmission sufficiently well to enable the neurochemical effects of neuroleptics on dopamine function to be evaluated.

3.4.2 Extracellular neuronal recording

The identification of essentially two types of spontaneously active neurons in the caudate putamen has been reported elsewhere (Skirboll & Bunney, 1979; Nisenbaum, Orr & Berger, 1988). The present results show that the distinguishing characteristics of Type I and Type II waveforms are independent of the degree of filtering, distance from the electrode tip, and type of microelectrode employed (tungsten or carbon-fibre tipped). The firing rates of Type I and Type II neurons are reported to be facilitated by cortical stimulation (Nisenbaum, Orr & Berger, 1988), presumably, by the release of glutamate, either, onto their cell bodies, or their dendritic processes (but see below). These neurons may represent functionally distinct populations of cells in the caudate putamen since each show different responses to dopamine and neuroleptic drugs. Thus, type II neurons are reported to be at least 5 times more sensitive to the inhibitory effects of iontophoretically-applied dopamine than type I cells (Nisenbaum, Orr & Berger, 1988) and haloperidol, when administered acutely, has been reported to increase the number of spontaneously active type II neurons, but when chronically administered, increases both the number of spontaneously active type I neurons, as well as the firing rate of both type I and type II neurons (Skirboll & Bunney, 1979). Unfortunately, the low, and often irregular, discharge of Type II neurons in the present study, frustrated any attempt to use them in meaningful studies.

Although two types of neurons were identified in the present study, as many as six different types have been observed in morphological studies (Chronister, Farnell, Marco & White, 1976; Preston, Bishop & Kitai, 1980). Type I units may represent the most abundant type of neuron found in the caudate putamen; a medium-sized, densely spiny neuron which has been shown to project to the substantia nigra and which receives a synaptic input from the cortex as well as from dopaminergic terminals (see David-Smith & Bolam, 1990). Since several type I neurons were invariably found in the same area, they must presumably, lie in close apposition. Indeed, whilst some cells in the caudate putamen appear to be distributed individually (Type II?), other, somewhat smaller cells, show a tendency to cluster and form dendritic bundles (Chronister, Farrell, Marco & White, 1976; Skirboll & Bunney, 1979).

Neurons in the prefrontal cortex receiving a dopamine innervation are thought to be located mainly within layers V and VI (Descarries, Lemay, Doucet & Berger, 1987). These neurons typically represent pyramidal cells in exhibiting large somas and an extensive arborization of apical and basal dendritic processes (Penit-Soria, Audinat & Crepel, 1987). These were characterised in the present study by large biphasic, or less generally, triphasic action potentials exhibiting initial-segment-soma-dendritic delays (see Gariano, Tepper, Sawyer, Young & Groves, 1989) and a wide array of basal activities (0.7-11.9Hz). The faster firing rates of these neurons (0.5-50Hz), reported recently by Sesack & Bunney (1989) using a low-cerveau isole preparation, probably reflects the absence of a general anaesthetic in that preparation. Unlike the caudate putamen, spontaneously active neurons in the cortex were abundant, and therefore it is not surprising that few, if any, studies employ excitatory substances like glutamate to enhance or maintain their activity.

The application of directly-acting (glutamate, NMDA), as well as indirectly-acting (bicuculline, leptazol, picrotoxin), excitatory agents near target neurons in the caudate putamen by dialysis, rather unexpectedly, resulted in an inhibition of spontaneous activity. These effects are presumably indirectly mediated since glutamate, when applied by microiontophoresis, is reported only to stimulate the firing of striatal neurons (see Wang, White, Mereu & Hu, 1987). Thus, in the striatum, excitatory glutamatergic receptors may also be present on presynaptic dopamine neurons. Indeed, glutamate has been reported to exert a facilitatory influence on dopamine release in this area (which may be expected to inhibit activity), either when perfused *in-vivo* (1mM) by microdialysis (Shimizu, Duan, Hori & Oomura, 1990), or, when superfused across striatal slices (Giorguieff, Kemel, Glowinski & Besson, 1977; Roberts & Sharif, 1978). Moreover, electrical stimulation of the cerebral cortex, which presumably activates a corticostriatal glutamatergic pathway (see Kornhuber & Kornhuber, 1986), is reported to induce a long-lasting activation of [^{3}H]DA release in the cat caudate nucleus (Nieoullon, Cheramy & Glowinski, 1978). This latter finding, however, is difficult to reconcile with the reported activation of striatal neurons observed following the electrical stimulation of the cerebral cortex (Nisenbaum, Orr & Berger, 1988). This discrepancy may be related to differences in the stimulation parameters employed as well as the area of cortex stimulated or it may indicate that dopamine released by glutamate does not inhibit the firing of striatal neurons. If this is correct then other mechanisms may contribute to the observed inhibitory effects of glutamate and NMDA in the present study. This might explain the

large difference in the concentration of glutamate needed in the present study to affect neuronal activity (10μM) with that found by Shimizu *et al* (1990) to affect dopamine release (1mM). This difference may of course simply relate to the inability of dialysis to directly monitor synaptic events (i.e., large glutamate concentrations may be needed in order to achieve sufficient synaptic overflow of dopamine to enable detection by dialysis). The general inhibitory effects of glutamate (and NMDA) on striatal firing seen in the present study might be taken as evidence that presynaptic glutamatergic mechanisms exert a relatively greater control over the firing of striatal neurons than postsynaptic glutamatergic mechanisms.

Further attempts to enhance the spontaneous activity of neurons in the striatum by attenuating GABAergic mechanisms also proved unsuccessful. Thus, although GABA and muscimol produced the expected inhibition of neuronal discharge, this effect, apart from only some exceptional cases, was also seen with bicuculline, picrotoxin and leptazol. These effects, like those of glutamate, could be indirectly mediated since GABA agonists are reported to enhance the spontaneous release of [^{3}H]DA from slices of the rat striatum (Giorguieff, Kemel, Glowinski & Besson, 1978).

When added to the perfusion medium, dopamine, amphetamine, 2-methyl-5-HT and CGS-15855A all produced an inhibition of striatal neuronal firing. Presumably, this effect is mediated by the direct (dopamine, CGS-15855A) or indirect (amphetamine, 2-methyl-5-HT) stimulation of dopamine receptors which may lie on intrinsic striatal neurons. Although L-ascorbic acid (10μM) has been reported to inhibit striatal adenylate cyclase activity (Thomas & Zemp, 1977), no effects of this compound were observed on the spontaneous discharge of striatal neurons even at 250μM. Since the selective dopamine D_2 receptor agonist CGS-15855A was considerably less potent than dopamine in inhibiting neuronal firing despite presumably not being taken-up into dopamine nerve terminals, it is possible that dopamine D_1 receptors mediate some of the inhibitory effects of dopamine on neuronal activity in this area. This conclusion has also been reached by other workers (see Hu & Wang, 1988; Ohno, Sasa & Takaori, 1987; Calabresi, Mercuri, Stanzione, Stefani & Bernardi, 1987). It was somewhat surprising that 2-methyl-5-HT produced complete cessation of striatal activity despite the report that 5-HT_3 receptors are only sparsely located in this area (Kilpatrick, Jones & Tyers, 1987). Presumably therefore, if dopamine release in the striatum is enhanced by 5-HT_3 receptor stimulation (Blandina,

Goldfarb & Green, 1988; see also chapter 8) then 2-methyl-5-HT may, nonetheless, still produce sufficient synaptic levels of dopamine to inhibit neuronal firing.

3.5 SUMMARY

The intracerebral microdialysis system developed in this chapter meets the criteria necessary to enable the actions of neuroleptic compounds on dopamine neurotransmission to be evaluated: (1) the efflux of dopamine in brain dialysates depends to some extent on calcium supply and neuronal activity (2) the dialysate levels of DA, DOPAC and HVA are measurable and (3) remain constant over many hours (4) DA, DOPAC and HVA levels respond in a predictable way to agents known to affect dopamine function.

Two distinctive action potential waveforms (Type I & II) were found in recordings from neurons in the rostral caudate putamen. The spontaneous activity of these neurons was low and generally exhibited burst firing. Type I neurons predominated and tended to exist in clusters. Spontaneously active neurons in the medial prefrontal cortex showed large biphasic or triphasic action potentials and exhibited faster rates than striatal neurons. Prefrontal neurons exhibited burst activity with a progressive within-burst diminution of spike amplitude. In general, recordings could be made from spontaneously active neurons in the caudate putamen for up to 3 hours, whereas neurons in the prefrontal cortex could be reliably monitored for at least 5-6 hours, and in some cases, up to 8 hours. This information provides a rational basis for selecting the experimental protocols adopted in chapter 6.

Attempts to enhance the firing rate of spontaneously active striatal neurons using microdialysis to locally administer excitatory agents were generally unsuccessful. The paradoxical inhibitory effects of these agents may have resulted from indirect interactions with neuronal afferents involved in the presynaptic control of dopamine release although other transmitter mechanisms may also be involved. Perfusion of dopaminergic agents by microdialysis produced concentration-related inhibitory effects on striatal neuronal firing. Since the selective dopamine D_2 receptor agonist CGS-15855A was considerably less effective than dopamine in these studies it was concluded that dopamine D_1 receptors possibly mediate some of the inhibitory effects of dopamine in this area.

CHAPTER 4

ACTIVITY OF TYPICAL AND ATYPICAL NEUROLEPTIC DRUGS AT $5\text{-}HT_3$ AND OTHER PERIPHERAL NON-DOPAMINERGIC RECEPTORS

4.1 INTRODUCTION

The aims of the work presented in this chapter were to evaluate the activity of a range of typical and atypical neuroleptic drugs at a variety of non-dopaminergic receptors, and to correlate these actions with the known clinical effects of these agents. Although both the antischizophrenic and the adverse motor effects of neuroleptics appear to arise through antagonism of central dopamine receptors, it has long been recognised that additional activity by neuroleptic agents at non-dopaminergic receptors could probably contribute to the relative expression of their clinical effects. Also in view of the widely differing molecular structures of these compounds it is likely that any non-dopaminergic effects could involve different neurotransmitters. Indeed, numerous studies have been dedicated toward elucidating the activity of neuroleptics at non-dopaminergic receptors (see Creese, Burt & Snyder, 1976; Jenner & Marsden, 1981; Richelson & Nelson, 1984; Hall, Sallemark & Jerning, 1986). However, many of these studies have assessed only their affinity for specified receptors using radioligand binding assays which gives no information on the outcome of any receptor interaction, i.e., whether the drug is acting as an antagonist, a partial agonist or as an agonist. It was for this reason, as well as the reports that 5-HT_3 receptors may be important in the regulation of central dopamine function (Costall, Domeney, Naylor & Tyers, 1987; Carboni, Acquas, Frau & Di Chiara, 1989; Hagen, Jones, Jordan & Tyers, 1990), that the present studies were undertaken. In this chapter a range of neuroleptic drugs were tested for activity at 5-HT_3, acetylcholine (muscarinic), tachykinin (substance P), alpha-1 and alpha-2 adrenergic receptors. This was achieved by evaluating the actions of these agents against the contractile effects of selective agonists on smooth muscle preparations (see section 2.2). It was hoped that an evaluation of neuroleptic activity for such non-dopaminergic receptors might help explain differences in the ability of these agents to produce their central effects.

There is now ample evidence to indicate that serotonin may have an important regulatory function on dopaminergic mechanisms in several forebrain structures such as the caudate putamen and the nucleus accumbens. *In vitro*, incubation of striatal slices and synaptosomes with 5-HT agonists has been shown to both inhibit (Westfall & Tittermary, 1982) and facilitate dopamine release (Blandina, Goldfarb & Green, 1989; De Belleroche & Bradford, 1980). Recently, the selective 5-HT_3 antagonist GR38032F (ondansetron), was found to block the hyperlocomotor response observed after the administration of either

amphetamine, or the infusion of dopamine into the nucleus accumbens (Costall, Domeney, Naylor & Tyers, 1987), and also to inhibit the hyperactivity (Hagen, Jones, Jordon & Tyers, 1990) and elevation of dopamine turnover in the nucleus accumbens (Hagen, Butler, Hill, Jordon, Ireland & Tyers, 1987) resulting from the injection of the neurokinin receptor agonist (DiMe-C7) into the rat ventral tegmental area. Since 5-HT_3 receptors appear to be differentially distributed throughout the CNS, with high numbers in cortical and limbic areas, and a relatively sparse number in the striatum (Kilpatrick, Jones & Tyers, 1987) it could be argued that selective 5-HT_3 receptor antagonists might be antipsychotic yet produce few adverse motor effects. This possibility is supported by the finding that these agents appear to modify the behavioural and neurochemical consequences of raised dopamine function in mesolimbic areas (see above) without blocking amphetamine-induced stereotypy, producing catalepsy or raising plasma prolactin levels in rats (Costall, Domeney, Kelly, Naylor & Tyers, 1987). In the present study a number of neuroleptic drugs were tested for 5-HT_3 receptor antagonist activity to gauge whether this is a feature common to only atypical neuroleptics.

Since antimuscarinic drugs have some beneficial effects in the treatment of Parkinson's disease any antagonistic activity of neuroleptic drugs at muscarinic acetylcholine receptors would be expected to reduce the likelihood of them causing extrapyramidal effects. Indeed, antimuscarinic agents, such as procyclidine and benzhexol are often co-administered with neuroleptic drugs to attenuate these adverse neurological effects. Little is known, however, of the role of substance P in the CNS, which is found in high levels in the mesencephalon, hypothalamus and preoptic area (Brownstein, Mroz, Kizer, Palkovits & Leeman, 1976). Substance P, when unilaterally injected into the substantia nigra can cause contralateral rotation (James & Starr, 1979) and an increased release of dopamine (Cheramy, Nieoullon, Michelot & Glowinski, 1977) in the ipsilateral striatum suggesting that substance P has a stimulatory action on the nigrostriatal pathway. Consistent with this view is the observation that iontophoretically applied substance P activates the firing of nigral neurons (Pinnock, Woodruff & Turnball, 1983). The actions of substance P, however, does not appear to be exclusively mediated in this system since it also stimulates the mesolimbic (Hagen, Butler, Hill, Jordan, Ireland & Tyers, 1987; Stinus, Kelley & Iverson, 1978; Hagen, Jones, Jordan & Tyers, 1990) and mesocortical (Bannon, Elliot, Alpert, Goedert, Iverson & Iverson, 1983) dopamine systems. Given the discrete regional distribution of substance P, close to the origin of the major ascending

dopamine pathways, and its many reported effects on dopamine neurotransmission, it would be of interest to know whether neuroleptic agents can interact directly with tachykinin receptors. Although haloperidol, has been shown to block the hyperactivity induced in rats, by the injection of the stable substance P analogue DiMe-C7, into the ventral tegmental area (Hagen, Butler, Hill, Jordan, Ireland & Tyers, 1987), it is likely that this action is mediated through antagonism of dopamine receptors, rather than at tachykinin receptors. It is anticipated that these indirect effects will be minimised in the present study by examining the actions of neuroleptic drugs against the contractile effects of substance P on isolated smooth muscle.

As well as blocking dopamine receptors, most neuroleptic drugs also antagonise noradrenergic receptors (Peroutka, U'Prichard, Greenberg & Snyder, 1977; Richelson & Nelson, 1984), but in contrast to dopamine receptor blockade, the noradrenaline blocking potencies of neuroleptics, as measured by adrenoceptor affinities, are not well correlated with their antipsychotic potencies (Richelson & Nelson, 1984; Hall, Sallemark & Jerning, 1986). Although the role of noradrenaline in the etiology of schizophrenia is unknown, there have been some reports of raised noradrenaline levels, estimated in both the limbic forebrain of postmortem schizophrenic brains (Farley, Price, McCullough, Deck, Hordynski & Hornykiewicz, 1978) and in the cerebrospinal fluid of chronic paranoid schizophrenic patients (Lake, Sternberg, Kammen, Ballenger, Ziegler, Post, Kopin & Bunney 1980; Sternberg, Van Kammen, Lake, Ballenger, Marder & Bunney, 1981). Neither propranolol (Peet, 1981), a non-selective β-adrenoceptor antagonist, or classical α-adrenoceptor antagonists such as phenoxybenzamine (Hornykiewicz, 1982) appear to produce any convincing antipsychotic effects. The α_2-adrenoceptor agonist, clonidine, however, may have some antipsychotic efficacy (Alder, Bell, Kirch, Friedrich & Freedman, 1982) and yohimbine, an α_2-adrenoceptor antagonist, has been noted to trigger latent psychotic processes (Holmberg & Gershon, 1961). There is some evidence to indicate that α_1-adrenoceptors may be involved in mediating the differential action of typical and atypical neuroleptics on A9 and A10 midbrain dopamine neurons (see chapter 1) since the α_1-adrenoceptor antagonist, prazosin, was found to prevent the inactivation of A9 dopamine neurons by chronically administered haloperidol (Chiodo & Bunney, 1985).

4.2.1 Neuroleptic activity at 5-HT_3 receptors

Both 5-HT (0.2-20μM) and 2-methyl-5-HT (1.0-40μM) caused concentration-related contractions of the isolated guinea-pig ileum with no significant differences noted in their maximal effects (Fig.4.1). The EC_{50} (± s.e.mean) values for 5-HT and 2-methyl-5-HT were 2.2 ± 0.7μM (*n*=20) and 6.8 ± 0.9μM (*n*=20) respectively. 5-HT_3 receptor antagonist potencies of neuroleptic compounds were assessed against 2-methyl-5-HT since this compound has been reported to be a selective 5-HT_3 receptor agonist (Richardson, Engel, Donatsch & Stadler, 1985). Metoclopramide, as well as the putatively selective 5-HT_3 receptor antagonists, GR38032F and ICS205930 (dose-response curves not shown), caused parallel, rightward shifts of the 2-methyl-5-HT concentration-response curve (Fig.4.2). This antagonism was assessed to be competitive from a Schild analysis of the data (Fig.4.8) and the pA_2 (± s.e.mean) values for GR38032F, ICS205930 and metoclopramide were 7.10 ± 0.12, 7.98 ± 0.08 and 5.31 ± 0.25 respectively. Of the neuroleptic drugs, clozapine blocked the response of 2-methyl-5-HT in an insurmountable manner (Fig.4.3). Despite this form of antagonism shown by clozapine, a Schild analysis of the data (Fig.4.8) revealed linearity (r^2=0.925), although the slope (± s.e.mean) was significantly greater than unity (1.39 ± 0.13 $p<0.01$). The pA_2 (± s.e.mean) value of clozapine was estimated to be 6.20 ± 0.14. Of the remaining atypical neuroleptic compounds, remoxipride and thioridazine both produced only weak antagonism of 2-methyl-5-HT's actions, and sulpiride, was found to potentiate the response at 1.0μM. This enhancement of response was also seen with the typical neuroleptic agent haloperidol at 1.0μM (Fig.4.2). Chlorpromazine produced only weak insurmountable antagonism, comparable to that of thioridazine (pA_2 < 5.3).

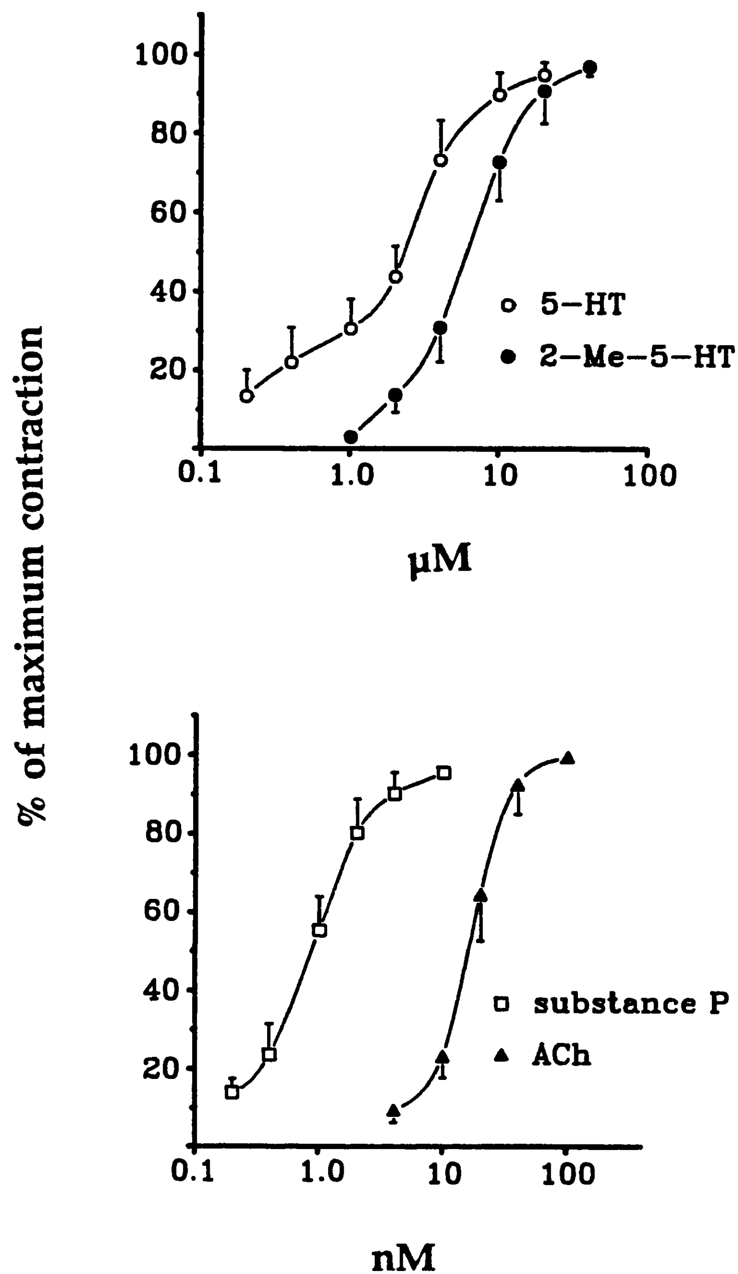
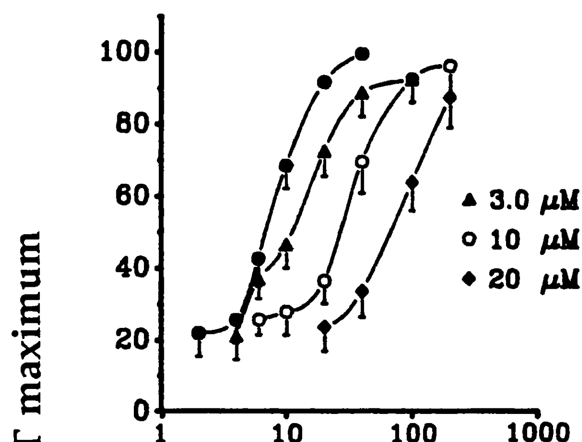
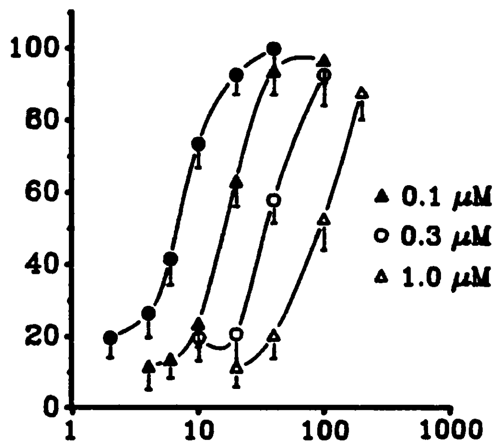


Figure 4.1 Concentration-dependent effects of various agonists on the isolated guinea-pig ileal preparation. Each data point (± s.e.mean, *n*=20) represents the contractile response (expressed as a percentage of maximum) of the tissue to either, 5-HT (○), 2-methyl-5HT (●), substance P (□) or acetylcholine (▲). The EC_{50} values (± s.e.mean, *n*=20) estimated for each agonist were 2.2 ± 0.7µM (5-HT), 6.8 ± 0.9µM (2-methyl-5-HT), 15.5 ± 0.9nM (ACh) and 0.91 ± 0.09nM (SP).

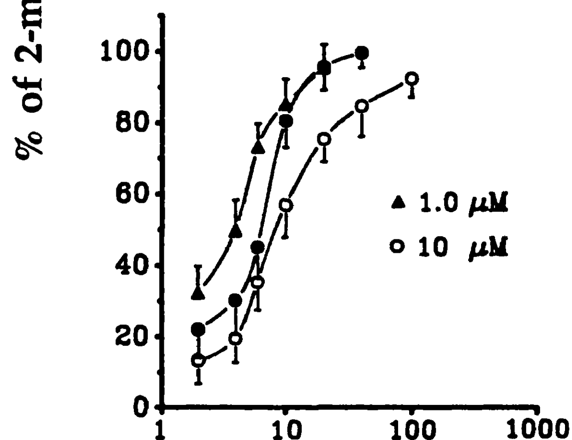
A. METOCLOPRAMIDE



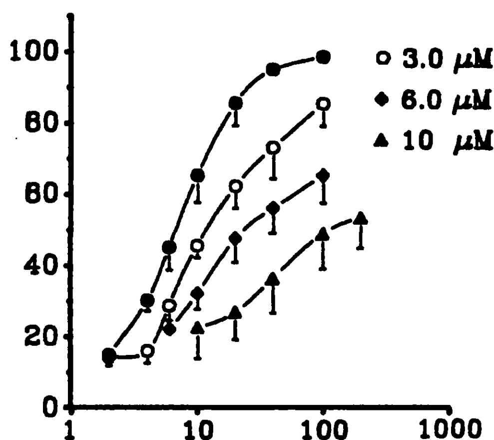
B. GR 38032F



C. HALOPERIDOL



D. CHLORPROMAZINE



% of 2-methyl-5HT maximum

2-methyl-5HT (μM)

Figure 4.2 Concentration-response curves showing the effects of metoclopramide, GR38032F (a selective 5-HT_3 receptor antagonist), haloperidol and chlorpromazine, on the contractile response of the isolated guinea-pig ileum to 2-methyl-5-HT. Each data point represents the mean (± s.e.mean, n=4-5) contractile response of the ileum, to either, 2-methyl-5-HT alone (●) (control), or to 2-methyl-5HT in the presence of increasing concentrations of each compound.

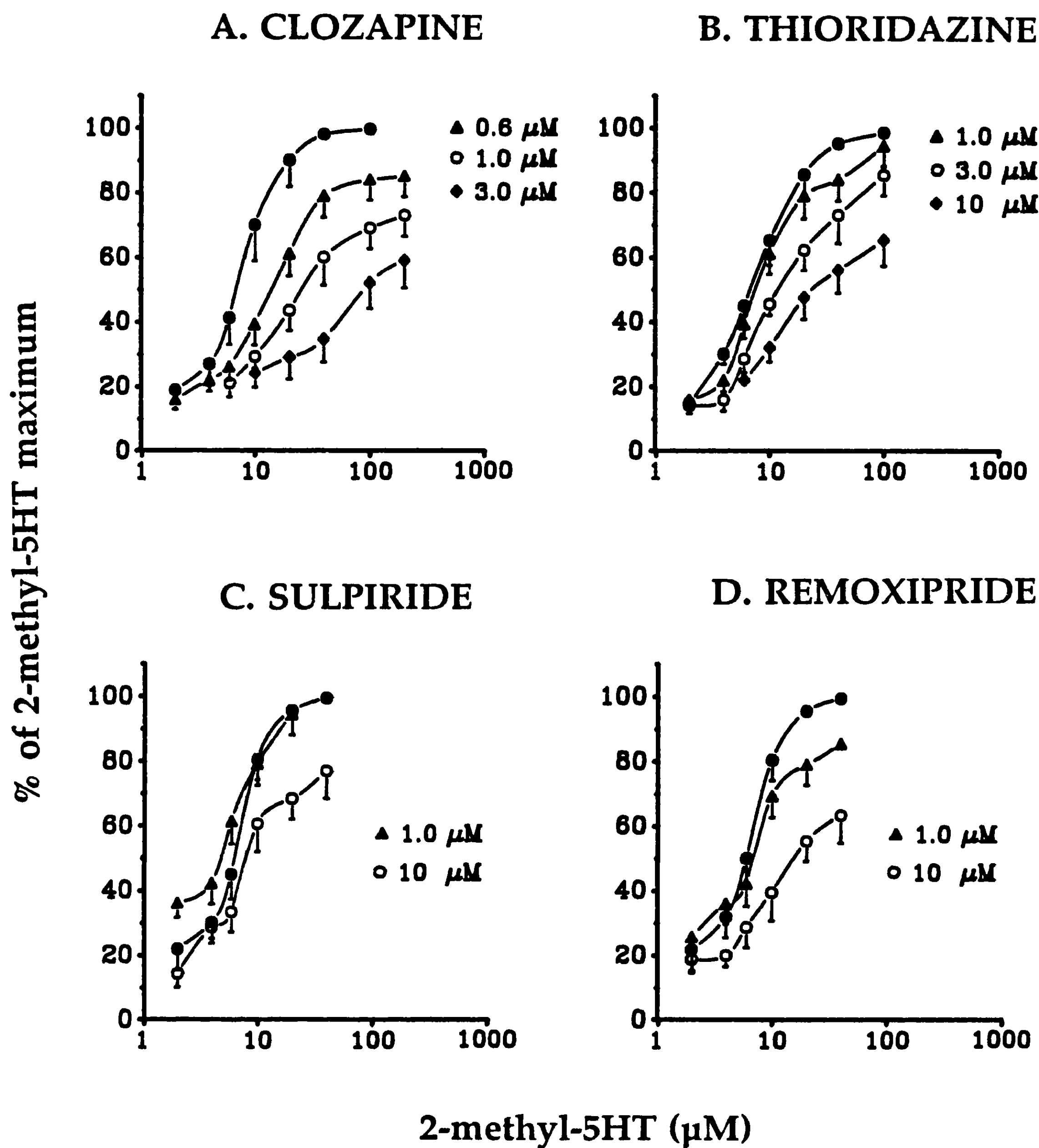


Figure 4.3 Concentration-response curves showing the effects of the atypical neuroleptic compounds, clozapine, thioridazine, sulpiride and remoxipride, on the contractile response of the isolated guinea-pig ileum to 2-methyl-5-HT. Each data point represents the mean (± s.e.mean, *n*=4-5) contractile response of the ileum, to either, 2-methyl-5-HT alone (●) (control), or to 2-methyl-5-HT in the presence of increasing concentrations of each compound.

4.2.2 Neuroleptic activity at muscarinic acetylcholine receptors

Acetylcholine contracted the ileum in a concentration-related manner (Fig.4.1) with an EC_{50} (± s.e.mean, n=20) of 15.5 ± 0.9nM. Atropine antagonised the actions of acetylcholine in a competitive manner (Fig.4.8) with a pA_2 value of 8.20 ± 0.17. Of the atypical neuroleptic compounds, clozapine and thioridazine both had marked antimuscarinic actions (Fig.4.4) with pA_2 values (± s.e.mean) estimated to be 7.06 ± 0.13 and 6.72 ± 0.13 respectively, however, sulpiride and remoxipride, were both inactive against acetylcholine. The typical neuroleptic chlorpromazine produced moderate antagonism of acetylcholine (pA_2 5.91 ± 0.78), and in increasing doses, diminished the maximal effect achievable (Fig.4.4) whilst haloperidol produced only weak antagonism, with an approximate pA_2 value (± s.e.mean) of 5.37 ± 0.35 (only 2 concentrations of haloperidol used in this estimation). Metoclopramide, as well as the putatively selective 5-HT_3 receptor antagonists, GR38032F and ICS205930, at concentrations of 10µM, were all devoid of antimuscarinic activity.

4.2.3 Neuroleptic activity at tachykinin (substance P) receptors

Substance P potently contracted the ileum with an estimated EC_{50} (± s.e.mean, n=20) of 0.91 ± 0.09nM. Its concentration-response curve was shifted rightward and in a parallel manner by the putative substance P antagonist, D-Pro^4, D-$Trp^{7,9,10}$ SP_{4-11}, at concentrations of 10 and 30µM (data not shown). The phenothiazine neuroleptics, unlike the remaining neuroleptics, blocked the actions of substance P, with thioridazine comparatively more effective than chlorpromazine, although for both compounds, the antagonism was insurmountable in nature. Antagonism of substance P appeared to be a feature of the phenothiazines since promethazine, an agent devoid of antipsychotic activity, was also effective against substance P, albeit only weakly. The pA_2 values (± s.e.mean) for thioridazine and chlorpromazine were estimated to be 6.24 ± 0.18 and 6.04 ± 0.19 respectively, however, these values are only approximations since only two concentrations (1.0 and 6.0µM) could be used in their estimation by Schild analysis. The antagonism of substance P by thioridazine and chlorpromazine was apparently competitive in nature since the slopes of the limited Schild plots, were not significantly different from unity. Chart recordings showing the antagonistic effects of thioridazine and clozapine on the ileal contractions induced by substance P and 2-methyl-5-HT are given in Fig.4.5.

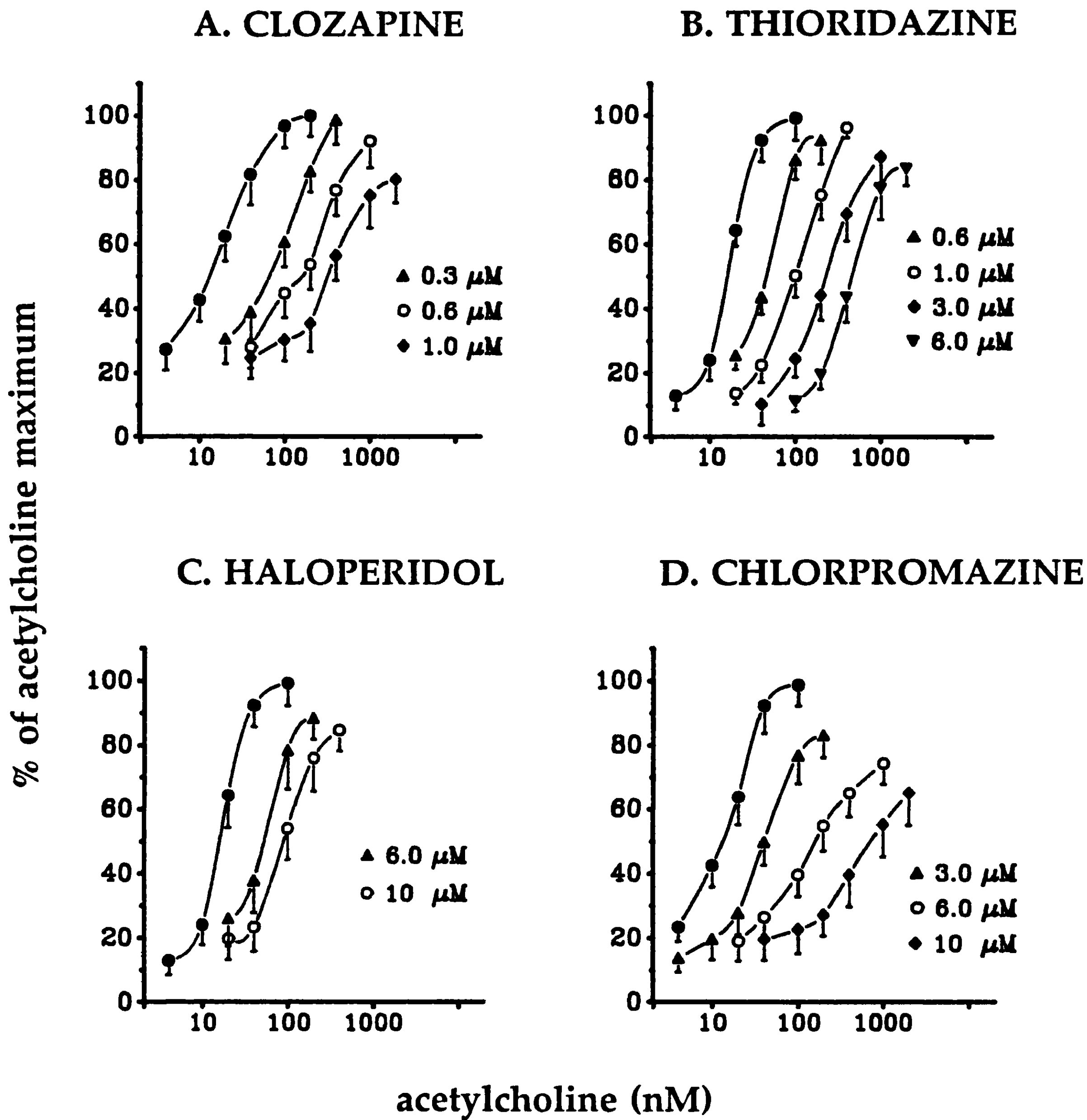


Figure 4.4 Concentration-response curves showing the effects of clozapine, thioridazine, haloperidol and chlorpromazine on the contractile response of the isolated guinea-pig ileum to acetylcholine. Each data point represents the mean (± s.e.mean, n=4-5) contractile response of the ileum, to either, acetylcholine alone (●) (control), or to acetylcholine in the presence of increasing concentrations of each compound.

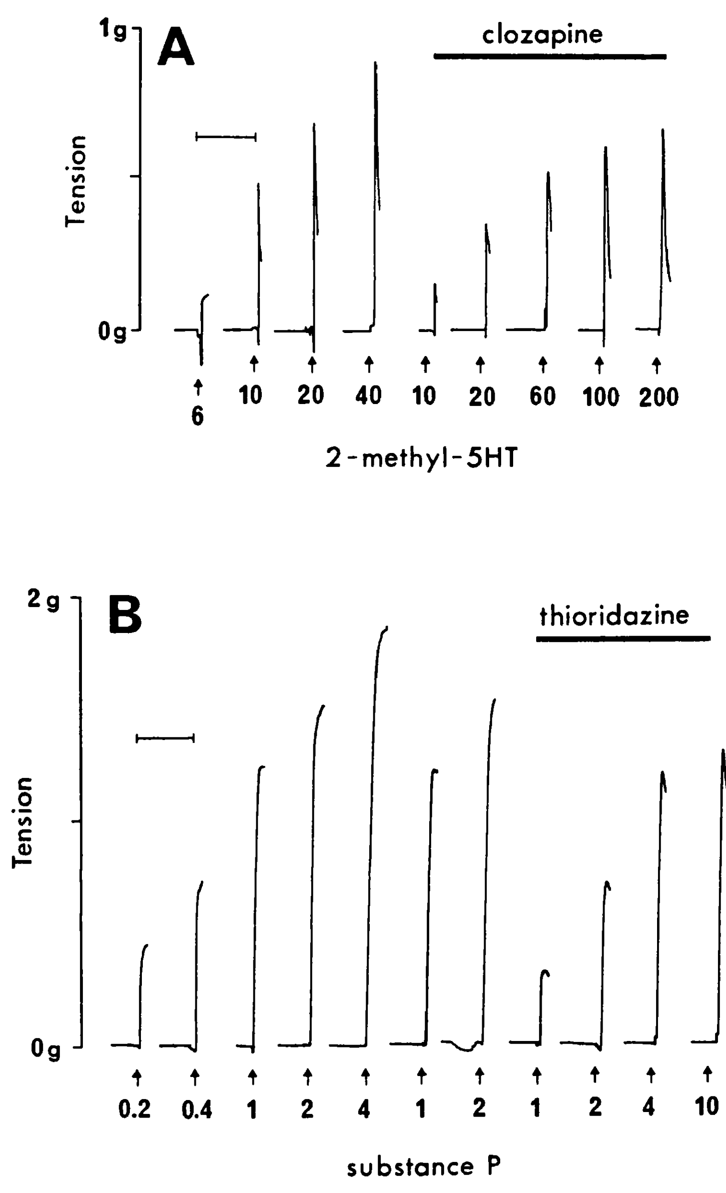


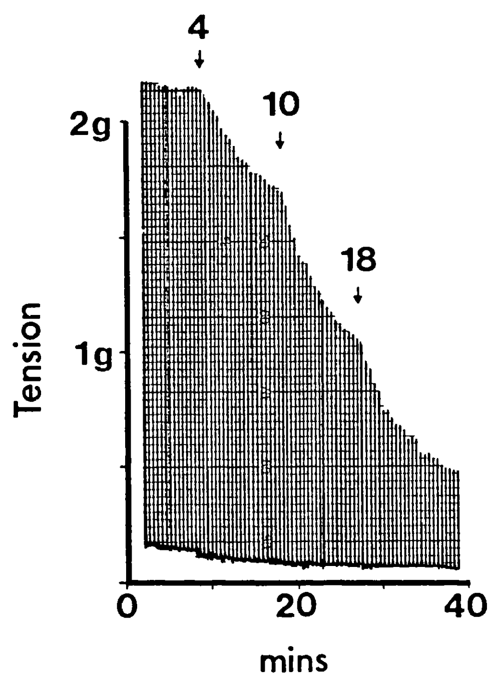
Figure 4.5 Chart recordings showing the effects of clozapine (1μM) and thioridazine (1μM) on the contractile response of the isolated guinea-pig ileum to 2-methyl-5-HT (μM) and substance P (nM) respectively. Clozapine and thioridazine (presence indicated by the bars) were pre-equilibrated with the tissue for 60 minutes before further concentrations of each agonist were applied. The horizontal calibration bars represent 15 minutes.

4.2.4 Neuroleptic activity at alpha-1 adrenoceptors

Adrenaline produced concentration-related contractions of the rat vas-deferens (propranolol present at 100nM) with an EC_{50} (± s.e.mean, n=20) of 9.67 ± 0.94μM. Prazosin potently and competitively antagonised the effects of adrenaline, and from the Schild plot given in Fig.4.8, its pA_2 value (± s.e.mean) was estimated to be 7.44 ± 0.60. Of the atypical neuroleptic agents, thioridazine (potently) and clozapine (moderately) antagonised the actions of adrenaline in a competitive manner, with estimated pA_2 values (± s.e.mean) of 6.92 ± 0.21 and 6.17 ± 0.34 respectively, however, sulpiride and remoxipride were both devoid of activity. Chlorpromazine and haloperidol both produced moderate antagonistic effects against adrenaline and the pA_2 (± s.e.mean) values estimated were 6.29 ± 0.65 and 5.62 ± 0.47 respectively. Metoclopramide, and the selective 5-HT_3 receptor antagonists, GR38032F and ICS205930, at concentrations of 10μM, were each, without significant activity in this preparation.

4.2.5 Neuroleptic activity at alpha-2 adrenoceptors

The selective alpha-2 agonist clonidine reduced the contractile response of the rat vas-deferens to electrical field stimulation in a concentration-dependent manner (Fig.4.6). The IC_{50} value (± s.e.mean, n=25) of clonidine in this preparation was found to be 9.08 ± 0.31nM. The cumulative concentration-response curve of clonidine was shifted to the right and in a parallel manner by yohimbine (data not shown) and from Fig.4.8, the pA_2 value (± s.e.mean) of this compound was determined to be 7.64 ± 0.09. Metoclopramide, at concentrations above 10μM, unlike all the other compounds tested, enhanced the contractile response of the vas-deferens to field stimulation. Metoclopramide showed only weak antagonism against clonidine (pA_2 5.30 ± 0.06), and neither GR38032F nor ICS205930, at concentrations of 10μM, produced any significant effects in this preparation. Of the typical neuroleptics, haloperidol was virtually devoid of antagonist activity (pA_2 < 5.0) but chlorpromazine was more effective in this respect (pA_2 5.6 ± 0.1). The most effective neuroleptic tested was clozapine which blocked the actions of clonidine in a moderately potent and competitive manner (Figs.4.7 and 4.8) (pA_2 6.23 ± 0.06). Of the remaining atypical neuroleptic compounds, thioridazine, showed weak to moderate activity (pA_2 5.65 ± 0.11), whereas, sulpiride (pA_2 4.92 ± 0.07) and remoxipride (pA_2 < 5.0) were less effective.



