THE RELATIONSHIP BETWEEN INTRATERMINAL POOLS OF DOPAMINE AND ITS RELEASE BY CHEMICAL STIMULI: AN IN VIVO MICRODIALYSIS STUDY

by

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Thesis submitted for the degree of Ph.D.

University of Edinburgh

November 1988

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Gordon Arbuthnott and Steve Butcher for their expert guidance, discussion and friendship throughout.

Many thanks to my friends and colleagues in the MRC Unit and the Department of Pharmacology for their moral and other support over the past three years.

Special thanks to Jean Hunter and her staff in the animal house for their patience with me and care of the animals.

I am sincerely grateful to Norma Brearley for her marvellous effort and expert typing of this thesis. She has saved me many a long and frustrating hour.

Finally, I should like to thank my Mother and Father and the rest of my family for their continuous support of the perpetual student.

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Chapter 3 Results

I declare that the studies presented in this thesis are the result of my own independent investigations.

This work has not been and is not being concurrently submitted for candidature for any other degree.

Candidate

Supervisors

List of Publications

Some of the results presented in this thesis have been published as follows:

Arbuthnott, G.W., Butcher, S.P., Fairbrother, I.S. & Kelly, J.S. (1986) *In vivo* microdialysis studies on dopamine release from the striatum evoked by nigrostriatal stimulation. Brit. J. Pharmac. 89: 878P.

Arbuthnott, G.W., Butcher, S.P., Fairbrother, I.S. & Kelly, J.S. (1986) On the mechanisms underlying amphetamine induced dopamine release: *in vivo* microdialysis studies. Brit. J. Pharmac. 89: 879P.

Butcher, S.P., Fairbrother, I.S., Kelly, J.S. & Arbuthnott, G.W. (1987) *In vivo* compartmentation studies using brain microdialysis: focus on dopamine. Neurosci. Lett. Supp. 29: 552P.

Fairbrother, I.S., Butcher, S.P., Kelly, J.S. & Arbuthnott, G.W. (1987) A physiological role for MAO-B in the metabolism of dopamine? Br. J. Pharmacol. 92: 612P.

Butcher, S.P., Fairbrother, I.S., Kelly, J.S. & Arbuthnott, G.W. (1987) Amphetamine induced dopamine release in the rat striatum: An *in vivo* microdialysis study. J. Neurochem. 50: 346-355.

Arbuthnott, G.W., Fairbrother, I.S. & Butcher, S.P. (1988) *In Vivo* assessment of the compartmentalisation of dopamine in striatal nerve terminals. Soc. Neuroscience. Abs. 99.2.

ABSTRACT

The technique of in vivo microdialysis was used to monitor the efflux of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxy-indoleaceticacid (5-HIAA) and 3-methoxytyramine (3-MT) from the striatum of halothane anaesthetised rats. Dialysis probes were acutely implanted and perfused with artificial cerebrospinal fluid. The concentrations of amines and metabolites in the perfusate were assayed with reverse-phase high performance liquid chromatography and electrochemical detection.

The actions of various DA releasing agents were studied. Pharmacological or biochemical intervention affecting the releasing actions of these agents allowed an evaluation of their mechanisms of action in vivo. Furthermore, information was obtained concerning the heterogeneous location of intraterminal pools of DA.

The effects of Ca^{2+} depletion and of the DA uptake inhibitor nomifensine on the action of releasing agents were compared. The results suggest that two mechanisms of release can be separated.

Amphetamine (AMPH), tyramine (TYR) and to some extent ouabain (OUAB), were blocked by nomifensine and so were deduced to be dependent on the DA uptake carrier. Their action was independent of changes in extracellular Ca^{2+}.

In contrast, high $[K^+]$ (KCl) and veratrine (VER)-induced release were sensitive to changes in extracellular Ca^{2+} and independent of the action of nomifensine on the carrier. Release was blocked by preventing Na^+ influx with tetrodotoxin (TTX). The action of OUAB was also partly inhibited by these agents.

Attempts to define the source of the released DA were less clear. The suggestion was made that release from a vesicular storage pool should be reserpine sensitive whilst release from a pool of newly synthesised DA should be differentially sensitive to α-methyl-p-tyrosine (AMT). The results show that all releasing agents are at least partly sensitive to both manipulations although to differing degrees suggesting that both pools are accessible to all the releasers. Thus, the order of degree of inhibition by reserpine (TYR > KCl > VER = OUAB = AMPH is in contrast to that for short term AMT (AMPH > OUAB > KCl > VER > TYR).

DOPAC efflux levels were altered by agents that preferentially released newly synthesised DA. The increased efflux of 3-MT does not happen unless reuptake and/or monoamine oxidase has been inhibited suggesting an extraneuronal source of this metabolite.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Dopamine: a neurotransmitter in the CNS

1.1.1 History

The neurotransmitter dopamine, is now recognised as one of the major transmitters in the vertebrate CNS and the anatomy, physiology and pharmacology of dopaminergic neurotransmission have been studied extensively.

For any compound to be defined as a neurotransmitter, it must satisfy certain criteria. Controversy surrounds the relative importance of the individual criteria but the schemes proposed by Werman (1966) and Orrego (1979) are considered to satisfy the definition of a neurotransmitter. These put forward six criteria: (1) the transmitter must be present intraneuronally (2) there must be a transmitter synthesising system (3) there should be an intraneuronal storage system (4) the transmitter should be released by a physiological or pharmacological stimulus in a Ca^{2+} dependent fashion (5) there must be an inactivation mechanism to terminate the action of the transmitter and (6) the transmitter should have an identifiable postsynaptic action.

Dopamine satisfies these criteria but it was to be five decades from the first reports of dopamine to its recognition as a neurotransmitter. The dopamine molecule was first synthesised in 1910 by Barger & Ewins and by Mannich & Jacobsohn and classified at the time as a weak sympathomimetic due to its weak vasodepressor action (see Hornykiewicz, 1984 for a review). Blaschko, in 1939, recognised DA as an intermediate in the synthesis of noradrenaline (NA) and proposed the now familiar route of the biosynthesis of NA and adrenaline (AD). The increasing frequency of reported measurements of DA in mammalian tissues and fluids awoke researchers

to the possibility of a separate physiological role for DA (see Anden, 1964). Attention was switched to the CNS when DA was reported to occur in equal concentrations to NA (Montagu, 1957; Carlsson et al., 1958). Carlsson also showed that, as well as depleting NA and 5-hydroxytryptamine (5-HT), reserpine also depleted DA in the CNS. Moreover, the precursor of DA, L-DOPA, increased the DA concentration in brain tissue after reserpine treatment. This helped to explain some of the CNS side effects of reserpine that could not be attributed to depletion of NA and 5-HT such as catalepsy and an extrapyramidal motor syndrome similar to Parkinson's disease.

Until this time, DA had only been described in the whole brain and not localised to specific areas. However, Bertler & Rosengren (1959) reported the occurrence of DA in high concentration in the absence of NA in the corpus striatum of the dog brain. This area was known to be a major part of the basal ganglia and a major constituent of the brain extrapyramidal motor system. This led them to conclude that DA is concerned with the function of the corpus striatum and so with central motor function thereby explaining the postulated DA depleting effects of reserpine upon the extrapyramidal motor system.

By the early 1960's, a considerable body of evidence existed for DA as a central neurotransmitter. A biosynthetic pathway had been proposed as well as the specific localisation of DA to certain areas of the brain. Moreover, the first evidence existed for a possible role of DA in the control of motor function.

1.1.2 The anatomy of dopaminergic systems

The advent of the Falck-Hillarp fluorescence technique in the early 1960's facilitated the specific location and visualisation of dopaminergic neurones. Prior to this, there was no method available that was specific for DA. The method relies on the conversion of DA to a highly fluorescent 5,6-dihydroxyindole which emits light over a specific spectrum. This was easily measured in the spectrophotofluorimeter. This method was used by Carlsson & Hillarp (1962), Dahlstrom & Fuxe (1964) and Fuxe (1965) to identify DA in nerve cell bodies and terminals. This resulted in the identification of three major dopaminergic pathways. Anden et al. (1966) and Ungerstedt (1971a) divided these into: (a) A major ascending nigrostriatal pathway from the DA neurones of substantia nigra pars compacta (cell group A9) to the larger part of the striatum, (b) a mesolimbic system from the medial mesencephalon (cell group A10) to the nucleus accumbens, olfactory tubercle, and several cortical areas within the entorhinal cortex and the frontal lobe. In later autoradiographic studies (Fallon & Moore, 1978; Beckstead et al., 1979), the major projection from cell group A10 was found to involve not only the nucleus accumbens and olfactory tubercle, but also the ventral quarter of the entire length of the striatum and (c) the final major dopaminergic pathway is the tuberoinfundibular pathway which is involved in the hypothalamic control of secretion of hormones from the anterior pituitary gland.

1.1.3 Synthesis of catecholamines

The synthetic pathway for catecholamines begins with the

essential amino acids phenylalanine and tyrosine. Generally, the dietary intake of tyrosine is adequate for catecholamine synthesis, but phenylalamine can be converted to tyrosine by phenylalanine hydroxylase (see Bowman & Rand, 1982). The first step is the addition of a hydroxyl group to tyrosine converting it from a phenol to the corresponding catecholamino acid, dihydroxyphenylalanine (DOPA) (Levitt et al., 1967). This is catalysed by the enzyme tyrosine hydroxylase, which requires Fe^{2+} and reduced pteridine as cofactors (Roth, 1979). Molecular oxygen is also required since during the oxidation process catalysed by the enzyme, one atom of oxygen is required for incorporation into tyrosine while the other is reduced to water. The two different fates of the oxygen atoms have led to the classification of tyrosine hydroxylase as a mixed function oxygenase.

The final stage in DA biosynthesis is the decarboxylation of DOPA to DA (Blaschko, 1950) (See Sourkes, 1979). This is carried out by the ubiquitous enzyme aromatic-L-amino acid decarboxylase, also referred to as DOPA decarboxylase. This is a non-selective enzyme and will decarboxylate many aromatic amino acids including 5-hydroxytryptophan to form 5-hydroxytryptamine (5-HT) (Sourkes, 1979). Noradrenaline (NA) is formed from DA by β-hydroxylation mediated by the enzyme dopamine β-hydroxylase. This is a copper requiring enzyme that also utilises molecular oxygen as a co-substrate and ascorbate as a co-factor.

Control of dopamine synthesis in the brain

The rate limiting step in the synthesis of DA and consequently NA is the conversion of tyrosine to DOPA by tyrosine hydroxylase (TH) (Nagatsu et al., 1964). Three levels of control are proposed

to exist for the modulation of DA synthesis and release, (1) end product inhibition (2) presynaptic DA autoreceptors and (3) the striatonigral negative feedback loop.

End product inhibition

This is an intracellular control mechanism whereby the products of the biosynthetic pathway (DA in dopaminergic neurones, NA in noradrenergic neurones), inhibit the action of TH so inhibiting further production of that transmitter. The mechanism that achieves this is thought to be competition between the product and the pteridine cofactor required by TH (Fernstrom, 1983). Anything that reduces the pool of transmitter mediating the inhibition such as nerve stimulation, reduces the inhibition and consequently stimulates production of the transmitter.

Activation of catecholaminergic cells induces the secretion of catecholamines and also induces the de novo synthesis of catecholamines to replenish the stores. This has been demonstrated in chromaffin cells, PC12 cells, superior cervical ganglia and in rat striatal synaptosomes (see Haycock, 1987). The increase in catalytic activity of TH is associated with a calcium dependent phosphorylation of TH at several sites upon the N-terminal region of the enzyme. This is likely to form part of the regulatory mechanism that determines the activity of TH.

DA autoreceptors

Dopamine autoreceptors are situated on the dopaminergic nerve terminals and on the cell body and dendrites (Roth et al., 1984). When stimulated by released DA they act to inhibit tyrosine hydroxylase activity so reducing DA synthesis. This has been demonstrated by the agonist induced reduction in the hydroxylation

of tyrosine using apomorphine (Walters & Roth, 1976). Accordingly, DA synthesis is also blocked. The effect is reversed by prior administration of DA receptor antagonists (Roth et al., 1984). An alternative technique is to drive the dopamine neurones by stimulating their axons and to measure the impulse induced increase in tyrosine hydroxylation. Using this model, the agonist induced reduction of this tyrosine hydroxylation has been used as an indication of the presence of synthesis modulating autoreceptors (see Roth et al., 1984). Electrophysiological techniques have been used to study cell body/dendritic DA autoreceptors (Aghajanian & Bunney, 1977; Skirboll et al., 1979). Using single unit recording techniques, the effects of DA and DA agonists upon the physiological activity of identified dopaminergic neurones has been used to demonstrate the presence and effects of DA autoreceptors. In addition, release modulating autoreceptors act to decrease DA release. DA autoreceptors have been classified as D_2 receptors, mainly due to the affinity of D_2 antagonists such as sulpiride for these receptors (see Woodruff, 1986). The role of DA autoreceptors was first postulated by Carlsson et al. (1972). They showed that transection of DA fibres acutely increased the rate of tyrosine hydroxylation and increased DA levels. The suggestion was that by removing the nerve impulse stimulated release of DA, the inhibitory autoreceptors are no longer occupied which removes this route of inhibition. Other workers using γ-butyrolactone (GBL) to diminish the firing rate of nigrostriatal DA neurones, also reported an increase in the rate of tyrosine hydroxylation and in DA levels (see Walters et al., 1976). This effect was blocked by the DA agonist apomorphine and by the DA releasing drug amphetamine. Accordingly,

the actions of these drugs were blocked by the DA antagonists haloperidol and chlorpromazine (Walters et al., 1976). A variety of DA autoreceptor selective agonists have been reported. These include N-0437 (Van der Weide et al., 1986), quinpirole (LY 17155) (Walters et al., 1984), 3-PPP (Hjorth et al., 1983) and B-HT 920 (Anden et al., 1983). Of these, B-HT 920 has been reported to be the most selective for autoreceptors and has been shown to inhibit DA release and synthesis (Jennewein et al., 1986; Pifl & Hornykiewicz, 1988).

Interestingly, there is evidence that synthesis modulating DA autoreceptors are absent on terminals of certain mesocortical DA neurones (Roth et al., 1984). It was noticed that mesocortical systems showed a different responsiveness to acutely and chronically administered antipsychotics as well as to mild stress and conditioned fear when compared to nigrostriatal and mesolimbic systems (see Roth et al., 1984). Using biochemical and electrophysiological methodology, Roth and co-workers demonstrated a lack of synthesis modulating DA autoreceptors in the prefrontal cortex, cingulate cortex and entorhinal cortex. The significance of this is not clear, but it endows certain properties upon these DA systems such as a higher rate of physiological activity (firing) with a different pattern of activity (more bursting), a higher turnover rate of transmitter, diminished biochemical and electrophysiological responsiveness to DA agonists and antagonists and a transmitter synthesis that is more readily influenced by the availability of tyrosine (see Roth et al., 1984 for review).

The striatonigral pathway

Due to the fact that DA receptor antagonists were seen to

increase the synthesis and metabolism of DA, several groups proposed that in addition to end product inhibition, DA receptor mediated control of dopaminergic neurotransmission occurs (Carlsson & Lindqvist, 1963; Kehr et al., 1972; Anden, 1972). An inhibitory striatonigral pathway was proposed as a return loop reciprocating the nigrostriatal pathway. This return pathway is via non-dopaminergic neurones that synapse directly onto dopaminergic nigrostriatal neurones (see Nauta & Domesick, 1984). In this way, it is suggested that postsynaptic DA receptors feed information backwards via this pathway to control the activity of the nigrostriatal neurones (Carlsson & Lindqvist, 1963; Bunney & Aghajanian, 1975). This mechanism is often known as the striatonigral negative feedback theory and persists even after the discovery of DA autoreceptors which appear to achieve a similar result.

The existence of such a negative feedback pathway has been questioned by Garcia-Munoz et al. (1977) on the basis of lesion experiments. It was found that lesioning the striatonigral pathway caused no change in DA turnover on the lesioned side. Moreover, agonist and antagonist effects upon DA metabolites were the same for the control and lesioned sides. The authors proposed that this was evidence for the lack of a striatonigral negative feedback pathway and further proposed that this pathway may be the striatal motor output pathway.

In addition to these three mechanisms, it has also been suggested that tyrosine availability may also be a controlling factor for DA synthesis (Wurtman et al., 1974). This was not a popular concept at the time because it was believed that TH is

saturated at the normal tyrosine concentration found in brain (100-200μM). The K_m for TH was reported to be 50-90μM. So tyrosine levels exceed the K_m but not remarkably so. Wurtman injected tyrosine and caused a small but significant increase in DOPA accumulation. Later studies showed an increase in NA but not in DA (Ulus et al., 1977). Ulus reported that dopaminergic neurones had to fire rapidly to be sensitive to tyrosine levels. So the possibility exists that tyrosine availability may play a role in the modulation of DA and NA synthesis albeit a minor one.

1.1.4 Dopamine storage

Dopamine is stored in the presynaptic terminal within vesicular storage pools. The accumulation of DA within these pools is achieved by a vesicular uptake mechanism (Philippu & Beyer, 1973; Philippu et al., 1975). This uptake carrier is distinctly different to the plasma membrane uptake carrier. The vesicular uptake carrier is highly dependent upon ATP and Mg^{2+} that stimulates an ATPase to produce an electrochemical H^+ gradient across the membrane (Taugner, 1971; Johnson & Scarpa, 1979). This is used to transport DA across the vesicular membrane (Philippu et al., 1975). Vesicular uptake is also temperature dependent and has a K_m for DA uptake of 1.52×10^{-6}M (Philippu & Beyer, 1973).

DA uptake across the plasma membrane in contrast, is independent of Mg^{2+} and ATP (Holz & Coyle, 1974). The K_m for DA uptake is 1.3×10^{-7}M (Holz & Coyle, 1974). Reserpine and amphetamine are both competitive inhibitors of vesicular uptake (Carlsson et al., 1962; Kirshner, 1962; Lentzen & Philippu, 1977) whereas the process is not sensitive to inhibition by ouabain (Philippu & Beyer,

1973; Philippu et al., 1975) unlike the plasma membrane carrier (Holz & Coyle, 1974).

1.1.5 Dopamine release

Calcium entry into the nerve terminal is the trigger for neurotransmitter release. The dependence of the release process upon calcium was first proposed in the 'calcium hypothesis' of Katz & Miledi (1967), for the neuromuscular junction. Calcium entry is triggered by depolarisation of the nerve cell which opens voltage dependent calcium channels (see Raiteri et al., 1978). The resultant increase in the intracellular concentration of calcium facilitates the release of neurotransmitter substances.

The calcium dependency of DA release has been demonstrated by numerous groups (e.g. Baldessarini & Kopin, 1967; Blaustein, 1975; Holz, 1975; Lane & Aprison 1977; Drapeau & Blaustein, 1983; Nachshen & Sanchez-Armass, 1987). Several of these groups have reported that Ca^{2+} entry is the rate limiting step for DA release during the first few seconds of depolarisation, a timescale which they considered to represent the physiological situation (Drapeau & Blaustein, 1983; Nachshen & Sanchez-Armass, 1987). These groups have demonstrated that dopamine release is directly dependent on the extracellular calcium concentration during the depolarisation of nerve terminals. Moreover, the change in the free cytosolic concentration of calcium induced by nerve stimulation is a linear function of the inward calcium current. However, they have also shown that the relationship between the external Ca^{2+} concentration and dopamine release is highly non-linear. This

non-linearity, they propose, can only be due to the exocytotic process.

The calcium dependence of the release process has been demonostrated pharmacologically. Thus, depolarising stimuli and the calcium ionophore A23187 increase the release of DA (Raiteri et al., 1979). The calcium channel agonist BAY K 8644 has also been demonstrated to increase DA release by increasing the voltage dependent entry of calcium into rat striatal symptosomes (Woodward & Leslie, 1986).

The recent development of in vivo voltammetric techniques has provided a technique with great spatial and temporal resolution for studying in vivo DA release. Electrical stimulation of the medial forebrain bundle has been shown to release DA in the rat caudate nucleus (Miller et al., 1985; Kuhr & Wightman, 1986). Pharmacologically evoked release using high potassium concentrations or neuroleptics, for example, have also been shown to increase DA release using voltam metric techniques (Blaha & Lane, 1983; Gerhardt et al., 1986, 1987; Lane & Blaha, 1987).

A second calcium independent release mechanism has been reported. This is thought to occur by a reversal of the membrane uptake carrier and has been demonstrated for NA in heart slices (Paton, 1973) and in hypothalamic synaptosomes (Raiteri et al., 1977). The possibility of the NA or DA passively diffusing across the membrane of the nerve terminal can be excluded since these molecules are largely ionised at physiological pH. The uptake carrier is responsible for the reuptake of released transmitter amines and will be described in the following section. Under certain pharmacologically induced conditions, it has been proposed

that the uptake carrier operates in the reverse direction to transport amines from inside the nerve terminal to the outside (Raiteri et al., 1979; Fischer & Cho, 1979). The involvement of the uptake carrier in this process is demonstrated by the fact that nomifensine, a potent inhibitor of the DA uptake carrier (Horn et al., 1971; Hunt et al., 1974), inhibits this elicited DA release. Similarly, desipramine, a NA uptake inhibitor, reduced NA release elicited by a Na^+-free medium, from heart slices (Paton, 1973) and from hypothalamic synaptosomes (Raiteri et al., 1977).

1.1.6 Transmitter inactivation

Termination of the extracellular action of monoaminergic neurotransmitters in the CNS is probably achieved by compartmental sequestration by specific reuptake into the nerve endings from which they were released. This was proposed for NA during the 1960's (see Coyle & Snyder, 1969) and was later shown to account for 70-80% of released NA in the periphery (Iversen, 1975). The existence of a similar mechanism for DA was also proposed during the 1960's when the accumulation of [^{3}H]-DA was observed in specific brain areas (Fuxe & Ungerstedt, 1968). Coyle & Snyder (1969) then reported that reuptake of released DA from synaptosomes accounts for the physiological inactivation of the transmitter. Since these early studies, it has been shown that specific uptake carriers exist for the reuptake of catecholamine neurotransmitters in the periphery and the CNS (Horn et al., 1971; Hunt et al., 1974; Kannengiesser et al., 1976; Hunt et al., 1979; Horn, 1979) and that this is the primary method for the inactivation of these released

transmitters. However, in addition to presynaptic reuptake, it is becoming clear that uptake of released neurotransmitters may involve postsynaptic sites and glial cells.

Glial uptake has been reported for amino acids (Fonnum, 1984; Mize et al., 1981) as well as monoamines (Henn & Hamberger, 1971; Hoffman & Vernadakis, 1979). In astrocyte cultures, high affinity uptake of [^{3}H]-NA and [^{3}H]-DA has been demonstrated with a K_m of 0.3μM, a dependence upon Na^+ and sensitivity to inhibition by tricyclic antidepressants (Semenoff & Kimmelberg, 1985). The functional significance of this type of uptake is unclear and in the absence of an in vivo technique for determination of the contribution made by glial cells in transmitter inactivation, only speculations have been made.

Non-dopaminergic cells have been reported to accumulate released DA in the substantia nigra (Kelly et al., 1985). This was demonstrated in vivo in nigral slices and is evidence of a possible non-dopaminergic postsynaptic uptake of DA. Once again, the significance of such an uptake process is not clear and may not be relevant under physiological conditions. However, the possibility exists that both glial and postsynaptic uptake sites are operative and may serve to inactivate released neurotransmitter that is not captured by presynaptic uptake.

Characteristics of neuronal dopamine uptake

The DA reuptake mechanism is temperature-sensitive and is linear for the initial two minutes in striatal synaptosomes (Holz & Coyle, 1974). Holz & Coyle also reported a concentration gradient of 1000:1 to be achieved in striatal synaptosomes. The system displays Michaelis-Menten kinetics with a K_m for DA = 1.3 x

10^{-7}M. The uptake process is sodium dependent, less dependent upon potassium and does not require calcium (Horn, 1978).

Various β-phenylethylamine analogues of dopamine are also good substrates for the uptake carrier. The structure activity relationship is similar to that for the NA uptake carrier (Iversen, 1971).

1. Phenolic hydroxyl groups in the para or meta position enhance the affinity for uptake.
2. α-methylation enhances uptake affinity.
3. β-hydroxylation decreases uptake affinity.
4. Mono or di N-methylation decreases uptake affinity.
5. O-methylation of the phenolic hydroxyl group decreases uptake affinity.

The uptake process is susceptible to metabolic inhibition by drugs or by artificial conditions that interfere with the Na^+ gradient of the plasma membrane. Thus, a lowering of the extracellular Na^+ concentration causes an increased release of [^{3}H]-DA from striatal synaptosomes that is thought to occur by a reversal of the DA uptake carrier (see Raiteri et al., 1978). An increase in the intracellular concentration of Na^+ caused by the Na^+–K^+ ATPase inhibitor ouabain or by the Na^+ channel opener veratridine also stimulates [^{3}H]-DA release from striatal synaptosomes (Raiteri et al., 1978).

Dopamine uptake inhibition

The therapeutic action of tricyclic antidepressants such as imipramine and desimipramine was suggested to be achieved by the inhibition of NA uptake (Fuxe & Ungerstedt, 1968; Coyle & Snyder, 1969; Samanin et al., 1975). This class of uptake inhibitors has

very little activity against DA uptake which is suggestive of a specific uptake carrier for DA distinct from that for NA (see Samanin et al., 1975). Further evidence for this was obtained with the discovery of a potent DA uptake inhibitor, nomifensine (see Hunt et al., 1974). This has an IC_{50} for inhibition of DA uptake of 1.45×10^{-7}M which is better than previously proposed DA uptake inhibitors such as benztropine, $IC_{50} = 3.1 \times 10^{-7}$M and amphetamine, $IC_{50} = 1.2 \times 10^{-6}$M. However, whilst nomifensine is a potent inhibitor of DA uptake, it is not specific for DA since it has been demonstrated to be more potent for the inhibition of NA uptake with an $IC_{50} = 1.2 \times 10^{-8}$M (Heikkila & Manzino, 1984). Since the discovery of nomifensine as a potent DA uptake inhibitor, another range of compounds have emerged with a greater potency than nomifensine and a much better specificity for DA uptake (Heikkila & Manzino, 1984). These are GBR 13069, GBR 13098 and GBR 12909. Their IC_{50} values for inhibition of DA uptake are 4×10^{-8}M, 4.3×10^{-8}M and 5.1×10^{-8}M respectively. Their specificity is apparent from their IC_{50} values for inhibition of NA uptake (8×10^{-7}M, 5.6×10^{-7}M and 2.6×10^{-6}M, respectively). These are the most specific compounds presently known for the inhibition of DA uptake. Nomifensine was used for the inhibition of DA uptake in experiments presented in this thesis. The experiments were performed in the striatum of the rat which receives very little noradrenergic innervation. Consequently, nomifensine was considered suitable as a DA uptake inhibitor in this region.

It has now been shown that the action of amphetamine is primarily as a releasing agent of DA and not as an uptake inhibitor (Heikkila et al., 1975; Hunt et al., 1979). Prior to this, the

situation was confusing since it had been proposed that an uptake inhibitor could be confused as a releasing agent. Thus, by inhibiting the reuptake of released transmitter, the extraneuronal concentration of transmitter will increase so resembling transmitter release. The situation was resolved somewhat by Hunt et al. (1979) who reported that it was possible to distinguish drugs acting purely as DA uptake inhibitors from those that mainly enhance DA release. In their experiments, they compared nomifensine with amphetamine. They used striatal synaptosomes preloaded with [^{3}H]-DA and found that even at a high concentration of 10^{-5}M, nomifensine only caused a small increase in the amount of radioactivity in the medium. Amphetamine on the other hand caused a much greater increase of [^{3}H]-DA in the medium. This effect was diminished when the two drugs were incubated simultaneously and not enhanced as it would be if both were acting as DA releasers. An alternative approach was adopted by Raiteri and co-workers (1975) who devised a superfusion technique to discriminate between an uptake inhibitor and a releaser. The rationale of their technique was that the released substrate is removed by the stream of superfusion fluid as soon as it is released, so preventing re-uptake. Consequently, a drug which does not exhibit any releasing activity in these conditions, can be considered as a pure uptake inhibitor. Using these criteria, Raiteri et al. (1978) classified nomifensine as a pure uptake inhibitor. They have also suggested that amphetamine has a mixed action in that it both releases and inhibits the uptake of [^{3}H]-DA in synaptosomes from rat striatum (Raiteri et al., 1975).

The identity of nomifensine as a DA uptake inhibitor has

provided an important method for the study and exploitation of the DA carrier and the consequences upon dopaminergic neurotransmission.

Dopamine metabolism

Dopamine is metabolised by the action of two enzymes, monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT). MAO cleaves the amine group from the DA molecule to form the corresponding acid metabolite, namely 3-4-dihydroxyphenylacetic acid (DOPAC) (see Tipton, 1979). COMT transfers an O-methoxy group to the 3-position of the phenyl ring of DOPAC to form 3-methoxy-4-hydroxyphenylacetic acid, usually know as homovanillic acid (HVA) (see Guldberg, 1979). COMT also acts upon DA to form 3-methoxy-4-hydroxyphenylethylamine or 3-methoxytyramine (3-MT). 3-MT can subsequently be metabolised to HVA by the action of MAO. The K_m values of MAO for the two substrates DA and 3-MT are 405 and 475μM respectively (Houslay & Tipton, 1976), although more recently, a much lower K_m for DA has been reported of 3μM (Schoemaker & Nickolson, 1983). The K_m value of COMT for DA was reported to be 160μM (Guldberg, 1979). DOPAC and HVA are also further metabolised to some extent to their corresponding sulphate conjugates which aids their removal from the brain (Dedek et al., 1979). 3-MT is not metabolised to a sulphate conjugate.

In vertebrate brain, as well as other tissues, MAO is present in two forms, MAO A and MAO B. This was first proposed by Johnston (1968) when he noticed a biphasic decline in MAO activity in the presence of increasing concentrations of the MAO inhibitor clorgyline. He associated this with two forms of MAO with different sensitivites to inhibition. Since this early report, many investigators have reported the presence of two forms of MAO

and attempted to localise these to different cell types within the CNS (Yang & Neff, 1974; Braestrup et al., 1975; Agid et al., 1973; Houslay & Tipton, 1976; Demarest et al., 1980; Schoepp & Azzaro, 1983; Francis et al., 1985; Kato et al., 1986; Kitahama et al., 1987; Ross, 1987; Liccione & Azzaro, 1988). Several groups have demonstrated that MAO A is located predominantly presynaptically (Agid et al., 1973; Demarest et al., 1980; Francis et al., 1985). These determinations were made by immunohistochemical and selective lesioning techniques. This led Francis and co-workers (1985) to propose that striatal MAO A was located 60% presynaptically and no more than 30% postsynaptically. MAO B,on the other hand, has been reported to be located predominantly postsynaptically and extraneuronally (Levitt et al., 1982; Francis et al., 1985). Levitt & Co-workers used kainic acid to selectively lesion striatal afferents and interneurones and demonstrated MAO B in postsynaptic and glial cells with only a small proportion located presynaptically.

MAO A and MAO B have different substrate specificities and different inhibitor sensitivities (see Houslay & Tipton, 1976 for review). This allows the use of selective inhibitors to achieve selective pharmacological effects. NA and 5-HT are MAO A preferring substrates whilst benzylamine and phenylethylamine are MAO B preferring substrates. DA and 3-MT are mixed substrates for both enzymes (Houslay & Tipton, 1974). Clorgyline is a relatively selective inhibitor of Type A MAO (Johnston, 1968) whilst selegeline is a relatively selective inhibitor of type B MAO (Knoll & Magyar, 1972).

The existence of two forms of MAO suggests a functional duality of the enzymes. However, it is still not clear whether or not MAO

B plays a role in striatal DA metabolism.

1.1.7 Classification of DA receptors in the CNS

During the mid-1970's, several groups identified binding sites for DA based upon the binding of tritiated DA agonists or antagonists to brain membranes and their subsequent displacement by cold ligand (Creese et al., 1976; Burt et al., 1976; Seeman et al., 1976; Creese et al., 1977). It was realised that a subclass of DA receptors were linked to the enzyme adenylate cyclase which was thought to be the second messenger system mediating dopaminergic transmission. On the basis of this biochemical evidence, Seeman (1977) and Kebabian & Calne (1979) proposed the existence of two classes of DA receptor, the D_1 receptor, associated with adenylate cyclase and the increased formation of cAMP, and the D_2 receptor, which they proposed was not linked to adenylate cyclase.

Since this original classification of DA receptors, other groups have proposed the existence of as few as one or as many as four distinct DA receptor subtypes. These conclusions were largely based on binding studies and different affinities of receptors for ligands. It eventually became accepted that these multiple forms were different affinity states of just two DA receptors and the consensus now is that there are only two distinct types of DA receptor (see Kebabian et al., 1986).

The original proposal made by Kebabian & Calne has since been modified to state that D_2 receptors inhibit the activity of adenylate cyclase (Stoof & Kebabian, 1984). Moreover, peripheral subtypes of DA receptors have also been identified and are known as DA_1 and DA_2 receptors (see Woodruff, 1986).