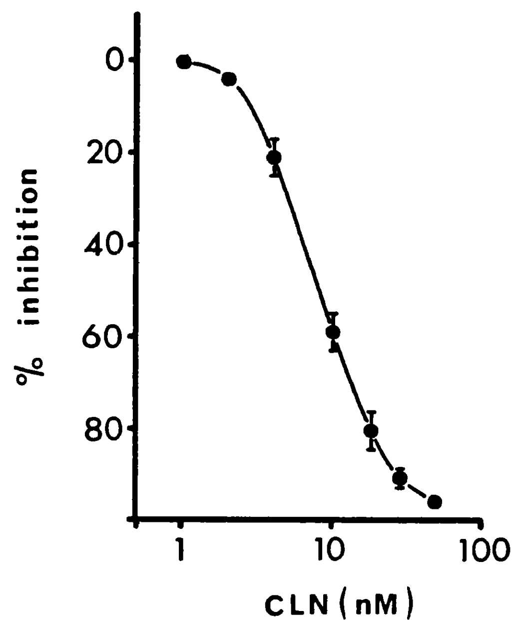


Figure 4.6 Concentration-dependent inhibitory effects of clonidine (CLN) on the field-stimulated isometric contraction of the rat vas-deferens. (A) Typical chart recording showing the effects of clonidine (given cumulatively in nM [4, 10, 18] at approximately 10 minute time intervals) on the response of the vas-deferens to field stimulation. (B) Concentration-response curve from which the IC_{50} value (± s.e.mean, n=20) of clonidine was estimated to be 9.08 ± 0.31nM. The error bars represent s.e.means.

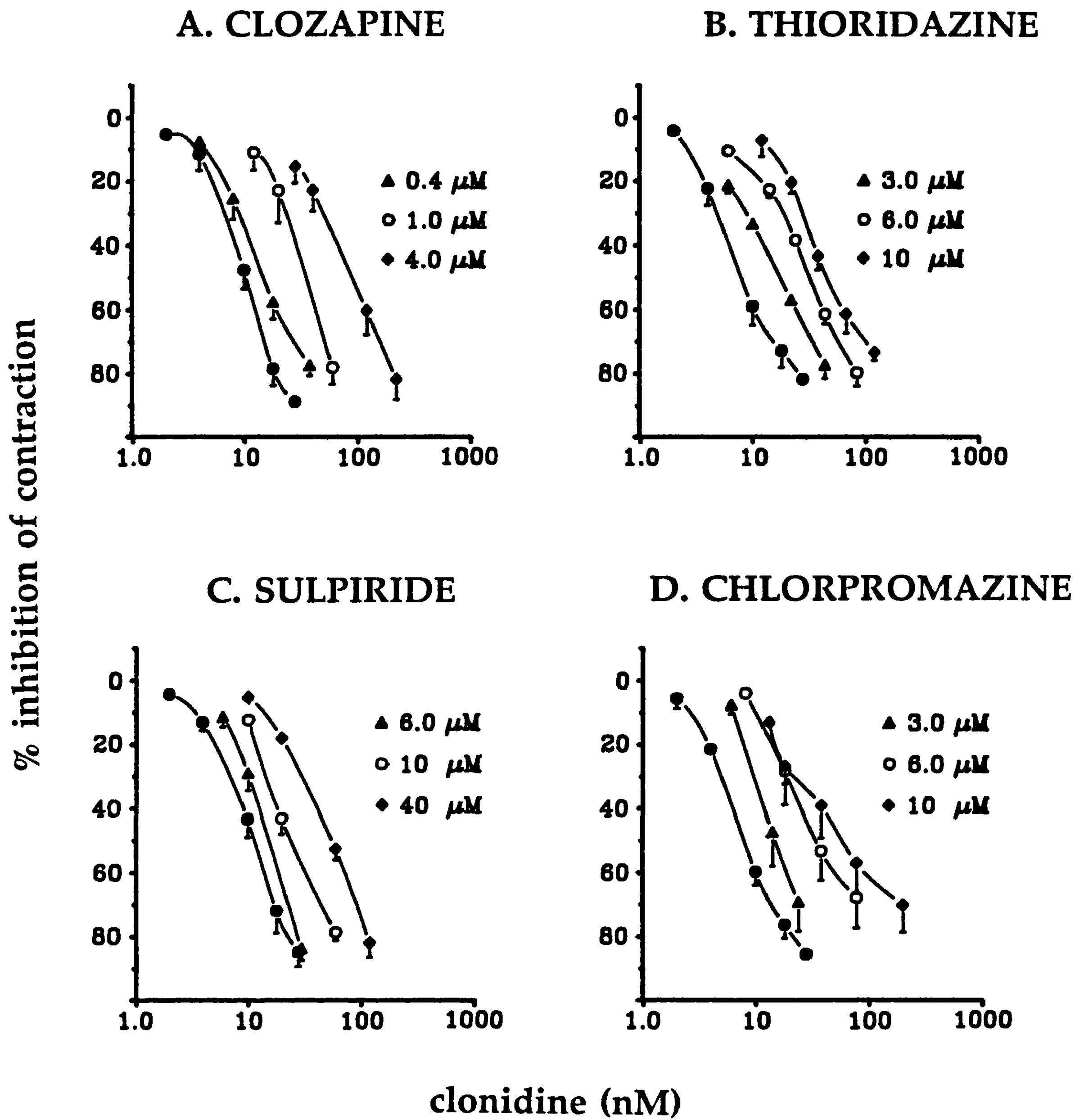


Figure 4.7 Concentration-response curves showing the effects of clozapine, thioridazine, sulpiride and chlorpromazine on the field-stimulated contractile response of the rat vas-deferens to clonidine. Each data point represents the mean (± s.e.mean, n=3-4) contractile response of the vas-deferens, to either, clonidine alone (●) (control), or to clonidine in the presence of increasing concentrations of each compound.

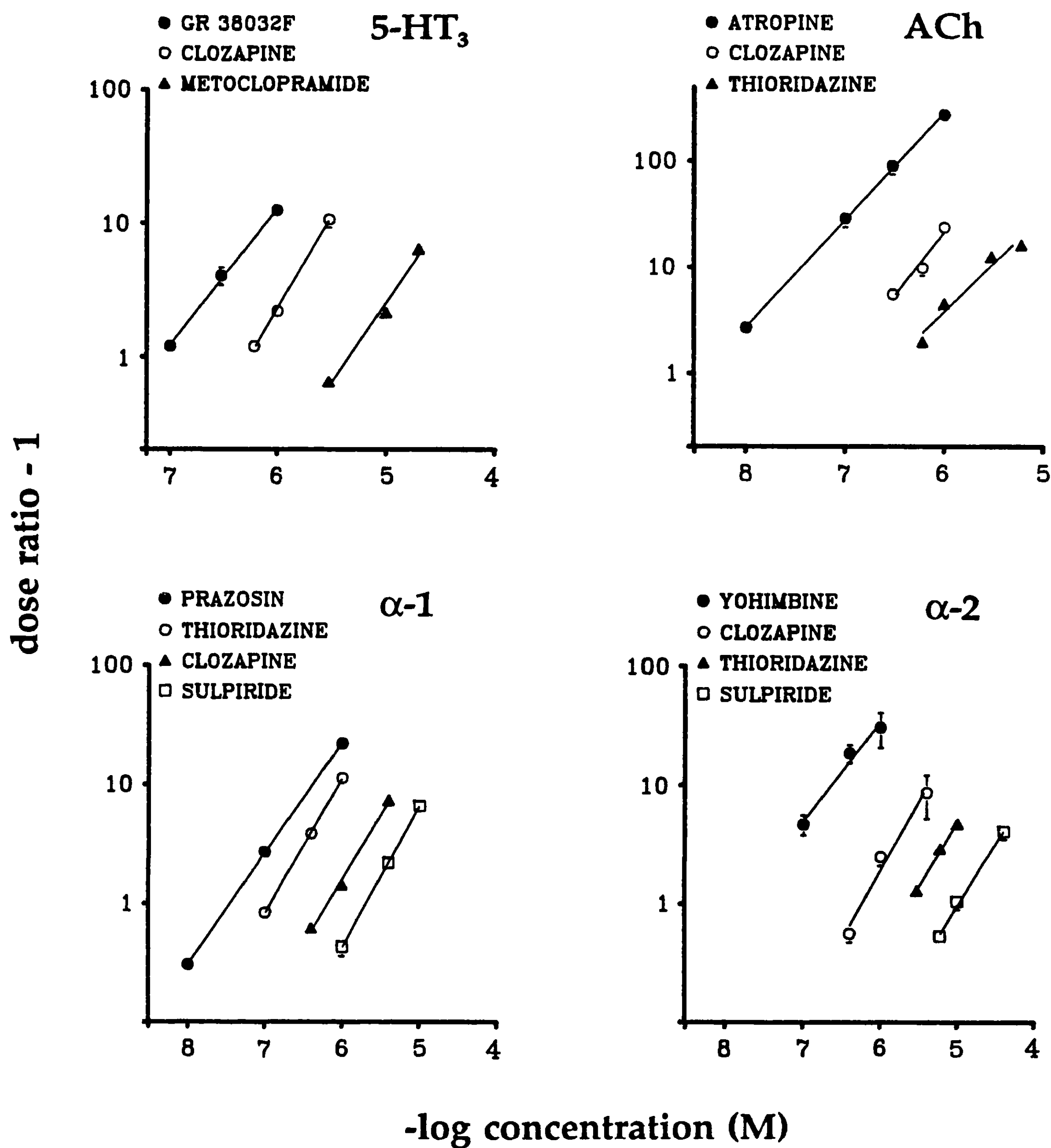


Figure 4.8 Antagonism by neuroleptics and selective agents of 5-HT_3, muscarinic, α-1 and α-2 adrenergic receptors. Dose ratios calculated from concentration response curves given in Figs.4.2, 4.3, 4.4 and 4.7 are plotted according to the method of Arunlakshana & Schild (1959). Linear regression (least squares) was used to fit a straight line through the data (± s.e.mean, *n*=3-5) of which only clozapine against 2-methyl-5-HT (1.39 ± 0.13) was significantly greater than unity ($p<0.01$). The estimated pA_2 values obtained from these "Schild" plots are listed in Table 4.1.

Table 4.1 (following page) Activity spectrum of typical and atypical neuroleptics, selective $5\text{-}HT_3$ receptor antagonists, and metoclopramide at dopaminergic, muscarinic, tachykinin, serotoninergic and adrenergic receptors (see "abbreviations and units" section). The data listed in the table were derived either from results obtained in the present studies (pA_2 values with s.e.means [*n*=4-5] given in brackets), or from the literature (receptor binding affinities expressed as either K_i or IC_{50} values). Table references and further explanatory notes are given in the key below:

(i) Inhibition constants (nM). [^{3}H]-SCH23390 and [^{3}H] spiperone were used to label rat striatal D_1 and D_2 dopamine receptors respectively (from Seeman & Grigoriadis (1987).

(ii) Inhibition constants (nM). [^{125}I]-iodosulpiride was used to label D_2 and D_3 dopamine receptors expressed in CHO cells (from Sokoloff *et al.*, 1990).

(iii) IC_{50} values (μM). [^{3}H]-spiperone was used to label $5\text{-}HT_2$ receptors in the rat cortex (from Hall *et al.*, 1986).

(iv) From Hall *et al.*, (1986).

(v) pIC_{50} values; personal communication from Dr Rainer Gamse (Sandoz, Ltd Basle) although no details were given regarding the origin of these estimates.

(vi) GR38032F appears to have little or no direct activity at dopamine receptors since it does not block amphetamine-induced stereotypy, produce catalepsy, raise plasma prolactin levels or displace [^{3}H]-spiperone binding (Costall, Domeney, Kelley, Naylor & Tyers, 1987).

(vii) Only two antagonist concentrations used in the estimation of the pA_2 value.

(viii) Slopes of the Schild plots were significantly greater than unity.

(ix) pA_2 estimate taken from Butler *et al.*, (1988). (rat aorta preparation using 5-HT as the agonist)

compound	clinical profile	D-1[i] K_i(nM)	D-2[ii] K_i(nM)	D-3[ii] K_i(nM)	ACh pA_2	SP pA_2	5-HT_2[iii] IC_{50} (μM)	5-HT_3 pA_2	α-1 pA_2	α-2 pA_2
	atypical NLs									
CLZ		200	56	180	7.06 (0.13)	<5.2	0.019	6.20[viii] (0.14)	6.17 (0.34)	6.23 (0.06)
SUL	↓ psychosis ↓ EPSEs	28,000	9.2	25	<5.0	<5.0	>100	<5.0	<5.0	4.92 (0.07)
THIO		10	3.3	7.8	6.72 (0.13)	6.24[vii] (0.18)	0.055	<5.3	6.92 (0.21)	5.65 (0.11)
REM		weak[iv]	120[iv]	-	<5.0	<5.0	32.3	<5.0	<5.0	<5.0
	typical NLs									
HAL	↓ psychosis ↑ EPSEs	63	0.45	9.8	5.37[vii] (0.35)	<5.0	0.036	<5.0	5.62 (0.47)	<5.0
CPZ		178	2.8	6.1	5.91[viii] (0.38)	6.04[vii] (0.19)	0.031	<5.3	6.29 (0.55)	5.60 (0.10)
MET	? psychosis ↑ EPSEs	50,000	49[i]	-	<5.0	<5.0	3.75	5.31 (0.25)	<5.0	5.30 (0.06)
	$5HT_3$ agents									
ICS-205930		<5.0[v]	<5.0[v]	-	<5.0	-	-	7.98 (0.08)	<5.0	<5.0
GR-38032F	? psychosis ? EPSEs	weak[vi]	weak[vi]	-	<5.0	-	4.6[ix]	7.10 (0.12)	<5.0	<5.0

Table 4.1 Activity of neuroleptics and other compounds at dopaminergic and non-dopaminergic receptors

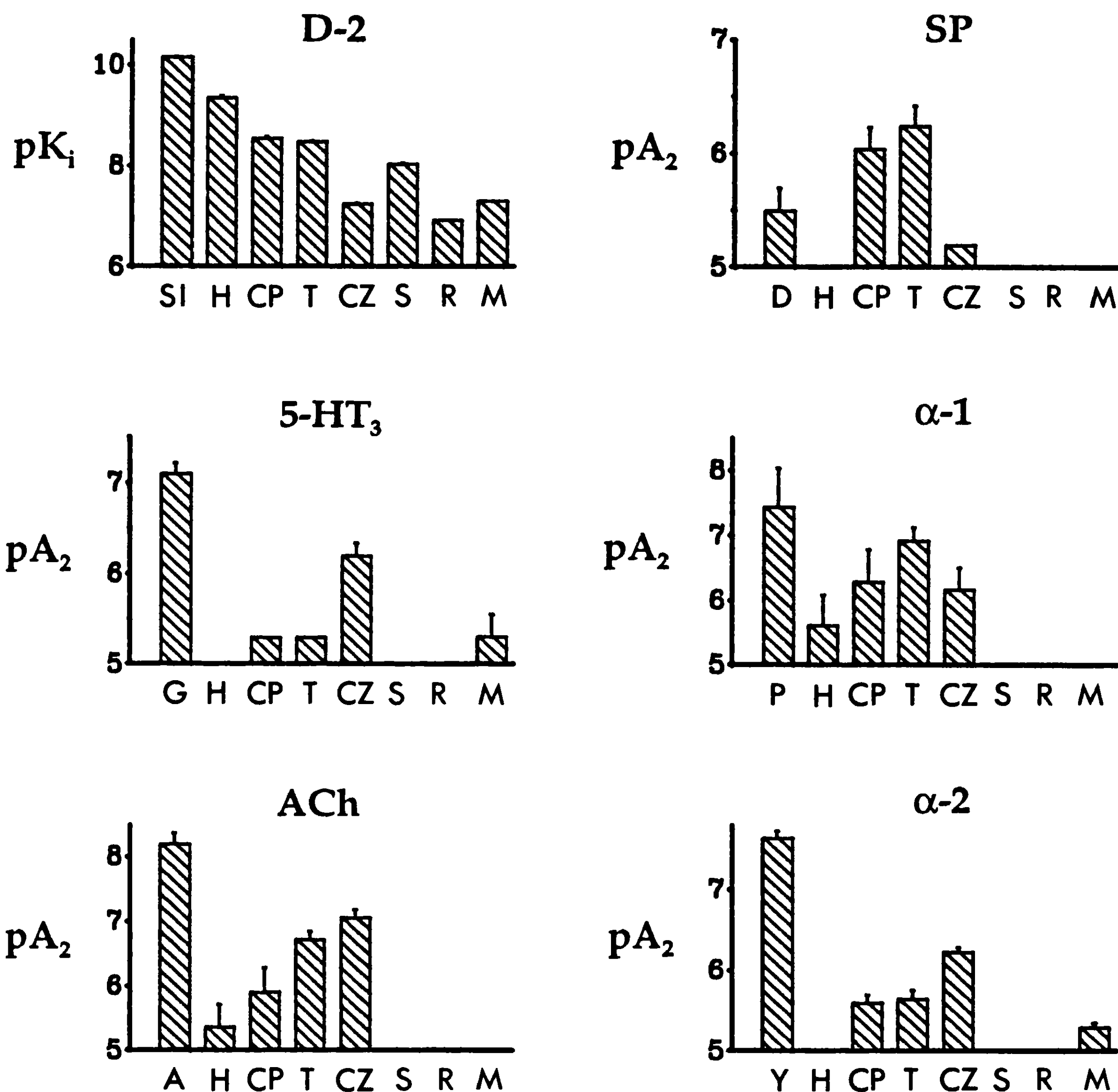


Figure 4.9 Histograms summarising the activity of several typical and atypical neuroleptic compounds at dopamine D_2, 5-HT_3, muscarinic (ACh), tachykinin (SP), alpha-1 (α-1) and alpha-2 (α-2) adrenergic receptors. pK_i and pA_2 values were derived from Table 4.1 and the error bars represent s.e.means (n=4-5). The pK_i of spiperone at dopamine D-2 receptors was obtained from Sokoloff *et al.*, (1990) and the following drugs (abbreviation) are included: spiperone (SI); haloperidol (H); chlorpromazine (CP); thioridazine (T); clozapine (CZ); sulpiride (S); remoxipride (R); metoclopramide (M); GR38032F (G); atropine (A); D-Pro^4, D-Trp^{7,9,10} SP_{4-11} (D); prazosin (P); yohimbine (Y).

4.3 DISCUSSION

In this chapter, several typical and atypical neuroleptic compounds were assessed for activity at various non-dopaminergic receptors. These receptor interactions may either produce unwanted peripheral effects, such as postural hypotension (anti-adrenergic), blurred vision and drying of mucosal secretions (anti-muscarinic), or modify the consequences of central dopamine receptor antagonism by neuroleptics, such as the development of extrapyramidal motor disorders and manifestation of their antischizophrenic effects. Although there is good agreement that the ability of neuroleptic drugs to bind to striatal dopamine (D_2) receptors *in vitro*, is well correlated with their clinical potency and tendency to induce adverse motor disorders (Creese, Burt & Snyder, 1976; Richelson & Nelson, 1984; Hall, Sallemark & Jerning, 1986), a causal relation between a functional dopaminergic blockade and antischizophrenic activity has not yet been established. Indeed, given the limitations of the dopamine hypothesis of schizophrenia (see section 1.2.1), particularly with regard to the irreducible time delay (2-3 weeks) required for the onset of antipsychotic activity, despite effective dopamine receptor blockade within hours of neuroleptic administration (Sedvall, Farde, Persson & Wiessel, 1986), it is perhaps not surprising, that other neurotransmitter systems, implicated in the regulation of dopamine function, have been widely studied.

The main aim of the present study was to identify common receptor interactions of either the typical or the atypical neuroleptic compounds which may account for their differential clinical profiles. Atypical neuroleptics, by definition, can alleviate the symptoms of schizophrenia without producing marked extrapyramidal side effects which are an inevitable consequence of typical neuroleptic therapy. In the present study, clozapine, thioridazine and remoxipride, could all, unequivocally, be defined as atypical neuroleptics (see Lieberman, Johns, Cooper, Pollack & Kane, 1989; Gerlach & Simmelsgaard, 1978; Andersson, Haggstrom, Nilsson & Widerlov, 1988). There appears to be some controversy, however, whether sulpiride can also be classed an atypical neuroleptic. Thus although, sulpiride differs from the typical neuroleptics, in causing few extrapyramidal effects (Mielke, Gallant & Kessler, 1977; Rama Rao, Bailey, Bishop & Coppen, 1981), does not block either apomorphine (Costall & Naylor, 1975) or amphetamine (Robertson & MacDonald, 1985) induced stereotypy in rodents, or produce catalepsy (Jenner, Elliot, Clow, Reavill & Marsden, 1978), similar to other atypical neuroleptics, it has been

reported to depolarise A9 dopamine neurons following chronic administration in rats (Chiodo & Bunney, 1983a) and in some exceptional cases, produce tardive dyskinesia (Sandyk, 1986). The depressant effects of chronic sulpiride on A9 dopamine neurons, however, have not been replicated by other workers (White & Wang, 1983b). Thus it appears there is some justification for classifying sulpiride as an atypical neuroleptic.

As discussed in section 4.1 5-HT_3 receptors, may serve an important regulatory function on central dopaminergic mechanisms, and have been implicated as potential targets for novel antischizophrenic drugs, devoid of extrapyramidal side effects. In the present study, clozapine was found to have quite marked actions at peripheral 5-HT_3 receptors (pA_2 6.2), unlike the remaining atypical and typical neuroleptic compounds tested. The antiemetic and weakly antipsychotic agent, metoclopramide, was also active at this receptor, albeit weakly (pA_2 5.3), although this has been reported previously (Butler, Hill, Ireland, Jordan & Tyers, 1988). The pA_2 values quoted for GR38032F and ICS205930 at 5-HT_3 receptors (Table 4.1) are in close agreement with a recent study, which also utilised the isolated guinea-pig ileum preparation (Butler, Elswood, Burridge, Ireland, Bunce, Kilpatrick & Tyers, 1990). Since this work was completed, the 5-HT_3 receptor blocking action of clozapine, has been confirmed by other workers (Watling, Beer & Stanton, 1989; Hoyer, Gozlan, Bolanos, Schechter & Hamon, 1989; Watling, Beer, Stanton & Newberry, 1990). These workers report a pA_2 value for clozapine of 7.1 (limited Arunlakshana-Schild plot) against the depolarising action of 2-methyl-5-HT on the rat superior cervical ganglion, and an pK_i value (displacement of [^{3}H]Q ICS205930 to rat cortical membranes) of 7.0. These data, together with my own, suggest that clozapine is a functional antagonist at the 5-HT_3 receptor, however, the significance of this action, is unknown, since all the other atypical neuroleptics tested were devoid of activity in this system, and metoclopramide, which carries a high risk of extrapyramidal side effects, was also active. Moreover, it appears from a recent study, that chronic administration of selective 5-HT_3 receptor antagonists in rats, may decrease the number of active dopamine neurons originating from both the A9 and the A10 brain regions, suggesting that these compounds may not be devoid of extrapyramidal effects (Sorenson, Humphreys & Palfreyman, 1989). Although the role of the 5-HT_3 receptor in the CNS remains to be fully clarified, it is possible that the blocking action of clozapine at the 5-HT_3 receptor mediates some of the unique clinical features of this compound (see section 8.4).

The atypical neuroleptics, clozapine and thioridazine, were both found to have strong anti-muscarinic actions, although, this was not a feature of the other atypical neuroleptic drugs tested. Clearly, it is vital to determine whether an anticholinergic effect, either inherent in the neuroleptic drug, or resultant from concomitant anti-Parkinson treatment, may protect not only against the initial neurological side effects (acute dystonic reactions and pseudoparkinsonism), but also against the subsequent development of tardive dyskinesia. It has long been recognised that neuroleptics with potent anti-muscarinic activity produce few Parkinsonian side effects (Snyder, Greenberg & Yamumura, 1974; Millar & Hiley, 1974). The relative contribution of cholinergic mechanisms to these clinical findings have, however, been disputed (Ljungberg & Ungerstedt, 1979). These authors examined to what extent the ability of haloperidol, but not clozapine, to antagonise certain behaviourial responses in rats, induced by apomorphine, could be accounted for by their widely differing anti-muscarinic potencies. In this study, combining haloperidol with the anticholinergic drug, scopolamine, failed to mimic the clozapine-like effect on apomorphine-induced behaviours. This aside, it does appear that neuroleptic agents with strong antimuscarinic actions, are considerably less likely to produce tardive dyskinesia (Gerlach & Simmelsgaard, 1978), although, it is well recognised that anticholinergic drugs can accentuate pre-existing neuroleptic-induced tardive dyskinesia.

The phenothiazine neuroleptic, thioridazine and chlorpromazine, and the non-neuroleptic phenothiazine, promethazine, produced moderate antagonism of substance P-induced contraction of the ileal muscle. It is possible that this effect was not directly mediated at the tachykinin receptor, although confounding contributions from cholinergic, histaminergic, and serotoninergic mechanisms can be ruled out due to the presence of selective receptor antagonists. The putative substance P antagonist, D-Pro_4, D-$Trp^{7,9,10}$, $SP_{4\text{-}11}$, produced what appeared to be competitive antagonism of substance P, with an approximate pA_2 value of 5.5 (estimated from only 2 concentrations), which in comparison to the activity of other octapeptide antagonists in this system (see Regoli, Mizrahi, D'Orleans-Justi, Dion, Drapeau & Escher, 1985), is slightly less active. Although, selective dopamine D_1 and D_2 receptor antagonists have been reported to have mixed effects on the levels of substance P estimated in some rat brain areas (review; Stoessl, 1989), there appears to be no selective ability of the phenothiazines to modify these levels. The possible significance of the present results therefore, if any, require further study.

As well as anti-muscarinic activity, the atypical neuroleptics, thioridazine and clozapine, and the typical neuroleptic, chlorpromazine, shared antagonism at α_1 and α_2 adrenergic receptors. The remaining atypicals, remoxipride and sulpiride, and the typical neuroleptic, haloperidol, perhaps to a lesser extent, showed no significant activity at either muscarinic or adrenergic receptors. Antagonism at either of these receptors, inherent to some neuroleptic compounds, would be expected to offset the likelihood of extrapyramidal effects and perhaps tardive dyskinesia, since cholinergic and adrenergic mechanisms may be involved in their development (Chiodo & Bunney, 1985; Robinson, Berney, Mishra & Sulser, 1979). These mechanisms, however, may be insufficient to counter the effects of central dopamine receptor blockade by chlorpromazine, since this compound, although sharing similar affinity for the dopamine D_2 receptor as thioridazine (Table 4.1), lacks the strong actions of thioridazine at muscarinic and α_1 adrenoceptors. Since haloperidol lacks significant activity at either muscarinic or adrenergic receptors, but has strong affinity for the dopamine D_2 receptor, it is perhaps not surprising that this compound has a typical neuroleptic profile. Noradrenergic mechanisms may also be involved in the underlying cause of schizophrenia. Not only have raised levels of noradrenaline been reported in chronic paranoid schizophrenic patients (see section 4.1), but tricyclic antidepressants, which are known to raise the synaptic availability of noradrenaline in the brain, have been found to exacerbate schizophrenic symptoms (Kessler, 1978). The possibility exists, therefore, that some of the therapeutic actions of clozapine, thioridazine and chlorpromazine, are mediated through central antagonism of noradrenaline. This action might presumably be mediated through blockade of postsynaptic α_1 adrenoceptors, since antagonism of presynaptic α_2 adrenoceptors, which would increase the synaptic availability of noradrenaline, at least initially, would be expected to worsen schizophrenic symptoms. Indeed, this might be the mechanism underlying the reported effectiveness of clonidine in the treatment of schizophrenia and the ability of yohimbine to activate dormant psychosis (see section 4.1). Clozapine, however, was found to have significant activity at the α_2 adrenoceptor, which further complicates its mode of action.

Sulpiride and remoxipride were devoid of activity in all the systems tested. Both neuroleptics, have selective affinities for the dopamine D_2 receptor, albeit weaker than the typical neuroleptics. Since neither compound has significant activity at either muscarinic, adrenergic or 5-HT_2 serotoninergic (see later) receptors, their atypical neuroleptic profiles are difficult to account for. Remoxipride does however, have a high affinity for the opioid

sigma receptor (Largent, Wikstrom, Snowman & Snyder, 1988), which may be an important site mediating the psychotomimetic effects of some opioid derivatives (Martin, Eades, Thompson, Huppler & Gilbert, 1976; Su, 1982). Blockade of sigma receptors may be a promising strategy for the development of new antipsychotic agents since remoxipride, although devoid of adverse motor effects, is apparently effective in the treatment of schizophrenia (Lindstrom, Besev, Stening & Widerlov, 1985). The low incidence of extrapyramidal side effects seen with sulpiride, given the selective anti-dopaminergic activity of this compound, requires some explanation. Several plausible theories have been reported including the possibility that sulpiride acts selectively at a sub-population of postsynaptic cerebral dopamine receptors functioning independently of adenylate cyclase (Jenner & Marsden, 1981). These authors determined that the specific binding of [^{3}H]-sulpiride to rat striatal membranes differed from that of [^{3}H]-spiperone, which also binds to adenylate cyclase independent dopamine receptors, in showing an absolute dependence on sodium ions. Further studies by these same authors, and others, have revealed that sulpiride may bind selectively to dopamine receptors located on the terminals of corticostriatal neurons (Theodorou, Reavill, Jenner & Marsden, 1981; Sokoloff, Martres & Schwartz, 1980; Brown & Arbuthnott, 1983). It has also been suggested that sulpiride, unlike typical neuroleptic compounds, may have relatively greater actions at presynaptic rather than postsynaptic dopamine receptors, since sulpiride can potentiate both apomorphine (Puech, Simon & Boissier, 1978) and amphetamine (Robertson & MacDonald, 1985) induced stereotypy in rodents. It is rather surprising that metoclopramide, which although is structurally very similar to sulpiride and has been shown to act as a central dopamine receptor antagonist (Robertson & MacDonald, 1985; Jenner, Elliot, Clow, Reavill & Marsden, 1978) with selective affinity for the dopamine D_2 receptor (Table 4.1), is only antipsychotic at very large doses (Harrington, Hamilton, Brodgen, Linkewich, Romankiewicz & Heel, 1983) and has a strong tendency to induce both extrapyramidal side effects (Stanley, Lautin, Rotrosen, Gershon & Kleinberg, 1980) and tardive dyskinesia (Lavy, Melamed & Penchas, 1978). The apparently selective action of this compound on the nigrostriatal dopamine system is confirmed by the observation that metoclopramide, when administered acutely, preferentially stimulates the firing of only A9 dopamine neurons, unlike the effects seen with thioridazine, which stimulates only A10 dopamine neurons (Hand, Kasser & Wang, 1987). Indeed, when chronically administered, metoclopramide has been noted to reduce the number of spontaneously active A9 dopamine neurons, with little or no effect on the activity of dopamine neurons

arising from the ventral tegmental area (White & Wang, 1983b). It is probable, given the contrasting actions of metoclopramide, particularly with regard to the atypical neuroleptics, that elucidating the underlying mechanism(s) of this compound, will greatly assist in the development of antipsychotic drugs devoid of extrapyramidal side effects.

As well as interacting with central dopamine D_2 receptors, most typical and atypical neuroleptics, apart from the substituted benzamide derivatives such as sulpiride (and metoclopramide), also antagonise central dopamine D_1 receptors (see Table 4.1), which unlike dopamine D_2 receptors, are abundant in the cerebral cortex (De Keyser, Claeys, De Backer, Ebinger, Roels & Vanquelin, 1988). The dopamine D_1 receptor, which is positively linked to adenylate cyclase, is now believed to have an important role in the action of antipsychotic drugs (see Clark & White, 1987), and may be important in the mode of action of clozapine (Anderson & Braestrup, 1986). It also appears that the dopamine D_1 receptor affinity of neuroleptic drugs are closely correlated with the affinity of these agents for the 5-HT_2 receptor (see Table 4.1 and McQuade, Ford, Duffy, Chipkin, Iorio & Barnet, 1988). This is of interest since recent studies have suggested that blockade of 5-HT_2 receptors in the brain may reduce extrapyramidal side effects associated with the blockade of central dopamine D_2 receptors (Luthman, Fredriksson, Plaznik & Archer, 1991; Brougham, Conway & Ellis, 1991; Saller, Czupryna & Salama, 1990).

4.4 CONCLUSIONS

The strong antagonistic actions of the atypical neuroleptic compounds, clozapine and thioridazine, at muscarinic and α-adrenoceptors probably accounts, at least in part, for their reduced tendency to induce extrapyramidal side effects. Since sulpiride and remoxipride were devoid of activity at these receptors other mechanisms may be involved which would explain the atypical nature of these compounds. The antagonism shown by the typical neuroleptics, chlorpromazine (modest) and haloperidol (weak to inactive), at muscarinic and adrenergic receptors may be insufficient to overcome the more potent effects of these agents at central dopamine receptors.

The significance of the novel findings from the present studies, that thioridazine and chlorpromazine appear to be modest antagonists of substance P, and that clozapine is an effective antagonist of 5-HT_3 receptors, remain to be clarified.

CHAPTER 5

EFFECTS OF NEUROLEPTICS AND APOMORPHINE ON THE RELEASE AND METABOLISM OF DOPAMINE IN THE CAUDATE PUTAMEN AND MEDIAL PREFRONTAL CORTEX

5.1 INTRODUCTION

In the preceding chapter attempts were made to identify different non-dopaminergic receptor interactions of typical and atypical neuroleptic compounds which may account for their differing clinical effects. In this and the following chapter the possibility was examined that these drugs may also have differing actions on dopamine transmission in certain cortical and subcortical structures. It is believed that both the antischizophrenic and the extrapyramidal effects of neuroleptic drugs arise through their antagonism of central dopamine receptors even though there is evidence to suggest that these clinical effects may be manifested in discrete brain regions. Thus although the extrapyramidal effects of these agents probably arise from reduced dopamine function in the nigrostriatal dopamine system it is likely that their therapeutic effects arise from actions within the mesolimbic and/or the mesocortical dopamine systems. The possibility that the atypical neuroleptic compounds, which cause few extrapyramidal side effects, have reduced actions compared to the typical neuroleptics on dopamine function in the nigrostriatal dopamine system was tested in the present study. To this end intracerebral microdialysis was employed to compare the actions of haloperidol and clozapine on the release and metabolism of dopamine in the medial prefrontal cortex and caudate putamen (see section 2.5). These agents were either tested alone or against the effects of the non-selective dopamine agonist apomorphine in order to gauge the relative ability of these agents to block dopamine receptors. It was anticipated that since both these neuroleptics are effective in reducing the symptoms of schizophrenia, yet clozapine carries a lower risk of inducing extrapyramidal side effects than haloperidol, that any differences in their actions on dopamine function in the two areas might explain their differing clinical actions.

5.2 RESULTS

5.2.1 Effects of neuroleptics on the basal efflux of dopamine, DOPAC, HVA and 5-HIAA in the caudate putamen and medial prefrontal cortex

The basal efflux (pmoles/20 minutes) of dopamine and its major metabolites DOPAC and HVA, as well as the 5-HT metabolite 5-HIAA into dialysates of the caudate putamen and medial prefrontal cortex are given in Table 5.1. Apart from the somewhat higher basal efflux of dopamine in the medial prefrontal cortex (see section 5.3.1), these values agree

	CAUDATE PUTAMEN	PREFRONTAL CORTEX
DA	0.071 ± 0.012	0.052 ± 0.010
DOPAC	18.1 ± 1.4	1.14 ± 0.22
HVA	11.7 ± 2.0	2.3 ± 0.4
5-HIAA	1.7 ± 0.3	1.4 ± 0.2
DOPAC/DA	255	22
DOPAC/HVA	1.55	0.49

Table 5.1 Basal efflux (pmoles/20 minutes ± s.e.mean, $n=20$) of dopamine, DOPAC, HVA and 5-HIAA estimated in dialysates of the rat caudate putamen and medial prefrontal cortex. DOPAC/dopamine and DOPAC/HVA ratios are also shown.

with those obtained by other workers (see section 3.4.1). Unlike 5-HIAA, the monoamines all showed a clear regional variation in their basal efflux, with considerably lower levels detected in the medial prefrontal cortex relative to the caudate putamen. The most striking feature of these results, and particularly those obtained from the caudate putamen, was the much higher levels of DOPAC and HVA appearing in the brain dialysates compared to dopamine. As well as showing lower efflux levels of monoamines, the medial prefrontal cortex was also distinctive from the caudate putamen in liberating relatively more HVA in the extracellular fluid than DOPAC.

Both clozapine and haloperidol increased the efflux of DOPAC and HVA in the caudate putamen (Fig. 5.1). The peak efflux of DOPAC occurred after about 2 hours, whereas in comparison, HVA peaked later (about 3 hours), and to a greater extent. No significant differences were observed in the maximal effects of either clozapine or haloperidol on the levels of these metabolites in the caudate putamen (Table 5.2). Despite the prolonged rise in the efflux of DOPAC and HVA in the caudate putamen no significant effect (for either neuroleptic) was noted in the efflux of dopamine.

Some important differences were noted, however, in the effects of the neuroleptics on the efflux of monoamines in the medial prefrontal cortex (Fig. 5.2). Thus both clozapine and haloperidol increased the efflux of DOPAC and HVA in this brain area more than the caudate putamen (see Table 5.2) but the most striking feature of these results was the significant rise in dopamine efflux observed in the prefrontal cortex following the administration of clozapine. This increase in dopamine efflux peaked after about 1 hour with the levels returning to basal values after approximately 3 hours. Although haloperidol produced no change in the efflux of dopamine in the medial prefrontal cortex it had a greater effect than clozapine on the efflux of HVA. This was still increasing 5 hours after the administration of haloperidol to a significantly ($p<0.01$; Student's non-paired t-test) higher level than after clozapine. The efflux of DOPAC in the cortex, following clozapine administration, peaked earlier and to a greater extent compared to haloperidol perhaps as a result of enhanced dopamine efflux by clozapine. The increased efflux of DOPAC also preceded that for HVA by about 60 minutes after both neuroleptics. Surprisingly, clozapine produced identical changes in the efflux of HVA in both the caudate putamen and the medial prefrontal cortex despite its much greater effects on the efflux of dopamine and DOPAC in the medial prefrontal cortex.

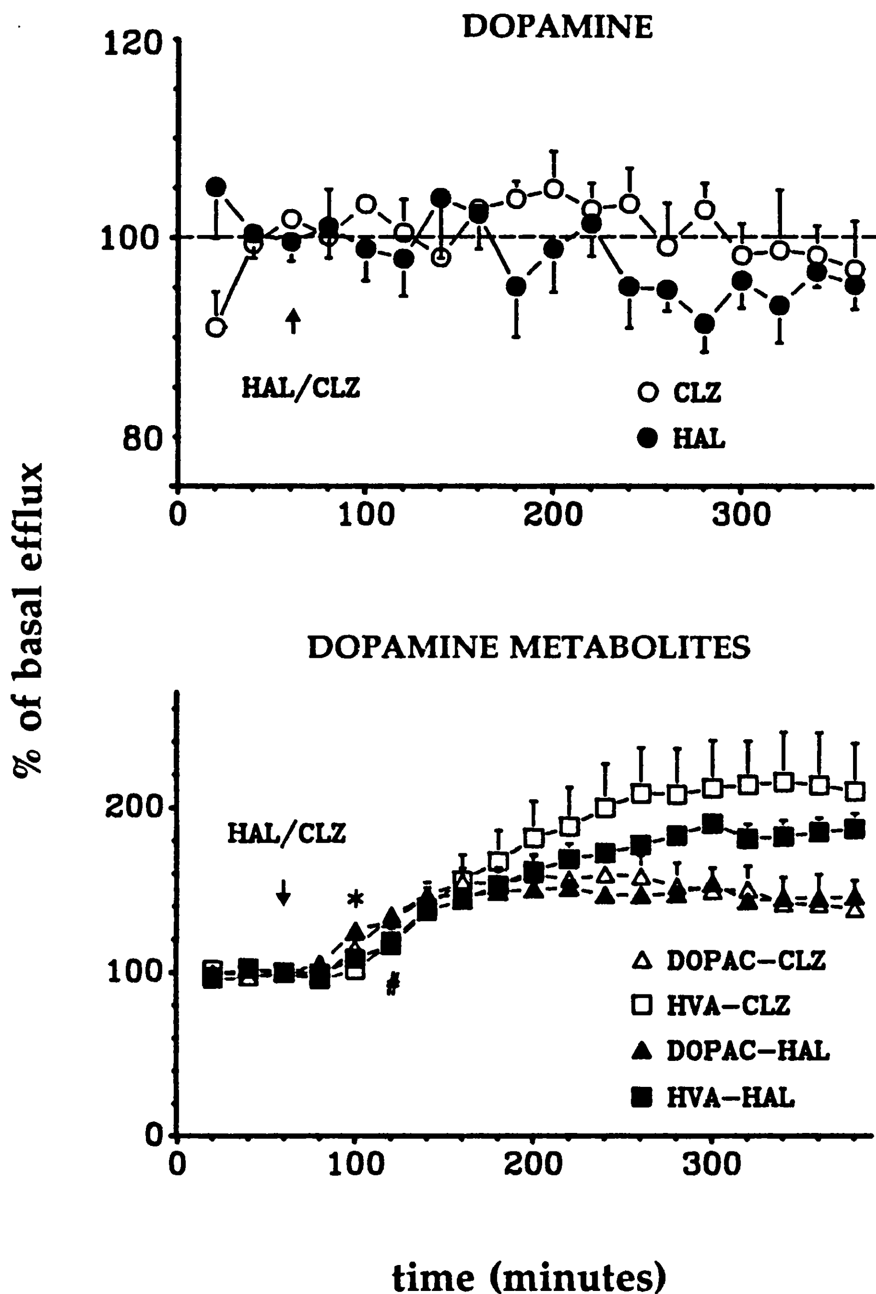


Figure 5.1 Effect of haloperidol (shaded symbols) and clozapine (open symbols) on the mean (± s.e.mean, *n*=5) efflux of dopamine and dopamine metabolites DOPAC and HVA in dialysates of the rat caudate putamen. Haloperidol (0.5 mg/kg) and clozapine (20 mg/kg) were administered parenterally (1.0 ml/kg i.p.) 60 minutes after the collection of the first basal sample. Statistical significance (versus basal data) was assessed using a Student's t-test (* DOPAC/# HVA and subsequent data $p<0.05$).

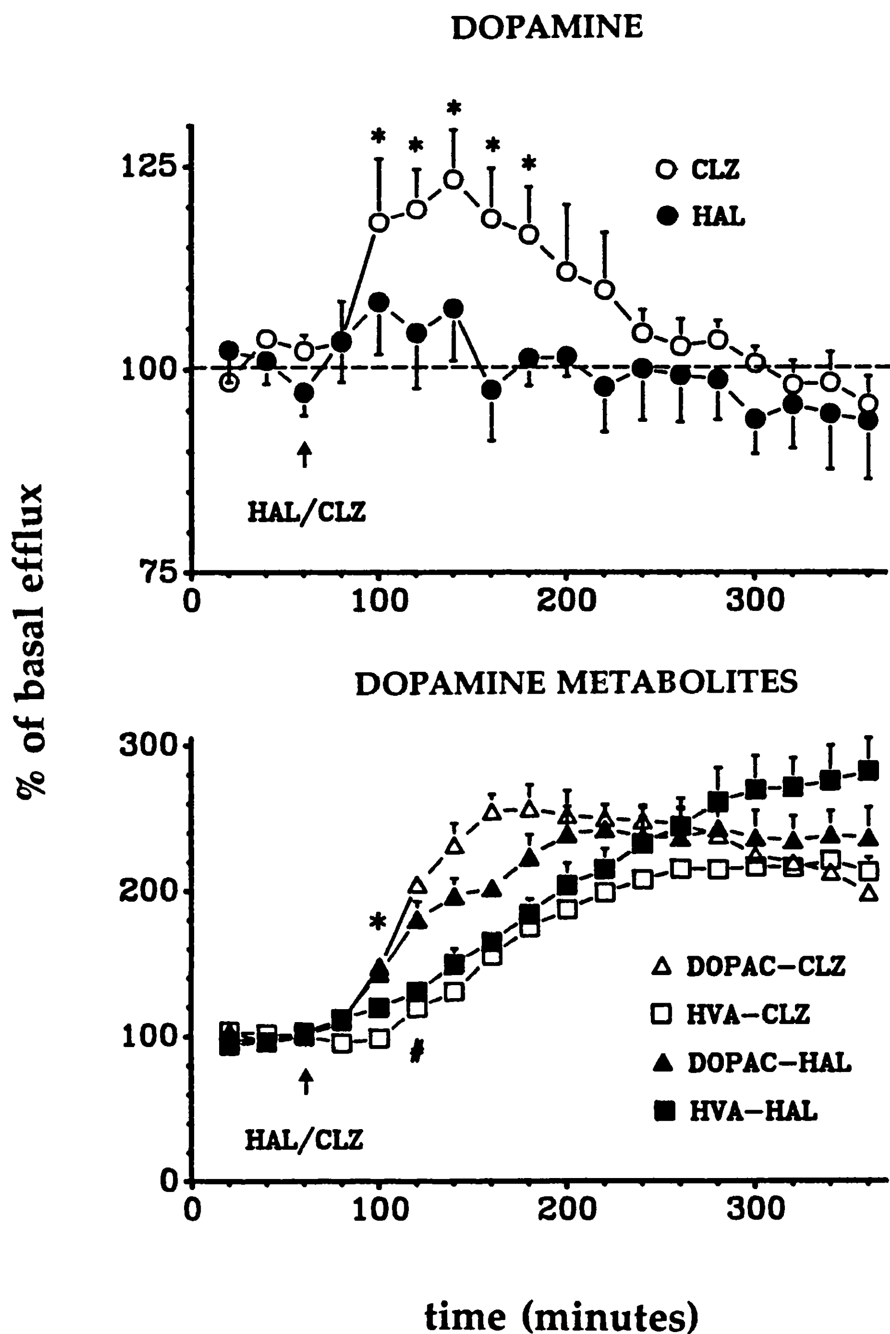
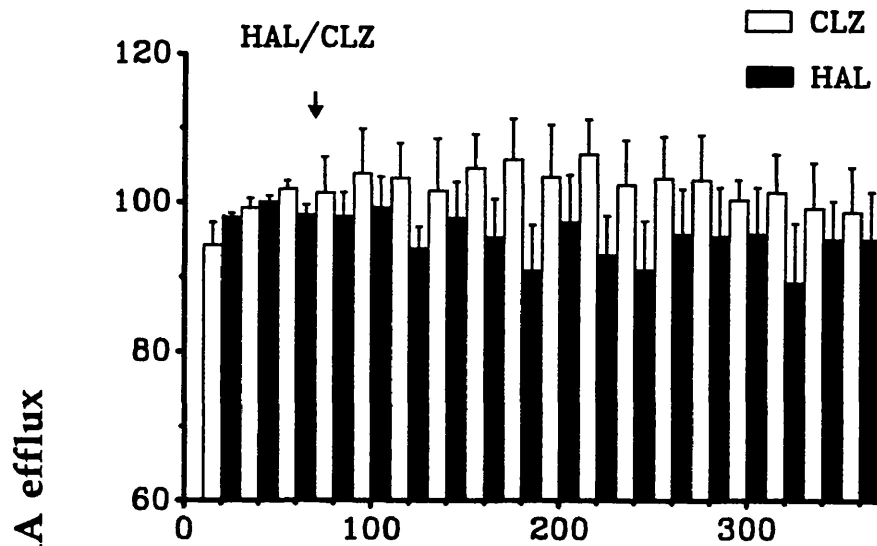


Figure 5.2. Effect of haloperidol (shaded symbols) and clozapine (open symbols) on the mean (± s.e.mean, *n*=5) efflux of dopamine and dopamine metabolites DOPAC and HVA in dialysates of the rat medial prefrontal cortex. Haloperidol (0.5 mg/kg) and clozapine (20 mg/kg) were administered parenterally (1.0 ml/kg i.p.) 60 minutes after the collection of the first basal sample. Statistical significance (versus basal data) was assessed using a Student's t-test (* DOPAC & DA; # HVA and subsequent data $p<0.05$).



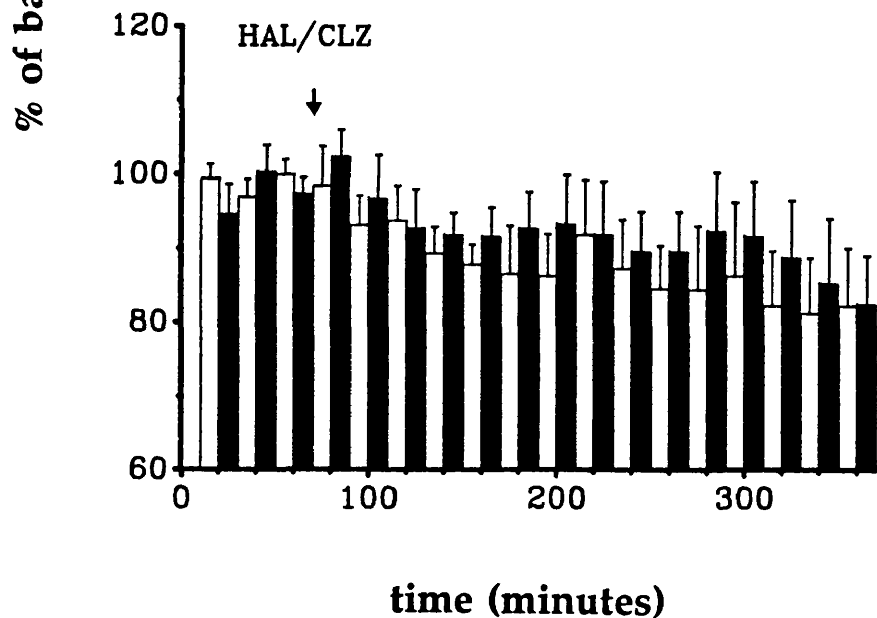


Figure 5.3 Effect of haloperidol (open symbols) and clozapine (shaded symbols) on the mean (± s.e.mean, n=5) efflux of 5-HIAA in dialysates of the rat caudate putamen and medial prefrontal cortex. Haloperidol (0.5 mg/kg) and clozapine (20 mg/kg) were administered parenterally (1.0 ml/kg i.p.) 60 minutes after the collection of the first basal sample.

Neither clozapine nor haloperidol produced any significant changes in the efflux of the 5-HT metabolite 5-HIAA in either the caudate putamen or the medial prefrontal cortex (Fig.5.3).

5.2.2 Effects of apomorphine on the basal efflux of dopamine, DOPAC, HVA and 5-HIAA in the caudate putamen and medial prefrontal cortex

The dopamine agonist apomorphine produced a progressive dose-dependent decrease in the efflux of dopamine, DOPAC and HVA in both the caudate putamen and the medial prefrontal cortex (Fig.5.4). Whilst the efflux of DOPAC and HVA (DOPAC before HVA) were decreased by 70-75%, that of dopamine was only reduced by 40% and then not until the higher doses of apomorphine (1.0-2.0mg/kg) were reached in the medial prefrontal cortex. The dose-response effects of apomorphine on the efflux of these neurochemicals, as well as 5-HIAA, in both brain areas are given in Fig.5.5. The ED_{50} values (± s.e.mean, n=5) of apomorphine and the maximum inhibitory effect (± s.e. mean, n=5) after a dose of 2.0 mg/kg of apomorphine were estimated to be 0.17 ± 0.03 mg/kg, 82.0 ± 1.0% (DOPAC) and 0.33 ± 0.08 mg/kg, 76.2 ± 3.4% (HVA) in the caudate putamen and 0.26 ± 0.07 mg/kg, 71.4 ± 4.8% (DOPAC) and 0.47 ± 0.11 mg/kg, 66.3 ± 7.2 % (HVA) in the medial prefrontal cortex. The efflux of 5-HIAA was only significantly decreased by apomorphine in the medial prefrontal cortex and only at the highest dose used (19.9 ± 3.8 % inhibition after a dose of 2.0 mg/kg).

5.2.3 Effects of neuroleptics on apomorphine-induced changes in dopamine, DOPAC and HVA efflux

Pretreatment with haloperidol completely blocked the inhibition of dopamine efflux in the caudate putamen produced by apomorphine but clozapine was much less active with its effect only reaching statistical significance at three sampling time intervals (Fig.5.6). No significant effects on dopamine efflux were noted in the medial prefrontal cortex upon concomitant administration of apomorphine with either clozapine or haloperidol although apomorphine did reverse the effects of clozapine on the efflux of dopamine in this brain area (Fig.5.2).

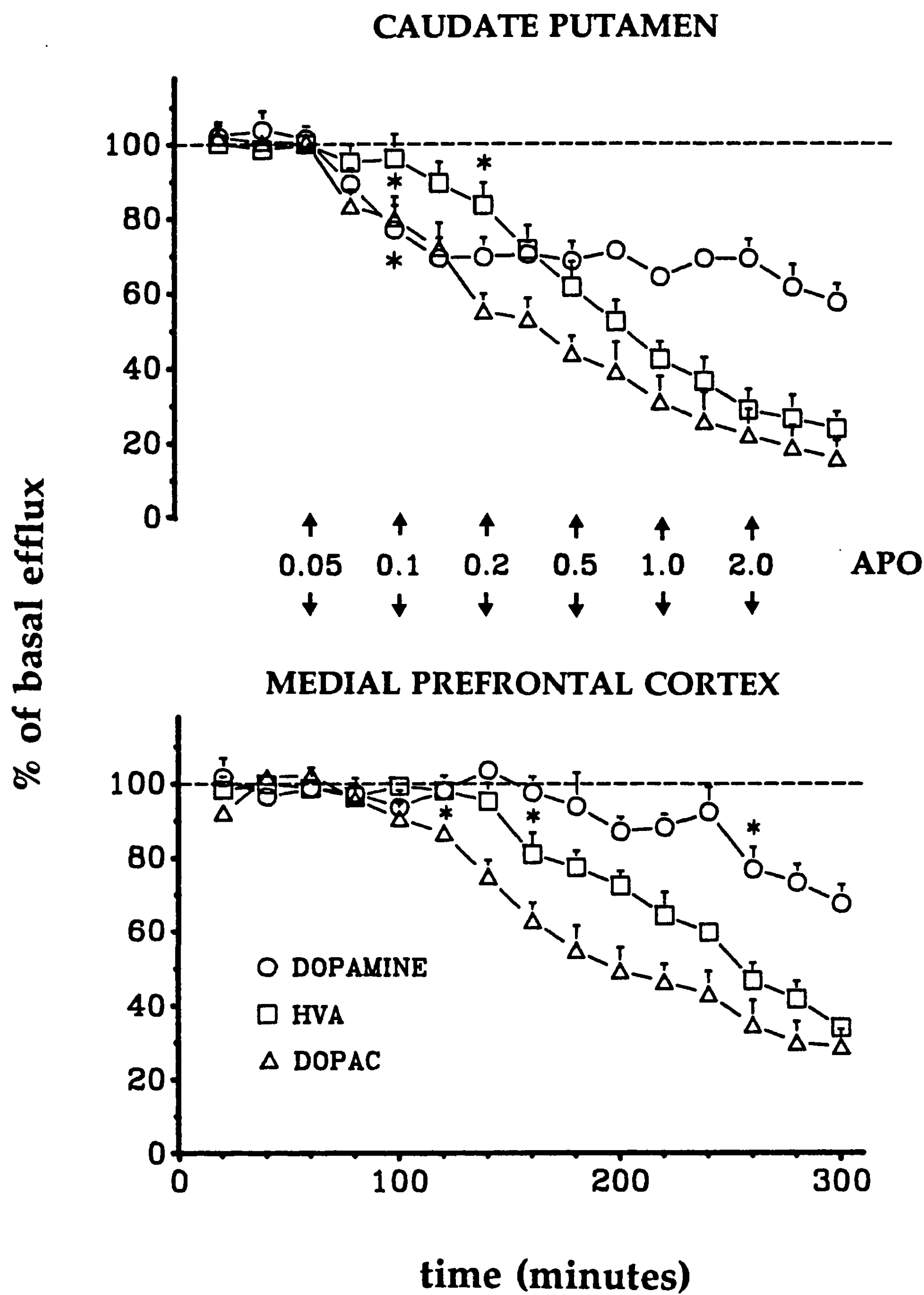
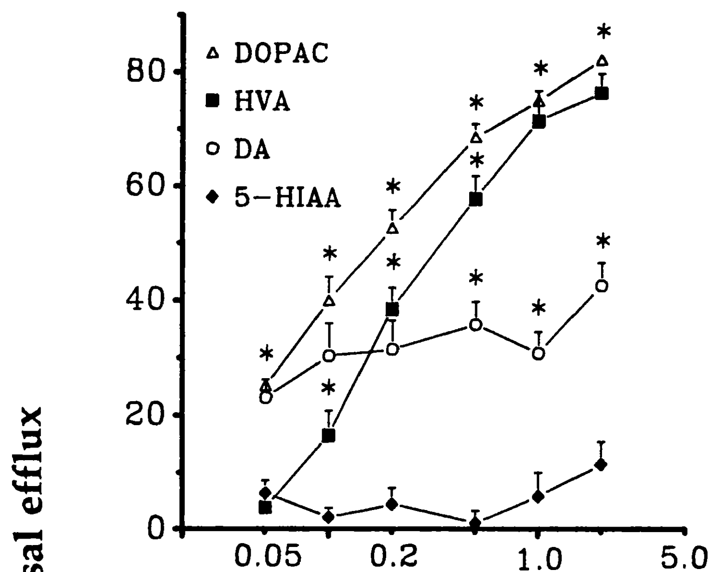


Figure 5.4 Effect of apomorphine (hydrochloride) on the mean (± s.e.mean, *n*=5) efflux of dopamine, DOPAC and HVA in the rat caudate putamen and medial prefrontal cortex. Apomorphine (mg/kg, 0.20 ml/kg i.v.) was administered 60 mins after the start of the first basal collection and in increasing doses every 40 minutes. Statistical significance (versus basal data) was assessed using a Student's t-test (* and subsequent data $p<0.05$).



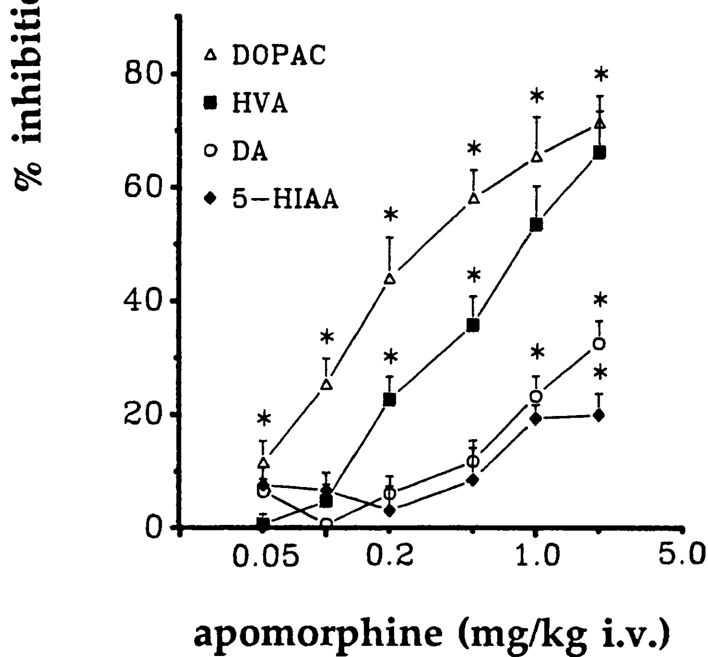
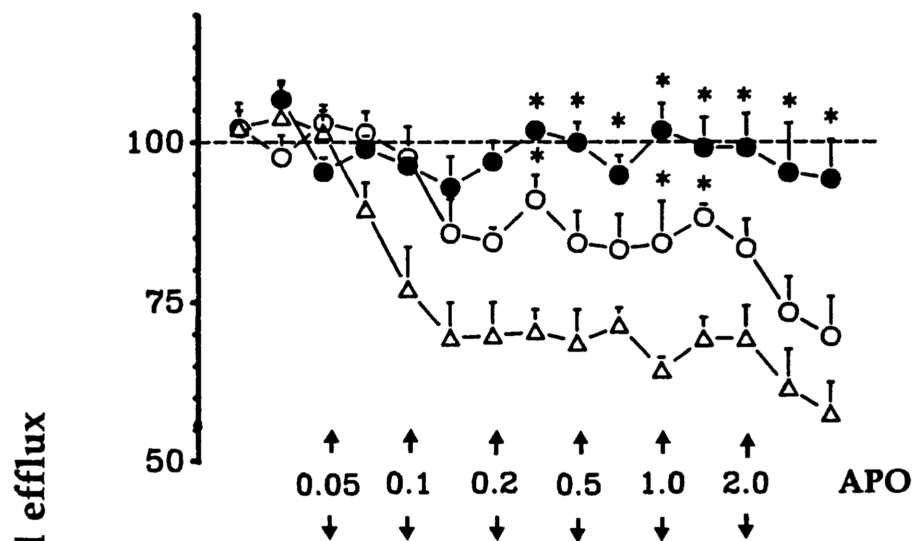


Figure 5.5 Dose-dependent inhibitory effects of apomorphine on the efflux of DOPAC (△), HVA (■), dopamine (○) and 5-HIAA (◆) in the rat caudate putamen and medial prefrontal cortex. The response was measured as the mean (± s.e.mean, *n*=5) maximum inhibition from basal efflux values and statistical significance (versus basal values) was assessed using a Student's t-test (*$p<0.05$).



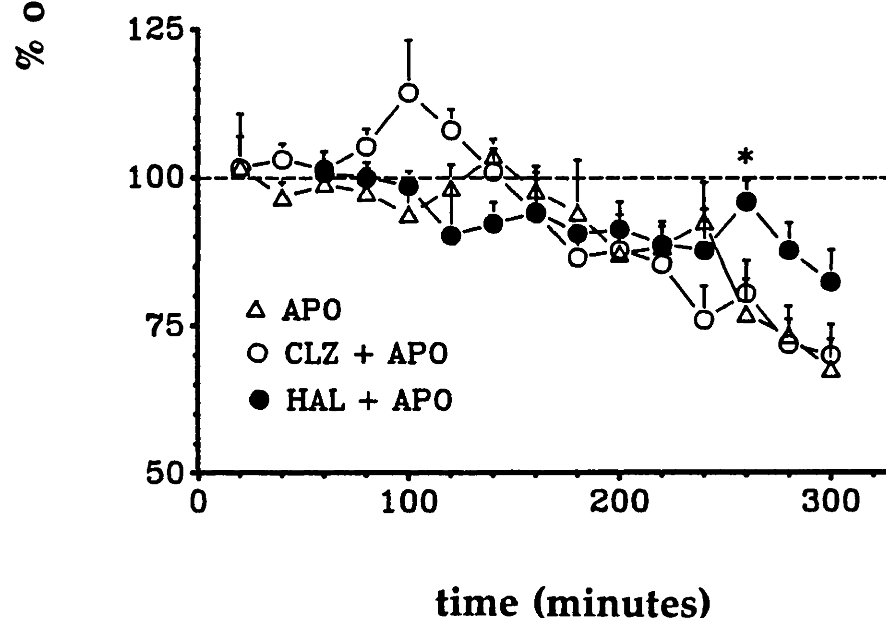


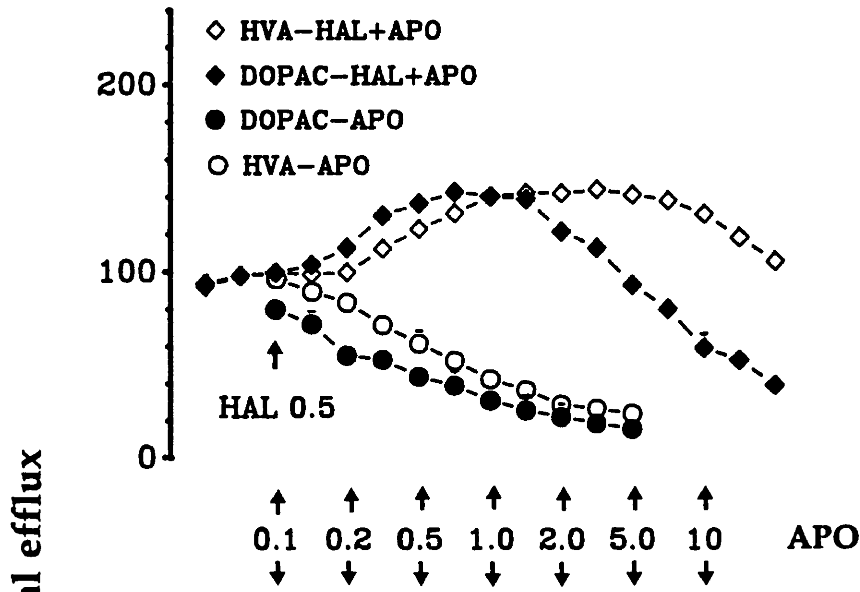
Figure 5.6 Effects of concomitant administration of either haloperidol (●) or clozapine (○) with apomorphine on the mean (± s.e.mean, n=5) efflux of dopamine in dialysates of the rat caudate putamen and medial prefrontal cortex compared with apomorphine alone (△). Clozapine (20 mg/kg) and haloperidol (0.5 mg/kg) were administered parenterally (1.0 ml/kg i.p.), 40 minutes before intravenously administered 0.1 mg/kg and 0.2 mg/kg apomorphine respectively. Statistical significance (versus apomorphine control) was assessed using a Student's non-paired t-test (*$p<0.05$).

Despite a much higher dose, clozapine produced only weak antagonism of apomorphine-induced changes in DOPAC and HVA efflux compared with haloperidol in both brain regions studied (Figs.5.7 & 5.8). A quantitative analysis of their relative potencies against apomorphine is compounded by the fact that these neuroleptics can themselves produce an elevation in the efflux of DOPAC and HVA (Figs.5.1 & 5.2). Nevertheless, some attempt has been made in Fig.5.9 to measure the relative ability of these agents to block the effects of apomorphine (and by implication, dopamine receptors) on DOPAC efflux. In this graph the ordinate values (percentage reduction of DOPAC efflux) were calculated with respect to either the basal efflux of DOPAC when apomorphine was dosed alone (see Fig.5.4), or to the time-matched effects of these neuroleptic compounds on DOPAC efflux (i.e., without apomorphine). From this graph the ED_{50} values (dose of apomorphine required to reduce the efflux of DOPAC by 50 % ± s.e.mean, $n=5$) in the presence of either clozapine or haloperidol were estimated in the caudate putamen to be 0.37 ± 0.11 mg/kg and 3.61 ± 0.09 mg/kg respectively, and in the medial prefrontal cortex to be 0.51 ± 0.08 mg/kg and 4.60 ± 0.22 mg/kg respectively.

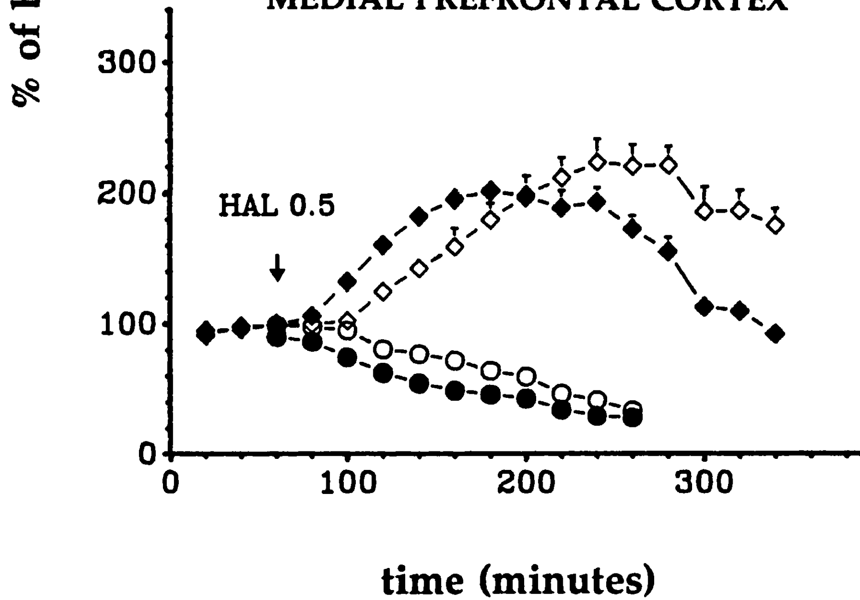
From the ED_{50} values calculated above, the activity ratios (ED_{50}[HAL]/ED_{50}[CLZ]) in the medial prefrontal cortex and caudate putamen are about 9.0 and 9.8 respectively. Thus, assuming that apomorphine mediates its actions exclusively at central dopamine receptors, and taking into account the 40 fold difference in dose, clozapine appears to be about 360 (cortex) to 390 (caudate putamen) fold less potent than haloperidol in blocking central dopamine receptors.

The weak apomorphine antagonism of clozapine relative to haloperidol in both brain areas studied is summarised in Fig.5.10. This histogram shows the effects of 2 doses of apomorphine (0.2 and 2.0 mg/kg) on the efflux of dopamine and its metabolites, either dosed alone, or following pretreatment with either clozapine or haloperidol. The most notable feature of these results, apart from the weak apomorphine antagonism shown by clozapine, is the relatively greater action of clozapine (compared to haloperidol) against the effects of the low dose of apomorphine on DOPAC and HVA efflux in the medial prefrontal cortex compared to the effects of this compound in the caudate putamen. The maximum response (percentage change of basal efflux) to apomorphine (1.0 mg/kg) before and after each neuroleptic and the maximum responses to each neuroleptic in both brain areas are given in Table 5.2.

CAUDATE PUTAMEN



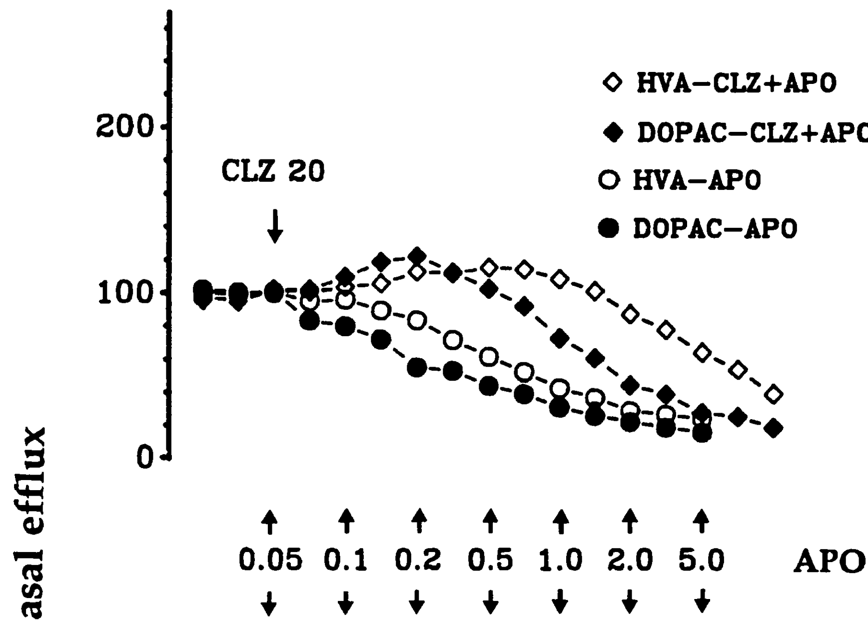
MEDIAL PREFRONTAL CORTEX



time (minutes)

Figure 5.7 Effects of concomitant administration of haloperidol with apomorphine on the basal efflux (± s.e.mean, *n*=5) of DOPAC (shaded symbols) and HVA (open symbols) in dialysates of the rat caudate putamen and medial prefrontal cortex compared with apomorphine alone. Haloperidol (0.5 mg/kg) was given parenterally (1.0 ml/kg i.p.), 40 minutes before the administration of increasing doses of apomorphine (mg/kg i.v.).

CAUDATE PUTAMEN



MEDIAL PREFRONTAL CORTEX

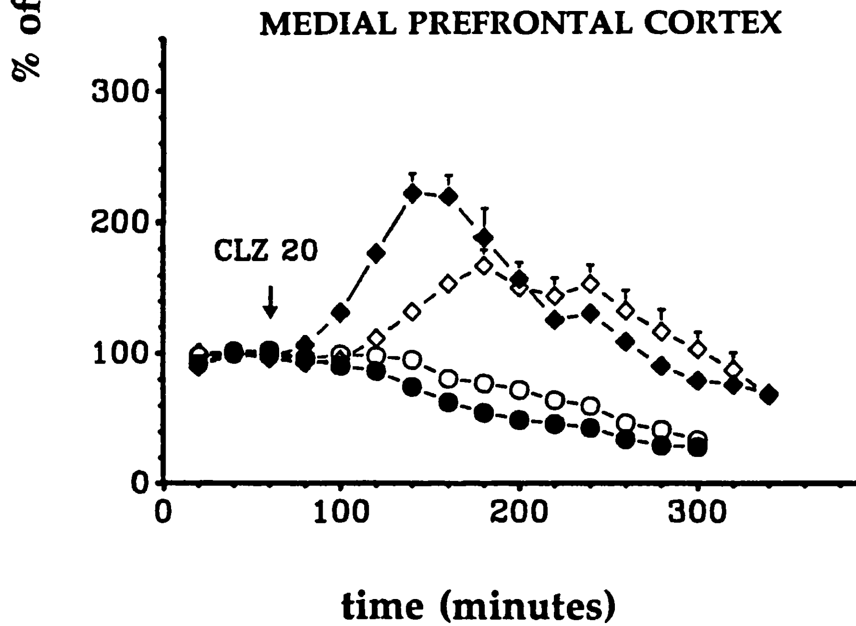
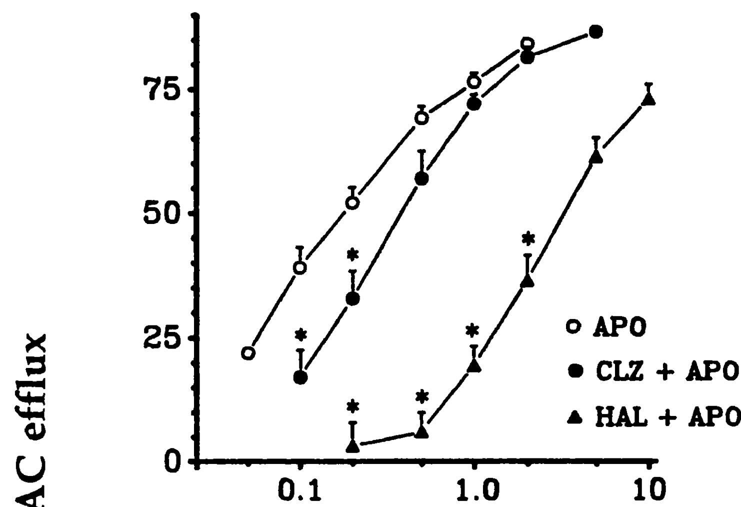


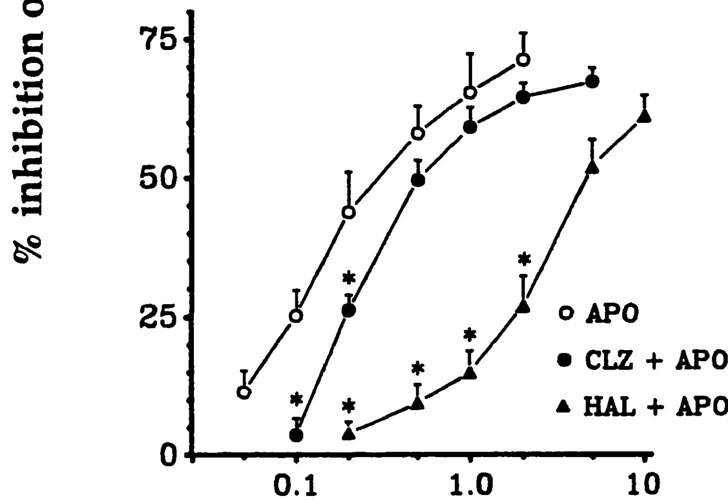
Figure 5.8 Effects of concomitant administration of clozapine with apomorphine on the efflux (± s.e.mean, *n*=5) of DOPAC (shaded symbols) and HVA (open symbols) in dialysates of the rat caudate putamen and medial prefrontal cortex compared with apomorphine alone. Clozapine (20 mg/kg) was given parenterally (1.0 ml/kg i.p.), 40 minutes before the administration of increasing doses of apomorphine (mg/kg i.v.).

CAUDATE PUTAMEN



% inhibition of DOPAC efflux

MEDIAL PREFRONTAL CORTEX



apomorphine (mg/kg i.v.)

Figure 5.9 Dose-response effects of apomorphine, either dosed alone (○), or following the administration of either clozapine (●) or haloperidol (▲) on the efflux of DOPAC in dialysates of the rat caudate putamen and medial prefrontal cortex. The response (± s.e.mean, n=5) to apomorphine alone was measured as the percentage inhibition of basal DOPAC efflux, whilst the effects of apomorphine in the presence of neuroleptics was measured as the percentage reduction in DOPAC efflux compared with that recorded at the same time after the administration of each neuroleptic alone. Statistical significance (versus apomorphine control) was assessed using a Student's non-paired t-test (*$p<0.05$).

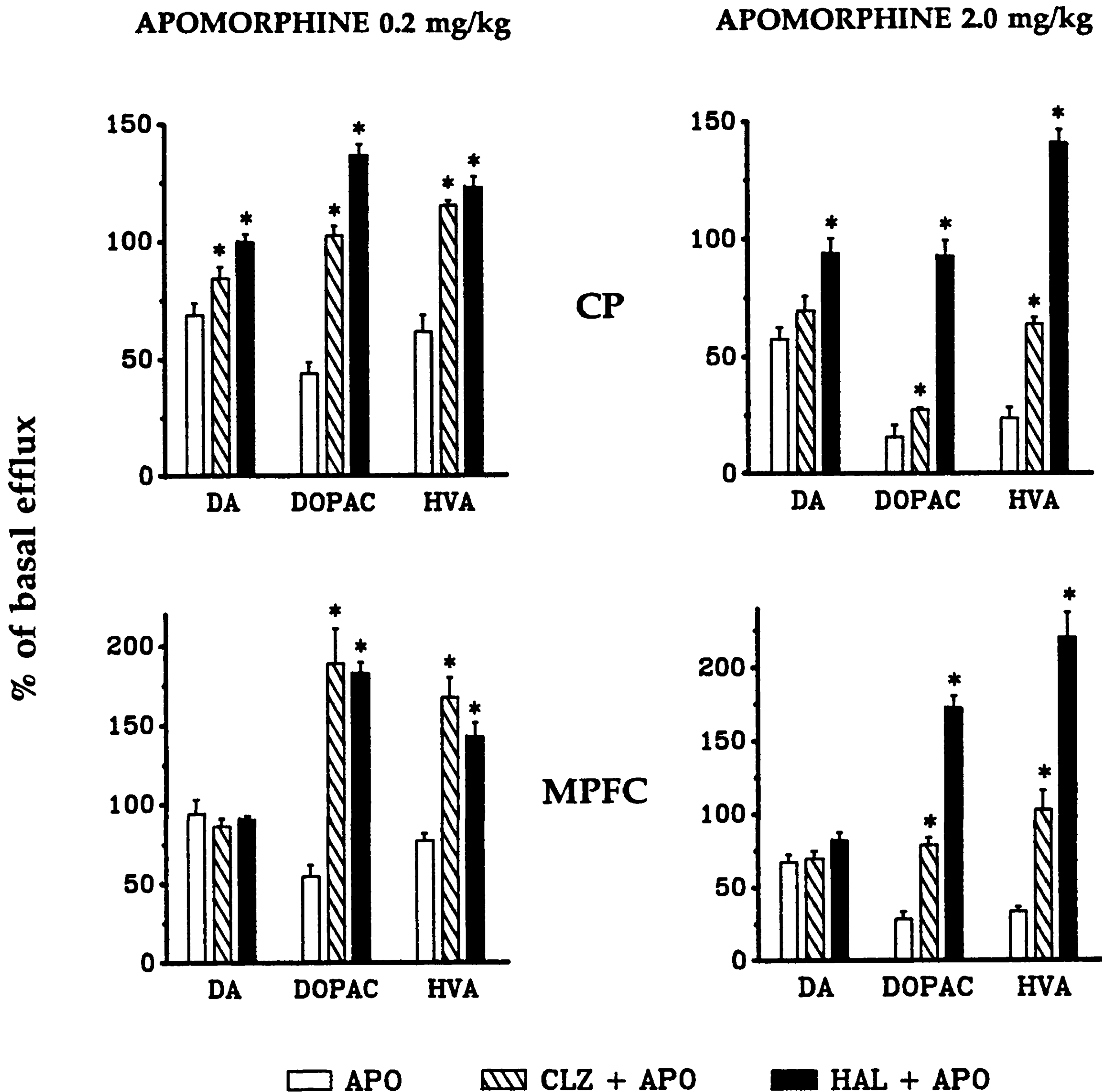


Figure 5.10 Histograms summarising the maximum effects of apomorphine, at two dose levels, either alone (open bars), or following the administration of either clozapine (striped bars) or haloperidol (shaded bars) on the mean (± s.e.mean, n=5) efflux of dopamine, DOPAC and HVA in the rat caudate putamen (CP) and medial prefrontal cortex (MPFC). Statistical significance (versus apomorphine alone) was assessed using a Student's non-paired t-test (* $p<0.05$).

	CP			MPFC		
	DA	DOPAC	HVA	DA	DOPAC	HVA
CLZ 20mg/kg	+4.8±1.2	+59.0±11.2*	+115.2±21.2*	+23.3±6.2*	+156.0±16.9*	+116.3±7.8*
HAL 0.5mg/kg	+3.9±1.9	+50.8±12.7*	+90.2±7.3*	+4.5±3.2	+142.2±30.7*	+174.1±23.5*
APO 1mg/kg	-33.7±5.2*	-72.7±13.1*	-59.5±12.8*	-23.2±2.7*	-65.5±7.1*	-53.5±5.0*
CLZ & APO	-12.5±8.1#	-55.8±2.1#	-13.1±2.4†	-30.1±6.3	+8.7±6.9†	+32.5±11.1†
HAL & APO	-0.9±4.8†	+21.4±6.1†	+41.8±5.3†	-11.4±4.4#	+72.2±8.2†	+120.5±16.9†

Table 5.2 Summary of the effects of apomorphine (1.0 mg/kg) administered alone or after either haloperidol (0.5 mg/kg) or clozapine (20 mg/kg), as well as the effects of these neuroleptics alone, on the efflux of dopamine, DOPAC and HVA in dialysates of the rat caudate putamen and medial prefrontal cortex. The values listed give the maximum percentage change in basal efflux (± s.e.mean, *n*=5) recorded for each combination of drugs used. * $p<0.01$ versus basal values, # $p<0.05$, † $p<0.01$ versus apomorphine 1.0 mg/kg alone (Student's t-test).

5.3 DISCUSSION

5.3.1 Acute effects of haloperidol and clozapine on the efflux of dopamine, DOPAC, HVA and 5-HIAA in the caudate putamen and medial prefrontal cortex

In keeping with the results from other studies both clozapine and haloperidol elevated the levels of the dopamine metabolites DOPAC and HVA in brain dialysates of the caudate putamen (also shown by O'Conner, Drew & Ungerstedt, 1989; Westerink, Damsma, De Vries & Koning, 1987; Imperato & Di Chiara, 1985; Zetterstrom, Sharp & Ungerstedt, 1985) and medial prefrontal cortex (Hernandez & Hoebel, 1989). Unlike all these other studies (except that of Zetterstrom, Sharp & Ungerstedt, 1985), however, neither haloperidol nor clozapine enhanced the efflux of dopamine in the caudate putamen. This inconsistency may be related to the use of a general anaesthetic agent (this is discussed in more detail below).

The most important finding of this chapter was that clozapine can facilitate the efflux of dopamine in dialysates of the medial prefrontal cortex. This effect has been substantiated recently by other workers (Egan, Karoum & Wyatt, 1991; Karoum & Egan, 1992) using the levels of the dopamine metabolite 3-methoxytyramine (3-MT) as an index of dopamine release. Since 3-MT is formed directly from released dopamine (see Wood & Altar, 1988) and appears to parallel the changes in dopamine levels following several pharmacological manipulations (Westerink & Spaan, 1982), this dopamine metabolite may provide a good measure of synaptic dopamine release (see Wood & Altar, 1988), although this claim has been disputed (Waldmeier, Lauber, Blum & Richter, 1981). Further support for the stimulation of dopamine release by clozapine in the prefrontal cortex stems from another brain dialysis study (Moghaddam & Bunney, 1990) which compared the actions of some neuroleptics on dopamine efflux in rats anaesthetised with chloral hydrate. Although these authors report a significant elevation in the efflux of dopamine in the prefrontal cortex after clozapine (5 & 10 mg/kg) and haloperidol (0.1 & 0.5 mg/kg) administration, they also observed similar increases, for both drugs, in the efflux of dopamine in both the caudate putamen and nucleus accumbens.

It is accepted that any differences observed in the results of microdialysis studies may be due, entirely, or in part, to the use of a particular general anaesthetic agent, even though

in rodents some anaesthetic agents appear to be better than others when studying central dopamine function (see Ford & Marsden, 1986). In this regard, chloral hydrate may not be a first choice anaesthetic agent since it has been shown to reduce the extracellular levels of both dopamine (Zhang, Tilson, Stachowiak & Hong, 1989) and DOPAC (Ford & Marsden, 1986) in the rat striatum, as well as reduce the rise in striatal DOPAC formation following the administration of haloperidol (Ford & Marsden, 1986). In contrast to the depressant effect of chloral hydrate on dopaminergic activity it appears that halothane facilitates the extracellular levels of dopamine in the rat striatum (Spampinato, Girault, Danguir, Savaki, Glowinski & Besson, 1986; Stahle, Collin & Ungerstedt, 1990). This finding may account for the relatively higher basal dialysate levels of dopamine detected in the medial prefrontal cortex (0.052 pmoles/20 minutes) compared to the basal levels reported in studies using chloral hydrate (0.012 pmoles/20 minutes: Bean, During & Roth, 1989; Moghaddam & Bunney, 1990). This assumption is supported by Sharp, Zetterstrom & Ungerstedt (1986), who also used halothane, and also noted a higher basal efflux of dopamine (0.030 pmoles/20 minutes).

The lack of any significant effects of either clozapine or haloperidol on the efflux of dopamine in the caudate putamen was surprising since other studies have reported small but significant rises (O'Conner, Drew & Ungerstedt, 1989; Imperato & Di Chiara, 1985; Moghaddam & Bunney, 1990). The absence of an effect by either of these neuroleptics in the caudate putamen may well be due to the use of halothane which has been reported to block the increase in the spontaneous release of [^{3}H]-dopamine observed after haloperidol (2.0 mg/kg) administration in rats (Spampinato, Girault, Danguir, Savaki, Glowinski & Besson, 1986). Moreover, the stimulation of dopamine release in the caudate putamen by neuroleptics may be critically dependent upon the stimulation of dopamine cell firing (Imperato & Di Chiara, 1985). In this regard, halothane is known to suppress the stimulatory effects of haloperidol on the firing of nigral dopamine neurons (Mereu, Fanni & Gessa, 1984). Apart from the effects of halothane, the doses of haloperidol and clozapine employed in the present study may be insufficient to elevate the efflux of dopamine in the caudate putamen. This is a possibility since in another brain dialysis study in which awake freely moving animals were used, a significant elevation in the efflux of dopamine in the caudate putamen was only noted at a haloperidol dose of 2.0mg/kg (Zetterstrom, Sharp & Ungerstedt, 1985). The fact remains though, that despite any possible attenuation of the ability of neuroleptic drugs to promote dopamine release

due to halothane, clozapine was still able to facilitate the efflux of dopamine in the medial prefrontal cortex. Clearly these studies need to be repeated in awake freely moving animals using both acute and chronic doses of clozapine. In addition it would be of value to assess the dose-dependency of this response and whether other atypical neuroleptic compounds behave like clozapine rather than haloperidol.