Progress has also been made in terms of selective DA receptor agonists and antagonists (see Stoof & Kebabian, 1984; Woodruff, 1986). Selective agonists for the D_1 receptor include SKF 38393 which is more potent than DA for stimulating the production of cAMP by adenylate cyclase (Stoof & Kebabian, 1984). Also included is SKF 82526 which is less potent than SKF 38393 and has slightly more D_2 agonistic activity. The development of a selective D_1 receptor antagonist SCH 23390 was an important development since it has contributed to our scant knowledge of the function of D_1 receptors. SCH 23390 has little effect on D_2 receptors, being at least 950 times more effective on D_1 receptors than D_2. Selective D_2 agonists include RU 24926, RU 24213 and LY 141865, whilst D_2 antagonists include domperidone and sulpiride (see Stoof & Kebabian, 1984).

Physiological and biochemical roles have been attributed to the two types of DA receptor. D_1 stimulation has been shown to enhance hormone release from bovine parathyroid gland and to enhance the firing of neurosecretory cells in the CNS of Lymnaea stagnalis (Stoof & Kebabian, 1984). D_1 receptors have also been implicated in the behaviour of rodents, in particular in its role in passive avoidance behaviour in rats (Woodruff, 1986).

D_2 receptors have been implicated in the inhibition of prolactin and α-MSH release from the pituitary, the inhibition of acetylcholine ACh and β-endorphin release, and the autoinhibition of DA release and turnover in the striatum.

1.1.8 Postsynaptic effects and functions of dopamine in the CNS

Since there is a widespread distribution of dopaminergic

terminals within the striatum, nucleus accumbens, hypothalamus and cortical areas, there is likely to be a multiplicity of DA functions. Consequently, factors affecting dopaminergic neurotransmission as a whole are likely to affect many functions subserved by the areas where DA neurones terminate. This point should be borne in mind when determining experimental data, especially in behavioural studies.

DA has been implicated in many functions such as motility, exploration, sensory and motor control, feeding, sexual arousal, aggression, drive, sleep, stress and learning and attention (Ungerstedt, 1979, 1984). Thus, apomorphine (a DA agonist) and amphetamine (a DA releaser) result in behavioural excitation such as increased motor activity and also in various stereotyped behaviours including repetitive head and forelimb movements, sniffing, grooming, chewing and gnawing (Moore, 1978; Costall & Naylor, 1979). The depletion of DA with reserpine or DA receptor blockade by antagonists results in hypokinesia and catalepsia. Lesion of the nigro-striatal pathway with 6-hydroxydopamine results in akinesia, adipsia and aphagia (Ungerstedt, 1971b). These are some of the examples of the effects of excess or decreased levels of DA in the CNS.

As mentioned previously, the nigrostriatal pathway is a major dopaminergic pathway. The degeneration of this pathway has long been recognised as a causal factor of Parkinson's disease (Ehringer & Hornykiewicz, 1960) (see Anden, 1979). Thus, the DA content of human post-mortem brains of Parkinsons disease patients is virtually abolished. The symptoms of the disease are temporarily relieved by the DA precursor L-DOPA (Birkmayer & Hornykiewicz, 1961) (see Anden,

1979), so implicating DA in the control of extrapyramidal motor function. However, despite the recognised importance of the nigrostriatal pathway in the mediation of this disease, the precise function of the pathway is not understood. A role in the control of motor behaviour is clear, but it is not known whether the nigrostriatal pathway is excitatory or inhibitory or both. Electrophysiological, pharmacological and morphological methods have been used to try and establish this (see Freund et al., 1984). Electrophysiological work has described both inhibition and excitation in the striatum after stimulation of the substantia nigra (see Walters et al., 1984). Stimulation of D_2 autoreceptors in the substantia nigra has been shown to have an inhibitory response upon cell firing which has been associated with an increase in K^+ conductance. This causes a hyperpolarisation of these cells (Bunney et al., 1973; Lacey et al., 1987). A similar phenomenon has been reported in striatal neurones (Freedman & Weight, 1988). Freedman used whole cell current clamp and voltage clamp upon dissociated striatal neurones, and demonstrated that DA and the D_2 agonist quinpirole opened K^+ channels in the neuronal membrane. This action was antagonised more potently by the D_2 antagnoist spiperone than by the D_1 antagonist SCH 23390. Freedman suggests that such a D_2 mediated effect upon K^+ conductance in striatal neurones may mediate inhibitory responses associated with D_2 receptors in the striatum. However, he does not rule out the involvement of other ion channels since D_2 receptors also mediate excitatory responses in the striatum. Pharmacological studies are not clear because of the lack of discrimination between direct actions of the nigrostriatal pathway or indirect actions involving

other striatal neurones. Morphological methods have been used in attempts to identify the neurones receiving input from nigrostriatal fibres. It was long held that cholinergic neurones received a large input but the methods used have since been shown to be non-specific (see Freund et al., 1984). So there is still no consensus with any of the methods.

Freund and co-workers (1984) have suggested a more subtle effect of dopamine in the striatum based upon their morphological work that studies synaptic contacts upon striatonigral neurones. They propose that DA released from nigrostriatal terminals can directly influence the functioning of striatonigral neurones by means of different synaptic specialisations located on anatomically distinct parts of striatonigral neurones. Through these specialisations they propose that D_1 receptors might mediate hyperpolarisation so leading to the attenuation of cell firing and that D_2 receptors mediate an increase of membrane conductance to certain ionic species that slightly depolarises the neurone. This may selectively cut off the input from a particular group of afferent neurones. Consequently, the overall effect of DA upon striatonigral neurones may not simply be one of excitation or inhibition, but rather a selective control of the pattern of firing. In this fashiion, the striatum may convert a highly diverse input into a specific pattern of motor output.

1.2.1 Neurotransmitter compartmentalisation

There was, and still is, considerable research effort devoted to the subject of vesicular storage of ACh in nerve terminals. The same is true for two of the monoamine transmitters, NA and DA, to

which the research expanded and encompassed.

Compartmentalisation of a transmitter concerns the heterogeneous location of a transmitter within the nerve terminal. The concept has been applied to ACh (see Zimmermann, 1979), NA (see Bavisch & Trendelenburg, 1987; Eisenhofer, 1988) and DA (see de Belleroche & Bradford, 1978).

Zimmermann (1979) in a commentary summarises the early evidence for not only a heterogeneity of ACh storage, but specifically a vesicular heterogeneity. The current controversy at the time was whether or not ACh was located solely in the cytoplasm or was heterogeneously distributed between the cytoplasm and a vesicular store. Using radiolabelled ACh in the electric organ of Torpedo, Zimmermann showed that not only was ACh taken up and stored in vesicles, but that there was also a heterogeneity of vesicular pools. He reported the appearance of smaller vesicles in the nerve terminal following electrical stimulation. These vesicles had a higher density than the larger group and contained newly synthesised ACh. This was apparent from the high specific activity of the radiolabelled ACh that rapidly appeared in those vesicles as opposed to the low levels in the larger vesicles. Thus Zimmermann proposed that ACh is preferentially released from and taken back up into the smaller vesicles. This does not deny the existence of a cytoplasmic pool of ACh as argued by Dunant & Israel (1979). However, the evidence was that the rapidly turning over pool of vesicular ACh represented the functional pool of transmitter.

This has also been demonstrated in sympathetic ganglia, the cerebral cortex and Torpedo electric organ (see de Belleroche & Bradford, 1978) where the most recently synthesised ACh was released

in response to stimulation.

Parallels are thought to exist for monoamine transmitter storage and release. Thus, Eisenhofer et al. (1988) report the distribution of [^{3}H]-NA in rat vas deferens into two pools, one containing a high specific activity and the other a low specific activity of radiolabel. Eisenhofer studied the effects of tyramine, electrical stimulation and elevated K^+ concentration upon [^{3}H]-NA and [^{3}H]-DHPG (the deaminated metabolite of NA) release from isolated rat vas deferens. He proposed the existence of two intraneuronal pools of NA, a fast release labile pool that preferentially contains newly synthesised NA and NA from reuptake, and a slow release pool that contains most of the tissue NA and which serves mainly as a storage pool.

Other workers using rat vas deferens demonstrated the release of NA from two intraterminal pools, a vesicular pool and a cytoplasmic pool (Langeloh et al., 1987; Langeloh & Trendelenburg, 1987).

Burn (1932) was the first to suggest that catecholamines might be taken up into storage sites in tissue. However, it was not until radiolabelled catecholamines became available that Axelrod et al. (1959) and Whitby et al. (1961) injected [^{3}H]-NA i.v. which was seen to be taken up into peripheral tissues. The first direct evidence for the accumulation of DA in brain tissue was obtained by Glowinski & Iversen (1966). They injected [^{3}H]-DA intraventricularly and observed accumulation in the following areas in order of decreasing concentration: striatum > hypothalamus > hippocampus > midbrain > medulla oblongata > cortex and cerebellum. This early work initiated numerous studies of DA storage and metabolism in nerve cells. This rapidly became a

controversial issue relating to catecholamine neurotransmission, the major controversy centering around the state of the transmitter stores in the neurone. There were two views on this problem. The first proposed that catecholamines are stored in a single open compartment or pool from which they are released by action potentials or drugs. The second proposed the existence of more than one compartment, a functional pool where transmitter is synthesised at a fast rate and from where it is preferentially released, and a second storage pool.

Both possibilities were studied during the late 1960's into the 1970's. The most direct method at the time for the in vivo determination of the disposition of an endogenous compound was to inject a tracer dose of the radioactive precursor of that compound and to follow the kinetic changes of the specific activity of the compound as a function of the specific activity of the precursor. Using this method, Neff et al. (1971) demonstrated that the curves expressing the time changes of the specific activities of brain DA and the precursor tyrosine were interrelated according to the model describing a direct product-precursor relationship in a single open compartment. Also, in earlier work, Costa & Neff (1966) followed the decline in tissue DA concentration after AMT was given. They obtained half lifes for DA utilisation of 2 hours in whole rat brain and 2.1 hours in rabbit caudate nucleus. DA levels were observed to decline in a monophasic fashion and on the basis of these results they assumed a single storage pool of DA. However, both sets of results were later disputed by other workers. Groppetti et al. (1977) highlighted the drawbacks associated with the use of [^{3}H] tyrosine as a marker for DA synthesis. Firstly, tyrosine is

involved in other metabolic pathways and so it has to be assumed that the various tyrosine compartments reach identical specific activities. The results presented by Neff neglected this fact. Secondly, the labelled precursor method only allows the measurement of overall changes and will not detect the existence of heterogeneous compartments unless each is large enough to form a significant portion of the pool of transmitter. The significance of this is that a functionally important pool which may be small in size with a high transmitter turnover rate may be overlooked when using the labelled precursor technique.

This fact was pointed out by Javoy & Glowinski (1971). They injected AMT and observed a biphasic decline in tissue DA levels. A similar phenomenon was reported by Iversen & Glowinski (1966) when they injected [^{3}H]-DA intraventricularly and observed a biphasic disappearance of this tracer. Within five minutes of injecting AMT, Javoy & Glowinski observed a rapid decline of DA levels in rat striatum with a t1/2 of 9 min. This was followed by a slower decline from 30 min onwards with a t1/2 of 124 min. The authors suggested that this multi exponential decline is indicative of the heterogeneous location of DA in the striatum. They proposed a two compartment model, compartments A and B. AMT caused the rapid depletion of DA in compartment A. This contains at least 23% of the DA and is regarded as the functional compartment from which DA is preferentially released. Compartment B is the main storage compartment from which DA is utilised at a much slower rate. The authors suggest it is this compartment that Costa & Neff measured in their experiments on whole rat brain and rabbit caudate with DA half lifes of 2 hours and 2.1 hours respectively. From the rate of DA

decline, Costa & Neff calculated the rate of synthesis of DA in rabbit caudate nucleus to be 2.85μg/g/h. This was estimated on the basis of a single compartment. Javoy & Glowinski estimated a DA turnover rate of 13.2μg/g/h whilst Javoy et al. (1970) estimated the in vivo synthesis rate of DA in striatum on the basis of the initial rise in DA after MAO inhibition to be 24μg/g/h. These two values suggest a rate of synthesis at least four times that of Costa & Neffs value. Javoy & Glowinski suggested that this large discrepancy demonstrated that the hypothesis for a distribution of DA in a single compartment could not be retained.

Further credence was given for the existence of more than one DA storage compartment by the results of Groppetti et al. (1977). They adopted an alternative approach by looking at the levels of DOPAC and 3MT. If the single compartment model is valid, they hypothesised that they would see the same product-precursor relationship postulated by Neff et al. (1971). So by injecting labelled tyrosine intravenously and intraventricularly, they measured the specific activities of [^{3}H]-DA, [^{3}H]-DOPAC and [^{3}H]-3MT. If only one compartment exists, then the product-precursor relationship should be valid and this being the case, the specific activity of DA should never be lower than that of the metabolites DOPAC or 3MT. But [^{3}H]-DOPAC and [^{3}H]-3MT had much higher specific activities than [^{3}H]-DA suggesting DA is stored in more than one compartment.

Post-mortem studies in rats also suggested a heterogeneity of pools. Carlsson (1974) reported a rapid increase in 3MT levels due to post-mortem metabolism of DA. This increase was more evident for endogenous DA than for labelled DA since labelled 3MT increased

by only 50% over 20 min whereas endogenous 3MT increased 35-fold over the same time. So this 3MT was converted from a pool of DA that was poorly labelled by the radioactive precursor.

The results obtained by Javoy & Glowinski (1971) describing a biphasic decline of DA following administration of AMT were later disputed by Costa and co-workers (Doteuchi et al., 1974). Costa did not dispute the likelihood of multiple pools of DA as he had earlier, but rather the incorrect results drawn from the methodology used by Javoy & Glowinski. This is probably worth mentioning since it highlights the problems associated with using DA synthesis inhibitors as a tool for determination of DA levels.

Costa argues that the biphasic decline in DA levels is due to (1) an early incomplete inhibition of tyrosine hydroxylase and (2) an increased release of DA during the first 20 min of AMT stimulated by the metabolites of AMT, para-hydroxyamphetamine and para-hydroxynorephedrine. Costa states that both of these factors served to confuse the results and mislead the authors. The early rapid decline in tissue DA was due to the DA releasing actions of the metabolites. The levels of these decline after the first few minutes and by 40 min AMT inhibition of tyrosine hydroxylase is almost complete. When applying the principle of steady state kinetics to the decline of neurotransmitter after synthesis inhibition, the inhibition must be complete and instantaneous. These requirements are not fulfilled by AMT which negates the figures proposed by Javoy & Glowinski but not the principle of multicompartments in the DA neurone.

The fact that DA seemed to be heterogeneously distributed, led to the speculation that the different pools of DA were functionally

distinguishable. Javoy & Glowinski (1971) and Glowinski (1975) reported that newly synthesised DA was preferentially released from striatal synaptosomes. The authors used [^{3}H] tyrosine to label [^{3}H]-DA pools within the synaptosomes. They found that the rapidly turning over pool of DA became labelled with [^{3}H]-DA and that this [^{3}H]-DA was preferentially released. Other groups reported similar findings. Thus, de Belleroche & Bradford (1978) used [^{3}H] tyrosine to differentially label pools of synaptosomal DA. They found that stimulation released newly synthesised [^{3}H]-DA as did other groups (Raiteri et al., 1978, 1979; Katz et al., 1976; Kapatos & Zigmond, 1977). So in synaptosomes at least, DA is heterogeneously located in two or more compartments and is preferentially released from one of them.

The newly synthesised releasable pool of DA was proposed to be a vesicular pool (de Belleroche & Bradford, 1978; Raiteri et al., 1978; 1979). This was based upon the sensitivity of this pool to reserpine. A second, cytosolic pool of DA was reported to be the pool where DA is accumulated in the presence of a MAO inhibitor (Raiteri et al., 1978) and from where the carrier mediated release of DA occurs (Raiteri et al., 1978, 1979; Fischer & Cho, 1979). This cytosolic compartment is not reserpine sensitive (Schoemaker & Nickolson, 1983). The transport of DA to the reserpine sensitive pool was reported to follow Michaelis-Menten kinetics (Schoemaker & Nickolson, 1983) which is in agreement with the transport kinetics found for DA uptake by isolated rat striatal synaptic vesicles (Philippu & Beyer, 1973). This is further evidence for the vesicular location of the releasable pool of DA.

Finally, the <u>in vitro</u> work of Schoemaker & Nickolson (1983)

using striatal synaptosome preparations yielded further results in support of a multi compartmental DA storage mechanism. They studied the kinetics of DA transport into and out of striatal synaptosomes. They reported that under steady state condition, the transport rates of DA are independent of time and follow first order kinetics. The data obtained fitted significantly better to a three compartment model (two intrasynaptosomal and one extrasynaptosomal) than to a two compartment model (one intrasynaptosomal, one extrasynaptosomal). They then considered the distribution of these compartments and the nature of the DA uptake into them. The possibilities are a parallel uptake from the medium into each compartment or an in series uptake where DA is first taken into one compartment from where it can then pass to the second compartment.