What mechanisms underlie the facilitatory action of clozapine on dopamine efflux in the prefrontal cortex? Perhaps, as suggested by Karoum & Egan (1992), clozapine stimulates the release and metabolism of dopamine in the medial prefrontal cortex by feedback activation following the blockade of postsynaptic dopamine D_1 receptors. This may be a plausible mechanism since SCH-23390, a selective dopamine D_1 receptor antagonist, has been reported to elevate the efflux of dopamine in dialysates of the rat caudate putamen when added to the perfusion medium (10μM) (Damsma, Robertson, Tham & Fibiger, 1991). However, at this concentration, it is possible that SCH-23390 also blocked dopamine autoreceptors (an action which would raise the extracellular level of dopamine). Nonetheless, the prefrontal cortex, like other cortical areas, contains a considerably higher concentration of dopamine D_1 receptors than dopamine D_2 receptors (De Keyser, Claeys, De Backer, Ebinger, Roels & Vanquelin, 1988; Boyson, McGonigie & Molinoff, 1986). Indeed the actions of clozapine may be predominantly mediated through dopamine D_1 receptor mechanisms since pretreatment with a selective dopamine D_1 receptor agonist (CY 208-243), but not a selective D_2 receptor agonist (LY 17555), has been shown to abolish the rise in striatal dopamine and DOPAC efflux induced by the administration of clozapine in the unanaesthetised rat (see Coward, Imperato, Urwyler & White, 1989). The fact that clozapine enhances the efflux of dopamine in the medial prefrontal cortex through D_1 receptor mechanisms is perhaps surprising since dopamine release in this brain area is also regulated by presynaptic autoreceptors (Galloway, Wolf & Roth, 1986; Altar, Boyar, Oei & Wood, 1987) which are of the D_2 subtype (Cubeddu, Hoffman & Talmaciu, 1990). It is of course unlikely that clozapine is interacting directly at autoreceptors to produce its effects since haloperidol, which has a higher affinity for the dopamine D_2 receptor (Table 4.1), did not enhance the efflux of dopamine in this area. It is possible that local non-dopaminergic mechanisms in the medial prefrontal cortex may by responsible for mediating the facilitatory action of clozapine on dopamine efflux in this area. These need not necessarily relate to the ability of clozapine to block 5-HT_3 receptors (see chapter 4), since although these receptors are abundant in the prefrontal

cortex (Gehlert, Gackenheimer, Wong & Robertson, 1991), it is apparent, at least in the striatum and the nucleus accumbens, that selective 5-HT_3 receptor antagonists inhibit the drug-stimulated release of dopamine (Blandina, Goldfarb & Green, 1988; Carboni, Acquas, Frau & Di Chiara, 1989). It seems equally unlikely that noradrenergic mechanisms are involved in the clozapine-induced enhancement of dopamine efflux in the prefrontal cortex. Thus although clozapine can enhance the release of noradrenaline from slices of the rat cerebral cortex (Grob, Joachim & Schumann, 1980) there appears to be no evidence, at least in the nucleus accumbens (Bull & Sheehan, 1991), that selective α_1 and α_2 adrenoceptor agonists can increase the extracellular levels of dopamine. Clearly there is inadequate data to provide a satisfactory explanation of clozapine's action in the medial prefrontal cortex. Nevertheless, whether clozapine interacts at postsynaptic dopamine receptors or at release-modulating auto- or heteroreceptors its ability to preferentially facilitate the efflux of dopamine in the medial prefrontal cortex may account for some of the unique clinical effects of this and perhaps other atypical neuroleptic agents. These possibilities are considered in section 5.3.4.

Although both haloperidol and clozapine increased the levels of the dopamine metabolites appearing in brain dialysates of the caudate putamen and the medial prefrontal cortex they had no significant effect on the efflux of 5-HIAA in these regions. This absence of an effect by these neuroleptics on 5-HT metabolism agrees well with a previous brain dialysis study (Zetterstrom, Sharp & Ungerstedt, 1985).

From the outset it was anticipated that this study would reveal regional differences in the effects of typical and atypical neuroleptics on the turnover (release and metabolism) of dopamine and it was predicted that this would be most apparent in the nigrostriatal system. In fact, in this regard, clozapine and haloperidol shared similar actions and only in the medial prefrontal cortex were any noticeable differences observed. Bartholini (1976) provided the first evidence for a selective effect of atypical neuroleptic compounds on mesolimbic dopamine neurotransmission. In this early study the effects of sulpiride, clozapine and thioridazine, in the presence of probenecid, caused a greater accumulation of HVA in the nucleus accumbens than either haloperidol or chlorpromazine. In a later study, using the same technique but without probenecid, a preferential metabolic effect of atypical neuroleptics in mesolimbic areas was again noted (Westerink, Lejeune, Korf & Van Praag, 1977). In view of the reported development of tolerance in both the

nigrostriatal and the mesolimbic systems to the effects of chronically administered neuroleptic drugs (see section 1.3.2) the clinical relevance of the above data is questionable. In the present study, apart from the effects of clozapine on HVA efflux (see below), both haloperidol and clozapine produced more marked effects on the efflux of dopamine metabolites in the medial prefrontal cortex than the caudate putamen. Surprisingly, clozapine produced a similar maximum effect on the efflux of HVA in both brain areas despite a much greater effect on the efflux of DOPAC in the medial prefrontal cortex. This excess in DOPAC efflux may be derived from the enhancement of dopamine release in this area by clozapine. Presumably, the more pronounced effects of these compounds on dopamine metabolism in this area are due to the much higher turnover of dopamine in the frontal cortex (Bannon, Bunney & Roth, 1981). Other studies have also demonstrated marked effects of neuroleptic drugs on dopamine metabolism in this area (Laduron, De Bie & Leyson, 1977; Hernandez & Hoebel, 1989). There are some discrepancies, however, in the results obtained using different sampling techniques. Thus, my results agree with those of another brain dialysis study by Hernandez & Hoebel (1989) in awake freely moving rats (haloperidol 0.5 mg/kg) suggesting that anaesthesia is not important but studies on whole tissue levels of DOPAC and HVA report either reversed (metabolite levels greater in the caudate putamen) or comparable changes in the caudate putamen and the prefrontal cortex after acute neuroleptic treatment (Matsumoto, Uchimura, Hirano, Soo Kim, Yakoo, Shimomura, Nakahara, Inoue & Oomagari, 1983; Bacopoulos & Roth, 1981; Bannon, Wolf & Roth, 1983; Bannon, Reinhard, Bunney & Roth, 1982; Westerink & Korf, 1976; Scatton, 1977). Although some reports have suggested a greater sensitivity of the mesocortical dopamine system (versus the nigrostriatal system) to the acute effects of haloperidol (Matsumoto *et al.*, 1983; Laduron, De Bie & Leysen, 1977) this only appears to occur at doses considerably smaller than the dose used in the present study. Perhaps, as alluded to above, the discrepancy in results may result from the method used to estimate dopamine metabolite levels (brain homogenation versus brain dialysis). Although there does not appear to be any difference in the ability of either of these techniques to reflect the relative quantities of DOPAC and HVA in both the caudate putamen and the prefrontal cortex (see Table 5.3) it is not inconceivable that brain dialysis preferentially samples either free or conjugated forms of DOPAC and HVA. Indeed this has been recognised as a potential source of variation between estimations of DOPAC and HVA content in brain homogenates (Bacopoulos & Roth, 1981). Since in my own studies, apomorphine depressed the extracellular levels of DOPAC and HVA in

	ref	DOPAC	HVA	DOPAC/HVA	mean
	1	0.218*	0.637*	0.34	
	2	0.098	0.114	0.86	
TISSUE-cortex	*3*	0.081	0.097	0.84	0.62 §
(μg/g)	*4*	0.100	0.130	0.77	(0.12)
	5	0.071	0.226	0.31	
	5	0.63	2.24	0.28	
DIALYSATE-cortex	*6*	3.85	2.78	1.38	0.72
(pmoles/20mins)	*7*	1.14	2.30	0.49	(0.34)
	1	10.99*	8.97*	1.23	
	2	0.93	0.59	1.58	
TISSUE-caudate	*3*	1.89	1.12	1.69	1.28 §
(μg/g)	*4*	1.51	1.36	1.10	(0.16)
	5	2.48	3.01	0.82	
	5	23.21	20.91	1.11	
DIALYSATE-caudate	*6*	11.08	8.53	1.30	1.32
(pmoles/20mins)	*7*	18.1	11.7	1.55	(0.13)

Table 5.3 Comparison of the levels of DOPAC and HVA estimated in either whole brain tissue (μg/g wet weight tissue; * μg/g protein) or in dialysates (pmoles/20 minutes) of the rat frontal cortex and caudate putamen. The relative levels of DOPAC and HVA estimated by these sampling techniques have been expressed as a ratio of DOPAC/HVA and the averaged ratios (± s.e.means) have been statistically assessed (cortex versus caudate putamen) using a Student's non-paired t-test (§ $p<0.01$). The data was derived from the following references: (1) Karoum & Egan (1992); (2) Scatton (1977); (3) Bannon *et al.,* (1983); (4) Bacopoulos & Roth (1981); (5) Sharp *et al.,* (1986); (6) Hernandez & Hoebel (1989); (7) own data (Table 5.1).

the prefrontal cortex, comparable to the results of Westerink & Korf (1976) who measured only free acid metabolite levels, but different to the results of Bacopoulos & Roth (1981) who collectively measured free and conjugated metabolite levels, it is possible that brain dialysis only samples free acid metabolite levels.

It is apparent from Table 5.3, that unlike the caudate putamen, the medial prefrontal cortex contains relatively more HVA than DOPAC. The abundance of HVA in the prefrontal cortex may result from either reduced clearance and/or the lack of synthesis-modulating autoreceptors (Chiodo, Bannon, Grace, Roth & Bunney, 1984) and enhanced turnover of dopamine in this area (see above). More specifically, the higher efflux of HVA compared with DOPAC in the cortex may reflect a higher synaptic availability of dopamine (either from increased synaptic release or reduced up-take; see also Sharp, Zetterstrom & Ungerstedt, 1986). Thus, compared with the caudate putamen, and even though MAO activity may be the same in the caudate putamen and the cortex (see Garrett & Soares-Da-Silva, 1990), relatively more extracellular HVA may be formed in the cortex by the deamination of 3-MT rather than by the O-methylation of DOPAC. Since in primates the frontal cerebral cortex is considerably larger than the basal ganglia, it is likely, as pointed out by Elsworth *et al.*, (1987), that this area contributes greatly to the levels of HVA appearing in the CSF despite a much higher concentration of HVA in the basal ganglia. Indeed there appears to be a good correlation between the levels of HVA in the frontal cortex and CSF, both in man (Stanley, Traskman-Bendz & Dorovini-Zis, 1985) and in primates (Elsworth, Leahy, Roth & Redmond, 1987).

5.3.2 Effects of apomorphine on the efflux of dopamine,DOPAC, HVA and 5-HIAA in the caudate putamen and medial prefrontal cortex

Apomorphine produced a progressive dose-related reduction in the efflux of dopamine, DOPAC and HVA in both brain areas studied with no apparent effects on the efflux of 5-HIAA except in the medial prefrontal cortex at the highest dose used. The effects of apomorphine on the levels of DOPAC and HVA in the caudate putamen, at doses which are likely to stimulate both pre- and postsynaptic dopamine receptors (> 0.1 mg/kg i.v.), are consistent with other studies using either whole tissue estimates (Westerink & Korf, 1976; Bannon, Wolf & Roth, 1983; Nicolaou, 1980) or estimates from brain dialysates (Zetterstrom & Ungerstedt, 1984). However, there are clear discrepancies in the prefrontal

cortex where little or no effect of apomorphine has been observed on the levels of DOPAC and HVA using whole tissue estimates (Bacopoulos & Roth, 1981; Bannon, Wolf & Roth, 1983). It is difficult to reconcile these findings in the prefrontal cortex with the marked effects of apomorphine observed in the present study although these differences may be critically dependent on the method of metabolite estimation (see section 5.3.1).

Although apomorphine reduced the efflux of dopamine it was not possible to achieve more than about a 35% inhibition from basal efflux values in the caudate putamen (23% in the prefrontal cortex). This finding is at variance with the results of another brain dialysis study in the caudate putamen where halothane was also used (Zetterstrom & Ungerstedt, 1984). Although these workers obtained similar results to my own at low doses of apomorphine (0.05, 0.1 and 0.2 mg/kg), they were able to show, in contrast to my results and those of Spampinato *et al.*, (1986), marked reductions in the efflux of dopamine (to undetectable levels) at a higher dose (0.5 mg/kg). Their interpretation of this reduction was that apomorphine at low doses stimulates dopamine terminal autoreceptors to reduce dopamine release whilst at higher doses it also stimulates postsynaptic dopamine receptors and thus activates feedback circuits to reduce dopamine release further by inhibiting impulse flow. Presumably, since low "autoreceptor" doses of apomorphine, as well as inhibiting dopamine release, also inhibits the firing of nigrostriatal dopamine neurons (Trulson, Trulson & Arasteh, 1987; Carlson, Bergstrom & Walters, 1986), striato-nigral feedback circuits are not exclusively involved in the effects of these "postsynaptic" doses of apomorphine. The discrepancy between the results of Zetterstrom & Ungerstedt (1984) and my own are difficult to reconcile. In the present study, and in the study of Zetterstrom & Ungerstedt (1984) at low apomorphine doses (see above), the efflux of DOPAC and HVA continued to fall without any discernable change in the efflux of dopamine. This indicates that the processes of dopamine release and metabolism in the caudate putamen and the medial prefrontal cortex may be independently regulated. Most likely, other mechanisms such as alterations in the synthesis of dopamine are involved. The efflux of dopamine in the medial prefrontal cortex was only significantly reduced at the higher doses of apomorphine used. This is surprising since dopamine agonists appear to reduce the stimulated release of dopamine from both striatal and prefrontalcortical slices with equal efficacy (Cubeddu, Hoffman & Talmaciu, 1990) although the maximum inhibition achieved in prefrontalcortical slices was considerably lower.

5.3.3 Effects of concomitant administration of either haloperidol or clozapine with apomorphine on the efflux of dopamine, DOPAC and HVA in the caudate putamen and medial prefrontal cortex

In these studies clozapine was assessed to be a considerably weaker antagonist of central dopamine receptors than haloperidol. Thus, despite using a much higher dose of clozapine than haloperidol, it was much weaker in blocking the neurochemical effects of apomorphine in both the nigrostriatal and the mesocortical dopamine systems. The overall difference in potency (about 400 fold) between haloperidol and clozapine (Fig.5.9) does not equate well with the difference in the daily maintenance dose of these agents required to stabilise the symptoms of schizophrenia (5-20 mg/day haloperidol versus 200-600 mg/day clozapine; see section 2.4.2). This would suggest that other mechanisms mediate the reported effectiveness of clozapine against both the positive and the negative features of schizophrenia (Kane, Honigfeld, Singer & Meltzer, 1988).

Consistent with the view that clozapine weakly antagonises dopamine receptors are the findings that clozapine, in clinically effective doses, does not, unlike typical neuroleptics, markedly increase plasma prolactin levels (mediated through dopamine D_2 mechanisms), or block the apomorphine-induced decrease in plasma prolactin levels (Meltzer, 1989). What is less clear is whether clozapine is producing its neurochemical effects (rise in DOPAC and HVA efflux) predominantly at either D_1 or at D_2 receptors. This uncertainty arises since apomorphine can stimulate both dopamine receptor subtypes although it has a more potent effect on D_2 mechanisms, and clozapine appears to elevate the levels of dopamine metabolites, at least in the caudate putamen, predominantly through dopamine D_1 mechanisms (Coward, Imperato, Urwyler & White, 1989). Nonetheless, since the D_1 antagonist SCH23390 (Hyttel, 1983) does not increase the levels of HVA in the rat striatum (Hietala, Lappalainen, Koulu & Syvalahti, 1990) it would appear that D_2 mechanisms must contribute to the increase of dopamine metabolism by clozapine. The weak antagonism shown by clozapine against apomorphine, together with its ability to release dopamine in the prefrontal cortex, implies that in some areas at least, clozapine may not provide sustained dopamine receptor blockade. Although this argument may account for the low tendency of this agent to induce extrapyramidal effects it would not explain why clozapine is an effective antipsychotic agent since these therapeutic effects are possibly mediated by dopamine antagonism in the mesocortical dopamine system.

5.3.4 Clinical considerations

Much interest has been generated by the recent finding that clozapine may be of value in schizophrenic patients who show poor response to conventional neuroleptic therapy (Kane, Honigfeld, Singer & Meltzer, 1988; Kane, Honigfeld, Singer & Meltzer, 1989; Angst, Stassen & Woggon, 1989). In these chronic schizophrenics, negative features such as blunted affect, poverty of speech and thought, apathy, social withdrawal and lack of initiative (see also Andreasen & Olsen, 1982) predominated. Collectively, these have been described as the most disabling features of this illness (Weinberger, 1987) since negative features are resistant to treatment by conventional dopamine receptor antagonists. They also tend to be over-represented in patients with structural abnormalities of the brain, such as cortical atrophy and dilated cerebral ventricles (see Waddington & Youssef, 1986), and in whom decreased cerebrospinal fluid concentrations of HVA have been found (Van Kammen, Mann, Sternberg, Scheinin, Ninan, Marder, Van Kammen, Rieder & Linnoila, 1983). Since depletion of dopamine in the prefrontal cortex of rhesus monkeys can cause similar cognitive deficits (Brozoski, Brown, Rosvold & Goldman, 1979) it has been argued that negative symptoms may arise from a relative loss of dopamine influence in some parts of the cortex (Weinberger, 1987). Herein lies one mechanism which may partly account for the unique clinical effectiveness of clozapine. By selectively releasing dopamine in the medial prefrontal cortex, clozapine may restore the dopaminergic balance in this region and thus counter the expression of negative schizophrenic symptoms. Evidence, albiet indirect, to support the claim that clozâpine may act in some brain areas by releasing dopamine comes from the use of the behavioural "despair" test (or forced swimming test) which is thought to predict anti-depressant activity (Porsolt, Anton, Blavet & Jalfre, 1978; Willner, 1984; Borsini & Meli, 1988). In this, both apomorphine (Porsolt, Bertin, Blavet, Deniel & Jalfre, 1979) and clozapine (Browne, 1979; Gorka & Janus, 1985), unlike typical neuroleptics (Porsolt, Bertin, Blavet, Deniel & Jalfre, 1979; Kawashima, Araki & Aihara, 1986) reduce the duration of immobility like anti-depressants. Clearly the interpretation of these results is compounded by the well recognised ability of both dopamine agonists and antagonists to modify motor activity in rodents. It is interesting to note, however, that another atypical neuroleptic, sulpiride, also shows anti-depressant activity both in this model (Kawashima, Araki & Aihara, 1986) and in clinical practice (Peselow & Stanley, 1982). Moreover, some have argued that since the presence or absence of tardive dyskinesias are prominently associated with the

presence or absence of negative symptoms (Waddington & Youssef, 1986; Waddington, Youssef, Dolphin & Kinsella, 1987; Csernansky, Kaplan, Holman & Hollister, 1983; Barnes, 1985) that drugs without extrapyramidal side effects (atypical neuroleptics) may be preferable in treating negative symptoms (Angst, Stassen & Woggon, 1989). The finding that clozapine appears to be superior in the treatment of these negative symptoms compared with chlorpromazine (typical neuroleptic) (see Kane, Honigfeld, Singer, & Meltzer, 1988) supports this claim. Since haloperidol produced potent antagonism of the neurochemical effects of apomorphine in the medial prefrontal cortex and since also it did not enhance dopamine efflux in this area it would be expected to be less effective against negative symptoms like chlorpromazine. The effectiveness of haloperidol and other neuroleptics (including clozapine) against the positive symptoms of schizophrenia may arise from dopamine receptor blockade in the mesolimbic system (see Weinberger, 1987). As noted in section 5.3.3 clozapine appears to be a considerably weaker dopamine receptor antagonist than haloperidol which may account for both its low incidence of extrapyramidal side effects and lack of any effects on the regulation of striatal dopamine receptors in rodents (Creese & Synder, 1980; Severson, Robinson, Simpson, 1984) and this latter effect may explain why this compound carries a lower risk of inducing tardive dyskinesia (see Gerlach & Casey, 1988; review of the pathogenesis of tardive dyskinesia). The weak dopamine receptor antagonism of clozapine may also underlie the rapid return of psychotic symptoms seen after the withdrawal of this compound (Ekblom, Eriksson & Lindstrom, 1984; Perenyi, Kuncz & Bagdy, 1985; Eklund, 1987).

5.4 CONCLUSIONS

The ability of clozapine to preferentially release dopamine in the medial prefrontal cortex may account for its reported effectiveness in the treatment of the negative symptoms of schizophrenia. The weak apomorphine (dopamine) antagonism shown by clozapine in the caudate putamen would account for the low incidence of extrapyramidal side effects seen with this compound although its weak action in the medial prefrontal cortex implies that its antipsychotic efficacy must stem, at least in part, from other actions.

CHAPTER 6

EFFECTS OF APOMORPHINE AND NEUROLEPTICS ON THE SPONTANEOUS ACTIVITY OF NEURONS IN THE CAUDATE PUTAMEN AND MEDIAL PREFRONTAL CORTEX

6.1 INTRODUCTION

The results from the preceding chapter established that clozapine is a considerably weaker dopamine receptor antagonist compared with haloperidol, and unlike haloperidol, can selectively enhance the release of dopamine in the medial prefrontal cortex. This chapter sets out to extend these findings by examining the effects of these agents, either alone, or against apomorphine, on the spontaneous activity of neurons in both the caudate putamen and the medial prefrontal cortex.

Whilst microdialysis can provide a measure of presynaptic dopamine function (with superimposed postsynaptic feedback effects) it is less likely to reveal changes in the function of postsynaptic mechanisms controlled by dopamine. Thus although the ability of neuroleptic drugs to block presynaptic dopamine receptors may contribute considerably to their stimulatory effects on dopamine turnover (Di Chiara, Porceddu, Spano & Gessa, 1977; Commissiong, Slimovitch & Toffano, 1990), these agents can also block postsynaptic dopamine receptors resulting in, among other effects, the stimulation of dopamine neuronal firing (see Mereu, Fanni & Gessa, 1984), the antagonism of apomorphine-induced hyperactivity and stereotypy (Magnusson, Fowler, Mohringe, Wijkstrom & Ogren, 1988; Ungerstedt & Ljungberg, 1977), the facilitation of striatal cholinergic function (Sethy & Van Woert, 1974; Guyenet, Agid, Javoy, Beaujouan, Rossier & Glowinski, 1975; Starke, Spath, Lang & Adelung, 1983; Baud, Arbilla & Langer, 1985; Compton & Johnson, 1989) and effects on other neurotransmitter systems. Indeed, a number of neuroleptic drugs have been shown to have equal antagonistic actions at both pre- and postsynaptic dopamine receptors (Starke, Spath, Lang & Adelung, 1983) indicating that activity at either of these receptors may, in part, underlie the clinical effects of these agents. In this chapter it is hoped to compliment the dialysis results of chapter 5 by recording the activity of postsynaptic neurons in dopamine-innervated areas. Many studies searching to elucidate the mode of neuroleptic drug action have examined the effects of these compounds (and dopamine agonists) on the activity of dopamine (assumed) neurons in the substantia nigra pars compacta (A9) and the ventral tegmentum (A10) (see section 2.1). This is rather surprising since presumably, it is the output of "effector" neurons (i.e., those neurons receiving a dopamine innervation) that will determine the outcome of neuroleptic-induced changes in the neurotransmission of dopamine. Although the neural circuits which interact with dopamine systems are poorly

understood and have yet to be fully characterised neurochemically, it would, nonetheless, be a useful start to test the effects of neuroleptics and dopamine receptor agonists on the activity of neurons which receive a dopamine innervation in the caudate putamen and medial prefrontal cortex. Ultimately, once the neural circuitry mediating neuroleptic-induced behavioral effects are known, it would be desirable to synchronously record from multiple neural sites in awake freely moving animals.

Despite a large number of studies, it remains controversial, particularly in the caudate putamen, whether dopamine acts as an inhibitory or an excitatory neurotransmitter. Thus when applied locally in the caudate putamen by iontophoresis, both dopamine and dopamine agonists are generally found to produce inhibitory effects on neuronal firing (Siggens, Hoffer & Ungerstedt, 1974; Yarbrough, 1975; Stone, 1976; Zarzecki, Blake & Somjen, 1977; Skirboll & Bunney, 1979; Brown & Arbuthnott, 1983) although excitatory effects have been observed with either exogenous dopamine, applied by iontophoresis (Bevan, Bradshaw & Szabadi, 1975; Ohno, Sasa & Takaori, 1986; Ohno, Sasa, Takaori, 1987) or endogenous dopamine, released by stimulating the medial forebrain bundle (Williams & Millar, 1990) or the substantia nigra (Preston, Bishop & Kitai, 1980). There is evidence, at least in the caudate putamen, that the inhibitory and excitatory effects of dopamine on neuronal activity are mediated by dopamine D_1 and dopamine D_2 receptors respectively, and that D_1 receptors, although possibly located on the same postsynaptic neuron as D_2 receptors (Ohno, Sasa & Takaori, 1987), lie proximally, but outside the dopamine synapse (Ohno, Sasa & Takaori, 1987; Hu & Wang, 1988; Akaike, Ohno, Masashi & Takaori, 1987; Williams & Millar, 1990). This evidence together with the findings that dopamine D_1 receptor stimulation may be an important pre-requisite for the expression of D_2 receptor mediated effects (Wachtel, Hu, Galloway & White, 1989) and that co-administration of selective dopamine D_1 and D_2 agonists can produce synergistic inhibitory effects on striatal activity (Hu & Wang, 1988) implies that the action of dopamine, in the nigrostriatal system at least, is more complex than perhaps initially anticipated. Indeed, some investigators have proposed a neuromodulatory role for dopamine in the caudate putamen especially with regard to the neurotransmission of some amino-acids (glutamate and GABA) in this area (Mercuri, Bernardi, Calabresi, Cotugno, Levi & Stanzione, 1985; Chiodo & Berger, 1986).

The situation in the medial prefrontal cortex is no less clear. Although some workers report that neurons in this area are particularly sensitive to the inhibitory effects of dopamine and dopamine agonists (Mora, Sweeney, Rolls & Sanguinetti, 1976; Bunney & Aghajanian, 1976; Peterson, St Mary & Harding, 1987), other workers report either excitatory effects (Penit-Soria, Audinat & Crepel, 1987) or inhibitory effects of dopamine itself but not of selective dopamine D_1 and dopamine D_2 receptor agonists (Sesack & Bunney, 1989). This latter observation together with the recent finding that some neuroleptics (sulpiride, fluphenazine, spiroperidol) but not others (haloperidol, levomepromazine) can block the inhibitory effects of VTA stimulation and dopamine application on neuronal firing in the prefrontal cortex (Thierry, Douarin, Penit, Ferron & Glowinski, 1986; Godbout, Mantz, Pirot, Glowinski & Thierry, 1991) raises the possibility that the inhibition of cells in this area by dopamine is mediated by a dopamine receptor subtype which is distinct from either a dopamine D_1 or a dopamine D_2 receptor.

In summary this chapter sets out to re-evaluate the effects of a dopamine agonist (apomorphine) on the spontaneous activity of neurons in the caudate putamen and medial prefrontal cortex and to show whether these effects can be antagonised by clozapine and haloperidol. It will thus be possible to compare these actions at postsynaptic dopamine receptors with their effects against apomorphine on dopamine release and metabolism (chapter 5). In addition, this chapter aims to re-examine the controversial results of Thierry *et al.*, (1986) and Godbout *et al.*, (1991), given above, which suggest that haloperidol may be less effective as a dopamine receptor antagonist in the medial prefrontal cortex. Finally, and of particular interest, it is hoped to provide some electrophysiological evidence to support the finding that clozapine may act by preferentially releasing dopamine in the medial prefrontal cortex.

6.2 RESULTS

6.2.1. Effects of drug vehicles on the spontaneous discharge of neurons in the caudate putamen and the medial prefrontal cortex

Before examining the effects of apomorphine and neuroleptic compounds on the activity of neurons in the caudate putamen and the medial prefrontal cortex it was considered important to firstly establish the effects of the vehicles used to dissolve these compounds

on this activity. In these experiments normal saline (used as the vehicle for apomorphine hydrochloride) given intravenously did not significantly modify the spontaneous discharge of neurons in either the caudate putamen (Fig.6.1) or the prefrontal cortex (Fig.6.2) although there was some tendency for this vehicle to transiently decrease neuronal activity in the caudate putamen (Fig.6.2). Since the activity of neurons in the caudate putamen, without exception, returned to basal levels within 5 minutes following the administration of normal saline (1.0 ml/kg), it was decided to assess the effects of apomorphine on neuronal discharge between 5 and 10 minutes after its administration.

The vehicle used to dissolve clozapine and haloperidol (see appendix I) also did not significantly alter the spontaneous activity of neurons in either brain area studied (Fig.6.2). Whilst there was some tendency for this vehicle given intravenously to decrease neuronal discharge in the caudate putamen and increase it in the medial prefrontal cortex, this only occurred transiently and did not alter the ability of apomorphine (200 µg/kg i.v.) to inhibit the activity of neurons in the caudate putamen. Since in other studies, saline also stimulated cortical neurons, with an equally rapid onset, this effect was considered to be indirectly mediated via local i.p. mechanisms.

6.2.2. Effects of apomorphine and neuroleptics on the spontaneous activity of neurons in the caudate putamen

Apomorphine generally produced a progressive dose-dependent inhibition of neuronal activity in the rostral caudate putamen (Fig.6.3) with an ED_{50} (± s.e.mean, n=5) of 0.102 ± 0.007 mg/kg (Fig.6.8). The majority of neurons monitored exhibited type I action potentials (see chapter 3). Over 80% of these neurons tested (n=30) were inhibited by apomorphine with about 10% (of the total) showing only a stimulatory response (Fig.6.4). Some neurons were however, stimulated by the lower doses of apomorphine (25-50 µg/kg i.v.) but these were invariably inhibited by higher doses (50-500 µg/kg i.v.). A transient stimulatory response followed by prolonged inhibition was occasionally noted after a high dose of apomorphine (Fig.6.1). The stimulation of neuronal discharge by apomorphine (without inhibition at higher doses) was more often than not confined to ventrally located neurons (4.5-6.0 mm below the cortical surface) which showed much larger action potentials than more dorsally located neurons. In contrast, apomorphine produced its inhibitory effects on neurons throughout the full dorso-ventral extent of the

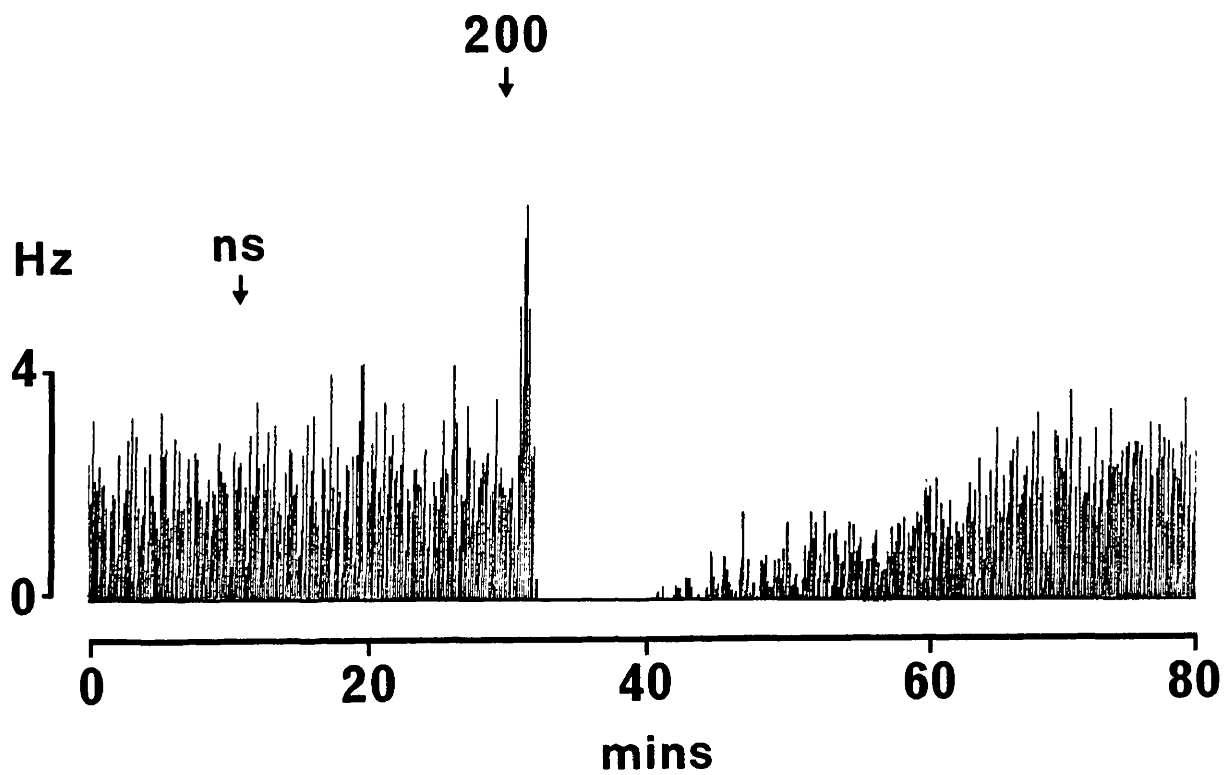


Figure 6.1 Representative rate recording showing the effects of intravenously administered normal saline (ns) (0.25mls) and apomorphine hydrochloride (200 µg/kg in 0.25mls normal saline) on the spontaneous activity of a type I neuron (see chapter 3) in the rostral caudate putamen.

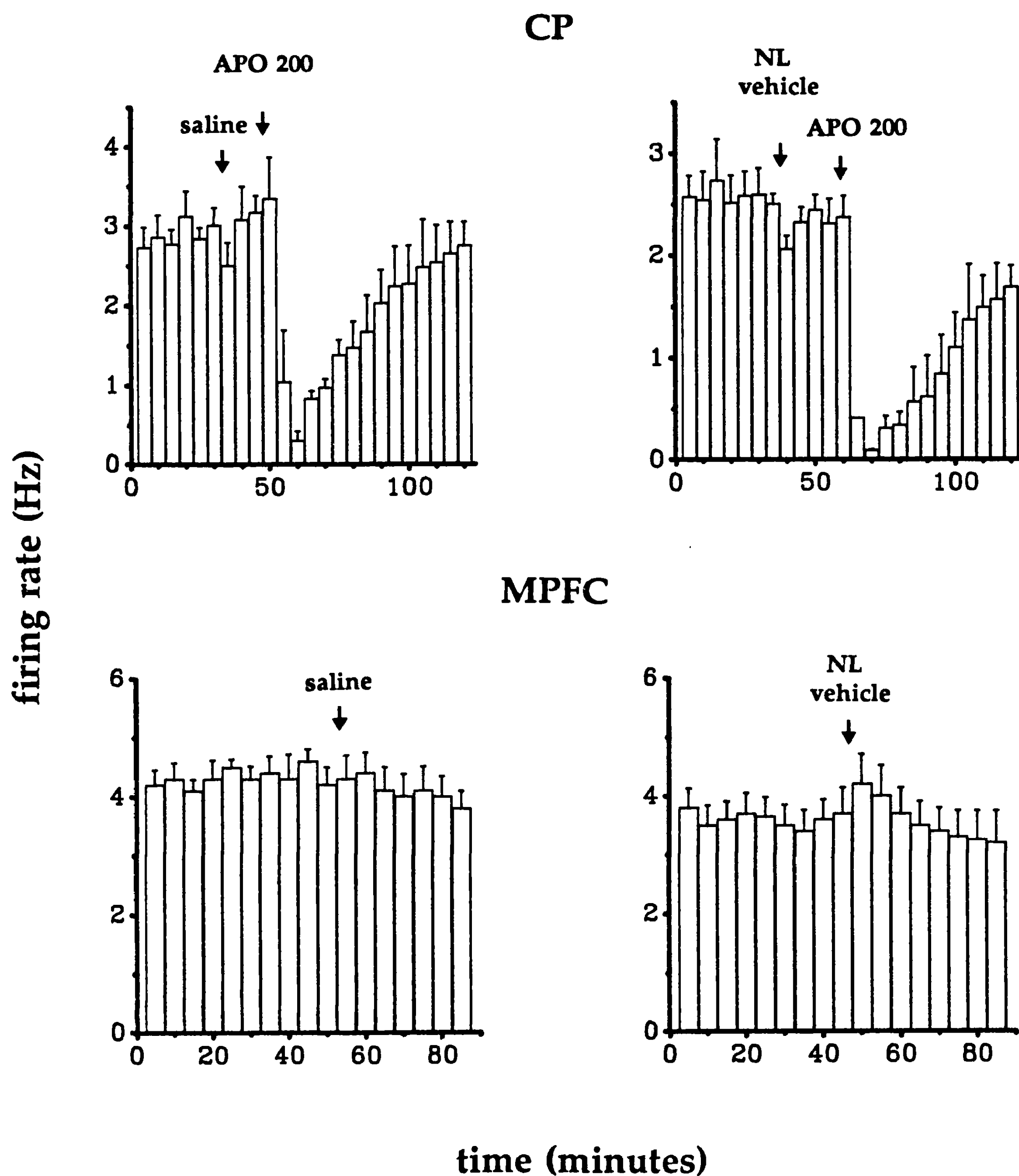


Figure 6.2 Control experiments showing the effects of drug vehicles (normal saline 1.0 ml/kg i.v., neuroleptic vehicle 1.0 ml/kg i.p.) and apomorphine (200 µg/kg i.v.) on the spontaneous activity of neurons in the rostral caudate putamen (CP) as well as the effects of drug vehicles on the activity of neurons in the medial prefrontal cortex (MPFC). Error bars represent s.e.means (*n*=4-5). The neuroleptic (NL) drug vehicle consisted of glacial acetic acid (25µl) in distilled deionised water (5mls) adjusted to pH 6.0 with 0.5M NaOH.

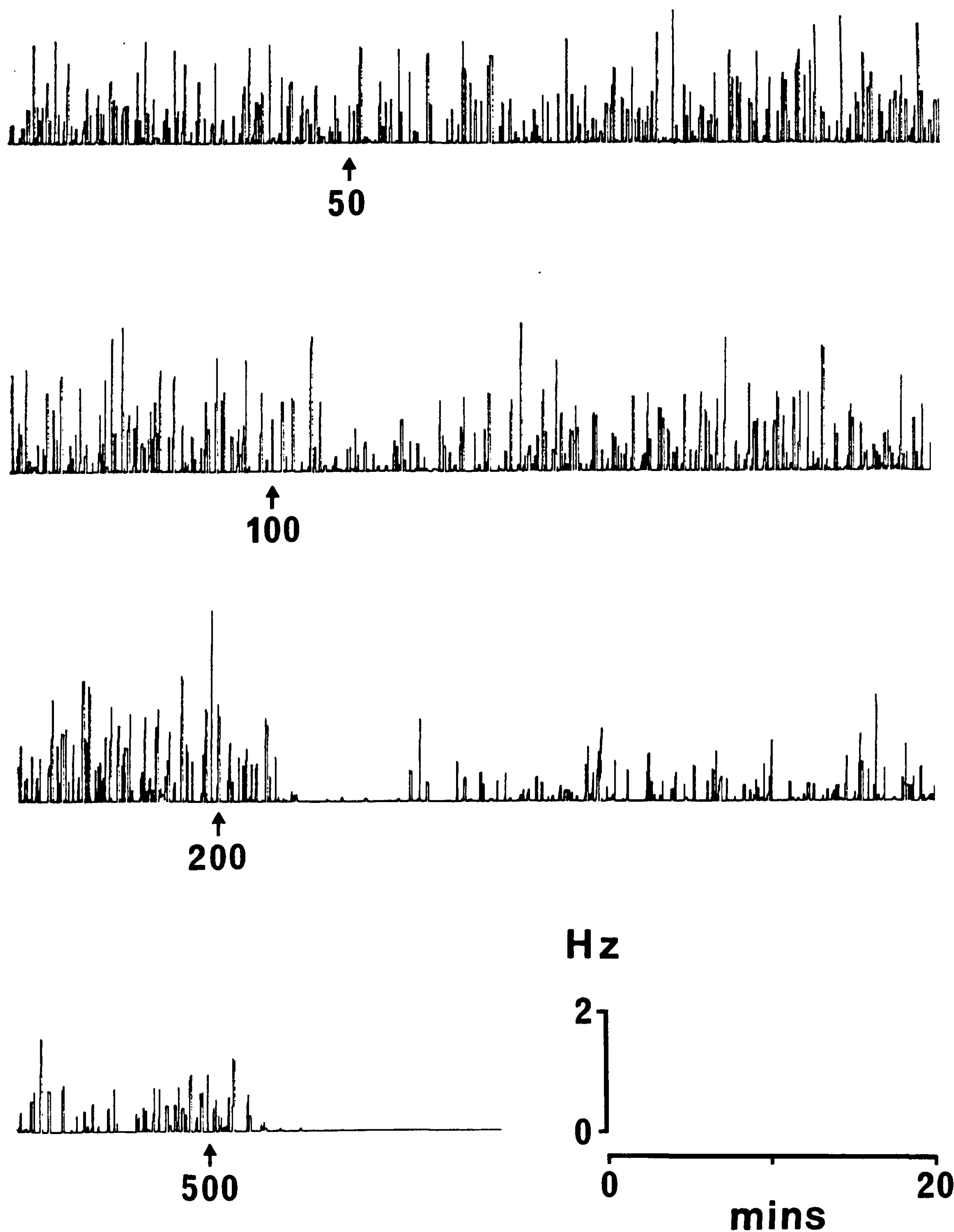
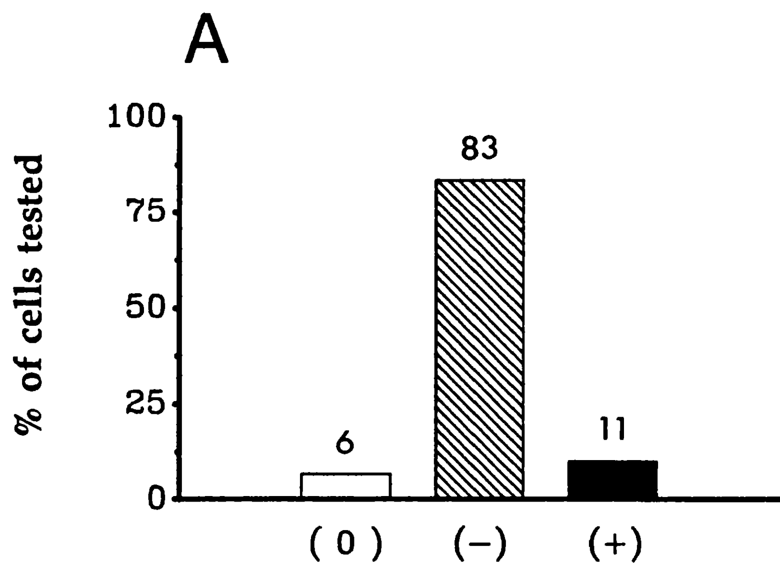


Figure 6.3 Representative rate recording showing the dose-dependent inhibitory effects of intravenous apomorphine hydrochloride (µg/kg 1.0 ml/kg) on the spontaneous activity of a type I neuron (see chapter 3) in the rostral caudate putamen.



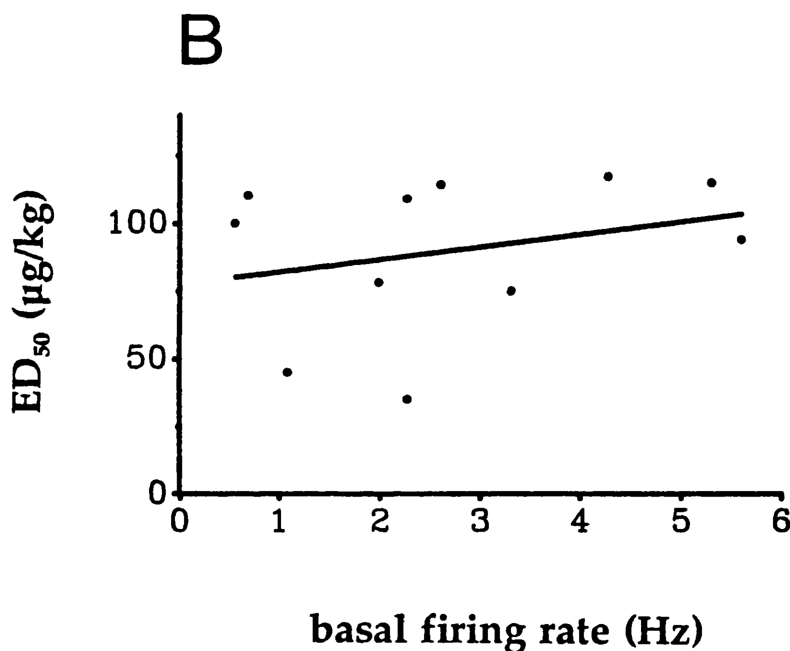


Figure 6.4 Graphs summarising the effects of apomorphine on the spontaneous activity of neurons in the rostral caudate putamen. The percentage of cells unresponsive (o) and responsive (- inhibition; + stimulation) to apomorphine (25-500µg/kg i.v.) is shown in graph (A) (*n*=30). The relationship between basal neuronal activity and the ability of apomorphine to inhibit the activity of neurons in the rostral caudate putamen (estimated by the dose required to produce a 50% decrease in neuronal activity [ED_{50}]) is given in graph (B). Linear regression was used to fit a straight line through the data (r^2 = 0.081).

rostral caudate putamen. The ability of apomorphine to inhibit the firing of neurons in this brain area did not depend on either their basal activity (Fig.6.4) or the pattern of neuronal discharge. Thus across the range of basal firing frequencies studied (0.4-5.8 Hz), the dose of apomorphine required to inhibit neuronal discharge by 50% (ED_{50}), did not vary appreciably from 100 µg/kg (i.v.).

Alone, clozapine (apart from some slight stimulation) and haloperidol (but see below), produced no significant effects on the spontaneous discharge of neurons in the caudate putamen (Fig.6.5). Haloperidol produced a significant inhibitory effect in 3 neurons tested (*n=10*), although the majority of cells were either not affected by this compound or showed modest stimulatory effects. No significant differences were noted in either the discharge rate of neurons recorded one hour after the administration of either clozapine or haloperidol compared with basal spontaneous rates (Fig.6.5B) or in the nature of neuronal activity (type of action potential and pattern of discharge) recorded following the administration of these neuroleptics. Since it was difficult to record the spontaneous activity of neurons in the caudate putamen for more than about 2-3 hours and since neither clozapine nor haloperidol appear to produce any consistent effects on neuronal discharge in this area it was decided to pretreat animals with these neuroleptics before testing the effects of apomorphine.

Haloperidol produced a marked antagonism of apomorphine-induced inhibition of striatal neuronal discharge but the effect of clozapine was much weaker (Figs.6.6, 6.7 and 6.8). The ED_{50} values (± s.e.mean n=5) for apomorphine in the presence of clozapine and haloperidol were 0.180 ± 0.036 mg/kg and 0.860 ± 0.163, respectively (both values significantly different [$p<0.05$] from the control apomorphine response), giving an approximate activity ratio of 4.8. This, together with the 40 fold difference in dose, suggests that haloperidol is about 190 fold more potent than clozapine in blocking postsynaptic dopamine receptors in the caudate putamen.

6.2.3. Effects of apomorphine on the spontaneous activity of neurons in the medial prefrontal cortex

Unlike its predominantly inhibitory effects in the caudate putamen (see section 6.3.2), apomorphine produced both inhibitory (41% of cells tested) and stimulatory effects (22%

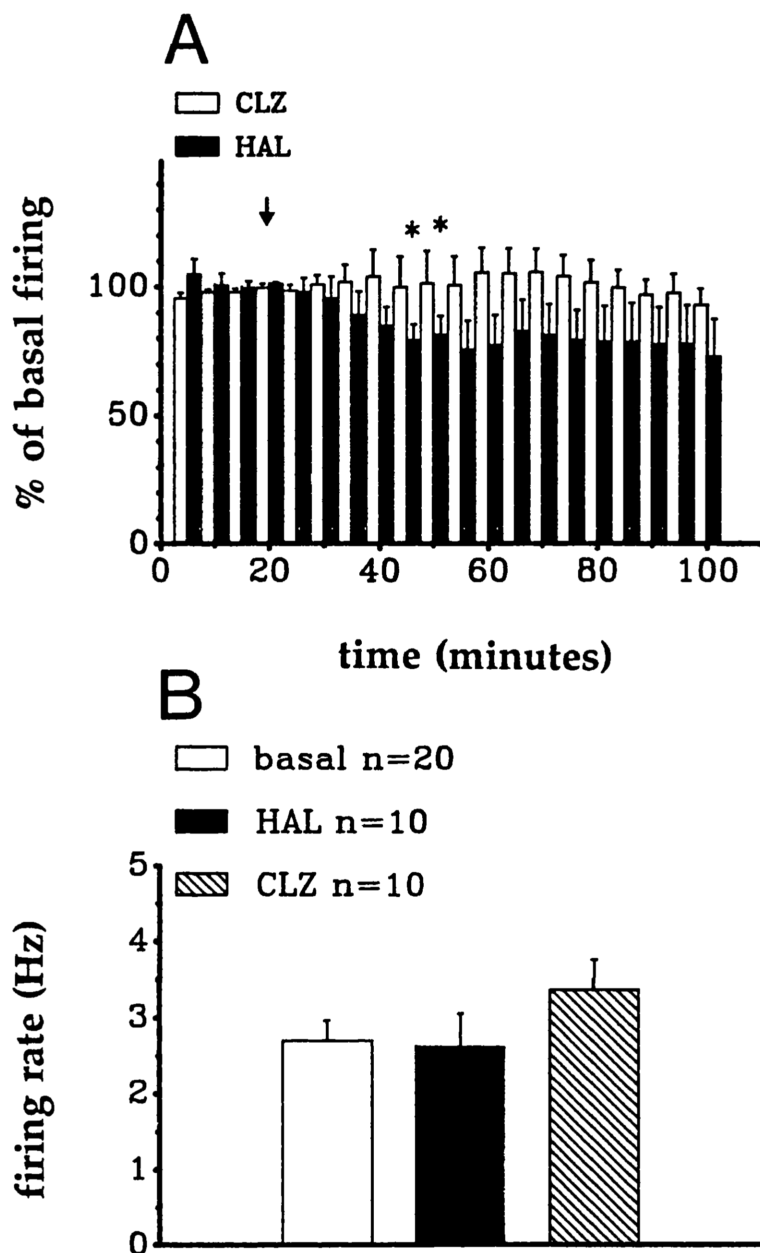


Figure 6.5 Effects of clozapine (CLZ) (20 mg/kg i.p.) and haloperidol (HAL) (0.5 mg/kg i.p) on the spontaneous activity of neurons in the rostral caudate putamen. The data (± s.e.mean) in graph (A) was averaged from 10 experiments and statistical significance relative to basal firing rates was assessed using a Student's non-paired t-test (* $p<0.05$). The firing rates (± s.e.mean) of neurons in the rostral caudate putamen recorded either before (2.69 ± 0.27 Hz *n=30*) or 60 minutes after treatment with either clozapine (2.61 ± 0.44 Hz *n=10*) or haloperidol (3.36 ± 0.40 Hz *n=10*) are given in (B).

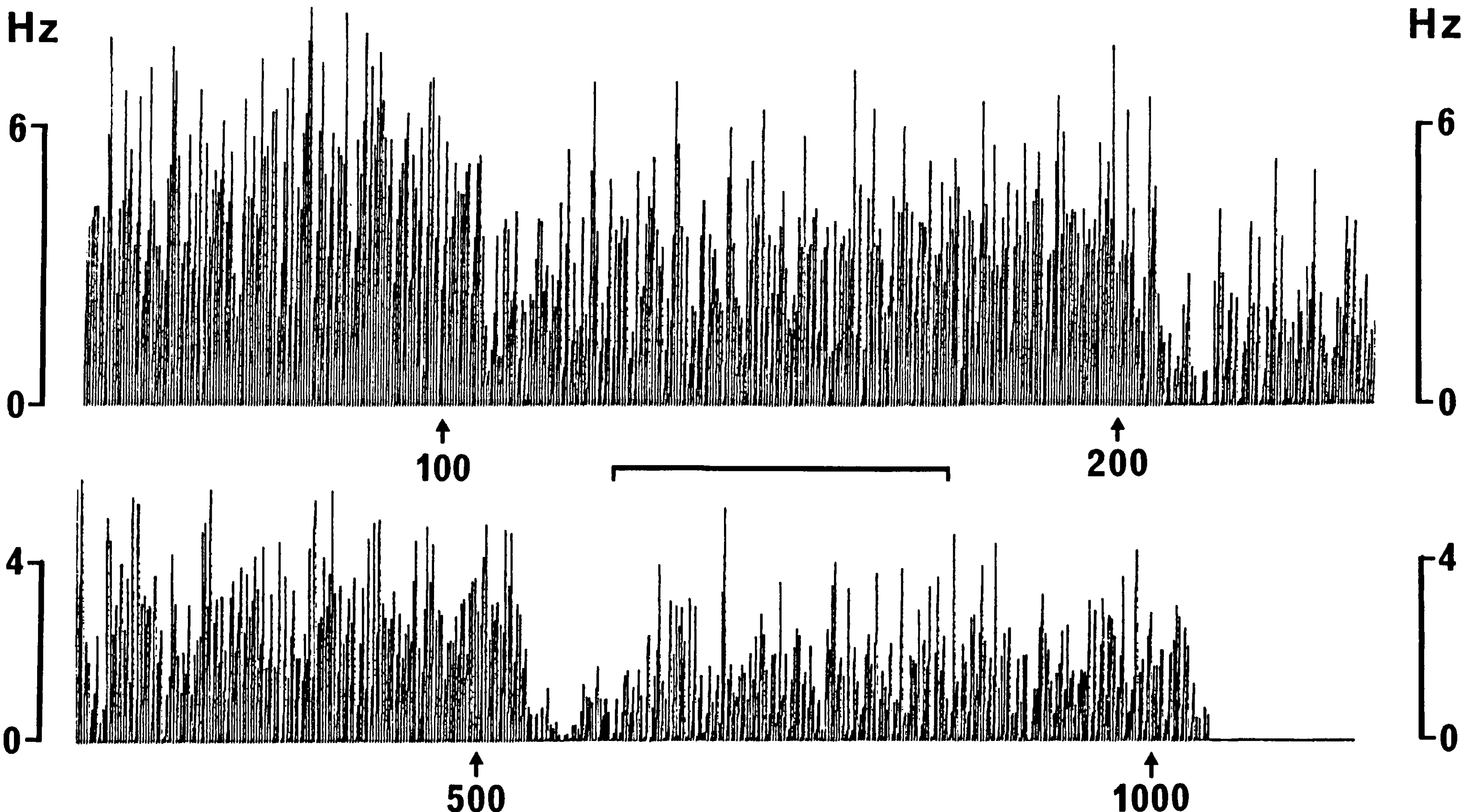


Figure 6.6 Representative rate recording showing the dose-dependent inhibition of striatal neuronal firing by apomorphine (μg/kg 1.0 ml/kg i.v.) after clozapine (20 mg/kg i.p.) administered 40 minutes before apomorphine (shown by arrows). See Fig.6.3 for typical control response to apomorphine. The time calibration scale represents 20 minutes.

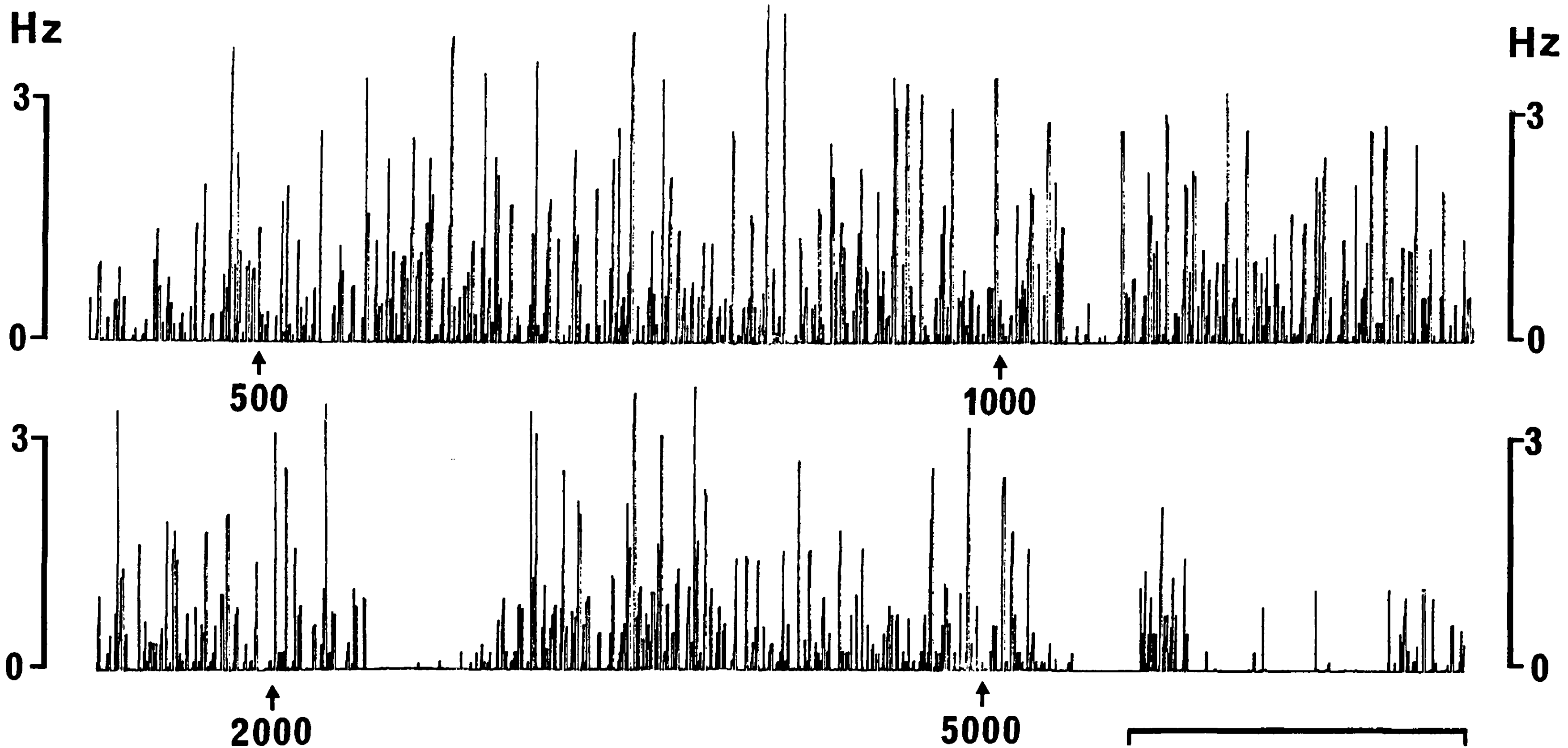


Figure 6.7 Representative rate recording showing the dose-dependent inhibition of striatal neuronal firing by apomorphine (μg/kg 1.0 ml/kg i.v.) after haloperidol (0.5 mg/kg i.p.) administered 40 minutes before apomorphine (shown by arrows). See Fig.6.3 for typical control response to apomorphine. The time calibration scale represents 20 minutes.

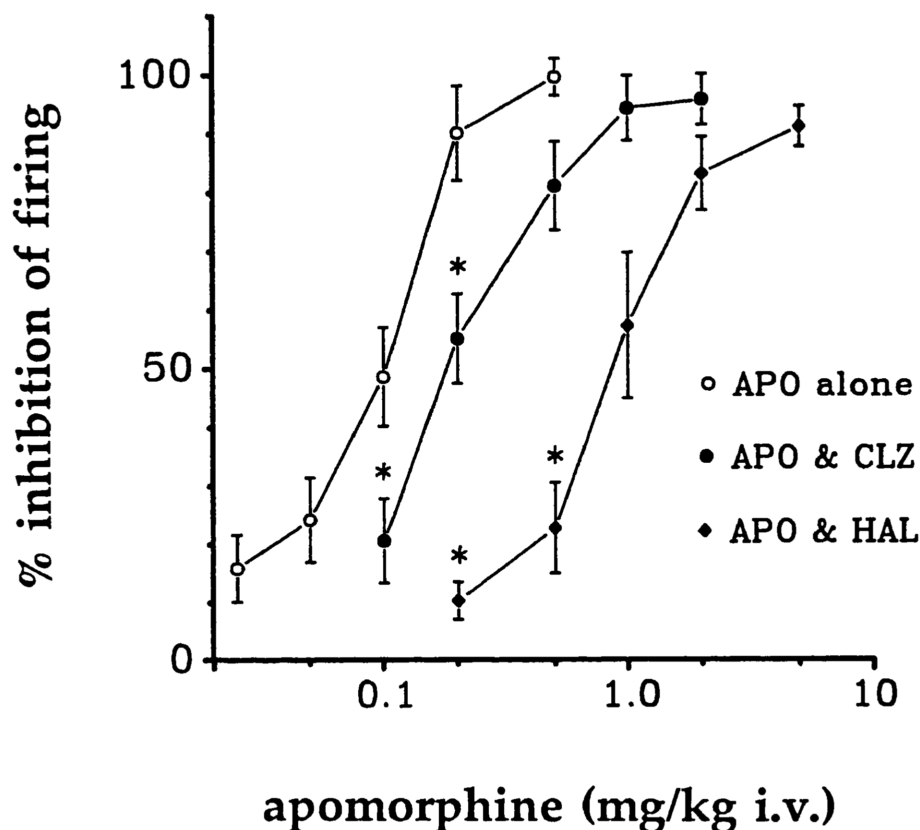


Figure 6.8 Dose-dependent inhibition of striatal neuronal firing by apomorphine (○) and its antagonism by pretreatment with either clozapine (20 mg/kg 1.0 ml/kg i.p.) (●) or haloperidol (0.5 mg/kg 1.0 ml/kg i.p.) (◆). Each neuroleptic was administered 40 minutes before apomorphine. Each data point represents the mean maximum percentage inhibition (± s.e.mean, n=5) from basal neuronal firing. Statistical significance between control and treatment responses was assessed using a Student's non-paired t-test (* $p<0.05$).

of cells tested) on the spontaneous activity of neurons in the medial prefrontal cortex (Fig.6.10). Although considerably fewer cells responded to apomorphine in this brain area, those that did (type I & III), were markedly more sensitive to the inhibitory effects of this agent (Fig.6.9 and Fig.6.10). Indeed the doses (± s.e.mean n=5) of apomorphine required to produce a 50% inhibition of the activity of neurons in the medial prefrontal cortex and the caudate putamen were estimated to be 0.023 ± 0.011 mg/kg and 0.102 ± 0.007 mg/kg respectively (Fig.6.10) ($p<0.05$). Unfortunately, however, those cells in which apomorphine produced complete inhibition were difficult to locate. Thus, in most cases, where apomorphine produced dose-related inhibition, only approximately 40 to 80% inhibition could be achieved. These neurons were, nonetheless, still more sensitive (3-4 fold) to the inhibitory effects of apomorphine compared with neurons in the rostral caudate putamen. Generally, cells with a large triphasic-potential in the prefrontal cortex (type III), which were located throughout the full dorso-ventral track, responded to the stimulatory effects of apomorphine, but this was not an exclusive response since, in some cases, they could also be dose-dependently inhibited by apomorphine.

6.2.4 Effects of neuroleptics and apomorphine on the spontaneous activity of neurons in the medial prefrontal cortex

Initially, as for the caudate putamen, it was hoped to test the relative abilities of clozapine and haloperidol to antagonise the effects of apomorphine in the medial prefrontal cortex. However, clozapine, but not haloperidol, appeared to mimic the effects of apomorphine in this brain area (see below). Thus it was only possible to evaluate the effects of haloperidol. Since the spontaneous discharge of neurons in the medial prefrontal cortex is considerably better maintained than in the caudate putamen (see section 3.5) it was possible to record the effects of apomorphine, both alone and after the administration of haloperidol, on the same neuron, and so obtain a direct measure of drug antagonism. In these experiments (see Fig.6.11), haloperidol produced potent antagonism of apomorphine-induced inhibition of cell firing with a dose ratio (± s.e.mean n=3) of 6.1 ± 1.2 (Fig.6.12). This data and that of section 6.2.2 suggests that haloperidol may act with comparable efficacy as an antagonist of postsynaptic dopamine receptors in both the caudate putamen and the medial prefrontal cortex.

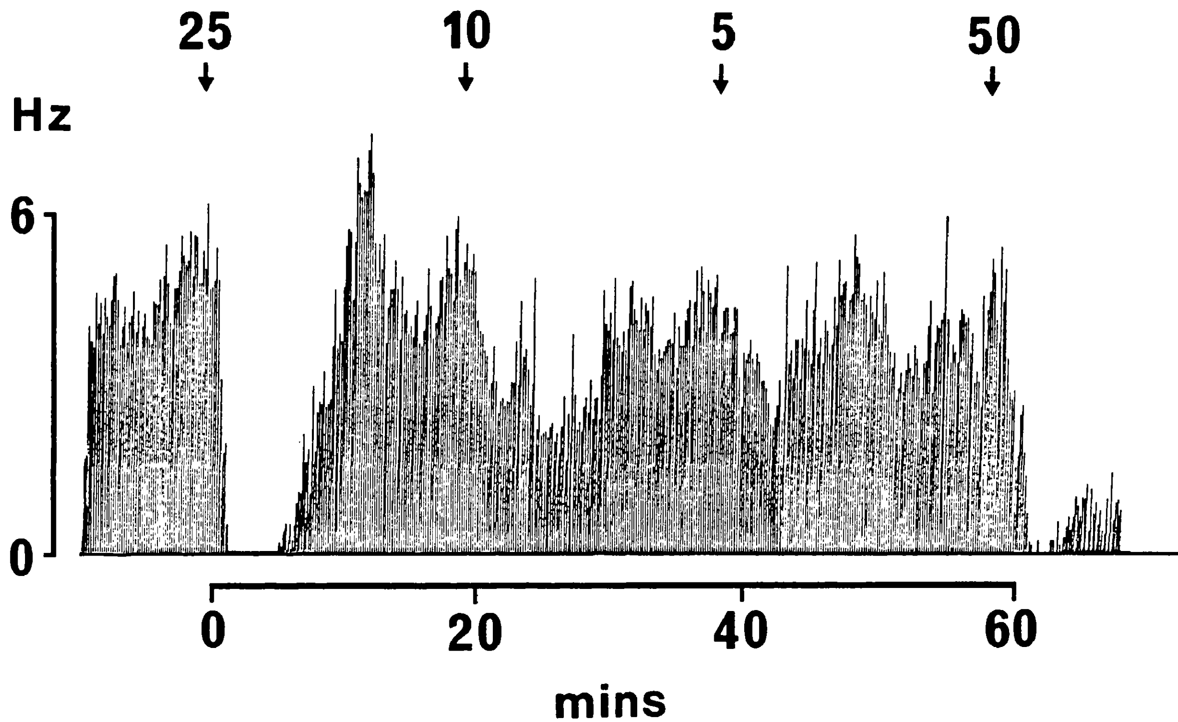


Figure 6.9 Representative rate recording showing the dose-dependent inhibitory effects of apomorphine hydrochloride (μg/kg 1.0 ml/kg i.v.) on the spontaneous activity of a neuron in the medial prefrontal cortex.

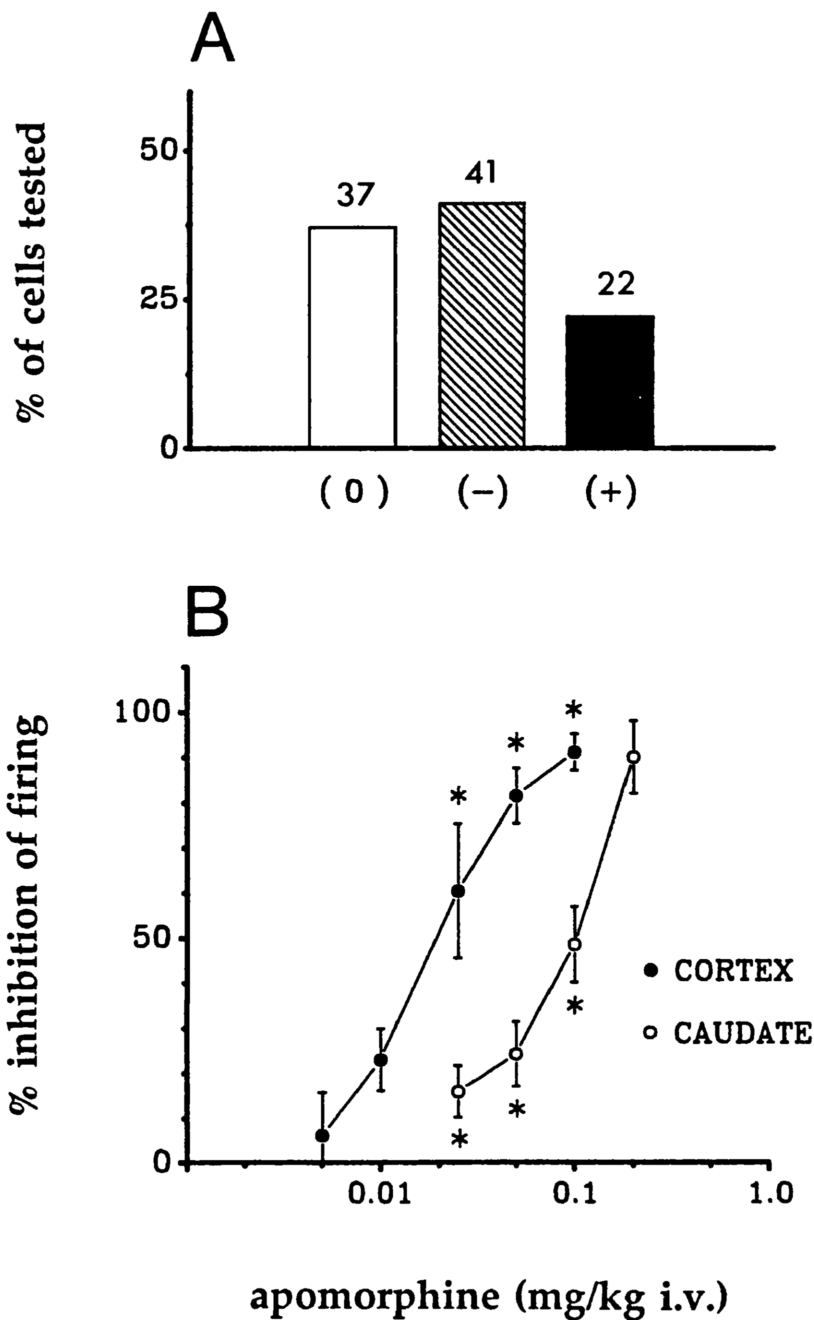


Figure 6.10 Effects of apomorphine on the activity of neurons in the medial prefrontal cortex. The percentage of neurons unresponsive (o) and responsive (- inhibition + stimulation) to apomorphine (5-100 µg/kg 1.0 ml/kg i.v.) is shown in graph (A) (*n*=*30*). The dose-dependent inhibitory effects of apomorphine on neuronal activity in the medial prefrontal cortex is shown in graph (B) together with its effect in the rostral caudate putamen for comparison. Error bars represent s.e.means (*n*=*5*) and statistical significance was assessed using a Student's non-paired t-test (* $p<0.05$).

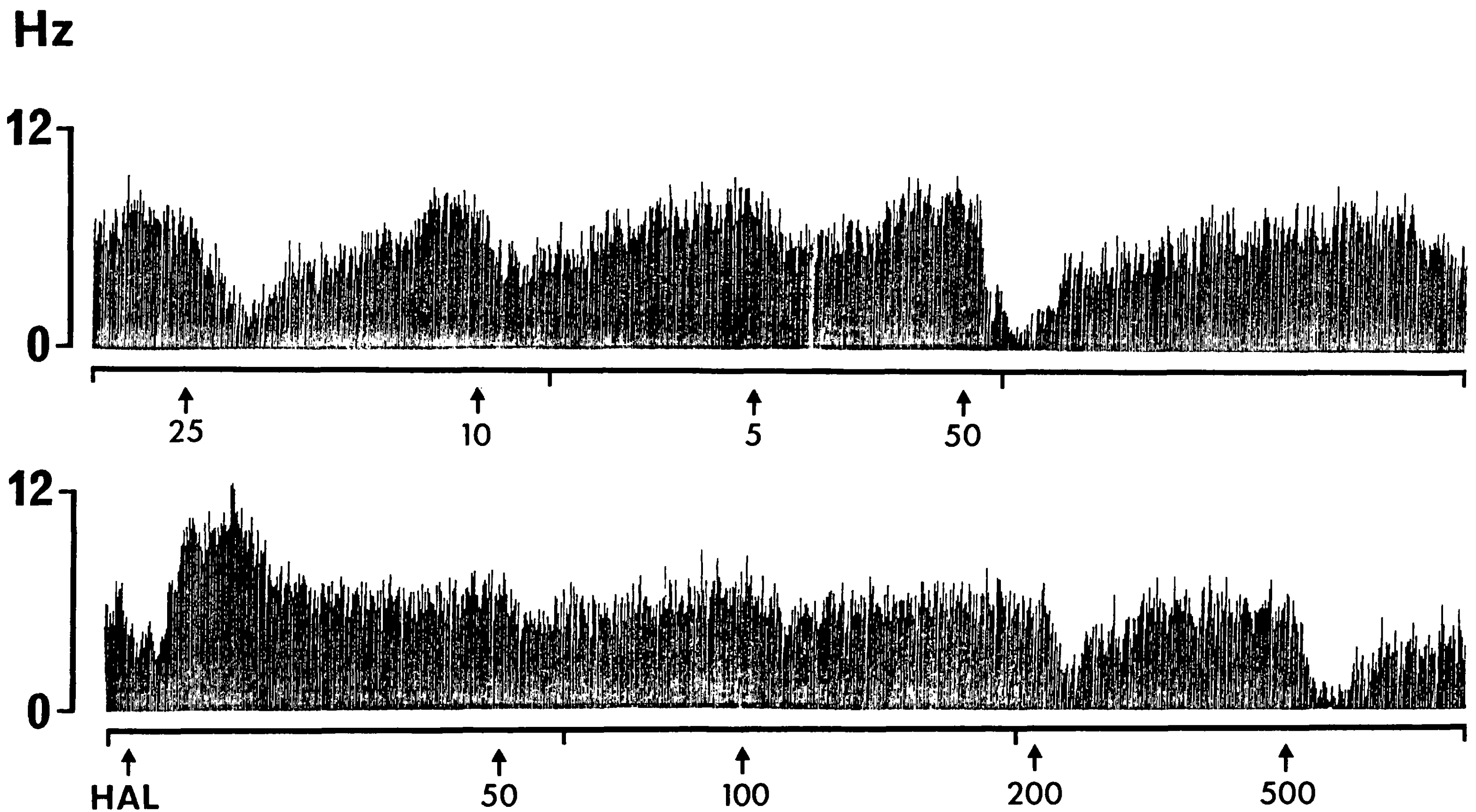


Figure 6.11 Representative rate recording showing the dose-dependent inhibitory effects of apomorphine hydrochloride (μg/kg 1.0 ml/kg i.v.) on the spontaneous activity of a neuron in the medial prefrontal cortex and its antagonism by haloperidol (HAL) (0.5 mg/kg 1.0 ml/kg i.p.). The calibration time scale beneath the tracing is divided into 50 minute time increments.

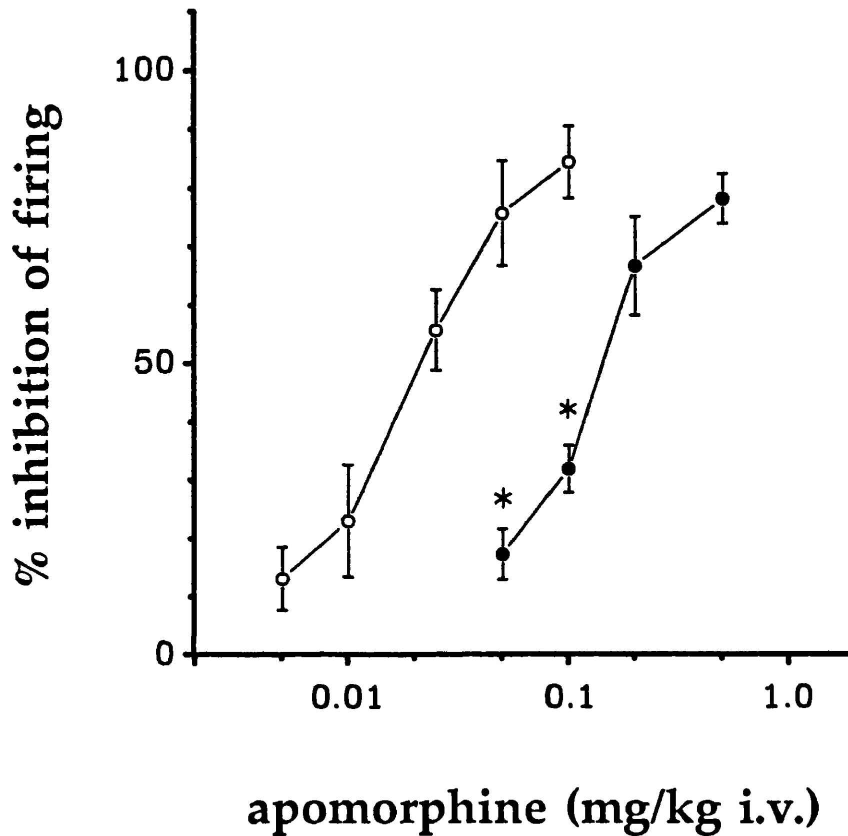


Figure 6.12 Dose-dependent inhibitory effects of apomorphine hydrochloride (o) on the spontaneous activity of neurons in the medial prefrontal cortex and its antagonism by haloperidol (HAL). In these three experiments the effects of apomorphine administered either before (control) or after haloperidol (0.5 mg/kg 1.0 ml/kg i.p.) were examined on the same neuron. Haloperidol was administered 40-50 minutes after the control apomorphine response (see Fig.6.11). Error bars represent s.e.means (n=3) and statistical significance (versus apomorphine control) was assessed using a Student's t-test (* $p<0.01$).

The results from representative experiments which summarise the effects of clozapine on the spontaneous discharge of neurons in the medial prefrontal cortex are shown in Fig.6.13. The most striking feature of these results is the ability of clozapine to mimic the actions of apomorphine in this brain area. Thus in the prefrontal cortex, clozapine both stimulated (peak increase occurring after about 20-30 minutes with levels returning to basal values over 1-2 hours), and inhibited neuronal firing, in approximately the same proportion of cells as apomorphine (Fig.6.16). Indeed, clozapine produced the same degree of inhibition as apomorphine, even in those cells where only partial inhibition of neuronal firing could be achieved. As well, the peak increase in the firing of neurons in the prefrontal cortex, noted after clozapine administration, which ranged between 100 and 200% (*n*=5), equates well with the peak stimulatory effect observed in some experiments after the administration of apomorphine in this area (*n*=4) (see Fig.6.14). Moreover, in those neurons which did not respond to the effects of apomorphine (about 40%), clozapine was also ineffective (*n*=3) (Fig.6.13). In some experiments, notably those in which clozapine produced inhibitory effects (but not exclusively so), clozapine enhanced the amplitude of the recorded action potential (see Fig.6.15).

The results from representative experiments which summarise the effects of haloperidol, either dosed alone or in combination with either apomorphine or clozapine, on the spontaneous activity of neurons in the medial prefrontal cortex are given in Fig.6.14. Unlike clozapine, but similar to its actions in the caudate putamen, haloperidol produced no consistent effects on the spontaneous discharge of neurons in this area. It did, however, block both the inhibitory (see also Fig.6.12) and the excitatory effects of apomorphine in this brain area. Moreover, this compound tended to have a "normalising" action by reinstating the basal level of spontaneous neuronal activity following the administration of either apomorphine (Fig.6.14) or clozapine (Figs.6.14 & 6.15). This effect was apparent irrespective of whether apomorphine or clozapine produced inhibitory or excitatory effects on neuronal discharge. As well, haloperidol also reversed the effects of clozapine on the amplitude of the recorded action potential (see above). A summary of the effects of apomorphine, clozapine and haloperidol, each administered alone, on the spontaneous discharge of neurons in the rostral caudate putamen and the medial prefrontal cortex are given in Fig.6.16. These graphs highlight the preferential ability of clozapine, but not haloperidol, to mimic the effects of apomorphine in the medial prefrontal cortex.

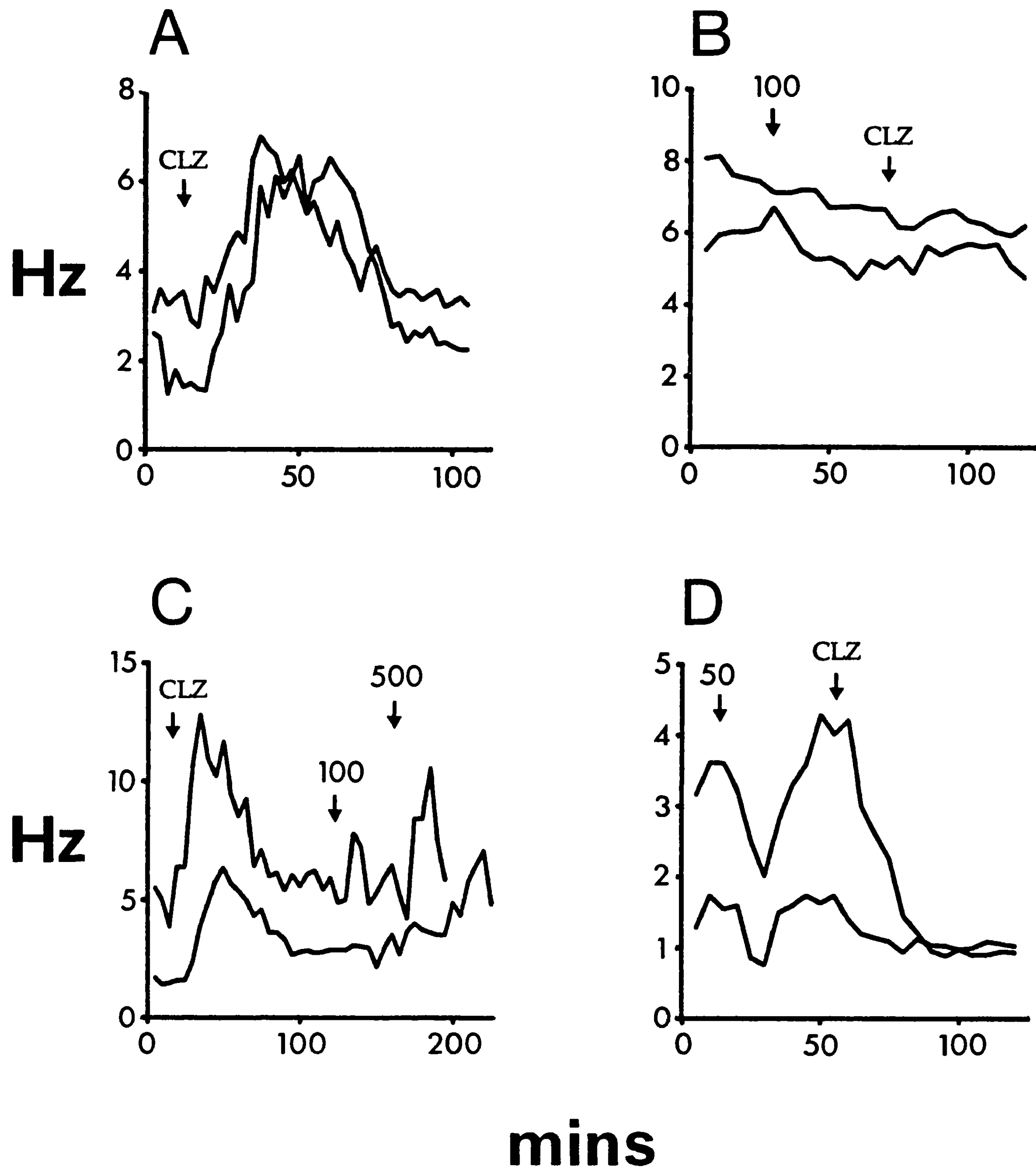


Figure 6.13 Graphs summarising the effects of clozapine (CLZ) (20 mg/kg 1.0 ml/kg i.p.), administered either alone (A), before (C) or after (B,D) the administration of apomorphine hydrochloride (µg/kg 1.0 ml/kg i.v.), on the spontaneous activity of neurons in the medial prefrontal cortex. The continuous lines in each graph represent the time-related effects of these agents on the firing of one neuron. Firing frequencies were calculated over 5 minute time intervals.

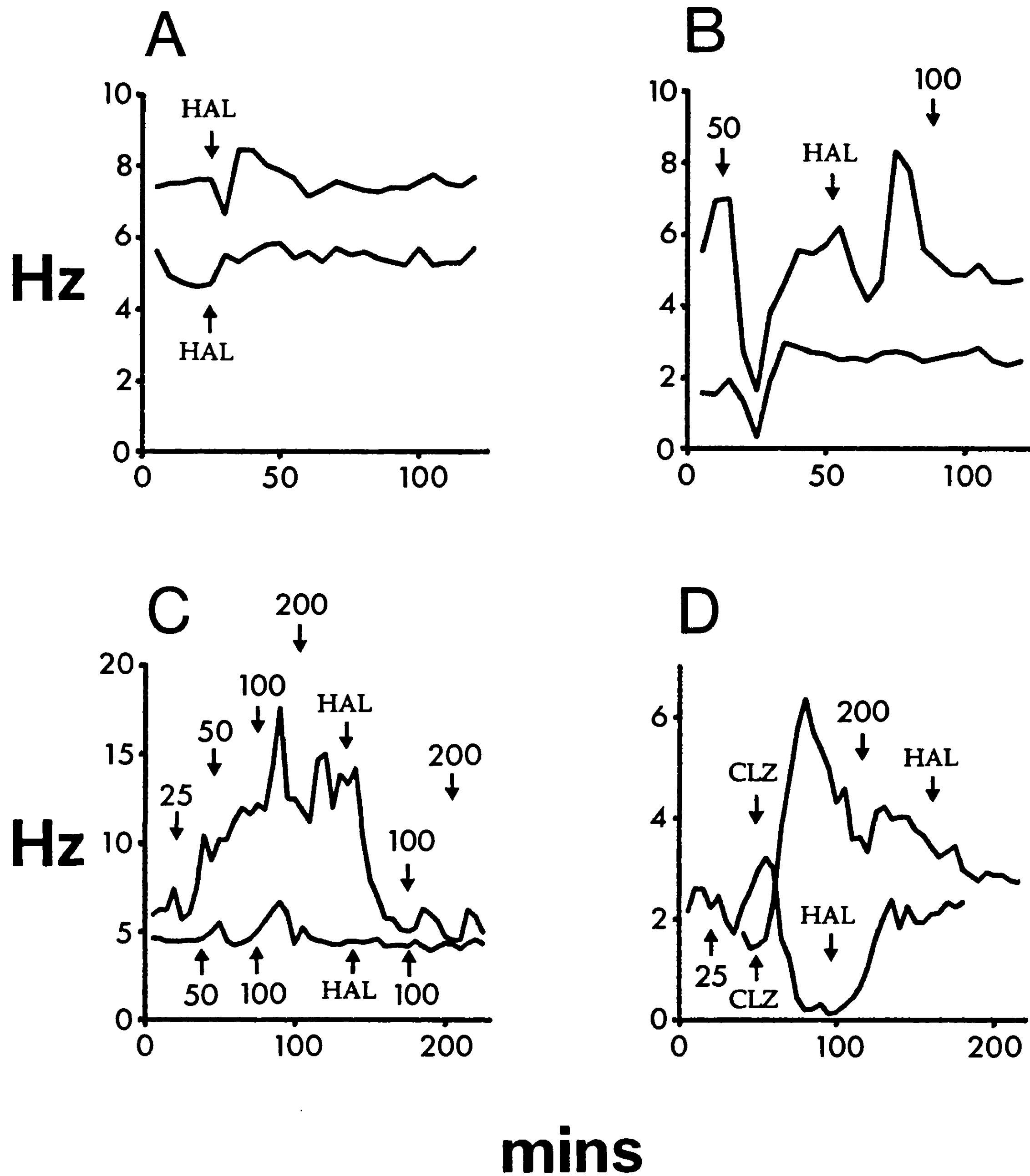


Figure 6.14 Graphs summarising the effects of haloperidol (HAL) (0.5 mg/kg 1.0 ml/kg i.p.), administered either alone (A) or after the administration of either apomorphine hydrochloride (µg/kg 1.0 ml/kg i.v.) (B,C) or clozapine (CLZ) (20 mg/kg 1.0 ml/kg i.p.) (D), on the spontaneous activity of neurons in the medial prefrontal cortex. The continuous lines in each graph represent the time-related effects of these agents on the firing of one neuron. Firing frequencies were calculated over 5 minute time intervals.