If the parallel uptake process occurred, then DA uptake would be biphasic due to the differing kinetic parameters for uptake. This process would fit a biphasic Hofstee plot of the initial DA uptake velocity. But DA uptake by rat striatal synaptosomes can be described by a single Michaelis-Menten process (Snyder & Coyle, 1968; Schoemaker & Nickolson, 1983). So they report that it is most plausible that the in series configuration represents the actual configuration of the kinetic compartments.

Since some of the early work quoted here, the question of multiple intraterminal compartments or pools of DA has been studied by several groups (e.g., Fischer & Cho, 1979; Kamal et al., 1981; Liang & Rutledge, 1982; Langer & Arbilla, 1984; Niddam et al., 1985; Herdon et al., 1985). The existence of two intraterminal pools of DA in the striatum was recently proposed by Leviel & Guibert (1987). By taking advantage of the reported differences in

the renewal speed or turnover of DA for the different pools, they reported the ability to preferentially label the functional pool of DA *in vivo* by perfusing with [^{3}H]-tyrosine. Thus, the functional pool which has a renewal time of 9 min contains a high specific activity of [^{3}H]-DA whereas the storage pool is relatively poorly labelled within the first few minutes since it has a renewal time of about two hours. They worked on the premise that [^{3}H]-DA release largely reflected release from the functional pool whereas total DA release including unlabelled endogenous DA reflected release from the storage pool of DA. AMT was given and a rapid decline in [^{3}H]-DA levels observed. Total DA levels decreased much more slowly. Their conclusion was that this was an indication of the differential involvement of the two intraterminal pools in DA release. Similar results were reported for AMPH stimulated release of [^{3}H]-DA (Miller & Shore, 1982; Niddam et al., 1985), where AMPH preferentially released [^{3}H]-DA.

It now seems to be generally accepted that DA, as well as other transmitters, exists within more than one intraterminal compartment. However, much speculation still exists in relation to the size and physiological significance of multiple pools of transmitter (see Paden, 1979). A discussion of these points will be presented in Chapter 4. In addition, another model has been proposed and tested by Justice et al. (1988)in which three intraterminal pools of DA exist. A free cytosolic pool, a releasable bound pool and an inactive bound pool. The implications of this will also be discussed later.

1.2.2 Compartmentalisation and organisation of DA metabolism

The precise locations of the DA metabolising enzymes is still a matter of controversy. MAO is thought to be predominantly located intraneuronally. Consequently, the major proportion of DOPAC is derived from the intraneuronal metabolism of DA. COMT is proposed to be located predominantly postsynaptically so implying a compartmentalisation of DA metabolism. This would explain why reserpine, which disrupts the storage of intraneuronal DA, gives rise to increased DOPAC levels before those of HVA indicating an intraneuronal formation of DOPAC (Guldberg & Broch, 1971; Roffler-Tarlov et al., 1971). This is achieved by the action of MAO which has been localised to presynaptic nerve terminals (Agid et al., 1973; Demarest et al., 1980; Francis et al., 1985). It was then considered that DOPAC could be used as an index of DA metabolism (Roffler-Tarlov et al., 1971). However, since these proposals, there has been an indication of an intraneuronal as well as an extraneuronal location of COMT (Guldberg & Marsden, 1975). This was also proposed by Korf et al. (1976) who observed an early rise of DOPAC followed by a secondary rise of HVA after reserpine and also after neuroleptic or cholinomimetic treatment and electrical stimulation, all of which increase DA release or metabolism. It now seems likely that DOPAC is the primary metabolite of intraneuronal DA metabolism whilst HVA is the secondary. HVA may be formed intraneuronally from DOPAC or postsynaptically from DOPAC. The possible locations of COMT outside the dopaminergic terminal are in postsynaptic cells, in glial cells or in endothelial cells of blood capillaries (see Kaakkola et al., 1987). HVA is also formed by the metabolism of DA

first to 3MT and then to HVA. The functional significance of this route of metabolism and indeed whether or not a significant proportion of DA is routinely metabolised to 3MT is still a matter of great speculation.

Following release, a fraction of the monoamine transmitter is metabolised during reuptake (see Westerink, 1979). The metabolic products do not re-enter the transmitter synthetic pathway. Neuroscientists have attempted to utilise this characteristic in attempts to determine changes in dopaminergic neurotransmission. Since during neuronal activity, a proportion of the released transmitter is not recovered, it is probable that steady state levels of the transmitter are maintained by new molecules of the precursor tyrosine entering the synthetic pathway. Consequently, the activity of tyrosine hydroxylase has been used as an assessment of the activity of dopaminergic neurones. As a further consequence, changes in dopamine metabolite levels have come to be equated with changes in dopaminergic neuronal activity. Thus, a neurochemical measure has been correlated with a neurophysiological response (e.g., Roth et al., 1976; Umezo & Moore, 1979; Waldmeier et al., 1981; Westerink & Spaan, 1982; Wood et al., 1987). The resulting literature is littered with controversy, speculation and contrary results. Before considering some of these results, it is important to elucidate the connections between monoamine synthesis, release and metabolism.

Firstly, what information is conveyed by monoamine metabolites? Korf et al. (1973) stated that in the normal untreated animal, monoamine metabolites are an indication of the metabolic intactness of the monoaminergic neurones. Under

experimental conditions, DOPAC and HVA reflect the metabolic functioning of dopaminergic neurones. It may be speculative to conclude any more than this. But, if DA metabolism is connected to DA release, then surely it may be used as an indication of release? However, studies of noradrenergic neurones in the spinal cord have suggested that NA is stored at a concentration of about 50% of the holding capacity of the nerves. If synthesis is stimulated by the addition of tyrosine or L-DOPA, the increased level of newly synthesised transmitter results in the immediate metabolism of this transmitter to maintain constant levels. No definitive evidence exists that this transmitter is released before it is metabolised (Commissiong, 1985). Commissiong also states that this seems to be the case for DA and 5-HT as well as NA in the spinal cord. This may hold for the brain as well.

A further problem in taking DA metabolites as indices of dopaminergic neurotransmission or release is that it has been shown that DA is synthesised and metabolised to DOPAC and HVA in non-monoaminergic cells, possibly in other neuronal cells, capillary endothelium or glial cells. This was surmised by Commissiong et al. (1984) and by Hefti et al. (1981) when DA synthesis and metabolism persisted even after the destruction of dopaminergic neurones.

It has already been suggested that increased levels of DA metabolites are not necessarily indicative of increased release of DA. Digiulio et al. (1978) reported increased levels of DOPAC but not HVA, which is indicative of increased intraneuronal metabolism, as a response to chloral hydrate. Here we have a situation again where there appears to be increased DA metabolism in the absence of

increased DA release. Certainly, when increased release is directly measured, there is also increased metabolism. The mistake would appear to be in assuming the opposite, that increased metabolism is indicative of increased release.

From the preceding discussion, it might appear that DA metabolites can convey very little information about the state of dopaminergic transmission. This is probably not the case. For example, an increase in DOPAC levels is indicative of the increased neuronal metabolism of DA but not of the increased release of DA for the reasons outlined above. Taking this further, several authors (e.g. Groppetti et al., 1977; Cubeddu et al., 1979) have proposed that DOPAC is formed intraneuronally from newly synthesised DA and not from vesicularly stored DA since vesicular DA is protected from metabolism by MAO. Thus, DA antagonist treatment, which increases DA synthesis and may result in increased metabolism of this newly synthesised DA, does not increase DA release (Zetterstrom et al., 1984). Conversely, drugs that release vesicularly stored DA should have no effect upon DOPAC levels. Thus methylphenidate and tyramine, which both release vesicular DA, had no effect upon DOPAC levels, whereas amphetamine and potassium chloride both decreased DOPAC levels suggesting that they released non-vesicular newly synthesised DA (Zetterstrom et al., 1988). These results suggest that DOPAC may be a useful indicator of intraneuronal DA metabolism and also may be useful as a determinant of the intracellular source of pharmacologically released DA.

Information conveyed by pharmacologically induced changes in levels of HVA is probably more limited than that for DOPAC. HVA is considered to be a secondary metabolite of DA and, as such, is a

less suitable indicator of changes in DA metabolism.

Much controversy surrounds the use of 3MT as an indicator of DA release. 3MT is a metabolite of DA but the physiological significance of this, and indeed, whether or not it represents a normal and significantly used route of metabolism for DA remains questionable. However, it has been proposed to be a good indicator of DA release (Wood et al., 1987; Westerink & Spaan, 1982). Other groups disagree with this (Waldmeier et al., 1981; Vulto et al., 1986).

The rationale for 3MT accumulation indicating DA release is that COMT is located predominantly outside the dopaminergic terminal. Therefore, 3MT is formed from extracellular DA that must first be released (Wood et al., 1987). These authors also report that 30% of extracellular DA is metabolised to 3MT. It is important to remember that in the normal situation in the absence of any pharamcological manipulation, most released DA is taken back up into the presynaptic terminal. Indeed, it may be necessary to artificially raise extracellular DA levels before seeing any rise in 3MT levels. Wood and co-workers also state that the turnover of 3MT is very rapid, being rapidly transformed to HVA by MAO. However, this is contrary to the results of Westerink & Spaan (1982). They report a turnover rate for DA of 20-30nmol g^{-1} h^{-1} and for 3MT of 2-3nmol g^{-1} h^{-1} in control rats suggesting a maximum of 10% of the metabolism of DA is via 3MT. Moreover, after neuroleptic treatment, DA formation was shown to increase 3-4 fold without a correlating increase in 3MT turnover (Westerink & Spaan, 1982). Westerink suggests that since the deamination of DA by MAO is much more efficient than the O-methylation by COMT, as

shown by the more rapid disappearance of 3-MT after COMT inhibition than the disappearance of DOPAC after MAO inhibition, this precludes an important functional route of DA metabolism via 3MT. He further suggests that since the reuptake of released DA is so efficient, very little DA will be present extracellularly to diffuse to sites where COMT is localised. This is supported by the observation that 3MT increases if DA release is stimulated in the presence of a MAO inhibitor which facilitates the metabolism of DA by COMT (Kehr, 1976). On the other hand, Westerink and co-workers propose that decreased DA release is followed by decreased 3MT levels (Westernik & Spaan, 1982).

Whatever the reality of the situation, it is important to consider several points when using DA metabolites as indices of dopaminergic neurotransmission or release. Firstly, it has been shown that the increased synthesis and metabolism of dopamine can be a purely pharmacological response and may bear little or no relationship to the state of dopaminergic neurotransmission. Secondly, care must be taken when applying experimental or pharmacologically induced conditions to the normal situation. It is easy to lose sight of the normal physiological state of neurotransmission. After all, any pharmacological intervention immediately departs from the physiological state and the risk exists that pharmacologically induced DA synthesis and metabolism completely by-passes the functionally important pool of DA i.e., that DA released by nerve cell depolarisation. However, by studying the metabolic fate of DA during physiological conditions and pharmacological intervention, the contribution of different pools of DA and different release processes may be further elucidated.

1.3 In vivo sampling techniques: an historical perspective

The extracellular space of the brain is a complex microenvironment serving roles both as a communication channel between neurones and in neuronal-glial interactions. The events taking place in this environment are a direct consequence of intracellular activities within cells of the CNS. Consequently, any technique that facilitates a direct or indirect assessment of these events may prove valuable as a research tool for the investigation of neuronal interactions. Several techniques have been developed for just this purpose and will, no doubt, continue to develop as technology provides researchers with progressively more accurate and sensitive techniques. A description of the major techniques is presented below, followed by a comparative criticism of these techniques.

1.3.1 The cortical cup

This is one of the earliest methodologies used to determine the release of a neurotransmitter *in vivo*. The technique was first applied by Macintosh & Oborin (1953) (see Moroni & Pepeu, 1984). These authors showed that ACh release was related to electrical activity of the cortex. Their work was extended by Mitchell (1961a,b and 1963) who demonstrated the electrically stimulated release of ACh from the cortical surface of sheep and cats. In the latter two papers, Mitchell also reported evoked cortical release of ACh by peripheral stimulation. The observed release was confirmed as neuronal release by removal of the cortex and by local cooling of the cortex both of which abolished release.

Principles of the technique

The cortical cup consists of a cylinder that is held in tight contact with the surface of the cortex. The cylinder is usually made of perspex, but silver and aluminium have also been used. The size of the cup has varied from as large as 3.0cm^2 (Mitchell, 1961) to as small as 0.2cm^2 (see Moroni & Pepeu, 1984). The cup formed by the cylinder and cortical surface is filled with a small volume of collecting fluid such as Ringer solution. The neurotransmitter is able to diffuse into this solution hopefully from neurones close to the surface of the cortex. In this fashion, the spontaneous and evoked release of transmitters in the cortex has been studied. It may be necessary to include an inhibitor in the collecting fluid to prevent the breakdown of the transmitter. This is particularly necessary for ACh which is rapidly hydrolysed in the absence of a cholinesterase inhibitor. The collecting fluid is removed and substituted periodically. An appropriate assay is then used for the transmitter collected in the fluid.

The size of the cylinder used will obviously affect the size of the area sampled. The smaller the cylinder is then the more discrete this area is. Constraints upon size are usually imposed by the sensitivity of the detection method available for the transmitter.

Applications of the cortical cup

Since the time of its inception up to the present day, the cortical cup has been used for the study of neurotransmitter release and interactions occurring within the cerebral cortex. The early work by Mitchell successfully demonstrated both spontaneous and stimulated neuronal release of ACh from the cerebral cortex of sheep

and cats. Rasmusson & Szerb (1976) correlated the cortical release of ACh with the behaviour of awake rabbits. Their results suggested the existence of two cholinergic systems projecting to or present within the cortex. Beani et al. (1978) demonstrated an effect of NA upon ACh release. The injection of NA into the cerebral ventricles decreased ACh outflow from the guinea pig parietal cortex. The adrenergic effect was confirmed when α-blocking agents were shown to counteract the effect of NA. More recently, Moroni et al. (1981) reported the release of endogenous GABA and glutamate using a cortical cup in rats. Their results highlighted one particular problem associated with the use of this technique in unanaesthetised animals. To avoid cortical damage, it is usually necessary to leave the dura intact when placing the cortical cup. This was found to have no effect upon spontaneous GABA release. However, the authors report differences in stimulated release. If 50mM KCl was placed into the cup on an intact dura, glutamate release increased and GABA release decreased. With the dura removed, the opposite was true, KCl decreased glutamate release and increased GABA release. However, KCl applied directly on the cortex will cause "spreading depression". This electrical silence may be associated with GABA release. Thus, it may be incorrect to conclude that it was the dura that caused this effect. However, the authors concluded that caution should be exercised when applying the technique to some neurotransmitters. This phenomenon was first noticed for ACh release (Beani et al., 1968). The ACh output from rabbits with an intact dura was approximately 70% of that measured after removing the dura. This was probably due to ACh leakage in the arachnoid

space and the diffusional barrier presented by the dura (see Moroni & Pepeu, 1984). However, despite this, the dura does not affect the stimulated increase or decrease of Ach or the size of drug induced changes. It would appear that the impairment caused by the dura depends upon properties of the transmitter and this should be borne in mind for each different transmitter.

Chronic superfusion of the cerebral cortex in unanaesthetised animals has also been performed by Bradford & Co-workers (see Abdul-Ghani et al., 1981). This group was able to follow the patterns of amino acid release from both visual cortex and sensorimotor cortex over periods of several days.

1.3.2 Ventricular Perfusion

Intraventricular perfusion involves the perfusion or sampling of the cerebrospinal fluid contained within the ventricles which is in contact with deep brain structures. Early applications of the technique were reported during the 1960's (Portig & Vogt, 1966, 1967; Portig et al., 1968). Portig & Vogt (1966) perfused artificial CSF through the anterior horn of one lateral ventricle of the cat. When the caudate nucleus was stimulated by noise, electrical stimulation of the sciatic nerves or by electrical stimulation of the substantia nigra, they were occasionally able to measure the release of DA and more consistently the release of ACh into the CSF. In a later paper, the same group reported that D-tubocuranine released DA from the caudate nucleus more reliably (Portig & Vogt, 1967). Later still, Portig et al. (1968) demonstrated that D-tubocurarine released DA and HVA from the caudate nucleus into the lateral ventricle of the cat.

Since these early experiments, intraventricular perfusion has been used to monitor stimulus-induced release of endogenous GABA and also neurotransmitter release from the cerebral cortex (see Abdul-Ghani et al., 1981).