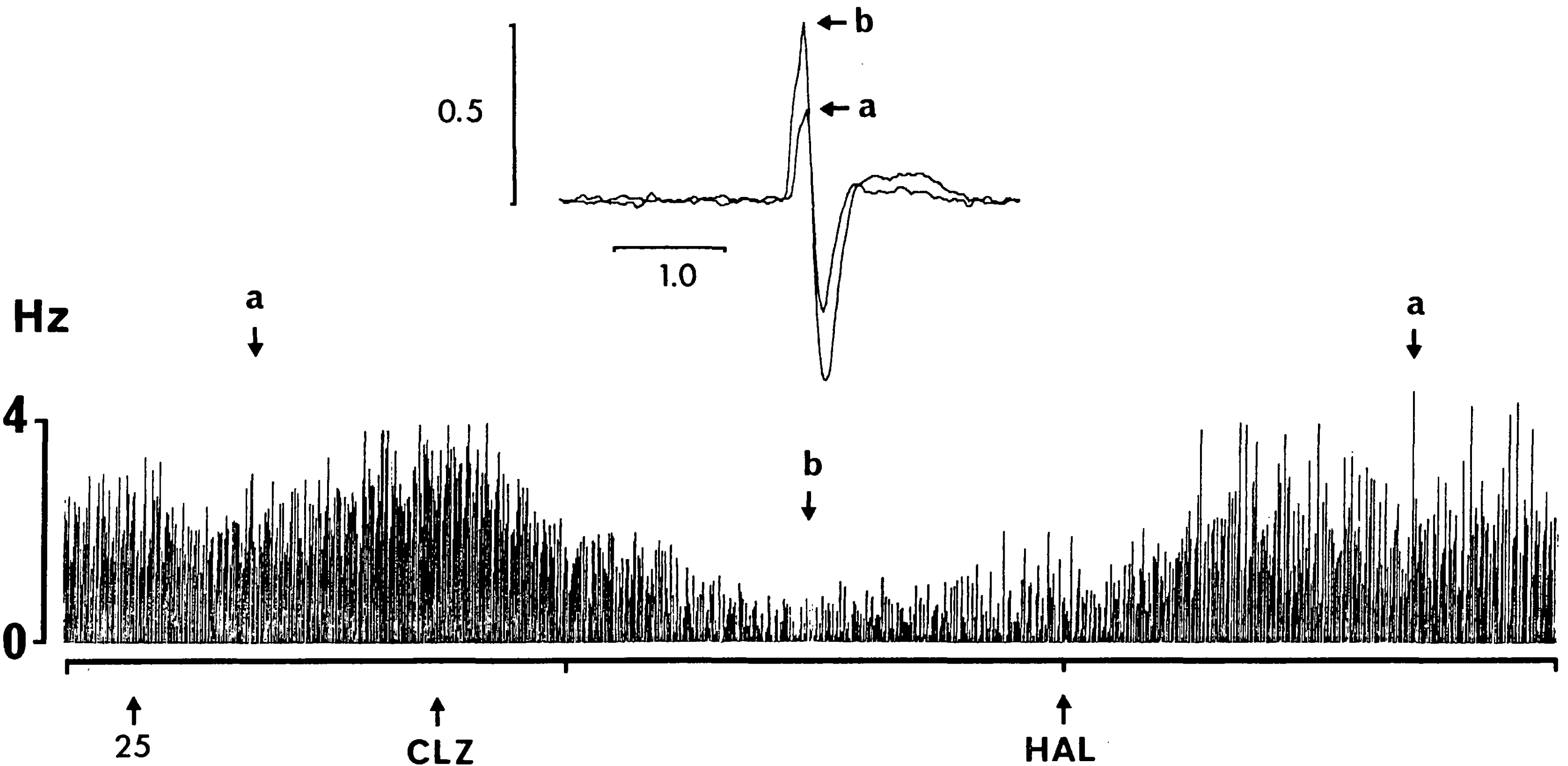
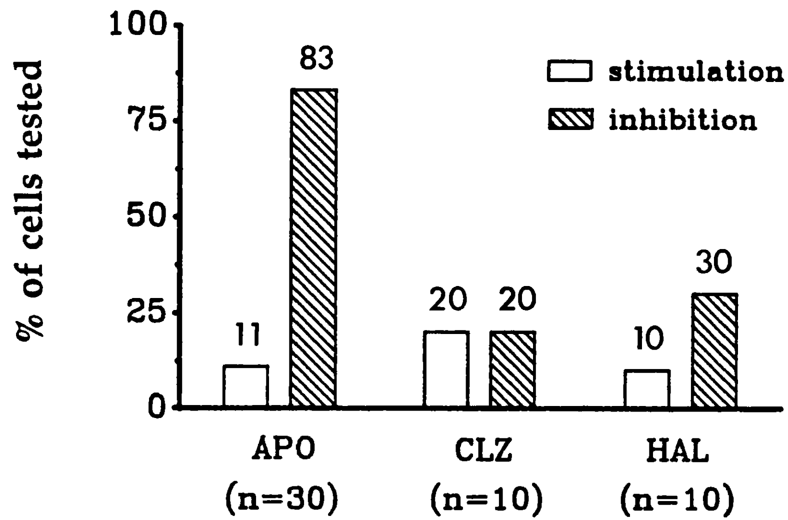


Figure 6.15 Cumulative rate recording showing the effects of apomorphine hydrochloride (25 µg/kg i.v.), clozapine (CLZ) (20 mg/kg i.p.) and haloperidol (HAL) (0.5 mg/kg i.p.) on the spontaneous activity of a neuron in the medial prefrontal cortex. The calibration time scale beneath the tracing is divided into 50 minute time increments. The extracellular potential recorded from this neuron either before (a), during (b) or after (a) clozapine treatment is also shown. The vertical and horizontal calibration bars are in units of mV and ms respectively.

CAUDATE PUTAMEN



MEDIAL PREFRONTAL CORTEX

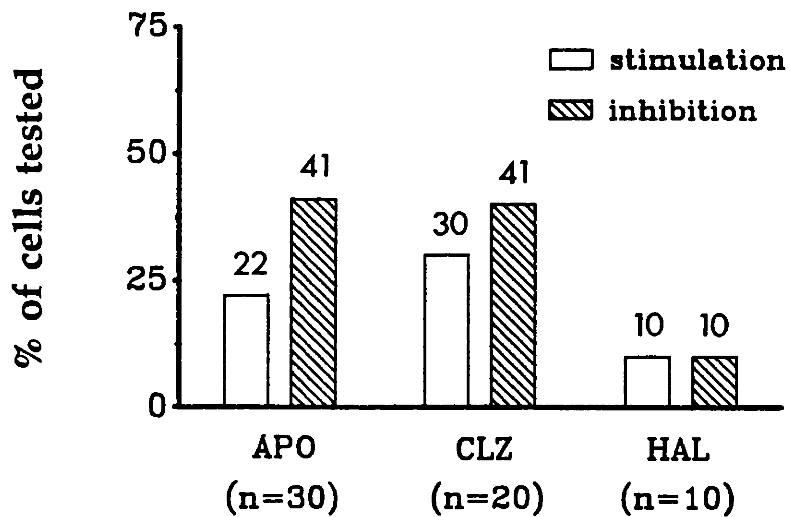


Figure 6.16 Graphs summarising the effects of apomorphine, clozapine and haloperidol on the spontaneous activity of neurons in the rostral caudate putamen and the medial prefrontal cortex. The percentage of neurons either stimulated (open boxes) or inhibited (striped boxes) by these agents are listed above each column.

6.3 DISCUSSION

6.3.1 Effects of apomorphine on the spontaneous discharge of neurons in the caudate putamen

Apomorphine was chosen as the postsynaptic dopamine receptor agonist in these studies since it is not taken up into dopamine nerve terminals (see Johnson, Hoffer & Freedman, 1986), it can cross the blood brain barrier (see chapter 7) and it can stimulate both dopamine D_1 and dopamine D_2 receptors (Creese, Sibley, Hamblin & Leff, 1983). Although this latter action may confuse the interpretation of results, it is evident, at least in the caudate putamen and the nucleus accumbens, that stimulation of both dopamine receptor subtypes may be necessary to express the full effects of dopamine receptor agonists on neuronal discharge in these areas. (Hu & Wang, 1988; Wachtel, Hu, Galloway & White, 1989).

The spontaneous firing of most neurons within the rostral caudate putamen, particularly those in the dorsal head of this structure, were inhibited by apomorphine in a dose-related manner. This is consistent with the generally accepted view that dopamine acts as a inhibitory neurotransmitter in the nigrostriatal system (see Wang, White, Mereu and Hu, 1987). Facilitation of neuronal discharge in the caudate putamen was also observed following the stimulation of dopamine receptors in this area, although this effect was not frequently encountered (but see below). In comparison with its potent inhibitory effects on dopamine neurons in the substantia nigra (Carlson, Bergstrom & Walters, 1986) and ventral tegmental area (VTA) (Trulson, Trulson & Arasteh, 1987) (with the ED_{50} value between 10 and 25 µg/kg i.v.), parenterally administered apomorphine, appears to be less effective in depressing the firing of neurons in the caudate putamen (ED_{50} 102 µg/kg i.v.). This discrepancy may arise since apomorphine is considerably less potent as an agonist at D_1 than at D_2 receptors (Creese, Sibley, Hamblin & Leff, 1983) and because inhibition of neuronal firing is believed to be mediated by dopamine D_1 receptors in the caudate putamen (Hu & Wang, 1988; Ohno, Sasa & Takaori, 1987; Calabresi, Mercuri, Stanzione, Stefani & Bernardi, 1987) and by dopamine D_2 receptors in areas A9 and A10 (see Wang, White, Mereu & Hu, 1987). Alternatively, the reduced ability of apomorphine to inhibit the firing of neurons in the caudate putamen compared with that in the substantia nigra and VTA may be because it inhibits the firing of neurons in this area by stimulating

postsynaptic dopamine D_2 receptors. These receptors, like those reported in the meso-accumbal system (see White & Wang, 1986), may be less sensitive to the inhibitory effects of dopamine agonists than presynaptic dopamine D_2 receptors (in the substantia nigra and VTA). This possibility is difficult to reconcile, however, with the findings that selective dopamine D_2 receptor agonists produce mainly stimulatory effects on striatal neurons (Ohno, Sasa & Takaori, 1986; Ohno, Sasa & Takaori, 1987) and only very weak inhibitory effects (Hu & Wang, 1988; see also Fig.3.18).

It would perhaps be unwise to neglect the stimulatory effect of dopamine and apomorphine on striatal neurons since this action may be relevant physiologically. Thus, there is some evidence that the facilitation of neuronal firing in the caudate putamen is mediated by the activation of synaptic dopamine D_2 receptors (see section 6.1). This view is supported by the findings that the stimulation-evoked release of dopamine invariably enhances the firing of neurons in the caudate putamen (Connor, 1970; Feltz, 1971; Norcross & Spehlmann, 1977; Norcross & Spehlmann, 1978; Williams & Millar, 1990), as do selective dopamine D_2 agonists (see above), but exogenously-applied dopamine, almost without exception, produces inhibitory effects (see section 6.1). Indeed, a low bath concentration of dopamine ($<1\mu M$), has been reported to depolarise and enhance the firing rate of neurons in slices of the caudate putamen (Akaike, Ohno, Sasa & Takaori, 1987) and this effect appears to be mediated by dopamine D_2 receptors (Uchimura, Higashi & Nishi, 1986; Akaike, Ohno, Sasa & Takaori, 1987). In contrast, dopamine D_1 receptors appear to mediate the hyperpolarising actions of higher concentrations of dopamine (100-500μM) resulting in a decrease in the firing rate of caudate putamen neurons (Akaike, Ohno, Sasa & Takaori, 1987). It is possible therefore that only dopamine D_1 receptors are activated by the high concentrations of dopamine produced by microiontophoresis (Bloom, 1974). Thus, any stimulatory effects of dopamine on these neurons may be overwhelmed by the inhibitory effects of dopamine acting on the more prevalent, and perhaps more accessible, dopamine D_1 receptor (Boyson, McGonigie & Molinoff, 1986).

The caudate putamen is a difficult area in which to record the spontaneous activity of neurons since many are either silent or fire very slowly (see Skirboll & Bunney, 1979). This problem is more than likely aggravated using a general anaesthetic agent. Nonetheless, the present result indicating that apomorphine is predominantly inhibitory

on neuronal activity in the caudate putamen is consistent with numerous other studies (see section 6.1) where the effects of iontophoretically-applied dopamine on either spontaneously active or glutamate-driven neurons were tested. The majority of neurons (about 75-90%) investigated in the present study could be classified as type I (see chapter 3) although of those few type II neurons tested, apomorphine still produced inhibition with an efficacy similar to its effects on type I neurons. The ability of apomorphine to inhibit the firing of neurons was also unaffected by their basal frequency although it was expected that since faster firing neurons would probably be under less tonic inhibition from dopaminergic innervation, and so probably contain fewer dopamine receptors, they would be less sensitive to the effects of apomorphine (and conversely for the less active neurons). This finding suggests that either, other, possibly non-dopaminergic mechanisms (glutamatergic?), control the firing rate of neurons in this area or that inhibitory dopamine (D_1) receptors are uniform in number on postsynaptic neurons and perhaps lie outside the dopamine synapse.

6.3.2 Effects of neuroleptics and apomorphine on the spontaneous activity of neurons in the caudate putamen

Consistent with the results of other studies which have also assessed the effects of systemically administered neuroleptic drugs on the activity of neurons in the caudate putamen (Ben-Ari & Kelly, 1976; Skirboll & Bunney, 1979; Johnson, Hoffer & Freedman, 1986), neither clozapine nor haloperidol, altered the spontaneous discharge rate of neurons in the caudate putamen. This is perhaps surprising since, if the dopamine innervation to this structure exerts a tonic inhibitory influence, then neuroleptic drugs would be expected to enhance the firing of these neurons. In fact this expected stimulation only seems to occur if neuroleptic drugs are perfused locally in the caudate putamen (Groves, Wilson, Young & Rebec, 1975). This would suggest that by stimulating the firing of dopaminergic neurons by blocking autoreceptors (Bunney, Walters, Roth & Aghajanian, 1973) and so enhancing the release of dopamine in the caudate putamen (Imperato & Di Chiara, 1985) the resulting inhibition can negate the expected stimulatory effects of systemically administered neuroleptics on striatal neuronal discharge (through antagonism of dopamine inhibition). Thus the increased synaptic availability of dopamine may be sufficient to competitively overcome the postsynaptic blockade of dopamine receptors by neuroleptics. This argument, however, is difficult to reconcile with the

results of chapter 5 where neither haloperidol nor clozapine significantly increased the efflux of dopamine in the caudate putamen. This discrepancy may be due though, to the inability of microdialysis to directly monitor the synaptic concentration of neurotransmitter substances (see section 2.1). Indeed, in the dialysis studies of chapter 5, both clozapine and haloperidol increased the efflux of DOPAC, at least some of which, could be derived from newly released dopamine.

Despite its higher dose clozapine produced only weak antagonism of apomorphine-induced inhibition of striatal neuronal firing whereas haloperidol was considerably more potent in this respect (about 180 fold). These results, like those of chapter 5, suggest that clozapine is only a weak dopamine receptor antagonist compared with haloperidol which may account for the low incidence of extrapyramidal side effects seen with this compound. There appears to be some tendency for haloperidol to be less effective as an antagonist of apomorphine-induced inhibition of striatal neuronal firing compared with its actions against the neurochemical effects of apomorphine (see chapter 5). This is perhaps not surprising since haloperidol has a higher affinity for dopamine D_2 rather than dopamine D_1 receptors (see Table 4.1), and that the inhibition of striatal neuronal firing appears to be mediated by dopamine D_1 receptors (see above) whereas both D_1 and D_2 mechanisms seem to regulate the release and metabolism of dopamine in this brain area (see section 1.2.3). It is surprising though, that clozapine is considerably less effective compared with haloperidol against the suppression of neuronal firing by apomorphine since this compound shares a similar affinity to haloperidol for dopamine D_1 receptors (Table 4.1). This may suggest that postsynaptic dopamine D_2 receptors are also involved in mediating the inhibitory effects of apomorphine on striatal neuronal firing. One should not rule out the possibility, however, that by stimulating dopamine D_1 receptors, apomorphine may facilitate its inhibitory actions at the dopamine D_2 receptor (see Wachtel, Hu, Galloway & White, 1989; Hu & Wang, 1988). Thus, the potency of haloperidol and clozapine in reversing the inhibitory effects of apomorphine on neuronal firing may depend on their respective antagonistic actions at both dopamine D_1 and dopamine D_2 receptors.

6.3.3 Effects of apomorphine on the spontaneous activity of neurons in the medial prefrontal cortex

It is hardly surprising that fewer neurons in the medial prefrontal cortex showed any response to apomorphine since this area receives a considerably reduced dopamine innervation compared with the caudate putamen (Tassin, Bockaert, Blanc, Stinus, Thierry, Lavielle, Premont & Glowinski, 1978). In contrast to its predominantly inhibitory effects in the rostral caudate putamen, apomorphine produced both stimulatory and inhibitory effects in the medial prefrontal cortex. Of the few studies which have assessed the effects of systemic apomorphine on the spontaneous firing of neurons in this brain area none have reported any stimulatory effects of this agent (Mora, Sweeney, Rolls & Sanguinetti, 1976; Peterson, St Mary & Harding, 1987). This is rather surprising since in the present study apomorphine produced a dose-related facilitation of neuronal firing in about 22% of neurons investigated. Moreover, this stimulatory effect of apomorphine, which most notably occurred with large triphasic-potential cells (amplitude >1mV), is consistent with the excitatory effects of dopamine reported on large pyramidal cells and other neurons recorded from slices of the rat prefrontal cortex (Penit-Soria, Audinat & Crepel, 1987). Dopamine has also been reported to exert an excitatory effect on cells in the parietal cortex (Bevan, Bradshaw, Pun, Slater & Szabadi, 1978) and this action appears to be mediated via the stimulation of dopamine D_2 receptors (Bradshaw, Sheridan & Szabadi, 1985). The mechanism underlying the stimulatory action of apomorphine on some neurons in the medial prefrontal cortex is not clear but may, in part, be indirectly mediated via changes in the release of various modulating neurotransmitters secondary to the stimulation of presynaptic dopamine receptors.

Apomorphine produced a progressive dose-dependent inhibition of the firing of neurons in the medial prefrontal cortex in about 40% of cells tested. The proportion of neurons inhibited by dopamine receptor stimulation in this area is consistent with other studies (Mora, Sweeney, Rolls & Sanguinetti, 1976; Bernardi, Cherubini, Marciani, Mercuri & Stazione, 1982). In about 20 to 30% of neurons (of those inhibited by apomorphine) apomorphine produced potent and complete inhibition of action potential discharge. Other workers have also reported that some neurons in the medial prefrontal cortex are particularly sensitive to the inhibitory effects of apomorphine (Mora, Sweeney, Rolls & Sanguinetti, 1976; Bunney & Aghajanian, 1976; Peterson, St Mary & Harding, 1987). It is

tempting to speculate that the inhibitory effects of apomorphine on these cells may be mediated by a dopamine receptor subtype which is similar to, but distinct from, either a dopamine D_1 or a dopamine D_2 receptor. This receptor may resemble a dopamine D_2 receptor since selective D_2 receptor agonists, but not selective D_1 receptor agonists (but see Sesack & Bunney, 1989), can mimic the effects of iontophoretically-applied dopamine in this area (Parfitt, Gratton & Bickford-Wimer, 1990; Godbout, Mantz, Pirot, Glowinski & Thierry, 1991). There is evidence, at least in the meso-accumbal system, that dopamine D_2 autoreceptors are 5 to 100 fold more sensitive to dopamine and dopamine agonists than postsynaptic dopamine D_2 receptors (White & Wang, 1986; Wang, White, Mereu & Hu, 1987). Thus it is possible, that apomorphine activates a postsynaptic dopamine D_2 receptor in the medial prefrontal cortex, similar in characteristics to a mesolimbic dopamine D_2 autoreceptor (dopamine D_3 receptor?), to produce its inhibitory effects on the firing of some neurons in this area.

Whilst the activity of the majority of neurons tested in the rostral caudate putamen was completely abolished by apomorphine this effect was not always encountered in the medial prefrontal cortex where only partial inhibition could sometimes be achieved. This may suggest that there are fundamental differences in the level of control that the dopamine innervation exerts on the firing rate of neurons in these areas. Alternatively, as suggested for other dopamine agonists in the prefrontal cortex (Sesack & Bunney, 1989), apomorphine may be acting as a partial agonist on some neurons in this brain area.

6.3.4. Effects of haloperidol and apomorphine on the spontaneous activity of neurons in the medial prefrontal cortex

Haloperidol produced no consistent effects on the firing of neurons in the medial prefrontal cortex. It did, however, potently block, and in some cases reverse, both the inhibitory and the excitatory effects of apomorphine. This ability of haloperidol to sustain the basal (drug-naive) discharge rate of neurons in the prefrontal cortex, mediated presumably through its antagonism of dopamine receptors, may relate to its efficacy as an anti-schizophrenic agent (assuming that altered dopamine function in this area contributes to the symptoms of this disorder).

Haloperidol potently antagonised the inhibitory effects of apomorphine on the firing of spontaneously active neurons in the medial prefrontal cortex. There was no evidence from these results to support the claim that haloperidol acts with reduced efficacy as a dopamine receptor antagonist in this brain area (see Thierry, Douarin, Penit, Ferron, Glowinski, 1986; Godbout, Mantz, Pirot, Glowinski & Thierry, 1991). In the studies of Thierry and co-workers the spontaneous discharge of neurons in the medial prefrontal cortex was suppressed by either stimulating the ventral tegmental area or by applying dopamine directly to these neurons using microiontophoresis. The ability of several neuroleptics, notably haloperidol and sulpiride, to antagonise this inhibition of neuronal firing was tested after either their systemic or their local (iontophoresis) administration. Since sulpiride, but not haloperidol, consistently blocked the inhibitory effects of dopamine and VTA stimulation on prefrontal cortical cells, the authors concluded that the inhibition of these neurons is mediated via a subtype of dopamine receptor which is particularly sensitive to the benzamides. The differential effects of sulpiride and haloperidol are likely to be related to differences in the sensitivity of postsynaptic dopamine receptors, but not of dopamine autoreceptors, because both sulpiride and haloperidol have been shown to block the inhibitory effects of dopamine agonists on the release of dopamine from slices of the rat prefrontal cortex (Hoffman, Talmaciu, Ferro & Cubeddu, 1988).

The discrepancy between my results and those of Thierry and co-workers may be due to methodological differences. It would be unsafe to over-emphasis the significance of the microiontophoretic results of Thierry and co-workers since this technique is predisposed to interpretative difficulties (see section 2.1 and Johnson, Hoffer & Freedman, 1986). Moreover, although it is widely reported that neuroleptic drugs can antagonise the inhibitory effects of dopamine on striatal and accumbal neurons when these agents are administered by microiontophoresis (see Siggens, Hoffer & Ungerstedt, 1974; Ben-Ari & Kelley, 1976; Stone, 1976; Akaike, Sasa & Takaori, 1983), very few of these studies, have tested the effects of haloperidol. Thus it is difficult to assess whether there is not some problem associated with the application of haloperidol by this method.

In support of my own results, both spiroperidol (Mora, Sweeney, Rolls & Sanguinetti, 1976) and cis-flupenthixol (Peterson, St Mary & Harding, 1987), have been reported to antagonise the inhibitory effects of parenteral apomorphine on neurons in the prefrontal

cortex following their systemic administration. It could be argued though, that by only testing cells which are sensitive to apomorphine one may introduce a bias in the results. Thus it is possible that apomorphine activates a dopamine receptor in the medial prefrontal cortex which is sensitive to antagonism by most, if not all, neuroleptic drugs. This may not be true for dopamine (released by VTA stimulation or applied by microiontophoresis) which may stimulate other, perhaps apomorphine-insensitive, dopamine receptors in the medial prefrontal cortex, to initiate its inhibitory effects. It would be interesting to test whether or not haloperidol can block the inhibitory effects of VTA stimulation on the same neuron which also shows inhibitory effects to apomorphine.

6.3.5. Effects of clozapine on the spontaneous activity of neurons in the medial prefrontal cortex

In contrast to the actions of haloperidol, clozapine produced inhibitory and stimulatory effects on neurons in the prefrontal cortex in approximately the same proportion of cells as apomorphine. Moreover, clozapine was found to mimic the actions of apomorphine when these agents were tested on the same neuron in this brain area. These observations together with the findings that haloperidol can reverse the effects of clozapine, similar to its effects against apomorphine, provides further evidence, albeit indirect, that clozapine may act by selectively releasing dopamine in the medial prefrontal cortex (see chapter 5). Indeed, the time course of clozapine-induced stimulation of neuronal discharge (about 1-2 hours) is consistent with the time course of raised dopamine efflux induced by clozapine in this area (see Fig.5.2). Some caution needs to be exercised, however, in the interpretation of these electrophysiological results, since other neurotransmitter systems are also known to modify the firing rate of neurons in the medial prefrontal cortex. For example, noradrenaline, like dopamine, can produce inhibitory effects on neurons in this area (Bunney & Aghajanian, 1976; Godbout, Mantz, Pirot, Glowinski & Thierry, 1991) and there is evidence, at least in slices of the rat parieto-occipital cortex, that clozapine can also enhance the release of noradrenaline (Grob & Schumann, 1980). The fact though that haloperidol can block the effects of clozapine, despite having only weak antagonistic activity at α-adrenergic receptors (see Table 4.1), would argue against this possibility. Clearly, further experiments are necessary to confirm the dopamine-dependency of clozapine's action in the medial prefrontal cortex (e.g lesion studies using 6-OHDA).

The mechanism underlying the observation that clozapine can enhance the amplitude of the action potential recorded from some neurons in the prefrontal cortex is unknown. This is a particularly unusual effect since most neuroleptic drugs are reported to decrease the amplitude of the recorded action potential, which some suggest, may be due to local anaesthetic effects (see Ben-Ari & Kelly, 1976). Since haloperidol could reverse this effect it is possible that dopaminergic mechanisms are involved. It is interesting to note that in intracellular recordings made from rat hippocampal cells, dopamine (1μM) has been reported to modify the shape of the evoked action potential (Pockett, 1985). In this study, action potentials were generated either by a direct depolarising current, or synaptically, by stimulating the Schaeffer collateral-commissural pathway. The author suggests that dopamine may produce its effects on these cells by modifying a fast Ca^{2+}-activated K^+ current. It is possible that a similar mechanism operates on neurons in the medial prefrontal cortex and if so, this would be consistent with the ability of clozapine to enhance the extracellular concentration of dopamine in this area.

The observation that clozapine acts like apomorphine in the medial prefrontal cortex suggests that its postsynaptic dopamine receptor blocking action may be inadequate to reverse the effects of the enhanced release of dopamine in this area induced by this compound. If this were true, then in essence, clozapine would act in the medial prefrontal cortex like an indirect dopamine agonist rather than as a dopamine receptor antagonist like haloperidol. This mode of action may underlie the unique clinical profile of clozapine. If, as hypothesized by Weinberger (1987), the primary lesion in schizophrenia involves a reduced dopamine function in the prefrontal cortex (with a corresponding elevation in the function of dopamine in some subcortical dopamine-innervated areas), then clozapine may act by restoring dopamine function in the prefrontal cortex. Thus, unlike haloperidol, which may act directly by blocking raised mesolimbic(?) dopamine function to alleviate the symptoms (positive?) of schizophrenia, clozapine may act at the primary lesion site (as described above) to indirectly reduce raised mesolimbic dopamine function (assuming that this dopamine system is inversely regulated by the mesocortical dopamine system; see chapter 9). This action of clozapine (to restore mesocortical dopamine function) may account for the paradoxical ability of this agent to alleviate the positive symptoms of schizophrenia (thought to be due to raised mesolimbic dopamine function) despite only a weak ability to block dopamine receptors (see chapter 5). The ability of clozapine to maintain dopamine function in the prefrontal cortex may also underlie the reported

effectiveness of this agent to alleviate the negative features of schizophrenia (see section 5.3.4) and the absence of tardive dyskinesia (see chapter 9).

6.4 CONCLUSIONS

The results from this chapter indicate that dopamine acts predominantly as an inhibitory neurotransmitter in the caudate putamen (implied by the effects of apomorphine) and with mixed (inhibitory and excitatory) actions in the prefrontal cortex (although some caution is necessary in this interpretation of results since apomorphine may also have indirect effects).

Since neurons in the prefrontal cortex are more sensitive to the inhibitory effects of apomorphine than neurons in the caudate putamen, different, but possibly D_2-like receptors, mediate the inhibitory effects of dopamine in this area.

The relative inability of clozapine compared with haloperidol to antagonise the effects of apomorphine in the caudate putamen may reflect weak postsynaptic dopamine receptor antagonism and could be related to the low incidence of extrapyramidal effects seen with this compound.

The observation that clozapine, but not haloperidol, can mimic the effects of apomorphine on the activity of spontaneously active neurons in the medial prefrontal cortex, supports the finding of chapter 5 that clozapine may act by selectively releasing dopamine in the medial prefrontal cortex. This action possibly underlies the effectiveness of this compound in alleviating both the negative and the positive features of schizophrenia.

CHAPTER 7

STUDY OF POSSIBLE NEUROLEPTIC DRUG EFFECTS ON APOMORPHINE PASSAGE ACROSS THE RAT BLOOD BRAIN BARRIER

7.1. INTRODUCTION

The results presented in chapters 5 and 6 established that both haloperidol and clozapine, when dosed systemically, could antagonise the actions of apomorphine in the caudate putamen and medial prefrontal cortex, although clozapine was shown to be considerably weaker in this regard. Several studies have noted that some neuroleptic drugs, including haloperidol, may reduce the accumulation of apomorphine in various areas of the rat brain (Vetulani, Melzacka & Wiszniowska, 1978; Westerink & Horn, 1979). This would contribute to the reported ability of neuroleptic drugs to reduce the central actions of apomorphine and perhaps other DA agonists. According to the results of Westerink & Horn (1979), pretreatment with haloperidol (1.0 mg/kg s.c) produced a considerable reduction (about 50-60%) of apomorphine content estimated in the caudate putamen (in agreement with the results of Vetulani *et al.*, 1978), cortex and cerebellum. This effect was not thought to result from an interaction at central DA receptors since the observed reductions were of similar magnitude in both DA innervated (caudate putamen and cortex) and non-innervated (cerebellum) areas. Instead, the authors suggest that neuroleptic drugs may interfere with the passage of apomorphine across the blood brain barrier since an earlier study in the anaesthetised baboon (*Papio anubis*) noted that apomorphine could produce large increases in cerebral blood flow, possibly facilitating its entry into the brain, and that this effect could be reversed by the neuroleptic drug pimozide (McCulloch & Harper, 1977).

The aim of the work described in this chapter was to re-evaluate the effects of neuroleptic drugs on the entry of apomorphine into the brain. To achieve this the technique of intracerebral microdialysis was employed to monitor the time course of apomorphine in the extracellular fluid of the rat caudate putamen following its systemic administration. This novel approach should reveal any change in the passage of apomorphine into the brain resultant from pretreatment with either clozapine or haloperidol and thus indicate what contribution, if any, a diminished apomorphine entry into the brain, induced by neuroleptic agents, would make on the reported ability of these drugs to antagonise the actions of apomorphine. It is also anticipated that this approach will yield an approximation of the extracellular concentration of apomorphine in the caudate putamen which could then be directly related to its actions on dopamine neurotransmission.

7.2 METHODS

Dialysis probes (vitafiber) were stereotaxically placed into the rostral caudate putamen of halothane-anaesthetised rats and perfused with artificial CSF at 5 µl/min. Experiments were commenced 3 hours later when apomorphine was given in increasing doses (0.2, 0.5, 1.0, 2.0 and 5.0 mg/kg) every 40 mins via a femoral vein, either alone, or in animals pretreated (40 mins previously) with either clozapine (20 mg/kg i.p) or haloperidol (0.5 mg/kg i.p). Dialysates were collected every 10 mins and the apomorphine content was estimated using HPLC with electrochemical detection (see section 2.8.3). Preliminary *in-vitro* studies (see section 2.5.3) showed that for any given flow rate the relative recovery of apomorphine was smaller than for the catecholamines i.e., 1.5 ± 0.2% (mean ± s.e. mean, n=5) at 5 µl/min compared with 2.0-2.5% for catecholamines. The absolute recovery of apomorphine (pmoles/20mins) was directly related to its concentration over the range 2.5 to 50µM with a least squares regression equation (in the form $y = m.x + c$) of $y = 2.03x - 3.06$ (where y is the absolute recovery, m is the slope, x is the concentration of apomorphine and c is the y intercept) ($r^2 = 0.998$).

7.3 RESULTS

Apomorphine was detected in striatal dialysates following its systemic administration (Fig.7.1) and the total efflux of apomorphine collected was dose related – – to the dose of apomorphine (Fig.7.2). The peak concentration of apomorphine always occurred in either the first or the second 10 minute sample and disappeared from striatal dialysates in a mono-exponential manner (Fig.7.3). The time course of apomorphine efflux in striatal dialysates was not modified by pretreating animals with clozapine (20 mg/kg i.p.) and haloperidol (0.5 mg/kg i.p.) only significantly reduced the efflux at one apomorphine dose (2.0 mg/kg) ($p<0.01$) (Figs. 7.1 & 7.2). The total efflux data (Fig.7.2) with respect to apomorphine, dosed either alone, or following pretreatment with clozapine, appeared to show slight signs of saturating above the dose of 2.0 mg/kg. Whilst, the total efflux data for combined haloperidol and apomorphine administration, was more directly related to the dose of apomorphine across the entire range of doses studied (Fig.7.2). The effects of clozapine and haloperidol on the disposition of apomorphine in dialysates of the rat caudate putamen are summarised in Table 7.1. Although clozapine produced no statistically significant changes in apomorphine efflux

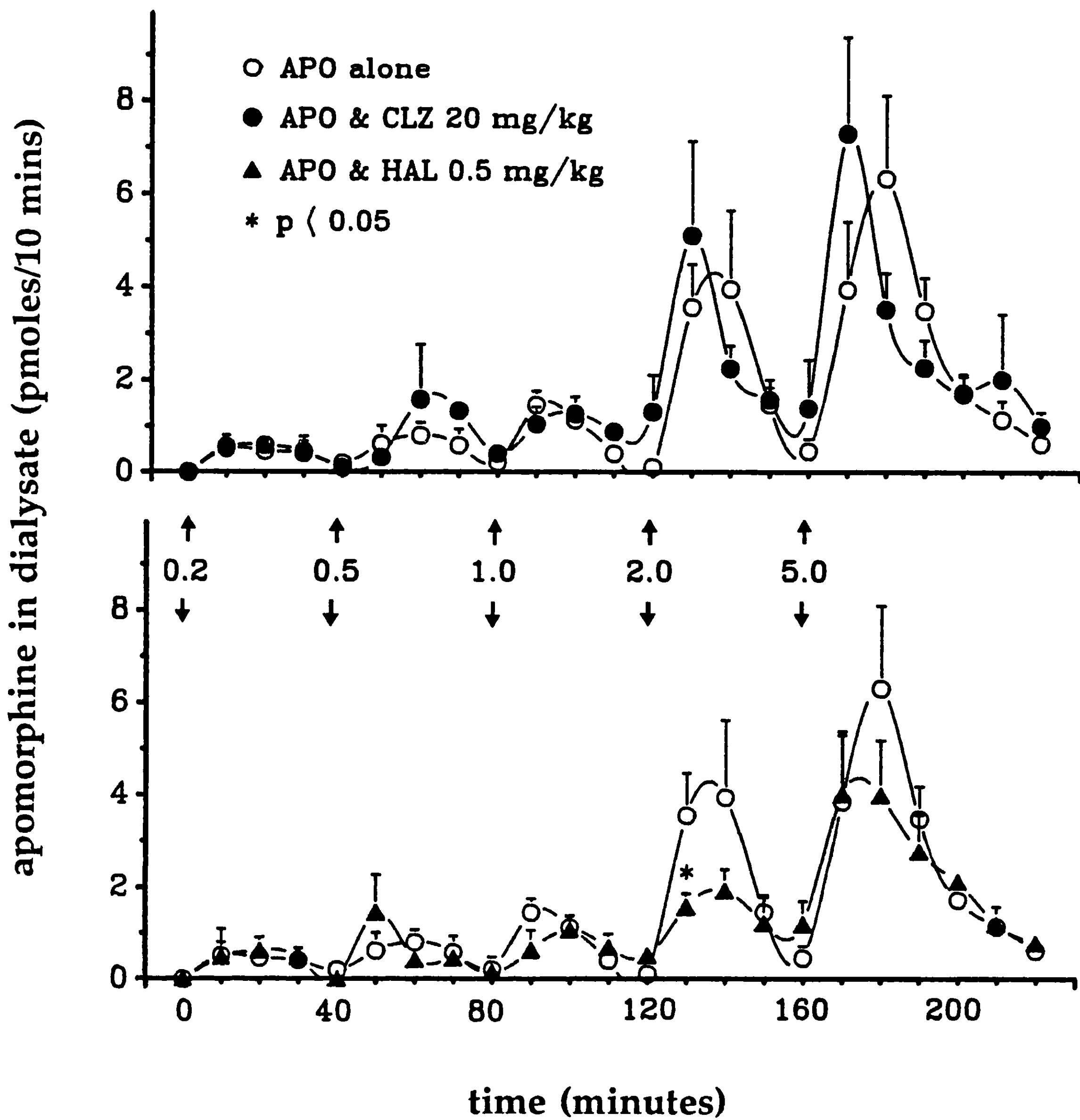


Figure 7.1 Effects of clozapine (20 mg/kg i.p.) and haloperidol (0.5 mg/kg i.p.) on the time course of apomorphine efflux in dialysates of the rat caudate putamen. Apomorphine was dosed every 40 minutes in increasing doses, either alone (○), or following pretreatment with either clozapine (●) or haloperidol (▲). The results (pmoles/10 mins) were averaged from 5 animals for each of the 3 treatment groups. The vertical error bars represent s.e.means and statistical significance was assessed using a Student's non-paired t-test.

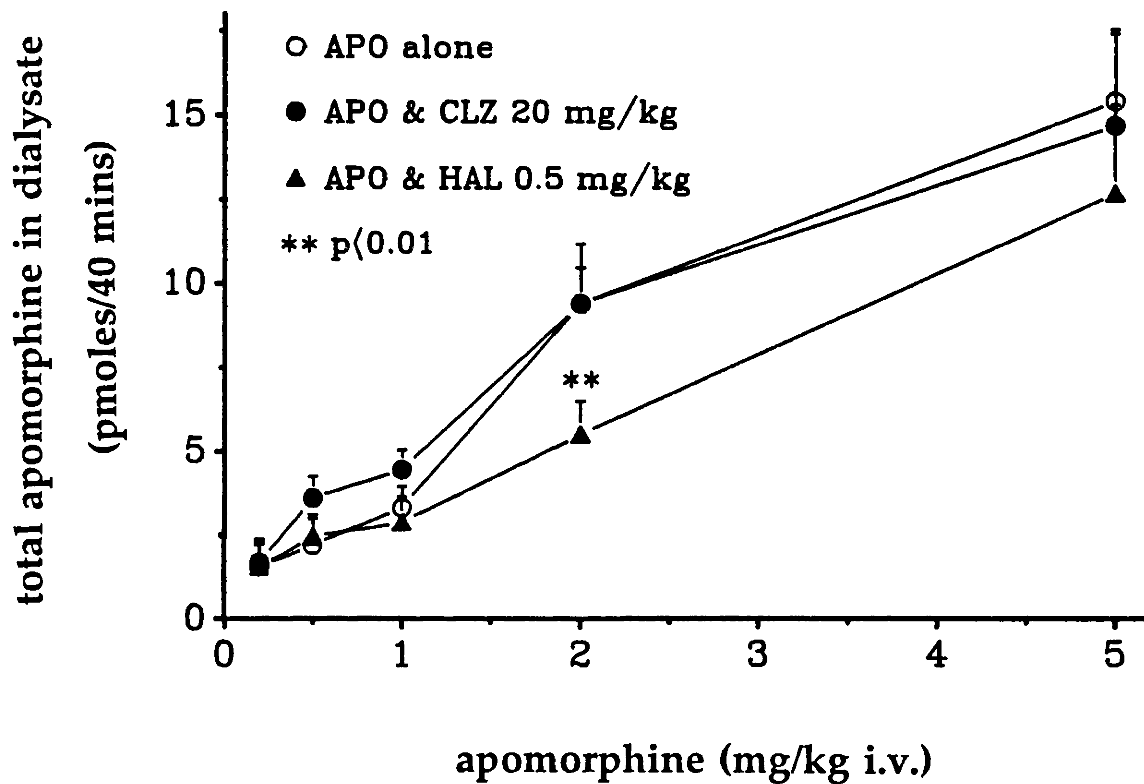


Figure 7.2 Effects of clozapine (20 mg/kg i.p.) and haloperidol (0.5 mg/kg i.p.) pretreatment on the total amount (pmoles ± s.e.mean, n=5) of apomorphine collected over 40 mins in dialysates of the rat caudate putamen following the systemic administration of apomorphine (mg/kg i.v). Statistical significance (versus apomorphine control) was assessed using a Student's non-paired t-test.

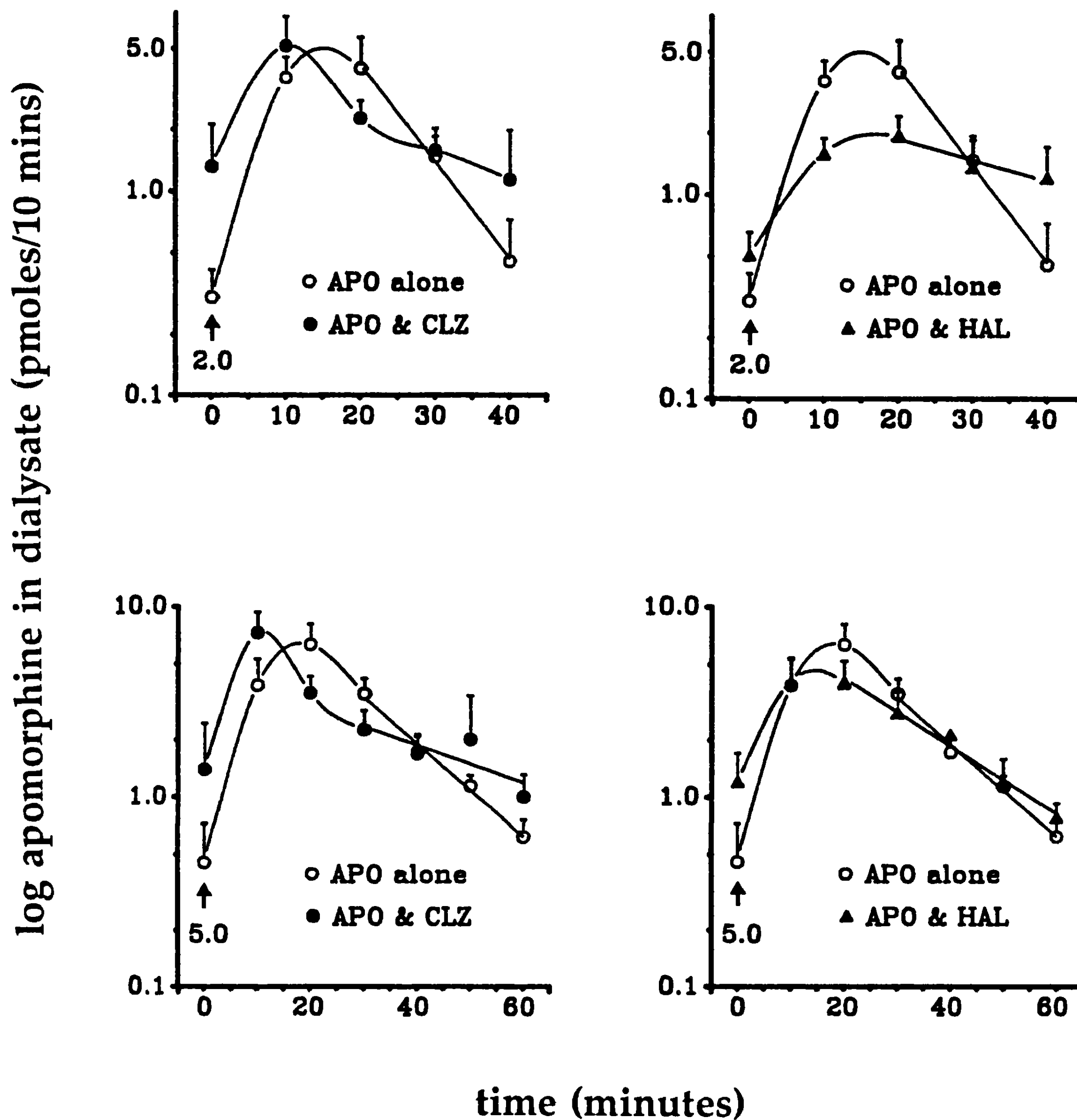


Figure 7.3 Log-linear graphs showing the effects of pretreatment with either clozapine (●) (20 mg/kg i.p) or haloperidol (▲) (0.5 mg/kg i.p) on the time course of apomorphine efflux (pmoles/10mins ± s.e.mean, n=5) in dialysates of the rat caudate putamen (○ apomorphine control) following the systemic administration of apomorphine (2.0 and 5.0 mg/kg i.v.). The origins of each plot (shown by the arrows) were derived from the preceding dose of apomorphine (see Fig.7.1). Linear regression was used to fit the data in the terminal elimination phase. The curve joining the data in the initial rising phase was generated using a standard curve fitting option (Sigma Plot version 3.1).

treatment	2.0 mg/kg			5.0 mg/kg				
	time peak efflux (mins)	peak efflux pmoles 10mins	total efflux pmoles 40mins	time peak efflux (mins)	peak efflux pmoles 10mins	total efflux pmoles 40mins	apparent half-life (mins)	(r^2)
apomorphine alone *n*=5	15.2±2.4	3.9±1.7	9.4±1.1	20.0±2.6	6.3±1.8	15.4±2.0	11.1±0.9	0.995
apomorphine and clozapine *n*=5	10.3±2.0	5.1±2.0	9.4±1.8	13.3±2.1	7.3±2.1	14.7±2.9	16.7±2.5	0.828
apomorphine and haloperidol *n*=5	20.0±3.2	1.9±0.5*	5.5±1.0**	18.0±3.7	4.0±1.4	12.7±2.6	14.9±1.0	0.862

Table 7.1 Effects of clozapine (20 mg/kg i.p.) and haloperidol (0.5 mg/kg i.p.) pretreatment on the disposition of systemically administered apomorphine (2.0 and 5.0 mg/kg i.v.) in the extracellular fluid of the rat caudate putamen. Values listed are means ± s.e.mean (*n*=5). * $p<0.05$; ** $p<0.01$ versus apomorphine control data (Student's non-paired t-test). The coefficient of determination (r^2) was derived from linear regressional analysis (least squares) of the log-linear plots given in Fig.7.4. The mean apparent half-life was derived using linear regressional analysis of individual time-course experiments.

there appeared to be a tendency for this compound to reduce the time to peak concentration (Fig 7.1) and also to alter the elimination of apomorphine from a distinctly mono-exponential to a multi-exponential (at least 2) decline. In contrast, however, haloperidol did not appear to modify either of these parameters. The apparent extracellular half-life of apomorphine (time required for the efflux of apomorphine to fall by 50%) was determined using linear regressional analysis (least squares) of data in the termination elimination phase (see Fig 7.3). Regression equations (in the form $y = m.x + c$) were obtained for individual time course experiments and the half-lives generated were averaged to yield the apparent half-life of apomorphine for each of the three treatment groups (listed in Table 7.1). The half-life of apomorphine dosed alone was determined to be 11.1 ± 0.9 minutes (mean ± s.e., *n*=5). At the apomorphine dose of 5.0 mg/kg, both clozapine and haloperidol tended to increase, but not significantly, the apparent half-life of dialysate apomorphine (16.7 ± 2.5 and 14.9 ± 1.0 minutes respectively). The half-life of apomorphine at the 2.0 mg/kg dose level was not calculated due to insufficient data in the terminal elimination phase, however, haloperidol appeared to produce an increase in the half-life of apomorphine under these conditions (Fig.7.3B).