More recently, CSF sampling rather than ventricular perfusion has been used to monitor levels of neurotransmitters and metabolites in cisternal CSF (Curzon et al., 1985; Hutson et al., 1985). Curzon and co-workers developed a technique whereby it was possible to make repeated withdrawals of CSF samples from the cisterna magna of conscious freely moving rats via an indwelling catheter. In this way, they determined the turnover rates of DA and 5-HT by measuring the accumulation of the metabolites DOPAC, HVA and 5-HIAA (Curzon et al., 1985). Similarly, using the same technique, this group determined the effect of tryptophan loading on 5-HT and 5-HIAA levels in the cisternal CSF.

Whilst the technique has without doubt provided some interesting and informative results, it is rather limited with respect to both temporal and spatial resolution. This arises from the fact that the CSF is present in a relatively large volume and is in contact with many distinct brain areas. Consequently, any compounds of interest within the CSF are not only diluted, but the source of these compounds may also be doubtful.

1.3.3 Push-Pull perfusion

The principle and application of the push-pull cannula was developed at a similar time to the cortical cup. Gaddum (1961) proposed the use of a push-pull cannula to limit the area of perfusion. This was based on a system developed by Fox & Hilton in 1958 (see Philippu, 1984) which consisted of two parallel needles

separated by 1.5-2cm. This arrangement was used for the perfusion of subcutaneous tissues. Gaddum adapted this for perfusion of small areas of the brain.

Principles of the technique

The push-pull cannula is based on the principle of a controlled infusion and simultaneous withdrawal of an artificial physiological fluid through the nervous tissue. This creates a stream of perfusing fluid that mixes with the extracellular fluid allowing bidirectional diffusion between the tissue and the exogenous fluid. Drugs and other chemicals affecting neurotransmission are added to the perfusing buffer and so are delivered to discrete brain areas. The reverse is also possible in that endogenous chemicals present in the extracellular fluid can be removed and assayed.

An advantage that is immediately apparent over the cortical cup and ventricular perfusion techniques is that very discrete areas deep within the brain can be perfused using the push-pull perfusion technique. The experimental scope is consequently greater as shown by the literature that quotes the use of this technique.

Applications of push-pull perfusion

The types of investigations that have been performed using push-pull perfusion are listed below with reference to some of the groups responsible.

1. The perfusion of ventricles of the brain with drugs or radiolabelled neurotransmitters and the subsequent determination of the release of endogenous or labelled compounds (Chase & Kopin, 1968; Sulser et al., 1969; Redgrave, 1978; Korf et al., 1976).

2. The perfusion of distinct brain areas with drugs and the study of their effects upon neurotransmission and behaviour. Tilson & Sparber (1972) perfused the psychoactive drugs amphetamine and mescaline and determined their effects upon the release of [^{3}H]-NA and [^{3}H] 5-HT. Bhargava et al. (1978) determined the peripheral effects upon cardiovascular control of drugs perfused into the CNS.
3. The injection of radiolabelled neurotransmitters such as NA, DA, 5-HT, GABA and glycine and the investigation of spontaneous and stimulated release of these transmitters and their metabolites. The use of electrical stimulation and drugs with this technique has been extensive (Chase & Kopin, 1968; Sulser et al., 1969; Myers & Mora, 1977; Redgrave, 1978; Kondo & Iwatsubo, 1978). One drawback with this approach is that the radiolabelled transmitter may be taken up and released from non-neuronal sites as well as neuronal. This is overcome by the use of transmitter precursors in most cases.
4. The perfusion of discrete brain areas with transmitter precursors such as [^{3}H] tyrosine, [^{3}H] tryptophan, or [^{3}H] glutamine and the determination of the release of the respective transmitters, DA or NA, 5-HT and GABA. This allows the study of resting and stimulated release of transmitter although it is necessary to separate the transmitter from the precursors and metabolites (Nieoullon et al., 1977; Cheramy et al., 1978, 1979; Hery et al., 1979, 1980). This technique excludes the possibility of release from non-neuronal sites since the transmitters are only synthesised intraneuronally.

5. The perfusion of discrete brain areas of anaethetised and conscious animals and the determination of the release of endogenous transmitters. Early experiments were limited due to the lack of sensitive assays for determination of the transmitters. Bioassays and fluorometric methods were the best available (Mckenzie & Szerb, 1968; Phillis et al., 1968). More recently, radioreceptor assays have been used for the determination of GABA (Dietl & Philippu, 1979) and with the advances in HPLC technology and the technique of electrochemical detection as well as radioenzymatic procedures, the accurate and sensitive determination of catecholamines has become possible (Bartholini et al., 1976; Stadler et al., 1975; Philippu et al., 1979, 1980, 1981; Elghozi et al., 1981). Other techniques such as radioimmunoassay have been used for determination of enkephalin (Cesselin et al., 1981) and substance P (Michelot et al., 1979). Mass fragmentography has been used for determination of glycine and glutamate (Wolfsenberger et al., 1981).

It is doubtless that the development of the push-pull perfusion technique has introduced a very versatile technique. Coupled with sensitive and accurate neurotransmitter assays, the method opened a new perspective for the study of the dynamics of neurotransmitter release in specific brain areas. However, no technique is perfect and there are drawbacks to the technique. These shall be discussed later.

1.3.4 In vivo voltammetry

The technique of in vivo voltammetry is a more recent

development along with that of brain microdialysis for the study of the extracellular compartment of the brain.

The appealing aspects of *in vivo* voltammetry are that endogenous compounds can be measured in the brain without the necessity of removing them and that real time measurements of transmitter levels are possible in response to physiological and pharmacological stimuli.

Principles of the technique

The technique relies on the placement of electrochemical detection apparatus within the brain. This is in the form of a miniature electrode, the size of which may vary between 1 and 1000μM in diameter. An oxidative potential is applied between this electrode and a reference electrode attached to the animal. The specificity of the technique relies on the fact that oxidisable species present in the extracellular fluid undergo optimal oxidation at characteristic potentials. The oxidation of the compound produces a current response, the size of which is proportional to the concentration of oxidisable material around the electrode.

The technique of *in vivo* voltammetry was pioneered by Adams and co-workers (1973) and by Lane et al. (1979) and has since been refined into several different techniques. These include fast cyclic voltammetry, differential normal pulse voltammetry, chronoamperometery and linear sweep voltammetry. The literature on the theory and application of these techniques is extensive and has recently been reviewed by Marsden et al. (1988).

1.3.5 *In vivo* brain microdialysis

The principle of brain microdialysis has been appropriately

described by Ungerstedt (1984) as an artificial blood vessel, represented by a dialysis membrane, implanted into the brain parenchyma. Substances present within the extracellular fluid are able to diffuse across the dialysis membrane into a perfusing medium and are thereby removed from the extracellular fluid without any nett transfer of fluid. Once contained within the perfusing medium, dialysates are collected and appropriately assayed for the compounds of interest. Chemicals and drugs can also be included in the perfusing medium and these enter the extracellular fluid by diffusion down a concentration gradient. Drugs and chemicals can, therefore, be directed into a specific region of the brain endowing anatomical specificity upon the technique.

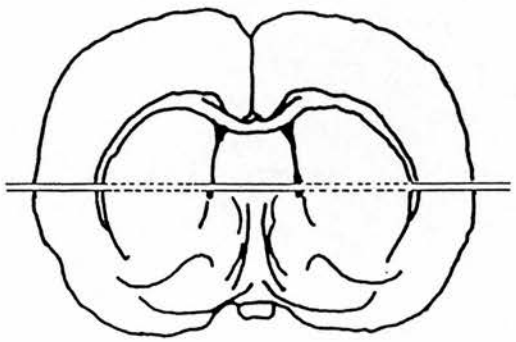
The earliest use of the dialysis principle was by Bito et al. (1966) who implanted small dialysis sacs filled with saline and dextran into the cerebral cortex of a dog between the cortical surface and the lateral ventricle. The sac was left in place for 10 weeks and then removed and analysed for amino acids. The next step was the development of a dialytrode (Delgado et al., 1972) which could be perfused thereby allowing continuous analysis of dialysates. The dialytrode consists of concentric cannulae forming a probe. The end face is formed from dialysis membrane giving a porous surface area of several mm^2. Later modifications included electrodes combined with the dialytrode for the recording of electrical activity wihtin its vicinity. Further refinements were made by Ungerstedt & Pycock (1974) who constructed dialysis probes less than 300μm in diameter using thin dialysis tubings. The technique had, therefore, advanced from the implantation of small dialysis sacs in non-specific brain areas, into the use of miniature

dialysis probes which can be accurately positioned within small brain areas. Brain microdialysis using this dialysis tubing has now become a widely used technique. The precise methodology employed varies with different groups. Ungerstedt & Pycock (1974), Tossman et al. (1983), Hamberger et al. (1985) and more recently Imperato et al. (1984,1988) used a trans-striatal dialysis device in which the dialysis fibre was inserted laterally through the brain, entering on one side of the skull and exiting on the other. The same groups have also used smaller, more discrete dialysis loops (see Fig. 1.3.1). The most recently developed technique is that of the dialysis probe. This is designed as a vertical probe of a single diameter, the size and design of which allow easy stereotaxic placement in small experimental animals compared with transverse probes. Several groups have used this probe design (Hamberger et al., 1985; Zetterstrom and coworkers, 1983, 1984, 1986; Church et al., 1987; Hernandez et al., 1986, 1987; Kendrick et al., 1986; Robinson et al., 1986). The dimensions of dialysis probes and loops can be varied so increasing or decreasing the dialysing area according to the size of the brain area of interest.

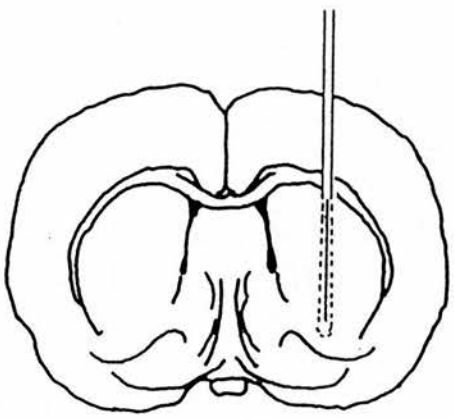
1.3.6 Critical assessment

Each *in vivo* sampling technique represents a different approach to similar problems and it is, therefore, worthwhile comparing and contrasting the four techniques described.

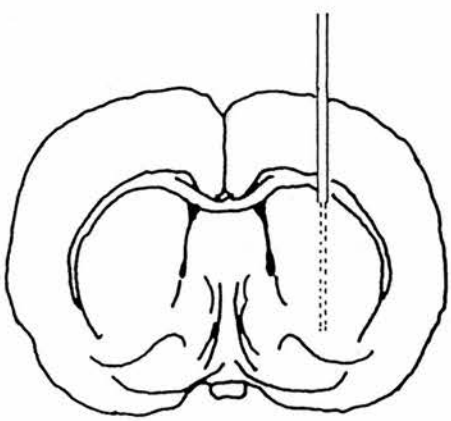
Both perfusion and voltammetric techniques work in a small pool of extracellular fluid. Both approaches measure neurotransmitter overflow rather than neurotransmitter release. This is an important point to remember and arises from the fact that there are



(a) Trans-striatal fibre



(b) Dialysis loop



(c) Dialysis probe

Fig.1.3.1 Illustration of the types of microdialysis devices used.

differences of several orders of magnitude between both the time resolution and size of the cannula/probe/electrode, and the synapse. For example, a synapse is between 1-10nm wide, voltammetric electrodes between 1-1000μm in diameter and the smallest dialysis probe about 200μm in diameter. As a result of this, it is the collected overflow of transmitters from many synapses rather than release from individual synapses that is monitored. Moreover, synaptic events occur in a sub-second timescale whereas most sampling techniques, with the possible exception of recent advances in voltammetric probes, cannot sample on this timescale.

The obvious drawback to the cortical cup technique is that it is only possible to determine transmitter output from relatively large, non discrete areas of nervous tissue that are close to or at the cortical surface. The study of deep brain regions is not possible with this technique. However, this is only a drawback if the researcher wishes to sample from deep brain areas. The technique is particularly amenable to the study of ACh overflow from the cortical surface.

Precautions must be taken with this technique as with the others to maintain the animal at the correct constant temperature. A fall in temperature will also cause a fall in transmitter overflow.

A technical difficulty associated with the cortical cup is the ease with which the surface of the cortex may be damaged during placement of the cup. Such damage may destroy cortical cells or release blood into the cup. This will effectively terminate the experiment.

A major drawback to both the cortical cup technique and the

push-pull perfusion technique is whether or not the overflow of transmitter, whether it is resting or stimulated overflow, is solely neuronal or derived from non-neuronal tissue also. This problem was highlighted by Chase & Kopin (1968). Using push-pull perfusion of the olfactory bulb, they demonstrated that olfactory stimulation caused by exposing the animal to an odour, increased the efflux of [^{3}H]-NA and [^{3}H] 5-HT in the push-pull perfusate. To determine whether or not this efflux was neuronal, they injected the metabolically inert substances ^{14}C-inulin, ^{14}C-urea and ^{14}C-α-aminoisobutyric acid with the ^{3}H amines. The ^{14}C compounds acted as extracellular markers since they are not taken up by cells. Odour stimulation was reported to increase the efflux of both ^{3}H and ^{14}C with the same response pattern. Manipulations of the experiment failed to dissociate the co-efflux of ^{3}H and ^{14}C. So in this example, it was not possible to determine if the stimulation induced alterations in NA and 5-HT efflux reflected physiological release from neuronal stores or just extracellular shifts of exogenous amine. The authors proposed that the push-pull perfusion technique seriously compromised the intra-extracellular barrier due to traumatised tissue. This complicates the interpretation of the efflux of exogenously applied transmitters.

Following this, Collier & Murray-Brown (1968) published work supporting the validity of the cortical cup technique for studying neuronal release of ACh. They perfused the surface of the cortex whilst stimulating the mesencephalic reticular formation and found that ACh was released independently of labelled urea.

This problem is overcome to a large degree if the efflux of endogenous transmitters is measured rather than exogenous. This is

not universally true though since large amounts of GABA and amino acids are present in the blood and in glial cells. Moreover, amino acids are also involved in non-transmitter functions. Consequently, the results of perfusion experiments measuring these transmitter substances should be interpreted with care.

When compared with the dialysis technique, the two major drawbacks of push-pull perfusion are that it is technically difficult and unreliable and that there may be a nett transport of perfusate to or from the tissue because the system is not closed. These technical difficulties arise from trying to balance the infusion and removal of liquid from the tissue. This is not easily achieved and any imbalance in flow rates may cause positive or negative alteration in hydrostatic pressure leading to mechanical damage to the tissue. Another common problem is the blockage of the cannula by tissue fragments. This may again cause accumulation of fluid in the brain leading to unreliable results and/or experimental failure. The perfusion of fluid through the extracellular space may lead to a nett transport of water to or from the tissue unless the osmolarity is carefully controlled and may also cause mechanical damage. Either of these will result in local biochemical interference, the effects of which may be long lasting.

The technique of <u>in vivo</u> microdialysis has several inherent advantages that overcome some of these problems. These are:

1. It is a closed system, i.e., there is no mixing of perfusing fluid and extracellular fluid.
2. The dialysis probe is made of soft material which reduces mechanical damage and helps make the probe an integrated part of the brain (Hamberger et al., 1985).

3. The dialysis probe can be pre-calibrated in vitro, enabling absolute concentrations of substances in the brain extracellular fluid to be estimated.

The assay of compounds of interest in the perfusate is simplified due to the diffusional barrier to large molecules. Perfusate samples are consequently 'clean' and may not require any extraction or purification prior to the assay.

A disadvantage of both the push-pull cannula and the dialysis probe is the poor resolution in time and space of neurochemical events. Both methods require sample volumes of several microlitres collected over a time scale of minutes, resulting in poor resolution of neurotransmitter dynamics. Advances have been made in this direction and will be described at the end of this section.

In vivo voltammetry lacks some of the methodological problems associated with perfusion techniques, but is not without problems of its own. Voltammetric electrodes range from 1 to 1000μm in diameter and are prepared as well as used in several different ways (see Marsden et al., 1988 for review). The technique has been used to measure DA release and changes in extraneuronal concentrations of DOPAC reflecting altered striatal dopaminergic activity (Cespuglio et al., 1981a,b; Conti et al., 1978; Yamamoto et al., 1982; Wightman et al., 1988). Also, 5-HT release and changes in levels of 5-HIAA have been measured (Cespuglio et al., 1981a,b; Kennet et al., 1982). However, drawbacks of the technique are that firstly, the ability of the electrodes to resolve multicomponent signals into separate electrochemical peaks is rather limited. Secondly, the sensitivity of the method is limited and the ability to measure basal DA levels is question able (see Marsden et al., 1988).

The poor resolution of single compounds is due to the presence of other compounds which oxidise at similar potentials in brain tissue. In an attempt to help overcome this, modified electrodes have been developed such as stearate modified electrodes used by Knott et al. (1985) and Lane & Blaha, (1986) or nafion coated electrodes as used by Gerhardt et al. (1984, 1986). These modifications have helped to overcome some of the selectivity problems particularly those associated with interference from ascorbate and DOPAC.

In vivo microdialysis coupled with HPLC-ECD overcomes the problems of selectivity since the individual substances are separated by HPLC before being measured electrochemically. In addition to this, it is possible to simultaneously measure basal and stimulated levels of DA, DOPAC, HVA, 3MT and 5-HIAA representing a great advantage over voltammetric techniques. This enables correlations of drug induced changes in transmitter and metabolite levels.

Having listed the most apparent advantages and disadvantages of each technique, the intention is not to say that one method is preferable to another, but rather to recognise the limitations of each technique and so realise and utilise the potentials of each. The advantages and potential uses of these techniques in the study of in vivo neurobiology are numerous. Despite the importance of chemically mediated neurotransmission and the general acceptance of it as the means of neuronal communication, there has been a scarcity of methodologies available for the study of the mechanisms mediating neurotransmitter release and metabolism. The techniques of in vivo microdialysis and in vivo voltammetry represent the best hopes for

future studies of these processes. The study of biochemical 'synaptology' in response to physiologically relevant as well as pharmacological stimulations appear to be an attractive use of these methods. However, the gap in time resolution to the real events is great, more so for dialysis than for voltammetry. This limits the role of dialysis techniques to the study of pharmacologically induced biochemical changes in defined CNS regions. In vivo voltammetry probably offers the greatest potential for monitoring real time (sub second) physiological phenomena. Improvements in selectivity and sensitivity of equipment may allow detection of rapidly changing concentrations of substances (e.g. Kuhr & Wightman, 1986). Both techniques are increasingly being used for the chronic measurement of neurotransmitters in awake freely moving animals (see Church et al., 1987; Hernandez et al., 1987; Westerink et al., 1982, 1986, 1987; Zetterstrom et al., 1984, 1986; Knott et al., 1985). This allows pharmacological, physiological and behavioural correlations of neurotransmitter release and metabolism to be made.

The most exciting prospect for in vivo microdialysis is its use with smallbore HPLC apparatus (see Carlsson et al., 1986). This involves using HPLC columns as narrow as 0.5mm internal diameter. The consequences of this are that injection volumes as small as 1µl can be used for the determination of catecholamines. This increases the temporal resolution of the microdialysis technique as well as enabling very slow perfusion speeds to be used thereby increasing the relative recovery of compounds from the extracellular fluid.

1.4 Analytical techniques for catecholamine and indoleamine analysis

Several techniques have been developed for the measurement of small quantities of catecholamines. The application of these to *in vivo* release methods has been possible because of their increased sensitivity compared with techniques used previously, such as bioassays and fluorometric assays. Examples are: (1) radioenzymatic procedures (2) gas chromatography alone or with mass spectrometry (3) HPLC with fluorimetric or electrochemical detection. The latter technique of HPLC-ECD provides the most useful method with regard to convenience and sensitivity in the present application. This approach was first applied by Kissinger et al. (1973) and rapidly adopted by others for the determination of catecholamines. The principle of electrochemical detection is fully discussed in the following section (2.3).

1.4.1 Reverse-phase ion-pair HPLC

For the prelminary separation of the catecholamines, reverse-phase ion-pair HPLC has become the method of choice. Reverse phase refers to the aqueous rather than organic mobile phase and the high organic carbon content of the stationary phase. The technique provides high resolution and rapid analysis times without the need for prior derivatisation. This is important for the measurement of catecholamines which are thermally labile and unstable at high pH. The method is also popular due to the high column stability and the ability to separate several different compounds in a single chromatographic run. Recently, a method was described for the separation of nine catecholamines and metabolites within a run time of 10 min (Murai et al., 1988).

Ionic strength of mobile phase

Increasing the ionic strength of the mobile phase using citrate

may cause slightly longer retention times since citrate ions can function as secondary ion pairing agents interacting with the column stationary phase and with the amine solutes (Krstulovic et al., 1981).

Concentration of organic modifier

Organic modifiers, such as methanol and tetrahydrofuran, serve to shorten the retention times of all the catecholamine solutes. This effect relies on the polarity and dielectric constant of the organic modifier along with its interaction with the ion pairing agent (Horvath et al., 1977).

Ion pairing agent

At the pH values used in reverse phase chromatography, DA, 5-HT, NA and AD are protonated and this charge diminishes the retention of these molecules. In order to overcome this problem, an ion pairing agent, such as sodium octanesulphonate, is included in the mobile phase. This attaches to the stationary phase and increases the retention times of these compounds. The precise mechanism by which the ion pair achieves the longer retention times remains a matter of controversy (see Krstulovic, 1982), but relies on the reversible binding of the ion pair to the hydrocarbon surface of the stationary phase. Dynamic ion exchange between the solute and the ion pairing agent then occurs and this retards the movement of the solute down the column. This effect requires that the solute molecule is charged for the interaction to occur. It is important to avoid high concentrations and excessively long chain lengths of the ion pairing agent since this will result in excessively long retention times and column equilibration times. Octanesulphonate was found to be ideal, the excessive retention times caused by its inclusion

being overcome by the inclusion of the organic modifiers mentioned above.

Effects of pH of the mobile phase

Retention times were most sensitive to the pH of the mobile phase. At lower pH values, the amino groups of the catecholamines are fully protonated whilst the carboxyl group of the metabolites is stabilised. This has the effect of enhancing the interaction between the solute molecules and the stationary phase due to the increased solute hydrophobicity. Thus, the acidic metabolites DOPAC, HVA and 5-HIAA are retained longer at lower pH values whilst the reverse is true for DA.

In the present study, the only mobile phase manipulation commonly used to ensure separation was varying the pH of the mobile phase. Occasional alterations of the ion pair concentration were used when this proved inadequate. This allowed the easy differentiation of the DOPAC, DA, HVA, 5-HIAA and 3MT peaks. Thus, either sodium hydroxide was added to differentially reduce the retention time of DOPAC when it co-eluted with DA, or perchloric acid added in order to retard HVA elution when it co-eluted with or close to DA.

On the whole, the system was very reliable and consistent, provided the HPLC apparatus was adequately maintained.

1.5 Electrochemical Detection

Electrochemical detection is based upon a process in which electroactive compounds are either oxidised or reduced. The resulting loss (oxidation) or addition (reduction) of electrons causes a change in potential difference between two electrodes.

This forms the basis for the detection of such compounds.

Electrochemical detection was first applied to catecholamine detection by Adams and co-workers (Kissinger et al., 1973). Since then, the technique has been used for the detection of many different chemicals, pharmaceuticals, dyes and carcinogens. It is now a popular technique for catecholamine detection due to its high sensitivity, being able to detect picogram quantities, and to its reliability and ease of use.

Electrochemical activity can be defined as the ability of a molecule to rearrange itself in such a way as to accommodate the loss or addition of one or more electrons. This must occur within certain potential limits which are defined by the electrode material and the properties of the solvent. The system commonly used for the detection of catecholamines consists of a carbon electrode and an aqueous solvent. This sets the potential limits between +1.2 and -0.8v with regard to a Ag-AgCl reference electrode.

Most aromatic amines and phenols are oxidisable between these potentials. Electrochemical oxidation converts catecholamines to the corresponding quinone. Serotonin is oxidised to a quinone-imine. O-methylated metabolites such as HVA and 3MT are more difficult to oxidise since the methoxy group must be cleaved. This requires a higher energy of activation and consequently, a higher electrochemical potential. Figure 2.3.1 shows the reaction that occurs at the electrode for a catecholamine and for an O-methylated catechol metabolite.

The current derived from the release of these electrons is proportional to the concentration of the species in solution. This is shown by the following expression for the current developed by

Levich (1962) (see Mefford, 1981). This is:

$$i_L = KnFV^aC_b$$

i_L is the limiting current obtainable from oxidation or reduction of a species.

K is the cell constant dependent upon the diffusion coefficient of the species of interest, the viscosity of the solvent and the geometry and area of the electrode.

n is the number of electrons involved in the oxidation or reduction of one molecule of the species.

F is Faradays constant

V^a is the velocity of the solution passing over the electrode surface to some power a.

C_b is the bulk concentration of the electroactive species present in solution.

For a given species under isocratic chromatographic conditions at a constant flow rate, n, V^a, F and K are all constant. So the equation simplifies:

$$i_L = BC_b$$

where B is a constant containing all the other constant terms.

The current obtained is, therefore, dependent only on the concentration of electroactive species in solution.

A greater selectivity is conferred upon the technique of HPLC-ECD by the fact that different electrochemically active species have different oxidation potentials. Some of these are listed in Table 1.5.1 (from Mefford, 1981).

TABLE 1.5.1

Compound	oxidation potential (volts)
DOPAC	+0.56
HVA	+0.76
3MT	+0.74
DA	+0.50
NA	+0.55
5-HT	+0.57
5-HIAA	+0.58

This fact can be used to validate the identity of a peak of a suspected compound by determining the relationship between the applied potential and the current response for that compound. The resulting curve is unique to that compound and is known as a hydrodynamic voltammogram (see section 2.2. and Fig. 2.2.2).

Electrochemical detector design

The detector used in all experiments presented here was the BAS LC-17 oxidative transducer. This is a thin layer electrode and is depicted in Fig. 2.2.1 (upper panel) and complete with the reference electrode in Fig. 2.2.1 (lower panel). It contains a glassy carbon electrode which is part of a channel wall formed by a gasket between a plastic and a metal block.

The primary concern in detector design are the thickness of the gasket which affects the volume of the electrode chamber and consequently the sensitivity, and the surface area of the electrode. The above expression for current produced by oxidation shows that the larger the electrode surface area, the greater the current derived from oxidation and the greater is the sensitivity of

the electrode. The same is true for the flow rate over the electrode. However, increasing either of these decreases the signal to noise ratio of the detector which is deleterious to detector performance since the increased 'noise' may obscure the peak of interest.

Baseline noise which is caused by the presence of electroactive species in solution is always present but should be minimised. The most common causal factors are dissolved oxygen and ferrous ions from the equipment. In addition to these, the mobile phase itself is electroactive so any variation in flow rate will cause baseline fluctuations.

To help overcome this, the following precautions were routinely taken:

1. Mobile phase was degassed under vacuum and helium bubbled through to remove dissolved oxygen.
2. A pulse dampener was included with the pump to reduce pulsations in the flow rate caused by the reciprocating piston.
3. Steel components of the HPLC apparatus were passified using 6M nitric acid.

CHAPTER 2

METHODS AND MATERIALS

2.1 Microdialysis Probe Construction and Characterisation

Microdialysis probes were constructed in the laboratory with the aid of a low powered binocular microscope.

The probes were made according to the method of Sandberg et al. (1986). Two lengths of plastic coated vitreous silica tubing (Scientific Glass Engineering; VS 170/110) were inserted into a 6mm length of dialysis membrane (Cuprophan B4 AH, Cobo Medical Supplies, Lafeyette, USA). The dialysis membrane has a molecular weight cut off of approximately 5,000, which prevents molecules of a greater molecular weight (e.g. proteins) diffusing across the membrane. The tips of the tubing were separated by 3mm, one being positioned 1mm from the end of the dialysis tubing and the second 3mm behind this. A length of teflon coated tungsten wire (Clark Electromedical, WT3) was then inserted alongisde the two pieces of tubing within the dialysis membrane in order to strengthen the probe. The tip of the wire was positioned to coincide with the open end of the dialysis tubing. Both ends were sealed with cyanoacrylate adhesive (Loctite Xtra). This assembly was then inserted into a short length of 21 gauge stainless steel tubing with the dialysis tubing protruding 10mm from this. This provided an anchoring point for mounting the dialysis probe in a stereotaxic probe holder. The structure of the dialysis probe is shown schematically in Fig. 2.1.1.

The completed dialysis probe has an outer diameter of approximately 0.3mm. The effective length of the dialysis membrane is 3mm since any membrane above the outlet capillary was coated in glue to prevent any diffusion above this point. This length was chosen to be suitable for placement in the particular area of the

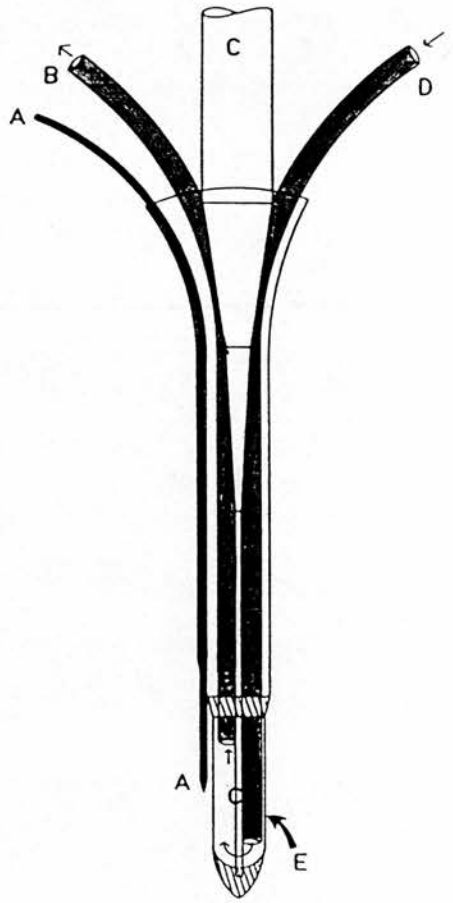


Fig.2.1.1 Structure of the microdialysis probe.

A:Optional stimulating electrode

B:Silica outlet capillary

C:Tungsten wire support

D:Silica inlet capillary

E:Dialysis tubing

striatum studied, which is greater than 3mm in depth at the point of insertion.

The use of this vitreous silica inlet and outlet tubes reduced the dead volume of the dialysis probe to an absolute minimum. This ensured that the responses measured correlated directly in time with drug or chemical application.

In vitro characterisation of dialysis probes

Determination of variations in probe efficiency

In order to assess the variation in efficiency between probes, four newly constructed probes were compared. The probes were perfused at a rate of 1.25µl/min in a standard solution containing DOPAC, DA, HVA, 5-HIAA and 3MT. The percentage recovery of each compound was determined for each probe and the values averaged. The results are shown in Table 2.1.1

TABLE 2.1.1

Variation in probe efficiency %

	Mean	S.E.M.
DA	7.9	0.4
DOPAC	10.8	0.8
HVA	12.4	0.9
5-HIAA	10.2	0.7
3MT	10.0	1.1

It is apparent from the results that variations in efficiency between probes were small.

Effect of perfusion speed on in vitro recovery

The effect of perfusion speeds on the recovery of DOPAC, DA, HVA, 5-HIAA and 3MT is illustrated in Fig. 2.1.2. This was performed by placing a dialysis probe into artificial CSF containing known concentrations of the above compounds. The probe was then perfused at rates of 0.5, 1.0, 1.25, 2.5, 5 and 10µl/min. Three 25µl samples were collected for each perfusion speed and the concentrations of DA, DOPAC, HVA, 5-HIAA and 3MT measured by HPLC-ECD. The results were averaged for each compound. On the basis of these results, a perfusion speed of 1.25µl/min was chosen for all experiments. At this speed, the % recovery varies between 7.9% for DA to 12.4% for HVA (see Table 2.1.1). Fig. 2.1.2 shows that at higher perfusion speeds than this, recovery rapidly decreases. Using a slower perfusion speed would necessitate longer collection times and so reduce the temporal resolution of the experiments.

2.2 HPLC and Electrochemical Detection

The separation of DA, DOPAC, HVA, 5-HIAA and 3MT was achieved using a reverse phase liquid chromatography system. An Altex 110A delivery pump set at a flow rate of 1.0ml/min was used. Samples were injected through a Rheodyne 7125 syringe loading sample injector fitted with a 20µl loop and an Altex 5µm Ultrasphere ODS C-18 column (250 x 4.6mm) was used. A thin layer oxidative transducer (BAS LC17) connected to an amperometric detector (BAS LC4A) was used for electrochemical detection of catecholamines and indoleamines. The detector output was recorded on a Bryans BS600 twin channel chart recorder.