7.4 DISCUSSION

In this chapter the technique of intracerebral microdialysis was used to evaluate the effects of two neuroleptic drugs, clozapine and haloperidol, on the passage of apomorphine into the rat brain. It was anticipated that the appearance of apomorphine in brain dialysates would reflect, albeit with some delay, the time course of apomorphine concentration in the extracellular fluid of the rat brain following its systemic administration. This appears to be the case since the results in the present study are in keeping with previously published observations (Bianchi, Landi & Garattini, 1986). These workers noted that the peak concentration of apomorphine in the caudate putamen, following a 5.0 mg/kg (i.p.) dose, occurred within about 10 minutes (apomorphine estimated in whole tissue) and declined, thereafter, in a mono-exponential manner with an estimated half-life of 11.2 minutes. Furthermore, these workers examined the effects of haloperidol pretreatment (0.25 mg/kg i.p.) on the entry of apomorphine into the rat brain. Their results indicate, in agreement with my own results, that haloperidol can reduce the levels of apomorphine estimated in the caudate putamen, but the effect appears to be considerably less marked than that claimed by earlier workers (Westerink

& Horn, 1979; Vetulani, Melzacka & Wiszniowska, 1978). Indeed, although Bianchi and co-workers (1986) noted a significant reduction of apomorphine content estimated in the caudate putamen with co-administered haloperidol, this only occurred at one sampling time (20 mins) and rather surprisingly, they could not show a corresponding reduction in the concentration of apomorphine in the plasma. Since in the present study, unlike in all the other studies mentioned above, the extracellular concentration of apomorphine is monitored, a strict comparison of results is difficult. This comparison is made even more difficult by the unknown influence of halothane on the passage of apomorphine into the CNS. Since apomorphine is not taken-up into nerve terminals (see Johnson, Hoffer & Freedman, 1986), this compound must presumably exist, either free in the extracellular fluid, or bound to dopamine and other tissue receptors. Thus, if as suggested by Westerink & Horn (1979), dopaminergic mechanisms such as dopamine receptor binding, are not involved in the ability of haloperidol to reduce the tissue content of apomorphine, it could be argued that microdialysis is the most suitable method available to test whether neuroleptics can modify the CNS entry of apomorphine.

The mechanism underlying the observed reduction of apomorphine efflux, albeit only at one haloperidol dose, is not at all clear. The dosing regime of apomorphine and haloperidol may influence the final outcome of any interaction. Since dialysis enables the time course of substances to be monitored in the same animal, the effects of multiple doses, as used in the present study, can be examined. Previous studies, however, have all assessed the effects of single apomorphine exposures in control and haloperidol treated animals. To my knowledge the influence of prior apomorphine exposure on its own passage into the brain is unknown. The dose of haloperidol, and perhaps less importantly, the timing of its administration, would also be expected to modify any interaction. Indeed, Vetulani and co-workers (1978) observed that haloperidol dose dependently (0.2-1.0 mg/kg s.c.) prevented the accumulation of apomorphine in the rat striatum, the effect becoming most apparent at 0.5 mg/kg.

It appears that the basal level of cerebral blood flow is not regulated by the activity of central dopamine neurons since McCulloch & Harper (1977) found it was not affected by selective DA antagonists such as pimozide. Nevertheless, they found that intravenous apomorphine produced an immediate and dose-dependent increase in cerebral blood flow, perhaps as a result of cerebral vasodilatation mediated by direct stimulation of DA

receptors located on these vessels. This vasodilatatory action of apomorphine might somehow enhance its passage across the blood brain barrier (perhaps by increasing the permeability of cerebral endothelial tight junctions; see Rapoport, 1976) and therefore, it would be expected that neuroleptic drugs could reduce or even reverse this effect. This may be a feasible mechanism since pimozide has been reported to block the cerebral vasodilatatory action of apomorphine in anaesthetized baboons (McCullock & Harper, 1977). On the basis of this assumption it is perhaps not surprising that clozapine, being a weak DA antagonist, did not to significantly alter the passage of apomorphine into the brain. This compound did, however, modify the elimination phase of apomorphine (Fig 7.4) although the significance of this effect remains to be clarified.

It is essential that in this type of study the blood brain barrier remains intact to avoid contamination from the systemic circulation. Although not thoroughly defined anatomically, the blood brain barrier represents an important permability barrier to the passage of substances from the blood stream into various regions of the CNS. This barrier probably arises from specialised endothelial cells located in brain capillaries, which unlike endothelial cells in peripheral capillaries, lack intercellular pores and are surrounded by a unique arrangement of glial cells (Rapoport, 1976). There is good evidence to indicate that the integrity of the blood brain barrier remains intact during dialysis. Although this barrier is obviously disrupted during probe insertion, it appears to be restored after about 30 minutes. Perhaps the most direct way to test the integrity of the blood brain barrier during microdialysis is to systemically administer substances which cannot pass across an intact barrier (presence of the substance in the perfusate would indicate some disruption). This approach has been adopted using sodium technetate ($Na^{99m}TcO_4$) intravenously administered in rats (Tossman & Ungerstedt, 1986). Since the ratio of perfusate to blood radioactivity was only 0.16 ± 0.03% (background 0.10 ± 0.03%) and this did not alter during the first four 20-min collection periods it was concluded that the blood brain barrier was not significantly damaged during cerebral microdialysis. In other studies (see Blasberg, Fenstermacher & Patlak, 1983; Benveniste, Drejer, Schousboe & Diemer, 1984), similar conclusions were reached using α-aminoisobutyrate, an inert neutral amino acid that does not cross the blood brain barrier rapidly.

A further aim of the present study was to estimate the extracellular concentration of apomorphine in the rat caudate putamen which could then be related to the actions of

this compound on DA neurotransmission. This can be achieved by *in-vitro* recovery studies although it is accepted that this approach only provides an estimate of the true extracellular concentration since there are marked differences in the natures of the extracellular brain environment and artificial cerebrospinal fluid (see Benveniste *et al.*, 1989). The passive transfer of substances in homogeneous matrices is governed by Fick's law of diffusion (1):

$$J = -D\nabla C \qquad (1)$$

where: J is the flux of substance (amount per unit time);
D is the diffusion coefficient;
∇C is the concentration gradient.

This equation forms the basis for describing the transfer of substances across a dialysis membrane and can be used to derive equation (2) (see Benveniste *et al.*, 1989 for derivation details). Equation (2) enables an estimate to made of the extracellular concentration of a substance in the brain and other tissues.

$$C_i^* = C_i \times \frac{C_o^*}{C_o} \qquad (2)$$

where: C_i^* = concentration in the extracellular fluid;
C_i = concentration in the artificial CSF;
C_o^* = concentration in the outflow solution from the probe when situated in the brain;
C_o = concentration in the outflow solution when positioned in artificial CSF.

In order to calculate the extracellular concentration of apomorphine in the caudate putamen, equation (2) was used in conjunction with the *in vitro* recovery data given in section 7.2. It is perhaps reasonable to assume that the peak extracellular concentration of apomorphine would provide the best correlation with the effects of this agent on dopamine neurotransmission (neurochemical and neuronal firing). In Fig.7.4 the peak

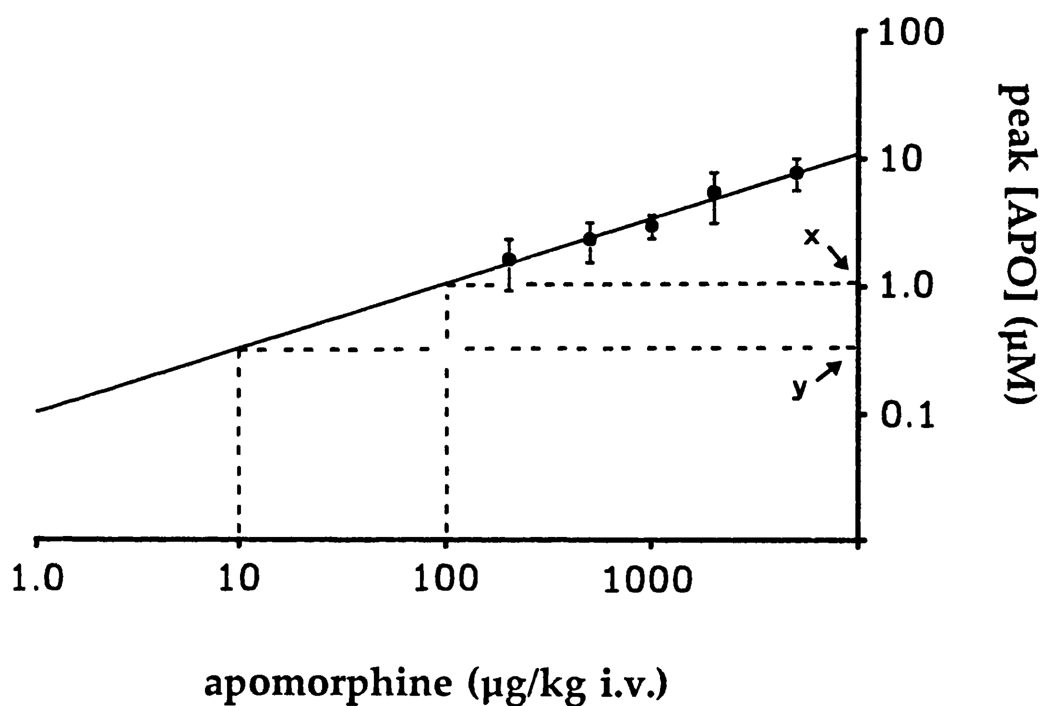


Figure 7.4 Log-log plot showing the relationship between the peak concentration of apomorphine (µM ± s.e.m., n=5) in the extracellular fluid of the rat caudate putamen and the dose of apomorphine administered parenterally (µg/kg i.v.). The peak concentration data were calculated as described in section 7.4 and fitted to a straight line (r^2 = 0.980 and slope 0.51) using linear regression (least squares). The peak concentrations (y) and (x) extrapolated from 10 and 100 µg/kg doses were determined to be 0.35µM and 1.0µM respectively.

extracellular concentration of apomorphine has been plotted against the dose of apomorphine (double log-axis scales). From this graph, the peak extracellular concentration arising in the caudate putamen, from doses of apomorphine (10-100 μg/kg i.v.) which can affect dopamine release and the firing of dopamine neurons (see chapters 5 and 6), may be estimated by extrapolation. Using this method the peak extracellular concentration of apomorphine in the caudate putamen after doses of 10 and 100 μg/kg (i.v.) would be about 0.3μM and 1.0μM respectively. These concentration estimates appear to be about an order of magnitude too high if these values are compared with either the affinity of apomorphine (K_i 24nM) for dopamine D_2 receptors (Sokoloff, Giros, Martres, Bouthenet & Schwartz, 1990) or to the IC_{50} of apomorphine (17nM) reported to inhibit the stimulated release (1 Hz, 120 pulses) of preloaded [^{3}H]-dopamine from rabbit striatal slices (Cubeddu, Hoffman & Talmaciu, 1990). A better correlation is obtained by comparing the affinity of apomorphine at the D_1 dopamine receptor where the inhibition constant (K_i) against [^{3}H]-SCH-23390 has been reported to be 0.68μM (Van Tol, Bunzow, Guan, Sunahara, Seeman, Niznik & Civelli, 1991). Low doses of apomorphine, however, are believed to stimulate only presynaptic dopamine receptors of the D_2 type (see Zetterstrom & Ungerstedt, 1984). Unfortunately, although the peak concentration of apomorphine estimated in the extracellular fluid of the caudate putamen was related to the dose of apomorphine ($r^2 = 0.980$; Fig.7.4), the slope was only about 0.5 rather than the expected slope of unity. This unexpected discrepancy may be related to the ability of apomorphine to stimulate cerebral blood flow and perhaps regulate its own passage across the blood brain barrier but the exact mechanism remains obscure.

7.5 CONCLUSIONS

It is evident from the results and discussion presented in this chapter that the technique of intracerebral microdialysis provides a unique means to assess the kinetics of drug disposition in the extracellular compartment of the rat brain. Clozapine failed to modify the amount of apomorphine appearing in dialysates of the rat caudate putamen at all doses employed and haloperidol only reduced the appearance of apomorphine at one dose (2.0 mg/kg i.v.). It is thus concluded that the ability of these neuroleptic drugs to antagonise the central actions of apomorphine does not appear to depend to any great extent on their ability to reduce the passage of apomorphine across the blood brain barrier.

CHAPTER 8

MODIFICATION OF SUBCORTICAL DOPAMINE RELEASE AND METABOLISM BY DRUGS ACTING ON 5-HT_3 RECEPTORS

8.1 INTRODUCTION

The results from chapter 4 provided evidence that clozapine acts as a moderate antagonist of peripheral 5-HT_3 receptors. Although this action was not shared by other atypical neuroleptics it would be useful to test whether clozapine also antagonises central 5-HT_3 receptors and so gauge whether this property contributes in any way to its atypical clinical profile. 5-HT_3 receptors are reported to be located in several dopamine-innervated brain structures such as the nucleus accumbens and medial prefrontal cortex (Gehlert, Gackenheimer, Wong & Robertson, 1991) and functionally, may be involved in the regulation of dopamine (Reith, 1990; Jiang, Ashby, Kasser & Wang, 1990) and acetylcholine (Barnes, Barnes, Costall, Naylor & Tyers, 1989) release. The observations in rodents that the selective 5-HT_3 antagonist GR 38032F (ondansetron) does not block amphetamine-induced stereotypy or produce catalepsy (Costall, Domeney, Kelly, Naylor & Tyers, 1987) but can reduce the hyperlocomotive response of intra-accumbal injections of dopamine and amphetamine (Costall, Domeney, Naylor & Tyers, 1987) are consistent with the relatively sparse number of 5-HT_3 receptors reported in the nigrostriatal system compared with the mesolimbic system (Kilpatrick, Jones & Tyers, 1987; Gehlert, Gackenheimer, Wong & Robertson, 1991). Thus, drugs which antagonise central 5-HT_3 receptors may be useful antischizophrenic agents devoid of extrapyramidal side effects. Unfortunately, this argument is tempered by the findings that 5-HT_3 receptor stimulation reportedly facilitates the release of dopamine from rat striatal slices in a concentration-dependent manner (Blandina, Goldfarb & Green, 1988) and that chronic administration of the selective 5-HT_3 receptor antagonist MDL 73,147EF reduces the number of spontaneously active A9 (and A10) dopamine neurons (Sorenson, Humphreys & Palfreyman, 1989). Indeed, evidence was provided in chapter 3 that stimulation of 5-HT_3 receptors may inhibit the firing of striatal neurones (see Fig.3.16). Taken together, these observations suggest that 5-HT_3 receptors also function in the regulation of dopaminergic mechanisms in the nigrostriatal system. This chapter attempts to re-evaluate this contentious claim by testing the effects of the selective 5-HT_3 receptor agonist 2-methyl-5-HT (administered by microdialysis; see 2.5.6) on dopamine release and metabolism in the caudate putamen and nucleus accumbens. By comparing the effects of clozapine against this agent with those of either haloperidol or GR 38032F it should be possible to determine whether clozapine also antagonises central 5-HT_3 receptors.

8.2 RESULTS

8.2.1 *In-vitro* calibration of 2-methyl-5-HT administration by microdialysis

The release of 2-methyl-5-HT *in-vitro* following its inclusion in the perfusate of cuprophan microdialysis probes (flow rate 2.5μl/min) was found to be directly related to its perfusion concentration (perfusion duration 20 mins) (Fig.8.1). Thus, this method of drug administration should enable the dose-dependency of its effects on central dopamine release and metabolism to be evaluated even though the extracellular concentration *in vivo* cannot be determined.

8.2.2 Effects of 2-methyl-5-HT on the release and metabolism of dopamine in the caudate putamen and nucleus accumbens

The basal efflux (± s.e.mean, $n=20$) of dopamine, DOPAC and HVA in accumbal dialysates were found to be 0.042 ± 0.017, 11.7 ± 2.1 and 4.8 ± 1.7 pmoles/20mins respectively. The effects of 2-methyl-5-HT on these levels as well as on their basal efflux in dialysates of the caudate putamen (see Table 5.1) are summarised in Figs.8.2, 8.3 and 8.4. Whilst this agent produced a significant and dose-related increase of about 40-60% in the efflux of dopamine in dialysates of the nucleus accumbens (Fig.8.3) this effect was not apparent in the caudate putamen where, at the higher concentration employed, 2-methyl-5-HT produced a significant decrease in the efflux of dopamine. The efflux of DOPAC and HVA were also significantly decreased in the caudate putamen during 2-methyl-5-HT perfusion (Fig.8.3) whereas in the nucleus accumbens there was a slight (not significant) increase in the efflux of HVA but not DOPAC.

In an attempt to test whether the effects of 2-methyl-5-HT on dopamine efflux in the caudate putamen were, in any way, attenuated by dopamine up-take into nerve terminals nomifensine was added to the perfusate (assuming that any effects of 2-methyl-5-HT are not mediated by carrier-mediated processes). Nomifensine produced a concentration-related increase in the efflux of dopamine appearing in striatal dialysates (paralleled by HVA but not DOPAC) which was strongly potentiated by haloperidol (1μM) (Fig.8.4). Despite the presence of a low concentration of nomifensine (10μM) 2-methyl-5-HT still reduced dopamine, DOPAC and HVA efflux in the caudate putamen (Fig.8.4).

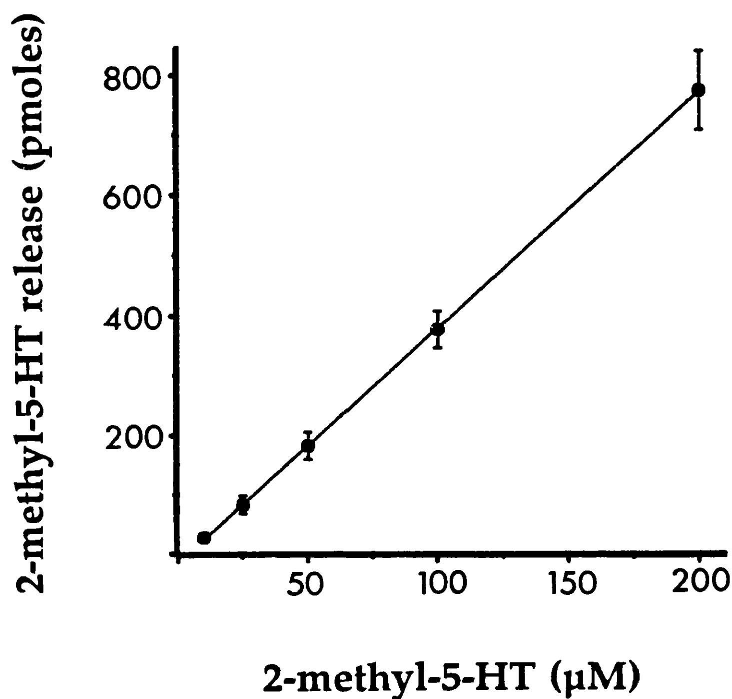


Figure 8.1 Calibration of 2-methyl-5-HT release from cuprophan microdialysis probes *in vitro*. 2-methyl-5-HT was added to the perfusion medium (flow rate 2.5 µl/min) in varying concentrations (µM) and pulsed for 20 minutes. The amount of 2-methyl-5-HT (pmoles) appearing in the surrounding medium (1.5mls artificial CSF; composition II [Appendix III], 37°C) was analysed using HPLC and electrochemical detection (see section 2.7). Error bars represent s.e.means (n=5) and linear regression (least squares) was used to fit a straight line through the data (r^2=0.9993).

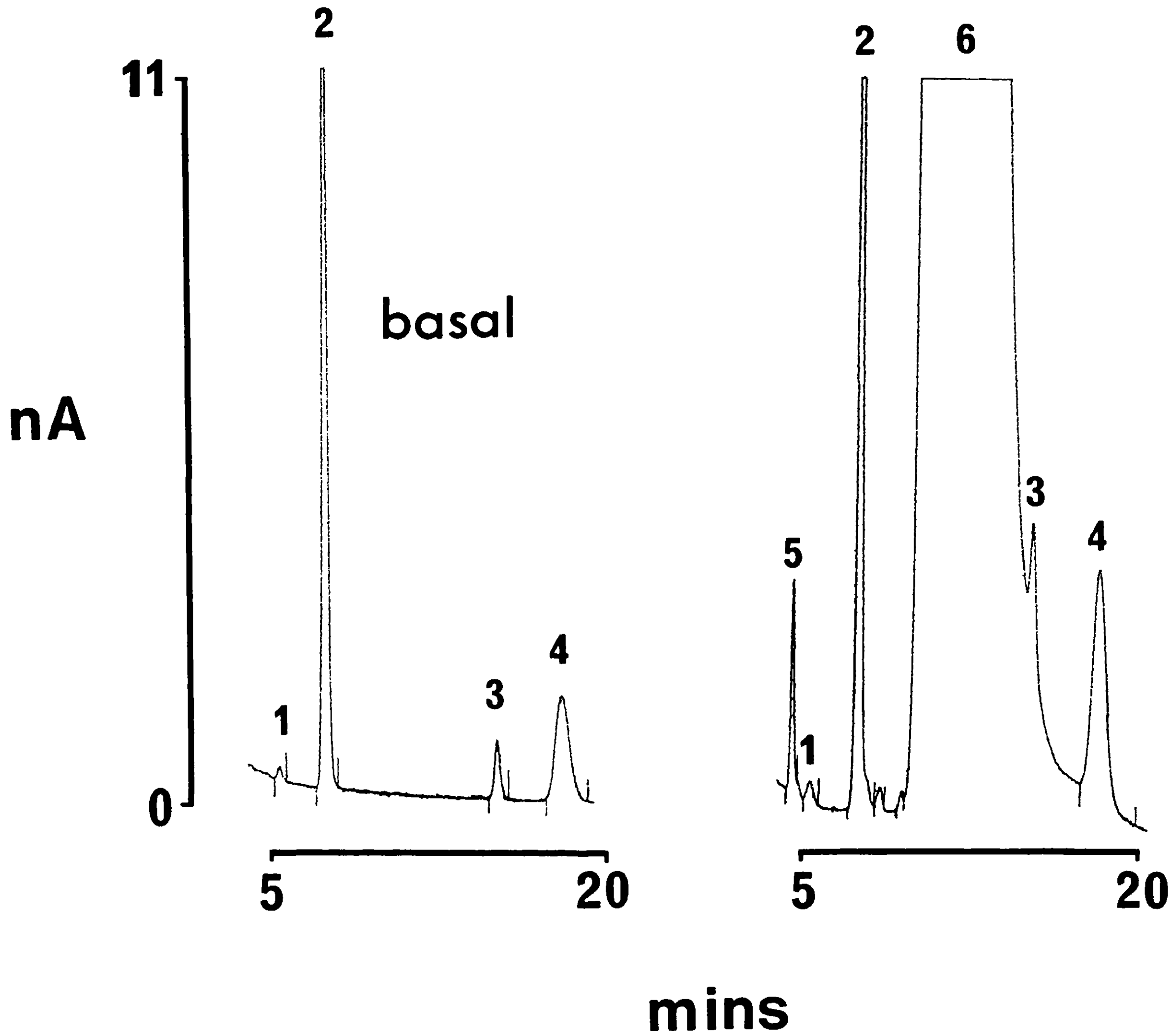


Figure 8.2 Representative chromatograms of accumbal dialysates showing the basal efflux levels of dopamine (1), DOPAC (2), 5-HIAA (3) and HVA (4) and the effects of 2-methyl-5-HT (100μM), administered by microdialysis (flow rate 2.5μl/min) over 20 minutes, on these levels. The elution peak of 2-methyl-5-HT shown in the right chromatogram (6) originates from the previous sample (i.e., its retention time is about 35 minutes). This chromatogram also reveals the elution of a further (but unidentified) substance (5) appearing in accumbal dialysates in response to the perfusion of 2-methyl-5-HT (see section 8.2.2). A detector gain of 750 with applied potentials of 20mV (D1) and 300mV (D2) was employed.

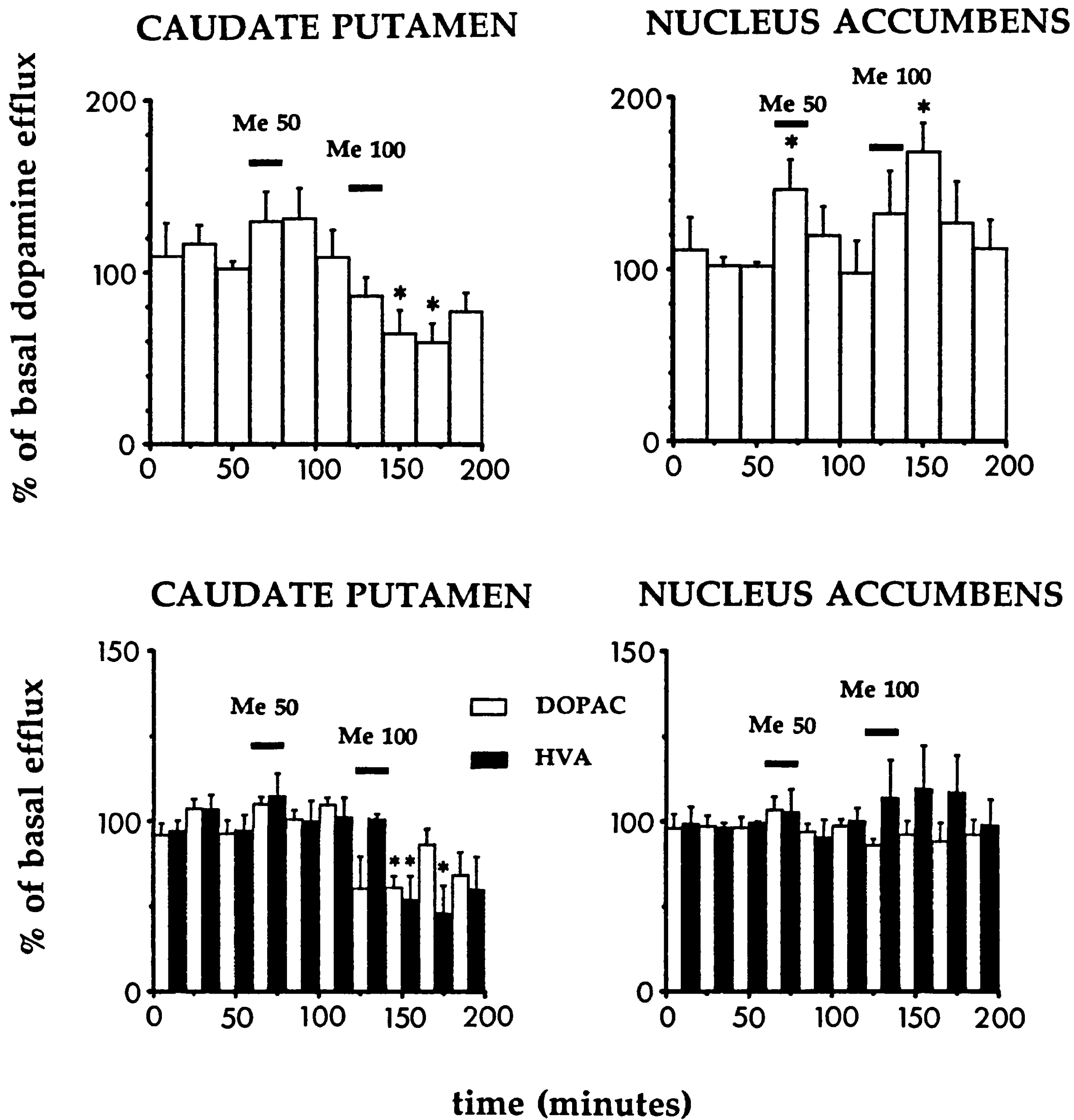


Figure 8.3 Effects of 2-methyl-5-HT (Me), administered by dialysis (flow rate 2.5µl/min), on the efflux of dopamine, DOPAC and HVA in dialysates of the rat caudate putamen and nucleus accumbens (horizontal bars indicate the period of drug administration and drug concentrations are in µM). Statistical significance (versus basal efflux) was assessed using a Student's t-test (*$p<0.05$). Error bars represent s.e.means (n=5).

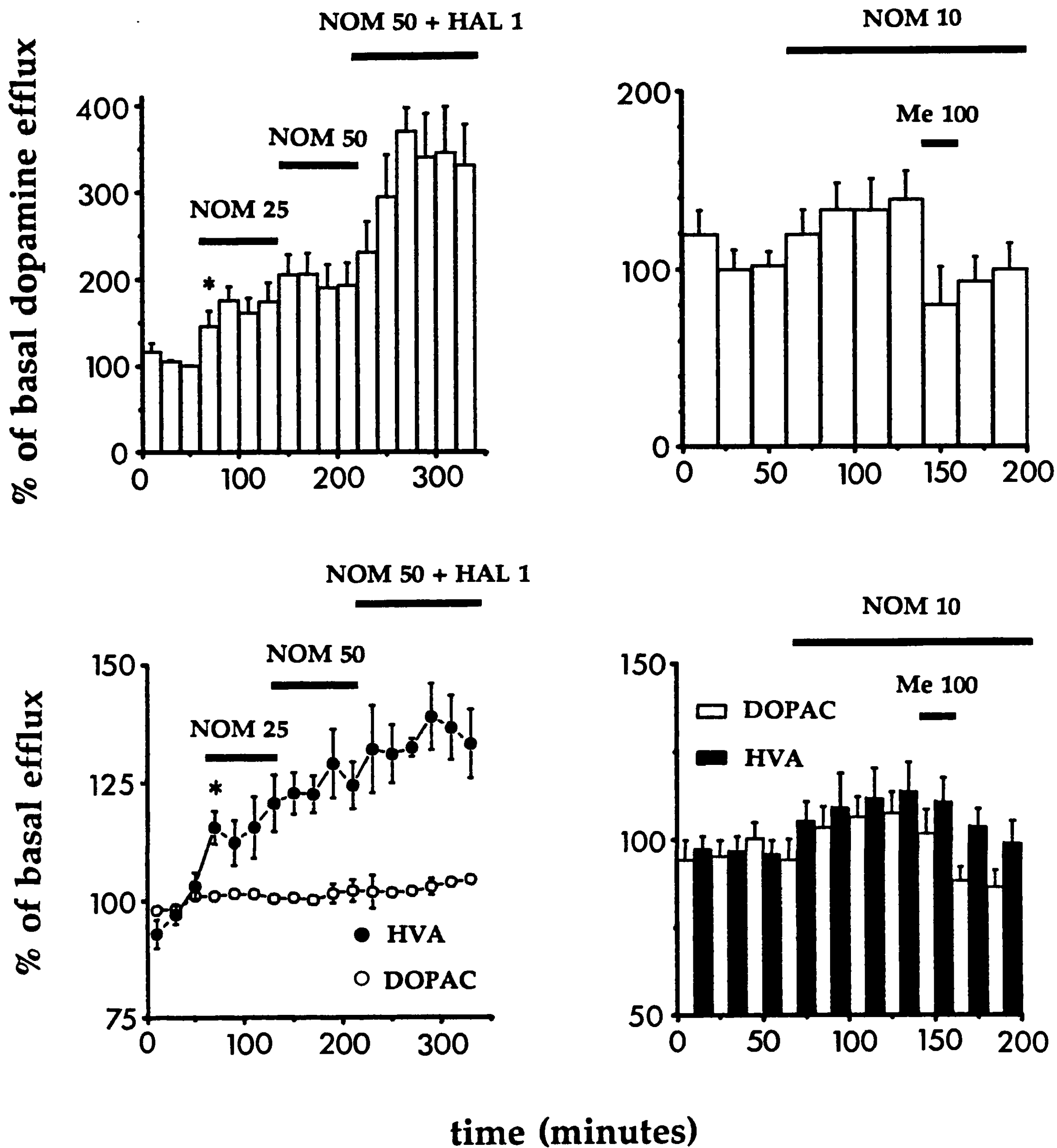


Figure 8.4 Effects of nomifensine (NOM), administered by microdialysis (flow rate 2.5µl/min), either alone, or combined with either haloperidol (HAL) or 2-methyl-5-HT (Me) on the efflux of dopamine, DOPAC and HVA in dialysates of the caudate putamen (horizontal bars indicate the period of drug administration and drug concentration units are in units of µM). Statistical significance (versus basal efflux) was assessed using a Student's t-test (* and subsequent data $p<0.05$). Error bars represent s.e.means ($n=5$).

As well as enhancing the efflux of dopamine in accumbal dialysates, 2-methyl-5-HT also produced a dose-related increase in the efflux of an unidentified substance which eluted immediately prior to dopamine (see Fig.8.2). This substance was not detected in basal dialysates and was not markedly evident in dialysates of the caudate putamen.

8.2.3 Effects of GR 38032F and neuroleptics on the release and metabolism of dopamine in the caudate putamen and nucleus accumbens

Fig.8.5 shows the effects of GR 38032F (0.1mg/kg i.p.), clozapine (20mg/kg i.p.) and haloperidol (0.5mg/kg i.p.) on the efflux of dopamine, DOPAC and HVA in dialysates of the rat caudate putamen and nucleus accumbens. GR 38032F did not modify the efflux of these neurochemicals in either area studied although at a higher dose (1.0mg/kg i.p.) it did show some tendency (not significant) to reduce the efflux of dopamine metabolites in the caudate putamen (data not shown). In contrast, clozapine and haloperidol, each to a similar extent, significantly raised the efflux of DOPAC and HVA in both brain areas without modifying the efflux of dopamine.

8.2.4 Effects of GR 38032F and neuroleptics on 2-methyl-5-HT-induced changes in the release of dopamine in the nucleus accumbens

The facilitatory action of 2-methyl-5-HT on the basal efflux of dopamine in dialysates of the nucleus accumbens (see Fig.8.3) was significantly attenuated by GR 38032F and clozapine but not by haloperidol (Fig.8.6). However, neither GR 38032F or clozapine completely blocked the effects of the higher concentration of 2-methyl-5-HT (100μM) and significant effects of 2-methyl-5-HT on basal dopamine efflux were still apparent after clozapine even at the lowest concentration employed (50μM). The antagonism of 2-methyl-5-HT by GR 38032F and clozapine in the nucleus accumbens was more striking against the efflux of the unidentified substance (see Fig.8.2) which appeared in accumbal dialysates in a dose-related manner during the perfusion of 2-methyl-5-HT (Fig.8.7). Although haloperidol reduced the 2-methyl-5-HT-induced efflux of this substance this effect was not marked and was only significant at two time points ($p<0.05$).

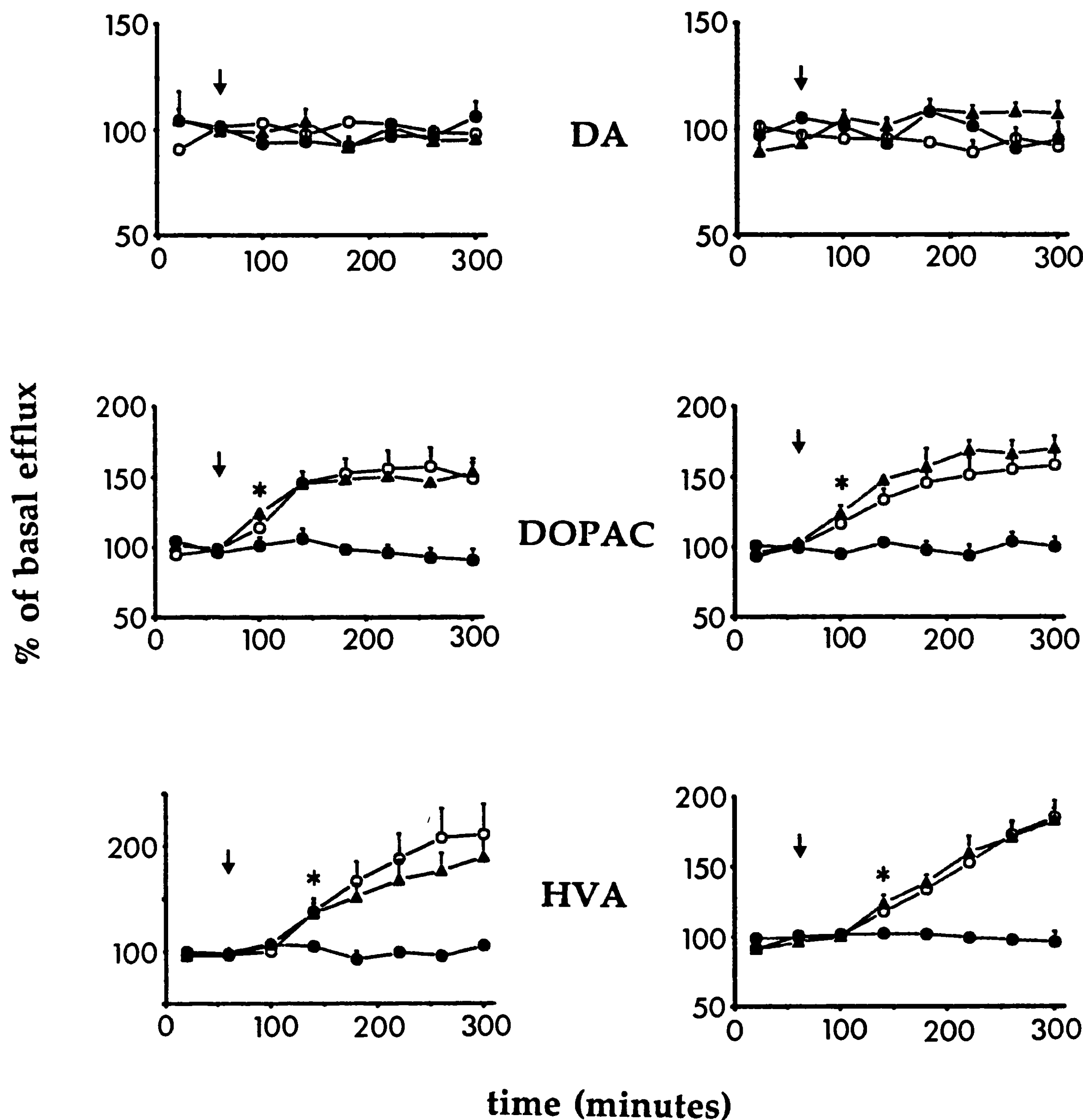


Figure 8.5 Effects of GR 38032F (0.1 mg/kg i.p.) (●), clozapine (20mg/kg i.p.) (○) and haloperidol (0.5mg/kg i.p.) (▲) on the efflux of dopamine (DA), DOPAC and HVA in dialysates of the rat caudate putamen and nucleus accumbens (administration time shown by arrows). Error bars represent s.e.means (*n*=5) and statistical significance (versus basal efflux) was assessed using a Student's t-test (* denotes $p<0.05$ for clozapine and haloperidol only).

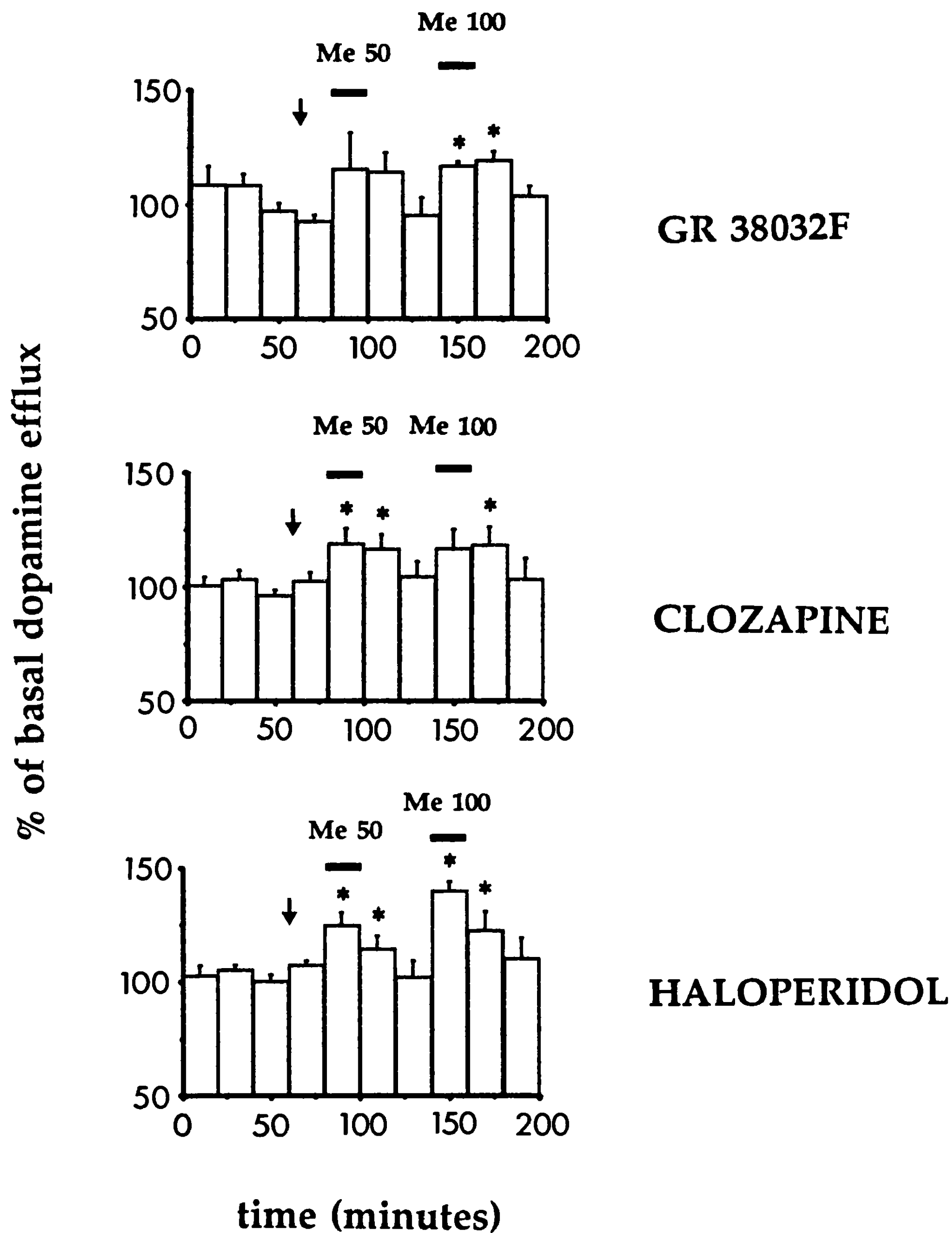


Figure 8.6 Effects of GR 38032F (0.1mg/kg i.p.), clozapine (20mg/kg i.p.) and haloperidol (0.5mg/kg i.p.) (administration time shown by arrows) on 2-methyl-5-HT-induced facilitation of dopamine efflux in accumbal dialysates (control response given in Fig.8.3). Error bars represent s.e.means (n=4-5) and statistical significance (versus basal efflux) was assessed using a Student's t-test (*$p<0.05$).