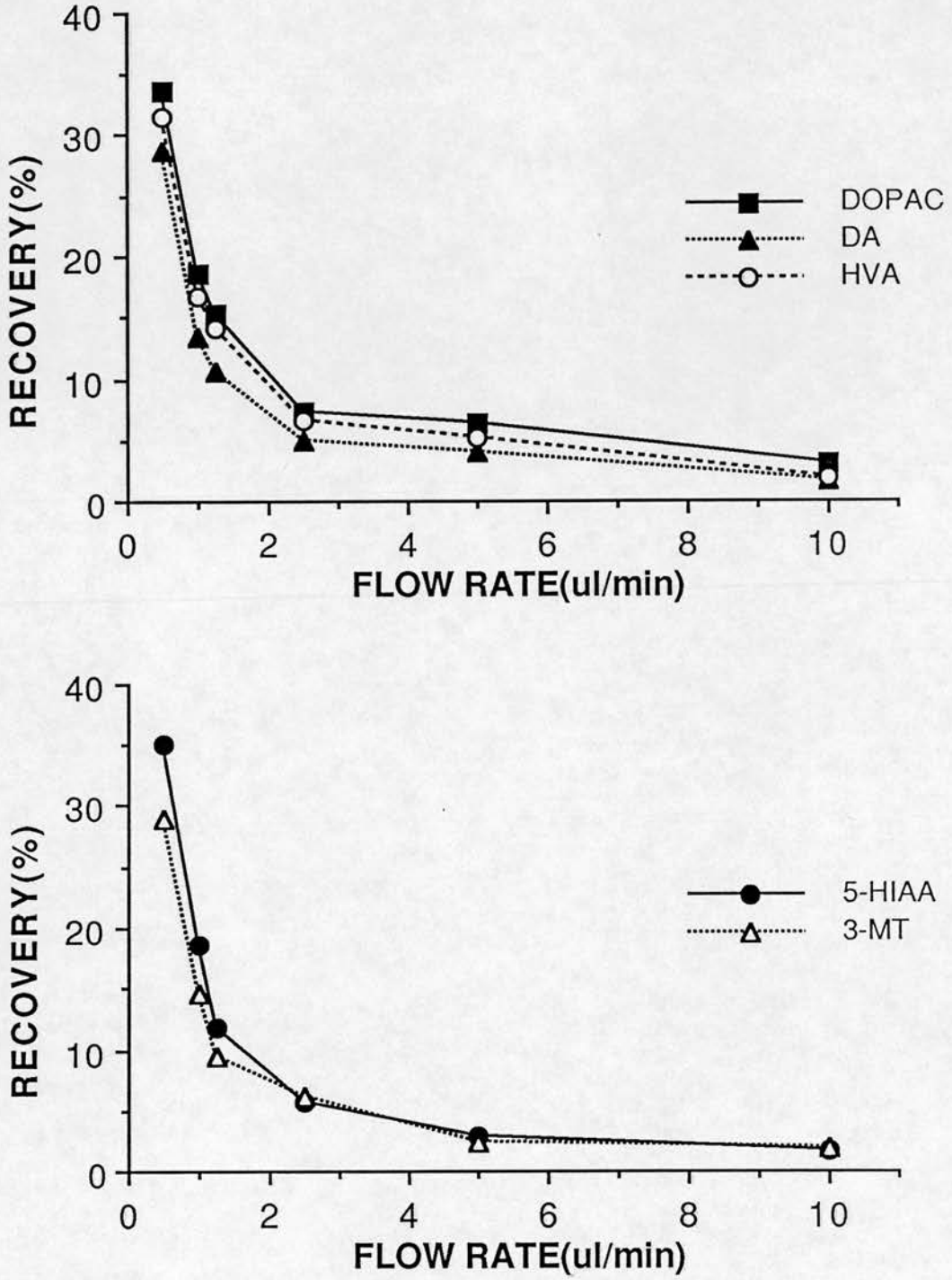


Fig.2.1.2 The relationship between perfusion speed and relative recovery in vitro of DA,DOPAC,HVA(upper panel),5-HIAA and 3-MT(lower panel).

Mobile phase

An isocratic mobile phase prepared according to the method of Sheward & Watts (1985) was used. This consisted of 100mM sodium citrate/acetate buffer (pH 5.2) containing 5% methanol, 2% tetrahydrofuran and 100µg/ml sodium octanesulphonate as an ion pairing agent. The mixture was degassed under vacuum prior to the addition of the tetrahydrofuran in order to remove dissolved gas. This is necessary to reduce interference from electroactive species such as dissolved oxygen at the working electrode of the detector. Experience showed that the mobile phase prepared in this way was stable and usable for several days.

HPLC/ECD Care and Maintenance

Occasional problems were experienced with the HPLC apparatus such as excessive pump pressure or leaks. These were all overcome or prevented by regular maintenance. Care was taken to ensure that no air bubbles or any other foreign material entered the system. To aid this, a pre-column was included (Spheri-5RP-18 OD-GU, 30mm x 4.6mm; Brownlee labs) which served as a guard for the main column. Other preventative maintenance included (i) piston and piston seal replacement in the liquid head of the pump (ii) frit renewal at the top of the column (iii) check valve cleansing

Between experiments and whilst not in use, the column was washed successively with water, methanol-water and finally methanol. This removed the ion pairing agent from the column as well as washing the system.

Electrode care and maintenance

The oxidative transducer electrode consists of a working

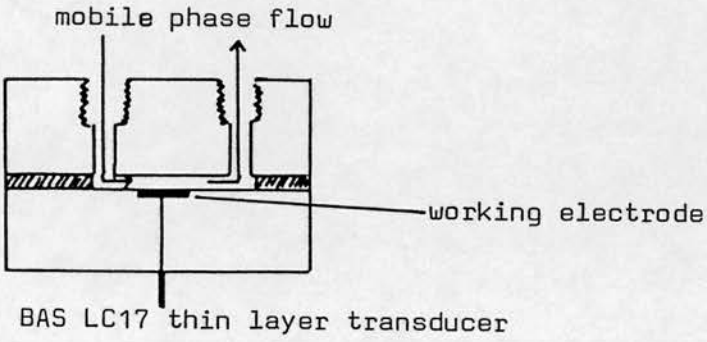
electrode, a reference electrode and an auxiliary electrode (see Fig. 2.2.1 for a schematic diagram). It is at the surface of the working electrode that the eluted solutes are oxidised. The working electrode is made of glassy carbon and has a long working life. Following prolonged use, this surface becomes contaminated thereby reducing the efficiency of the electrode. To prevent this, the electrode was disassembled regularly and polished with alumina according to the manufacturer's recommendations. Most importantly, care was taken not to scratch the surface of the electrode. The electrode was then sonicated and washed in methanol before reassembly.

The reference electrode is a sealed unit (BAS RE-1) consisting of a Ag/AgCl electrode containing 3M NaCl. It was important not to allow the tip of the electrode to dry out. A faulty reference electrode causes a drifting baseline due to deviations in the set potential of the electrode. This may be due to a leaking or cracked electrode. If this was suspected, the electrode was tested by placing it with a new electrode into a beaker containing 3M NaCl. The potential difference between the two electrodes was then measured with a voltmeter. If the meter reading exceeded ± 20mv, the electrode was faulty and was replaced.

A final precaution was to ensure that no bubbles of air were present in the reference chamber. This was done by removing the reference electrode and allowing the chamber to overflow whilst gently tapping the sides to dislodge any bubbles. The reference electrode was then re-inserted.

Construction of hydrodynamic voltammogram

The relationship between the applied potential and the current



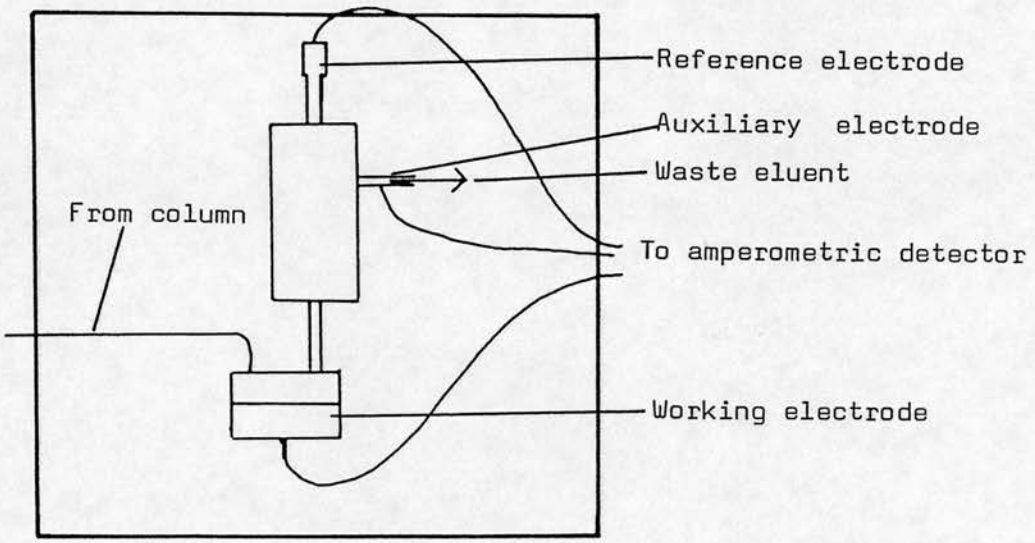


Fig.2.2.1 Schematic representation of the oxidative electrode used for electrochemical detection.

response was determined for DOPAC, DA, HVA, 5-HIAA and 3MT. The results are shown in Fig. 2.2.2. These show that at the voltages used, the detector was most sensitive for DA. The response for any electroactive species is characterised by a rapid increase in response as more voltage is applied which levels off to a plateau. An applied potential of +0.7V was used for all experiments which ensures optimum sensitivity for DOPAC, DA and 5-HIAA whose oxidation potentials are +0.56, +0.5 and +0.58V respectively. The O-methylated metabolites HVA and 3MT have higher oxidation potentials of +0.76 and 0.74V respectively which is illustrated by the rapidly rising current response for both compounds between 0.6 and 0.7V with HVA continuing to rise rapidly between 0.7 and 0.8V. The recommended voltage to use is at the beginning of the plateau phase within approximately 100mv of the oxidation potential. This ensures maximum sensitivity whilst keeping the background current to a minimum which also reduces the likelihood of interference from other solutes. Thus an oxidation potential of +0.7V was considered as suitable since DA and DOPAC were the primary compounds of interest.

Daily Operation of the HPLC

To allow the column to equilibrate with the mobile phase, and in particular, with the ion pairing agent, the mobile phase was prepared 12-18 hours in advance and pumped through the column overnight at a speed of 0.1ml/min. On the day of use, the pump speed was reset at 1.0ml/min and the electrochemical detector switched on. A large current signal due to charging and transient background current occurs each time the electrode is switched on. The signal gradually decays to a steady state background current

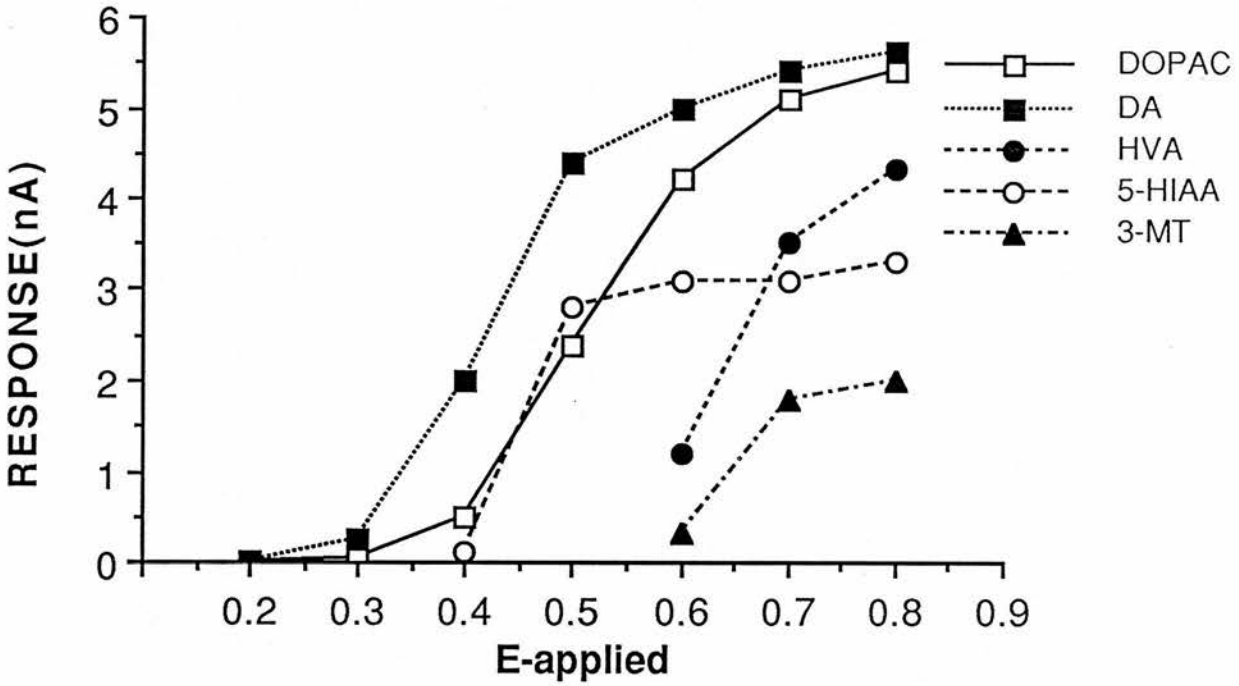


Fig.2.2.2 A hydrodynamic voltammogram for DA,DOPAC,HVA,5-HIAA and 3-MT.

after 5 min. The detector electrode was always switched on at least one hour prior to use for this reason.

Baseline drift caused by oxidative species present in the mobile phase was occasionally observed and this was corrected for using the offset control on the detector. After use, the detector electrode was always switched to standby to avoid unnecessary use of the electrode and subsequent deterioration over time. The electrode was regularly cleaned to ensure consistent performance and results.

Elution and identification of standards and samples

The buffer system used allowed the separation and identification of DOPAC, DA, HVA, 5-HIAA and 3MT with each injection of dialysate. Typical retention times for these compounds were as follows; DOPAC, 5 min; DA, 7 min; HVA, 8.5 min; 5-HIAA, 9.5 min; 3MT 11.5 min. Variations in these values occurred with variations or changes of mobile phase. In particular, the acid metabolites of DA, namely DOPAC and HVA as well as the serotonin metabolite 5-HIAA, are very sensitive to changes in pH of the mobile phase. The reasons for this are explained in 1.4. Any deviation in the pH of the mobile phase, therefore, altered the retention times of these compounds.

Dopamine, being uncharged, is relatively unaffected by alterations in pH of the mobile phase and the retention time is, therefore, relatively unaffected by altering buffer pH. Measurements of 3MT were not routinely performed since basal levels were usually close to the detection limit. However, measurements were taken if the applied drugs caused an increase in 3MT. Dialysate samples were collected for 20 min and since most experiments involved bilateral implantation of dialysis probes, two

samples were collected every 20 min. This reduced the storage time of dialysate samples to a maximum of 10 min.

Standard amounts of DA, DOPAC, HVA, 5-HIAA and 3MT were injected for calibration of the response to each. Standard solutions of 100µg/ml were prepared monthly in 0.2M perchloric acid and were stored at 4°C. These were mixed and diluted daily to a concentration of 100ng/ml and 20µl samples (i.e. 2ng of each compound) of this solution containing DOPAC, DA, HVA, 5-HIAA and 3MT were injected routinely before each experiment. The standard solution was injected until three identical responses were obtained. The mean of each signal was then used to calibrate the detector and so determine the concentrations of catecholamines in the dialysate samples by comparing the sample peak heights with those of the standards. Fig 2.2.3 shows a typical chromatograph of a standard solution and a chromotograph of a dialysate sample with basal levels of DOPAC, DA, HVA, 5-HIAA and 3-MT.

The basal values of the dialysate catecholamines and indoleamine overflow varied between experiments, but were always within a certain range. These are shown in Table 2.2.1.