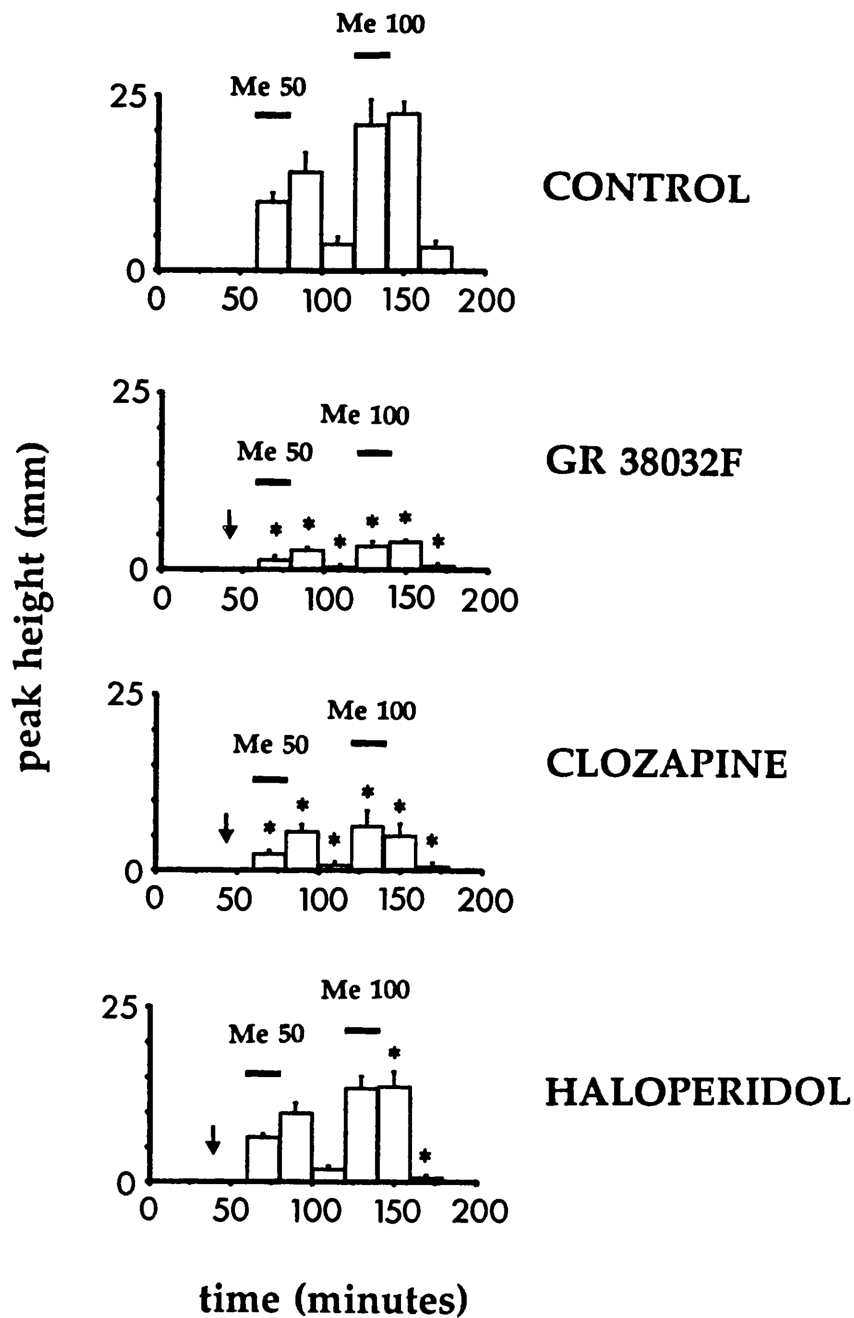


Figure 8.7 Effects of GR 38032F (0.1mg/kg i.p.), clozapine (20mg/kg i.p.) and haloperidol (0.5mg/kg i.p.) (administration time shown by arrows) on 2-methyl-5-HT-induced facilitation of neurochemical (5) efflux in accumbal dialysates (see Fig.8.2). Peak height (mm) was used to quantify the amount of this unidentified substance appearing in brain dialysates (detector conditions as described in Fig.8.2). Error bars represent s.e.means (*n*=4-5) and statistical significance (versus time-related control response) was assessed using a Student's t-test.

8.3 DISCUSSION

The lower efflux of dopamine, DOPAC and HVA detected in dialysates of the nucleus accumbens compared with the caudate putamen probably relates to the relatively smaller surface area of accumbal dialysis probes (see Fig.2.5). Despite this difference in probe construction there is some indication that the extracellular concentration of DOPAC (but not dopamine or HVA) is higher (by about 60-70%) in the nucleus accumbens than in the rostral caudate putamen (also observed by Sharp, Zetterstrom & Ungerstedt, 1986). This observation together with the fact that neither clozapine nor haloperidol modified the efflux of dopamine in either of these brain areas despite increasing the efflux of DOPAC and HVA suggests that neither basal nor drug-induced changes in the level of dopamine metabolism need reflect, to any great extent, changes in the release of dopamine. This dissociation of release from metabolism was again clearly evident when nomifensine was perfused in the caudate putamen. Thus, the efflux of dopamine and HVA were increased in a parallel manner without any discernible increase in the efflux of DOPAC. This rather striking effect may reflect a shift toward an extraneuronal metabolism of dopamine under these conditions (see also Butcher, Fairbrother, Kelly & Arbuthnott, 1988).

5-HT_3 receptors (originally termed 5-HT M-receptors since morphine blocks the indirectly mediated contractile response of 5-HT on guinea-pig ileum; see Gaddum & Picarelli, 1957) are believed to be located on neuronal elements in both the peripheral and the central nervous system where they mediate many of the excitatory responses to 5-HT. Thus, in addition to stimulating acetylcholine release from intramural cholinergic nerves leading to contraction of the guinea-pig ileum (Kilbinger, Kruel, Pfeuffer-Friederich & Wessler, 1982), 5-HT_3 receptors also activate the release of noradrenaline from sympathetic nerves innervating the isolated rabbit heart giving rise to a positive chronotropic effect (Fozard, Mobarok & Newgrosh, 1979; Richardson & Engel, 1986) and mediate the fast depolarising response to 5-HT in the rat superior cervical ganglion (Watling, Beer, Stanton & Newberry, 1990) and N1E-115 neuroblastoma cells (Henderson, 1990). 5-HT_3 receptors also participate in the vagally-mediated reflex bradycardia observed in anaesthetised rats following a bolus i.v. injection of 5-HT (Sanger & Nelson, 1989), a response known as the Bezold-Jarisch reflex. The function of centrally located 5-HT_3 receptors, however, have yet to be fully clarified. There is evidence that these receptors, which are present in limbic and cortical areas of the forebrain such as the entorhinal cortex, frontal cortex, amygdala,

nucleus accumbens and olfactory tubercules (Kilpatrick, Jones & Tyers, 1987) regulate the release of dopamine and acetylcholine (section 8.1) as well as directly control the firing of prefrontal cortical neurons (Ashby, Edwards, Harkins & Wang, 1989). 5-HT_3 receptors located in the area postrema may also be involved in the emetic response though there may also be some contribution from 5-HT_3 receptors located on visceral afferent neurons (Costall, Domeney, Naylor & Tattersall, 1986; Bunce, Tyers & Beranek, 1991).

The selective 5-HT_3 agonist 2-methyl-5-HT (but see below) when administered by dialysis produced a concentration-related increase in the efflux of dopamine in the nucleus accumbens. This effect was also observed in a recent *in vivo* chronocoulometric study where 2-methyl-5-HT was administered by intraventricular injection (Jiang, Ashby, Kasser & Wang, 1990). Thus, 5-HT_3 receptors appear to have a functional role in the control of dopamine release in this structure, and perhaps as well, in other mesolimbic projection sites where these receptors are equally numerous (Gehlert, Gackenheimer, Wong & Robertson, 1991). This may not be the case in the nigrostriatal system, however, since 2-methyl-5-HT produced only inhibitory effects on dopamine efflux in the caudate putamen. This lack of stimulation by 2-methyl-5-HT on striatal dopamine release was also observed in a recent *in-vitro* study where this agent was superfused across striatal slices (Schmidt & Black, 1989), although Blandina *et al.*, (1988), who also used a striatal slice preparation but with nomifensine added to the perfusion medium, showed that this agent caused a dose-related increase in the release of dopamine which could be blocked by the selective 5-HT_3 receptor antagonist ICS 205-930. Based on these observations it could be argued that the ability of 2-methyl-5-HT to enhance dopamine release in the striatum may be offset or attenuated by the greater number of dopamine neuronal up-take sites present in this area compared with the nucleus accumbens (Stamford, Kruk, Palij & Millar, 1988; Marshall, O'Dell, Navarrete & Rosenstein, 1990). In the present study, however, this proved not to be the case since nomifensine (10µM) did not modify the effects of 2-methyl-5-HT in this brain area. It was decided to employ a relatively low concentration of nomifensine to avoid the possibility of the increased dopamine efflux it produces overwhelming any effects of 2-methyl-5-HT on striatal dopamine release. Nomifensine (10µM) produced a small and steady accumulation of dopamine in striatal dialysates consistent with its reportedly potent inhibitory effects on dopamine uptake into rat striatal synaptosomes (IC_{50} 0.5-1.0µM; see Hunt, Kannengiesser & Raynaud, 1974). Since 2-methyl-5-HT reduced the efflux of dopamine in the caudate putamen (as well as

DOPAC and HVA) its seems unlikely that dopamine mediates the inhibitory effects of this agent on striatal neuronal firing (see Fig.3.16).

Although Schmidt & Black (1989) were unable to replicate the results of Blandina *et al.*, (1988) (see above) they were able to show a concentration-related increase in the release of dopamine from rat striatal slices using another putatively selective 5-HT_3 receptor agonist (phenylbiguanide). However, this effect was found to involve a carrier-mediated process which was independent of calcium supply and unrelated to the stimulation of 5-HT_3 receptors. This finding may explain why selective 5-HT_3 receptor antagonists fail to block both 5-HT-induced [^{3}H] dopamine release from rat striatal synaptosomes (Yi, Gifford & Johnson, 1991) as well as the enhanced behavioural response (indicative of dopaminergic activation) induced in rodents by phenylbiguanide and its chlorinated derivative (Higgins & Sellers, 1992).

The relative selectivity of 2-methyl-5-HT for 5-HT_3 receptors has recently been challenged since this agent also induces contractile effects of the isolated rabbit renal artery (pD_2 5.6), an effect thought to be mediated by a 5-HT_1-like receptor (Tadipatri & Saxena, 1992). Given that 5-HT_1 receptors have been reported to exert a facilitatory influence on dopamine release in the rostral striatum (Benloucif & Galloway, 1991) one cannot rule out the possibility that the observed effects of 2-methyl-5-HT on dopamine function in the nucleus accumbens are mediated, at least to some extent, by 5-HT_1-like receptors. Indeed, this may explain why GR 38032F did not completely block the effects of 2-methyl-5-HT in this brain area.

As well as enhancing the efflux of dopamine in accumbal dialysates, 2-methyl-5-HT also raised, in a concentration-dependent manner, the efflux of an unidentified substance from previously undetectable levels. This substance is unlikely to be derived from any catabolism of 2-methyl-5-HT since it was not apparent in studies of the caudate putamen. This substance may be structurally similar to dopamine since these compounds shared quite similar retention times although it doubtful, even despite a virtually identical retention time, that this elution peak represents L-dopa (see Fig.2.7). It is tempting to speculate that this unknown substance may in some way relate to an enhanced release of noradrenaline since this catecholamine is present in high concentrations in the nucleus accumbens (see Hornykiewicz, 1982), and, at least in the periphery, activation of 5-HT_3

receptors are known to enhance its release from sympathetic neurons (see above). Although noradrenaline itself elutes well before this unidentified substance, its metabolite MOPEG (3-methoxy-4-hydroxyphenyl glycol) elutes much closer to dopamine in this assay. Since this substance presumably shares comparable electroactive properties to dopamine the possibility exists that the Nafion-coated electrodes employed by Jiang *et al.*, (1990) (see above) may not be entirely selective for dopamine. The principal advantage of employing chromatographic procedures in neurochemical analysis is clearly evident in this situation. Whatever the identity of this substance it is conceivable that 5-HT_3 receptor mechanisms are involved to some extent in its regulation since GR 38032F potently blocked its appearance in accumbal dialysates.

The selective 5-HT_3 receptor antagonist GR 38032F did not modify either the release or the metabolism of dopamine in both brain areas studied. This finding is consistent with others (Koulu, Sjoholm, Lappalainen & Virtanen, 1989; Hagan, Jones, Jordan & Tyers, 1990) as well as the lack of any obvious behavioural effects such as stereotyped behaviour, or changes in locomotor activity, seen after the acute administration of this compound (Costall, Domeney, Naylor & Tyers, 1987). These observations together with the fact that these compounds are not reported to alter the firing rate of midbrain dopamine neurons after their acute administration (Sorensen, Humphreys & Palfreyman, 1989) indicates that 5-HT_3 receptor mechanisms do not appear to exert a tonic influence on central dopamine function.

The results from this chapter substantiate and extend the finding of chapter 4 that clozapine acts as an antagonist of 5-HT_3 receptors. Thus, like GR 38032F, but unlike haloperidol, clozapine also appears to antagonise central 5-HT_3 receptors. On the basis of radioligand binding studies (using [^{3}H] ICS 205-930) others have also reached this conclusion (Hoyer, Gozlan, Bolanos, Schechter & Hamon, 1989; Watling, Beer, Stanton & Newberry, 1990). It was somewhat surprising that GR 38032F did not completely abolish the effects of 2-methyl-5-HT since this dose is reported to considerably reduce both the increase in locomotor activity and the elevation of accumbal dopamine metabolism observed after the injection of the neurokinin receptor agonist DiMe-C7 into the rat ventral tegmental area (Hagan, Jones, Jordan & Tyers, 1990). This discrepancy may relate to the possibility that 2-methyl-5-HT also stimulates other serotonin receptor subtypes involved in the control of accumbal dopamine release (see above).

The ability of clozapine to antagonise central 5-HT_3 receptors may contribute to some of the unique clinical features of this compound. Of relevance to this argument is the reported effects of selective 5-HT_3 receptor antagonists on raised mesolimbic dopaminergic function in the rat and marmoset. Whether raised by injecting dopamine or amphetamine into the nucleus accumbens (Costall, Domeney, Naylor & Tyers, 1987), DiMe-C7 into the ventral tegmental area (Hagen, Jones, Jordan & Tyers, 1990), or drugs such as morphine, nicotine or ethanol which are known to increase dopamine release in the nucleus accumbens (Carboni, Acquas, Frau & Di Chiara, 1989), selective 5-HT_3 receptor antagonists (GR 38032F and ICS 250-930) all reduced mesolimbic dopamine function (locomotor behaviour, dopamine release and dopamine metabolism) to basal or control levels. This effect seems to depend on the ability of 5-HT_3 receptor antagonists to prevent the stimulation of dopamine release induced by drugs known to stimulate the firing activity of dopamine neurons (Carboni, Acquas, Frau & Di Chiara, 1989). As relatively few 5-HT_3 receptors exist in the nigrostriatal system (see section 8.1) and given the evidence in this chapter that these receptors mediate little, or at best, only modest effects on dopaminergic mechanisms in the caudate putamen, the low tendency of clozapine to induce extrapyramidal side effects may not be related to its ability to block 5-HT_3 receptors. It is perhaps more likely that this action contributes in some way to its ability to alleviate the positive symptoms of schizophrenia which may arise through a raised mesolimbic dopamine function (see section 1.1.2). This may explain why clozapine is a more potent anti-schizophrenic agent that what might be predicted from its apparently weak ability to antagonise central dopamine D_2 receptors (see section 5.3.3).

8.4 CONCLUSIONS

The results from this chapter indicate that 5-HT_3 receptors may function to regulate the release of dopamine in the nucleus accumbens, an action which may extend to other mesolimbic projection areas such as the hippocampus, amygdala and olfactory tubercles, but not to those areas innervated by the nigrostriatal system (i.e., caudate putamen).

The ability of clozapine to antagonise central 5-HT_3 receptors may account for its efficacy against the positive symptoms of schizophrenia despite relatively weak dopamine receptor antagonism compared with haloperidol.

CHAPTER 9

GENERAL DISCUSSION

9.1 GENERAL DISCUSSION

The objective of the experimental work recorded in this thesis was to investigate further and attempt to explain why some neuroleptic drugs produce fewer extrapyramidal side effects (EPSEs) in the treatment of schizophrenia than others. To this end a typical (haloperidol) and an atypical (clozapine) neuroleptic were compared for their ability to; (1) modify ongoing dopaminergic activity in the caudate putamen, prefrontal cortex and nucleus accumbens as measured by changes in the efflux and metabolism of dopamine, (2) counteract the effects of the dopamine agonist apomorphine on these measures as well as its ability to inhibit neuronal firing in the caudate putamen and medial prefrontal cortex. The involvement of $5HT_3$ (muscarinic and adrenergic) receptors in the activity of these neuroleptics were also investigated in both peripheral and central systems.

On the assumption that the antischizophrenic effects of neuroleptics are mediated by actions in the mesolimbic and/or mesocortical systems and their ability to induce EPSEs by activity in the caudate putamen their effects in these areas need comparison. Any explanation of the effectiveness of, and differences between, neuroleptics based simply on dopamine receptor antagonism must take account of the fact that although central dopaminergic systems are quite distinct anatomically (Fig.1.1) there is evidence that their function, particularly cortical versus subcortical systems, are coupled in an inversely-related manner. This possibility was first described by Pycock and co-workers (see Pycock, Carter & Kerwin, 1980; Pycock, Kerwin & Carter, 1980) who in several studies were able to show that by destroying dopaminergic terminals in the rat medial prefrontal cortex using 6-hydroxydopamine the metabolism of dopamine as well as the specific binding of tritiated ADTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene [a dopamine receptor agonist]) and spiperone were increased in the caudate putamen and nucleus accumbens. Further lesion studies (6-OHDA and bilateral ablation) in this brain area by these same authors (Carter & Pycock, 1980) and others (Scatton, Worms, Lloyd & Bartholini, 1982) revealed marked hyperactivity and an enhanced behavioural response to amphetamine and apomorphine indicative of an overactive subcortical dopamine function. Taken together these observations suggest that the prefrontal cortex, an area in which dysfunction has been implicated in the etiology of schizophrenia (Weinberger, 1987), may provide a tonic inhibitory influence on dopaminergic mechanisms in the nigrostriatal and mesolimbic systems. The prefrontal cortex sends monosynaptic

projections to the striatum (Kocsis, Sugimori & Kitai, 1977; Somogyi, Bolam & Smith, 1981; Frotscher, Rinne, Hassler & Wagner, 1981), as well as to such limbic nuclei as the amygdala, hippocampus, septum, and midbrain structures such as the substantia nigra and ventral tegmentum (Goldberg, Berman & Weinberger, 1989), thus providing potential routes through which this inhibitory regulation may be mediated. If these neural circuits arising from the prefrontal cortex utilise the excitatory amino-acid glutamate as their neurotransmitter (see Fonnum, Storm-Mathisen & Divac, 1981; Carlsson & Carlsson, 1990), and if a reduction of dopamine influence in the prefrontal cortex augments subcortical dopamine function, then the afferent dopaminergic innervation to the prefrontal cortex must be inhibitory. Such an influence has recently been reported by Godbout *et al.*, (1991). If the prefrontal cortex functions to regulate subcortical dopamine transmission it may exert considerable influence in more highly evolved species, since in man, but not in rodents, this area is considerably larger than the basal ganglia, occupying nearly 30% of the total brain area (Elsworth, Leahy, Roth & Redmond, 1987; Goldberg, Berman & Weinberger, 1989). The ability of the prefrontal cortex to regulate nigrostriatal and mesolimbic dopamine function may depend critically on a mesocortical dopamine function that is not easily modified. This view could explain why dopamine neurons which project to the cortex either lack or are deficient in impulse/synthesis-regulating nerve terminal autoreceptors (Chiodo, Bannon, Grace, Roth & Bunney, 1984). This argument is further developed in some of the remaining sections in an attempt to establish the relevance of the results presented in this thesis to the mode of typical and atypical drug actions.

In the medial prefrontal cortex both haloperidol and clozapine increased the efflux of the dopamine metabolites DOPAC and HVA but only clozapine produced a significant elevation in the efflux of dopamine. The fact that haloperidol and clozapine increased the metabolism of dopamine without modifying its release in the caudate putamen and nucleus accumbens (and haloperidol in the prefrontal cortex) suggests that these processes may not be directly coupled and that the efflux of DOPAC and HVA possibly provide a better index of dopamine synthesis rather than dopamine release. This would be supported by the fact that at those sites where neither neuroleptic affected dopamine release they did not modify the firing of neurons assumed to be innervated by dopamine terminals and inhibited by apomorphine. The absence of any changes in the efflux of dopamine in dialysates of the caudate putamen and nucleus accumbens may relate to a

reduced stimulation of dopamine neurons by these neuroleptics due to the presence of halothane (see section 2.1). Apart from the effects of clozapine on the efflux of dopamine in the prefrontal cortex it is clear that no marked differences exist between haloperidol and clozapine in their abilities to increase the level of dopamine metabolism in any of the three brain areas studied. However, considering the 40 fold difference in the doses of haloperidol and clozapine employed and the fact that these doses are reported to produce submaximal effects on dopamine metabolism (Nicolaou, 1980; Imperato & Di Chiara, 1985; O'Connor, Drew & Ungerstedt, 1989) it is apparent that haloperidol is more potent than clozapine in this respect. Presumably, since the level of dopamine metabolism, at least in the caudate putamen, is reported to be controlled by dopamine D_2 receptors (Zetterstrom, Sharp & Ungerstedt, 1986b), then the more potent effects of haloperidol compared with clozapine probably relates to the higher affinity of haloperidol for dopamine D_2 receptors (see Table 4.1).

In order to provide a more sensitive and a more direct measure of dopamine receptor antagonism these compounds were tested against the effects of the dopamine agonist apomorphine on central dopamine neurotransmission. In these studies clozapine (20 mg/kg) was found to be about 10 fold (i.e., 400 fold overall) less potent than haloperidol (0.5 mg/kg) in blocking the inhibitory effects of apomorphine on the metabolism of dopamine, as measured by DOPAC efflux in both the caudate putamen and the medial prefrontal cortex. This difference in potency was also reflected in their antagonism of apomorphine-induced inhibition of neuronal firing in the caudate putamen whilst in the medial prefrontal cortex although haloperidol remained a potent antagonist of apomorphine clozapine reduced neuronal firing like apomorphine. Such a difference in potency between the neuroleptics in the medial prefrontal cortex is somewhat surprising since if the neuroleptics were used in clinically equivalent doses (see section 2.4.2) and the medial prefrontal cortex is important in mediating their clinical antischizophrenic actions then their effects against apomorphine in the medial prefrontal cortex should be similar (despite the 40 fold dose difference). This discrepancy together with the fact that clozapine is reported to be about 100 (Meltzer, Matsubara & Lee, 1989) to 120 (Sokoloff, Giros, Martres, Bouthenet & Schwartz, 1990) fold less active than haloperidol in its affinity for dopamine D_2 receptors suggests that dopamine D_2 receptor antagonism alone cannot account for the antischizophrenic effects of clozapine. Indeed, if anything, clozapine shows even weaker dopamine receptor antagonism *in vivo* than what would

be predicted on the basis of its *in vitro* affinity for dopamine D_2 receptors. Clearly, therefore, other mechanisms must contribute to the therapeutic effects of clozapine in schizophrenia. The weak ability of clozapine compared with haloperidol to antagonise the inhibitory effects of apomorphine on DOPAC efflux and neuronal firing in the caudate putamen would, however, be consistent with the relatively low dopamine receptor antagonism of this compound. This would account for its atypical neuroleptic profile since it would be less likely to compromise dopamine function in the nigrostriatal system and produce EPSEs. This appears to be the case since chronic administration of clozapine is not reported to increase the number of dopamine receptors in the caudate putamen of rodents (Creese & Synder, 1980; Severson, Robinson & Simpson, 1984). The potent antagonism of dopamine receptors shown by haloperidol would account for its effectiveness against the positive symptoms of schizophrenia but this would also contribute to the development of EPSEs. In this regard it would be important to establish whether, under any conditions, typical neuroleptics can reduce the positive symptoms of schizophrenia without producing EPSEs. According to a recent study it appears that the doses of typical (high potency) neuroleptics such as haloperidol and fluphenazine normally employed in schizophrenics may be greatly excessive (at least 4-5 fold) compared with those used for low potency neuroleptics such as chlorpromazine and thioridazine (Baldessarini, Katz & Cotton, 1984). This would suggest that more conservative doses of typical neuroleptics may be associated with a reduced risk of EPSEs and yet still provide an adequate cover against the positive symptoms of schizophrenia. The fact, however, that typical neuroleptics produce EPSEs at all warrants the search for compounds which are devoid of these adverse motor effects.

The ability of clozapine to enhance the extracellular availability of dopamine in the medial prefrontal cortex may be an important mechanism underlying the reported effectiveness of this compound against the negative symptoms of schizophrenia (Kane, Honigfeld, Singer & Meltzer, 1988), since these symptoms, which predominate in chronic cases of schizophrenia (Andreasen & Olsen, 1982), are thought to be caused by a relatively underactive dopamine function (Chouinard & Jones, 1978; Karoum, Karson, Bigelow, Lawson & Wyatt, 1987; Meltzer, Matasubara & Lee, 1989). If, as suggested by Weinberger (1987), this dysfunction occurs in the prefrontal cortex, which, through a cortical/subcortical feedback pathway (see above) renders the mesolimbic dopamine system hyperactive so resulting in positive symptoms, then the ability of clozapine to

restore mesocortical dopamine function may account for its effectiveness against these positive symptoms despite only weak dopamine receptor antagonism. In contrast, haloperidol, by virtue of its potent dopamine receptor antagonism in the prefrontal cortex would not be expected to alleviate negative symptoms but this action in the nucleus accumbens would tend to reduce the likelihood of positive symptoms. The fact that phencyclidine (PCP) enhances the release of dopamine in the rat caudate putamen and nucleus accumbens (Vickroy & Johnson, 1982) and yet produces both positive and negative symptoms (Snyder, 1980) suggests that reciprocal subcortical/cortical circuits may also exist to regulate dopaminergic activity. The finding that clozapine also acts as an antagonist of $5HT_3$ receptors in the nucleus accumbens, which may serve to regulate dopamine release, provides yet another mechanism to account for the effectiveness of this compound against positive symptoms. Since haloperidol was devoid of any marked activity at limbic $5HT_3$ receptors its ability to alleviate positive symptoms is probably not mediated through any action at these receptors.

The principal aim of the work described in this thesis was to account for the benign EPSE profile of atypical neuroleptics. As discussed above the weaker dopamine receptor antagonism shown by clozapine compared with haloperidol may contribute, in part, to the fewer EPSEs seen with clozapine. Indeed, other atypical neuroleptics have also been reported to act as weak dopamine (D_1 and D_2) receptor antagonists when compared with typical neuroleptics (Meltzer, Matasubara & Lee, 1989) producing little, or only transient, effects on plasma prolactin levels in rats (Gudelsky, Nash, Berry & Meltzer, 1989; Saller, Czupryna & Salama, 1990) and weak to non-existent catalepsy (Costall & Naylor, 1975). This makes it difficult to account for their apparent anti-schizophrenic effects (see section 4.3), and does not explain why sulpiride and remoxipride (particularly the former), which have a comparatively high affinity for dopamine receptors, do not produce marked EPSEs (as discussed in section 4.3). To reconcile the anomaly that atypical neuroleptics can alleviate the symptoms of schizophrenia without producing conspicuous EPSEs it could be argued that somehow these agents block dopamine function only in those brain areas receiving a predominant mesolimbic and/or mesocortical dopamine innervation. The fact, however, that clozapine increased the level of dopamine metabolites in dialysates of the caudate putamen, nucleus accumbens and medial prefrontal cortex, as for haloperidol, and similar to sulpiride (Moghaddum & Bunney, 1990), an effect which is thought to be mediated through blockade of dopamine D_2 receptors (see above), tends to argue against

this possibility. If, as suggested by some workers (see Racagni, Groppetti, Parenti, Bugatti, Bruno, Maggi & Cattabeni, 1978; Commissiong, Slimovitch & Toffano, 1990), neuroleptics increase dopamine metabolism mainly by antagonising presynaptic dopamine receptors and since atypical neuroleptics are not reported to completely reverse either apomorphine- or amphetamine-induced stereotypy (Costall & Naylor, 1975; Robertson & MacDonald, 1985), produced through an activation of postsynaptic dopamine receptors (see Zetterstrom & Ungerstedt, 1984), it could be argued that atypical neuroleptics may be more selective at presynaptic rather than at postsynaptic dopamine receptors in the caudate putamen. This could allow unabated nigrostriatal dopamine transmission and a reduced risk of EPSEs. Unfortunately, although sulpiride may exhibit preferential activity at presynaptic dopamine receptors (Robertson & MacDonald, 1985), it is apparent from the results presented in chapters 5 and 6 as well as those reported by Seeman (1977) and Stamford, Kruk & Millar (1988) that clozapine and other atypical neuroleptics do not show preferential antagonism of dopamine autoreceptors in either the striatum or the nucleus accumbens.

Since neither haloperidol nor clozapine increased the efflux of dopamine in dialysates of the caudate putamen the low incidence of EPSEs seen with atypical neuroleptics is unlikely to be accounted for in this way. It is perhaps worth mentioning though, that clozapine has been reported to increase the efflux of dopamine in striatal dialysates of halothane-anaesthetised rats but only in the dorsolateral, and not in the rostral (fundus striati), aspects of this structure (O'Connor, Drew & Ungerstedt, 1989). This finding suggests that the caudate putamen may be functionally heterogeneous and so one cannot rule out the possibility that the low tendency of clozapine to induce EPSEs is due to some extent to a facilitation of dopamine release in some areas of the basal ganglia. Furthermore, the atypical neuroleptic sulpiride, at doses as low as 10 mg/kg, has also been reported to increase the efflux of dopamine in striatal dialysates of anaesthetised (Moghaddam & Bunney, 1990) and freely moving (Zetterstrom, Sharp & Ungerstedt, 1985) rats. These observations may explain why this compound is reported to potentiate amphetamine-induced stereotypy in rats (Robertson & MacDonald, 1985). It is doubtful, however, whether any increase in the release of dopamine in the caudate putamen would be sustained following the chronic administration of atypical neuroleptics given that tolerance to these effects is likely to occur (see section 1.3.2). This suggests that other mechanisms may contribute to the benign EPSEs of atypical neuroleptics.

The possibility that atypical neuroleptics produce less marked extrapyramidal side effects by antagonising, in addition to dopamine receptors, other cerebral receptors involved in the regulation of dopaminergic mechanisms was evaluated in chapter 4. In this regard, clozapine and thioridazine potently blocked muscarinic acetylcholine and alpha-1 adrenergic receptors and there is evidence that these actions may contribute to their atypical clinical profiles. Thus, the silencing of A9 dopamine neurons and reduction of extracellular striatal dopamine content produced by chronically administered haloperidol is reported to be reversed by the co-administration of the alpha-1 adrenergic receptor antagonist prazosin (Chiodo & Bunney, 1985; Lane, Blaha & Rivet, 1988) with trihexyphenidyl (anticholinergic) preventing the electrophysiological effects of this typical neuroleptic (Chiodo & Bunney, 1985). The observations that combining neuroleptics with anticholinergic drugs can reduce the incidence and severity of extrapyramidal side effects (Fann & Lake, 1976; Manos, Gkiouzepas & Logothetis, 1981) are consistent with an involvement of cholinergic mechanisms mediating these adverse motor effects. It is, of course, not clear where or how anticholinergic drugs act to produce their effects, though presumably, these may relate to restoring the balance of striatal dopamine and acetylcholine function. Since neither sulpiride nor remoxipride produced any antagonism of muscarinic or alpha-1 adrenergic receptors it is clear that other mechanisms mediate their atypical clinical profiles. These probably also do not relate to any antagonism of 5-HT_2 receptors (see Table 4.1) which would be expected to reduce the likelihood of extrapyramidal side effects since the selective 5-HT_2 receptor antagonist ICI 169,369 is reported to attenuate both the increase in the number of striatal D_2 receptors as well as the rise in plasma prolactin levels induced in rats by haloperidol (Saller, Czupryna & Salama, 1990). These observations suggest that 5-HT_2 receptors may function to enhance the activity of nigrostriatal and other dopaminergic neurons. The high affinity of clozapine for this receptor (pK_i 8.3; Meltzer, Matsubara & Lee, 1989) provides another mechanism to account for its atypical neuroleptic profile. Since haloperidol and chlorpromazine also share high affinity for 5-HT_2 receptors (see Table 4.1) yet produce disturbances of motor function it seems that balanced changes in dopaminergic and serotoninergic function, based mainly on D_2 and 5-HT_2 receptor antagonism, may be necessary if a drug is to be an atypical neuroleptic.

The ability of clozapine and perhaps other atypical neuroleptics to selectively augment mesocortical dopamine function may contribute to the virtual absence of tardive

dyskinesia seen with these compounds. Although there is no direct evidence to support such a claim there appears to be a close association between tardive dyskinesia and negative schizophrenic symptoms (Csernansky, Kaplan, Holman & Hollister, 1983; Barnes & Braude, 1985; Waddington, Youssef, Dolphin & Kinsella, 1987) suggesting that these disorders may share a common etiology. This may relate to a relative deficiency of cortical dopamine activity since negative symptoms are ameliorated to some extent by strategies which raise central dopamine function (see Gerlach & Luhdorf, 1975; Chouinard & Jones, 1978; Meltzer, Matsubara & Lee, 1989). Based on the evidence presented above it is conceivable that if dopaminergic activity in the cortex is relatively underactive in schizophrenia then hypersensitivity of the nigrostriatal dopamine system, in the form of raised dopamine receptor number, may develop. Although dopamine hypersensitivity is incongruous with a number of observations made in patients inflicted with tardive dyskinesia (i.e., no consistent increase in the number of dopamine receptors have been detected in post-mortem brain tissue in this disorder; see Ashton, 1987) and seems unlikely to be a primary causative influence (see section 1.3.4) it may still contribute to the appearance and severity of this disorder (Gerlach & Casey, 1988). Given this reasoning it is possible that by augmenting dopaminergic activity in the medial prefrontal cortex clozapine may conserve nigrostriatal dopamine function and thus avoid the appearance of tardive dyskinesia. A similar mechanism may account for the reported abilities of clozapine and remoxipride to either prevent or lessen the severity of neuroleptic-induced tardive dyskinesia (Sayers, Burki & Asper, 1975; Ashton, 1987; Andersson, Haggstrom, Nilson, Widerlov, 1988). The low to non-existent incidence of tardive dyskinesia seen with clozapine may also be attributable to weak dopamine receptor blockade which would tend to offset the development of nigrostriatal hypersensitivity.

9.2 SUMMARY OF RESULTS

The single most important finding of this thesis was that the atypical neuroleptic clozapine selectively enhances the efflux of dopamine in dialysates of the medial prefrontal cortex at least in halothane-anaesthetised rats (chapter 5). Its ability to mimic the actions of the dopamine agonist apomorphine in this area (chapter 6) supports the finding that clozapine (but not the typical neuroleptic haloperidol) increases the extracellular availability of dopamine in the medial prefrontal cortex. This effect may

explain why clozapine, unlike haloperidol, reduces the negative symptoms of schizophrenia which may be caused by reduced dopamine function in the medial prefrontal cortex. Such an action together with the weak ability of clozapine, compared with haloperidol, to directly antagonise the effects of apomorphine on central dopamine neurotransmission (chapters 5 & 6) challenges the view that all neuroleptics modify psychomotor behaviour in schizophrenia by directly reducing cerebral dopamine function. The fact that clozapine was found to act as an antagonist of peripheral 5-HT_3 receptors and those in the nucleus accumbens may account in part for the effectiveness of this compound against the positive symptoms of schizophrenia despite its weak dopamine receptor antagonism. Other studies designed to evaluate the activity of neuroleptics at peripheral non-dopaminergic receptors (interactions which may offset the development of extrapyramidal side effects) showed that clozapine and thioridazine potently blocked muscarinic acetylcholine and alpha-1 adrenergic receptors which may contribute to their atypical profiles (i.e., no EPSEs) even though other atypical neuroleptics tested such as sulpiride and remoxipride were devoid of activity at these receptors (chapter 4).

9.3 FUTURE CONSIDERATIONS

It will be clear from the above discussion that a single mechanism accounting for the reduced tendency of all atypical neuroleptics to produce motor-related side effects is unlikely. Future experiments should be dedicated toward characterising the receptor mechanisms mediating the clozapine-induced elevation of extracellular dopamine content in the prefrontal cortex. To understand whether these mechanisms contribute in any way to an atypical neuroleptic profile a wider range of atypical and typical neuroleptics should be tested. Ideally, these studies should be repeated after chronic dosing regimens and preferably in awake freely moving animals to negate any effects of general anaesthesia. In addition, attempts should be made to produce an animal model of schizophrenia (perhaps by lesioning dopaminergic afferents to the prefrontal cortex?) and to record synchronously from several dopamine-innervated regions and nuclei to assess the extent to which cortical and subcortical dopaminergic mechanisms are functionally coupled.

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Appendix I: Reagents and drugs

Reagents

The following were obtained from BDH Chemicals Ltd Poole:

L-Ascorbic acid
Calcium chloride
Disodium hydrogen orthophosphate
D-Glucose
Ethanol
Ethylenediamine-tetra-acetic acid (disodium)
Formaldehyde solution
Hydrochloric acid
Magnesium chloride
Magnesium sulphate
Orthophophoric acid
Perchloric acid
Potassium chloride
Potassium dihydrogen orthophosphate
Sodium chloride
Sodium heptane sulphonic acid
Sodium hydrogen carbonate
Sodium dihydrogen phophate
Sodium hydroxide

Other substances were obtained from the following sources:

Substance	Source
Acetic acid (glacial)	May and Baker Ltd.
Acetonitrile	May and Baker Ltd.
Citric acid	Fisons
Halothane	ICI
Methanol	May and Baker Ltd.
Nitric acid	Fisons
Potassium hydroxide	Sigma
Tween 80	Koch-Light Laboratories Ltd.
Urethane	Sigma

Water

All water used in these studies was distilled (re-condensed plant steam, U.C.L), deionised (Elga Spectrum, SC-1), carbon-swept (SC-6) and filtered (SC-20).

Drugs

The drugs used in this study were obtained from the following sources:

O-Acetylcholine iodide	BDH
γ-amino-n-butyric acid	Sigma
Apomorphine hydrochloride	Sigma
D-amphetamine sulphate	SKF (gift)
L-Aspartic acid	Sigma
Atropine sulphate	Sigma
(+)-Bicuculline	Sigma
CGS-15855A	Ciba-Geigy
Chlorpromazine	Sigma
Clonidine hydrochloride	Boehringer Ingelheim
Clozapine	Sandoz (gift)
3,4-Dihydroxy-phenylacetic acid	Sigma
L-β-3,4-Dihydroxy-phenylalanine	Sigma
Diphenhydramine	Sigma
L-Glutamic acid	Sigma
GR 38032F	Glaxo (gift)
Haloperidol	Sigma
4-Hydroxy-3-methoxy-phenylacetic acid	Sigma
5-Hydroxyindole-3-acetic acid	Sigma
3-Hydroxytyramine	Sigma
ICS 205-930	Sandoz (gift)
N-Methyl-D-Aspartic acid	CRB
2-Methyl-5-HT	Glaxo (gift)
Metoclopramide	Sigma
Methysergide	Sandoz
Nomifensine	Hoechst
Pargyline	Sigma
Pentylenetetrazole	Sigma
Picrotoxin	Sigma
Prazosin	Pfizer

D-Pro[4],D-Trp[7,9,10] SP_{4-11}	Peninsula
Propranolol	ICI
Raclopride	Astra (gift)
Remoxipride	Astra (gift)
Serotonin (creatinine complex)	Sigma
Substance P	Peninsula
Sulpiride	Sigma (gift)
Tetrodotoxin	Sigma
Thioridazine	Sandoz (gift)
Yohimbine	Sigma

Drug Vehicles

Apart from some neuroleptic compounds, all drugs were dissolved in either distilled deionised water, normal saline, Krebs Henseleit solution or artificial CSF. Water-insoluble neuroleptics were dissolved in a minimum quantity of glacial acetic acid and made to volume in either of the above listed mediums. The pH was adjusted to at least 6 with 0.5M NaOH or until obvious precipitation occurred.

Appendix II: *In vitro* bathing solutions

Krebs Henseleit solution

Compound	Concentration (mM)
NaCl	119
KCl	4.7
KH_2PO_4	1.1
$MgSO_4$	1.2
Glucose	11
$NaHCO_3$	25
$CaCl_2$	2.5
Atropine	10^{-4}
Propranolol	10^{-4}

Tyrodes solution

Compound	Concentration (mM)
NaCl	137
KCl	2.7
$CaCl_2$	1.8
$MgCl_2$	1.1
$NaHCO_3$	11.9
NaH_2PO_4	0.4
Glucose	5.6
Diphenhydramine	10^{-4}
Atropine	10^{-4}
Methysergide	10^{-4}

The bathing solutions were made to volume with distilled deionised water and the final pH ranged between 7.2 and 7.5. Calcium and magnesium chlorides were added as 1M solutions.

Appendix III: Artificial cerebrospinal fluid

In some experiments (see chapter 3) the following composition of CSF was employed:

Composition I

Compound	Concentration (mM)
NaCl	137
KCl	2.7
$CaCl_2$	2.2
$MgCl_2$	1.0
$NaHCO_3$	11.9
Na_2HPO_4	0.4
Glucose	10

In the remaining experiments the following composition of artificial CSF was employed:

Composition II

Electrolyte	Concentration (mM)
Na^+	147.0
Ca^{2+}	2.3
K^+	4.0
Cl^-	155.6

The solutions were made to volume with distilled deionised water. The pH values of compositions I and II were 7.6 and 6.0 respectively. Calcium and magnesium chlorides were added as 1M solutions.

Appendix IV: Statistical analysis

All statistical tests described in this thesis were computed using a statistics programme (" Pharmacological Calculation System " version 4.0, by R.J. Tallarida and R.B. Murray, Springer-Verlag, New York, 1986)

More appropriate tests of significance which should have been used in this thesis include analysis of variance (assuming that errors are normally distributed) followed (if significant differences are found) by either a Dunnett's test for time-related comparisons or by either a Tukey's or a Newman-Keuls test for comparisons between drug treatments.