TABLE 2.2.1

	pmoles/20µl dialysate	Molar concentration
DOPAC	18-24	0.08-1.2µM
DA	0.06-0.10	3-5nM,
HVA	7-9	0.35-0.45µM
5-HIAA	3-4	0.15-0.2µM
3MT	0.04-0.07	2.0-3.5nM

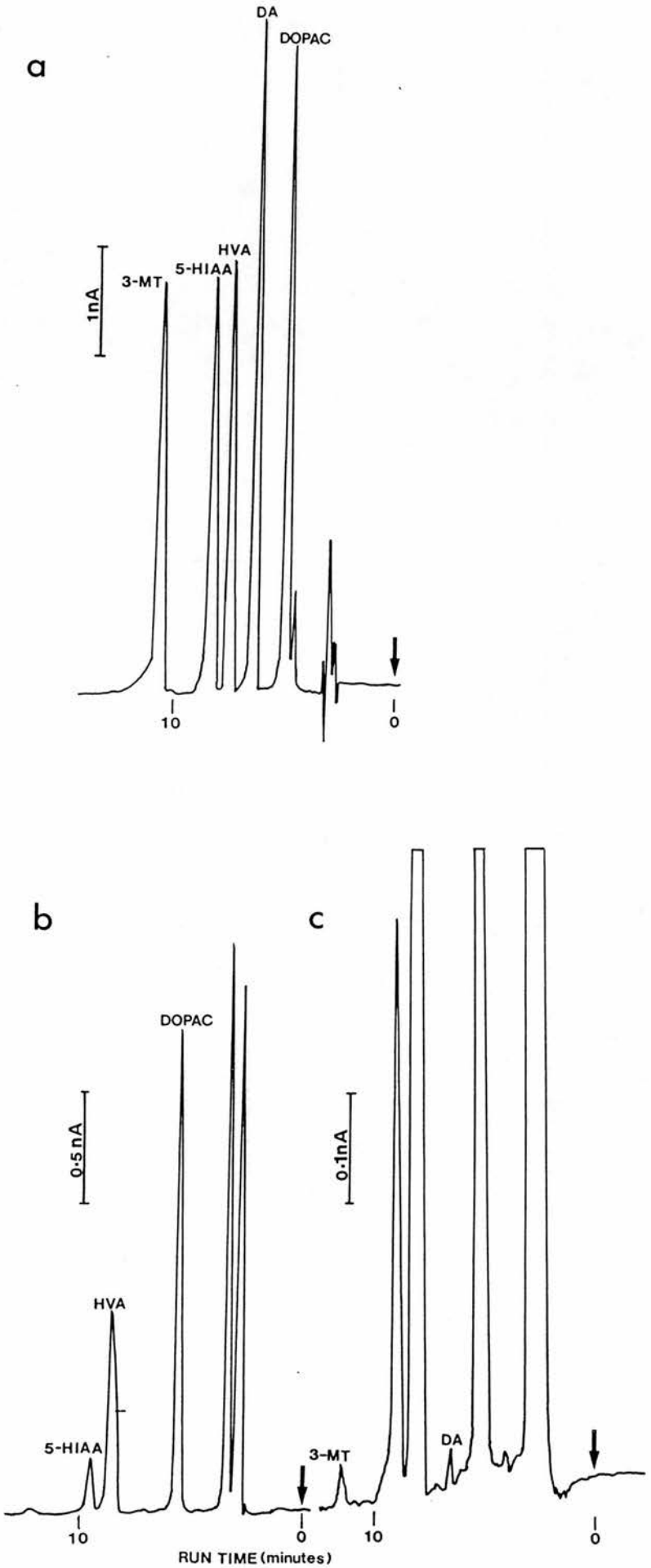


Fig.2.2.3 Elution profile of DA and monoamine metabolites separated by HPLC/ECD.a.Separation of a calibrating solution containing 2ng of each compound.b and c: experimental dialysate measured at two different sensitivities.

The values in Table 2.2.1 cover the normal range of basal concentrations of these compounds. Higher and lower values were occasionally encountered.

The detection limit for DA was approximately .03 pmoles i.e., 5.5pg for the reliable measurement of the DA peak (signal:noise ratio > 3). The usual basal value of DA was, therefore, approximately 2-3 times the detection limit.

DOPAC levels were the most variable between animals as the range shows in Table 2.2.1. Once again, this range was occasionally exceeded in either direction. HVA and 5-HIAA levels were more consistent.

Since there was a 200-fold or greater difference in concentrations of DOPAC and DA, a dual channel chart recorder was used. This enabled simultaneous measurement of all compounds over the entire range of concentrations encountered. DA and 3MT were measured on one channel with 2nA full scale deflection whilst the metabolites DOPAC, HVA and 5-HIAA were measured on a second, less sensitive channel with 20nA full scale deflection. This provided an ideal method of simultaneously monitoring all the compounds and also enabled a decrease in peak height to be accurately followed from the less sensitive to the more sensitive channel.

It was important to establish the identity of the chromatographic peaks. This was performed in two ways. Firstly, the retention times of the identifiable standards were compared with those of the experimental samples. These times could be determined quite accurately and, therefore, provided a reliable method. Fig. 2.2.3 shows a chromatograph of the standards compared to a chromatograph of a sample. The retention times are identical as

shown by those peaks indicated for the compounds of interest. Secondly, a dialysate sample was collected over a period of 40 min from a probe inserted into the striatum of an untreated rat. The sample was divided into two equal parts, one of which was 'spiked' with a known concentration of either DOPAC, DA, HVA or 5-HIAA. Each was then injected onto the HPLC system. The spiked samples have a larger peak which corresponds to the known amount of standard plus that in the unspiked sample. These methods prove beyond doubt the identity of the chromatogram peak.

In practice, problems with 'foreign' peaks co-eluting with any of the catecholamine peaks were not encountered. Noradrenaline and ascorbic acid both elute close to the solvent front whilst 5-HT elutes at approximately 20 min and is barely detectable due to peak broadening. Providing that the five peaks DOPAC, DA, HVA, 5-HIAA and 3MT were separated by at least one minute, no problems were encountered with peak resolution or interference by other peaks.

2.3 Surgical Procedures

Male Han-Wistar rats supplied by the Institute of Occupational Medicine (Edinburgh) were used throughout the study. These were housed in a 14 hour light:10 hour dark cycle with free access to water and lab chow. The weights of animals varied between 180 and 300g, typically being around 250g.

Rats were initially anaesthetised using Brietal (sodium methohexitone, Eli Lilly & Co., U.K.) at a dose of 75mg/kg i.p.. An incision was made in the neck and the parotid glands deflected to expose the trachea. A cannula was inserted into the trachea below the thyroid arch and secured in place. The animal was then mounted

in a David Kopf stereotaxic frame with the tooth bar set at 3.0mm below the interaural plane (according to Paxinos & Watson, 1982). Anaesthesia was maintained using halothane (< 1%) in air. Body temperature was maintained between 36-38°C using an electric blanket thermostatically controlled by a rectal probe. The surface of the skull was exposed and burr holes were drilled above both striata. The dura was carefully cleared to allow the insertion of dialysis probes. Each dialysis probe was mounted in a purpose built holder (supplied by Carnegie Medicine) and this assembly was held in a micro-manipulator mounted on the stereotaxic frame. Probes were then positioned above the striatum (AP +0.5, ML ± 2.5mm; relative to bregma; Paxinos & Watson, 1982) and were inserted to a depth of 7.5mm below the dura. The bottom 1mm of the probe therefore lies below the striatum but with the actual dialysing area centred in the striatum.

Each probe was perfused with freshly made oxygenated Krebs-bicarbonate buffer of the following composition in mM: NaCl, 124; KCl, 3.3; $MgSO_4$, 2.4; KH_2PO_4, 1.25; $CaCl_2$, 2.5; $NaHCO_3$, 25; pH 7.4.

Animals were frequently monitored for depth of anaesthesia.

2.4 Drug Delivery and Sample Collection

Following implantation of the dialysis probes, each was connected via a short length of small bore polythene tubing to a 1ml syringe mounted in a constant drive microinfusion pump (Carnegie Medicine, CMA 100). It was important to ensure that no air bubbles were present within the syringe or within the connecting tubing since these may become trapped within the probe and so reduce its

efficiency. Buffer solution was then perfused through the probes at a rate of 1.25µl/min. The probes were perfused in this fashion for approximately 60 min prior to the commencement of all experiments. This was regarded as a necessary equilibration period to allow recovery from the acute effects of implantation trauma. Basal levels of the compounds of interest were found to be steady after this initial washout period. Perfusate samples were collected by placing a small Eppendorf tube over the outlet tubing. Each sample was collected for 20 min which gave a volume of 25µl. At least three 20 min samples were collected before drug application for determination of basal efflux of catecholamines.

Dopamine is an unstable compound and is easily oxidised under certain storage and analytical conditions. However, the dopamine and metabolites of interest, DOPAC, HVA and 5-HIAA, were found to be stable when collected in air. The inclusion of 0.2M perchloric acid in the Eppendorf tube was found to be unnecessary since there were no differences in basal values of DA, DOPAC, HVA or 5-HIAA dialysate content in successive samples collected with and without the inclusion of perchloric acid in the Eppendorf tube. This was probably due to the short length of time (maximum 10 min) between sample collection and subsequent analysis and the minimal deadspace of the dialysis apparatus.

After collection, 20µl of the dialysate was injected onto the HPLC for determination of the levels of the compounds of interest. A 20µl HPLC injection loop was used and the entire 25µl of dialysate was injected, the excess being lost through the valve overflow. This ensured minimal sample to sample variation in injection volume.

Drugs and chemicals were given by two routes depending upon the

experimental procedure. These were intraperitoneally (i.p.) or via the dialysis probe. When given via the probe, account must be taken of the efficiency of the probe when considering the amount that actually reaches the extracellular fluid of the rat striatum. The probes used had a recovery of between 10-15% suggesting that a similar percentage of the drug/chemical will enter the extracellular fluid by passive diffusion from the probe. The solution containing the drug/chemical was simply exchanged with the perfusing buffer either by disconnecting the connection to the probe or by using a liquid switch (Carnegie Medicine, CMA 110). The advantage of using the liquid switch is that the flow of perfusate through the probe is uninterrupted. Although changing the tubing connected to the probe involves interrupting the flow, no change-over artefacts were noticed in the levels of the compounds of interest. Obviously, the ideal method is to use the liquid switch but this was not always possible since a liquid switch and pump are required for each probe. Many of the experiments involved bilateral implantation of dialysis probes so requiring two pumps and two liquid switches which were not available.

Basic experimental protocol

Details of the combinations and duration of administration of drugs and chemicals are shown in the results for each experiment. Table 2.4.1. below indicates the route of administration and the dose range of all the drugs and chemicals used. All drugs given intraperitoneally were dissolved in saline (0.9% w/v) except for reserpine. The procedure for dissolving reserpine was as follows: citric acid (125mg) and reserpine (125mg) were dissolved in 1ml benzyl alcohol by warming and stirring. 5ml Tween 80 were added

with continued stirring. The volume was then made up to 50ml with 0.9% saline. The final concentration of reserpine was 2.5mg/ml.

Nomifensine was found to be virtually insoluble and so was injected as a fine suspension.

All drugs and chemicals given via the dialysis probe were mixed or dissolved in the perfusing buffer solution.

TABLE 2.4.1

Drug/Chemical	Route of Administration	Dose range
Amphetamine	IP and Probe	2-16mg/kg and 5μM
Ouabain	Probe	10μM-1mM
Veratrine	Probe	10μg/ml-1mg/ml
Nomifensine	IP and Probe	20mg/kg, 1-100μM
α-Methyl Tyrosine	IP	250mg/kg
Reserpine	IP	5mg/kg
Clorgyline	IP	10mg/kg
Pargyline	IP	75mg/kg
Selegeline	IP	10mg/kg
Tetrodotoxin	Probe	2μM
KCl	Probe	30-120mM
Tyramine	Probe	1μM-100μM
EGTA	Probe	20mM

2.5 Tissue levels of catecholamines

In order to validate the conclusions based on the dialysate results concerning drug effects upon DA, DOPAC, HVA, 5-HIAA and 3MT in the nerve terminal, it was necessary to determine tissue levels of those same compounds following drug administration. This will then allow the correlation of the pharmacological effects of these

drugs upon catecholamine efflux with their effects upon intracellular levels of these compounds. Each drug was administered to anaesthetised animals for sufficient time to allow maximal effect and this time was equivalent to that used in dialysis experiments. The following drug paradigms were used:

1. Amphetamine

Amphetamine was administered 30 min prior to decapitation. The maximal effect of amphetamine upon DA efflux occurs during the second dialysate collection period, that is, between 20 and 40 min. It is at this point that tissue levels of DA and metabolites are most relevant.

A dose of 4mg/kg i.p. D,L,-amphetamine was chosen for its relevance to behavioural experiments (see Zetterstrom et al. 1986). This allows behavioural and biochemical correlations of amphetamine to be drawn.

2. α-Methyl-p-tyrosine

The effects of AMT upon tissue levels of DA and metabolites were determined at two time points, 20 min and 120 min. At the shorter time point, DA synthesis has been reported to be decreased by up to 80% (Widerlov & Lewander, 1978), whilst the reserpine sensitive pool is unaffected. This suggests that DA synthesis is largely inhibited without depleting tissue stores of DA. The longer time point was used in order to obtain a 100% inhibition of DA synthesis. A dose of 250mg/kg over 2h was reported to cause a complete inhibition of DA synthesis (Scheel-Kruger, 1971). However, at this time point, tissue levels of DA are reported to be reduced by more than 50% (Papeschi, 1975; Widerlov & Lewander, 1978). This is borne out by the results in section 3.1.

3. Reserpine

A dose of 5mg/kg of reserpine over 3h was chosen based on the histochemical results of Dahlstrom & Fuxe (1964). Using the Falck-Hillarp fluorescence technique to study monoamine stores in various brain regions, they demonstrated that a dose of 5mg/kg of reserpine strongly reduced or abolished any fluorescence within 2h. In accordance with this, Umezu & Moore (1979) obtained a 95% decrease in tissue DA levels 2h after the i.p. administration of reserpine (2mg/kg). DA levels remained decreased by more than 90% for 24h.

4. Pargyline

Pargyline is widely used and well documented (see Westerink, 1978; Kato et al., 1986; Sharp et al., 1986). Sharp and co-workers used a dose of 100mg/kg and demonstrated that DOPAC levels in tissue decreased to 30% of control and 5% of control at 20 and 60 min respectively. They also reported > 95% reduction in DOPAC levels in perfusates collected from rat striatum over 120 min.

In this study, a dose of 75mg/kg was injected 120 min prior to pharmacological stimulation. This was shown to almost entirely inhibit DOPAC formation in tissue whilst greatly elevating 3MT levels indicating a significant inhibition of monoamine oxidase.

5. Selegeline

The MAO-B inhibitor selegeline was administered at a dose that reportedly almost entirely blocks MAO-B activity (see Demarest et al., 1980). Demarest and co-workers used a dose of 1mg/kg i.v. which is thought to be equivalent to 10mg/kg i.p. This caused a 90% inhibition of phenylethylamine (PEA) deamination and a 50% inhibition of DA deamination. PEA is an MAO-B selective substrate

whilst DA is a substrate for both MAO-A and MAO-B (Houslay & Tipton, 1976).

Similar results were obtained by Christmas et al. (1972). Using benzylamine as an MAO-B selective substrate and 5-HT as a MAO-A selective substrate, they demonstrated almost 100% inhibition of MAO-B using 10mg/kg orally administered selegeline whilst the same dose caused a 25% inhibition of 5-HT deamination.

So a dose of 10mg/kg i.p. was used in an attempt to completely inhibit MAO-B with the knowledge that MAO-A would also be inhibited to some extent at this dose.

6. Nomifensine

A dose of 20mg/kg i.p. nomifensine was used. Nomifensine was given simultaneously with other drugs since the effects upon uptake blockade are rapid in onset.

Determination of tissue levels of catecholamines

The following groups of aminals in Table 2.5.1 were treated with the drugs indicated. Twelve animals were grouped in a large box at one time where they were held under halothane anaesthesia for the required length of time. The rats were then decapitated and the brain rapidly removed. The two striata were dissected out and frozen in liquid nitrogen. After weighing, the tissue samples were stored at -70°C until they were assayed for catecholamine content.

Each tissue sample was homogenised in 1ml 0.2M perchloric acid at 4°C. 100ng of n-acetyl dopamine was added in a volume of 20µl as internal standard. The sample was centrifuged at 2000g for 2min. 100µl of the supernatant was then filtered through cellulose filters under centrifugation at 5000g for 5 min. The supernatant was injected directly onto the HPLC for determination of

tissue levels of DOPAC, DA, HVA, 5-HIAA and 3MT.

TABLE 2.5.1

Treatment	Dose, mg/kg i.p.	Duration (min)	Number of Animals
Control unanaesthetised	-	-	6
Control anaesthetised	-	20	4
	-	120	4
	-	180	4
amphetamine	4	30	6
α-methyl-p-tyrosine	250	30	6
α-methyl-p-tyrosine	250	120	6
nomifensine	20	20	6
pargyline	75	120	6
selegeline	10	60	6
reserpine	5	180	6
reserpine vehicle	-	180	6

Materials

All drugs were supplied by Sigma Chemical Co. except for α-methyl-P-tyrosine supplied by Aldrich. The following were generously donated by the parties mentioned:

1. Nomifensine hydrogen maleate; Hoechst Pharmaceuticals
2. Selegeline hydrochloride; Britannia Pharmaceuticals Ltd.

All chemicals used were analar grade and supplied by British Drug Houses except for methanol and tetrahydrofuran from Rathburn Chemicals and octanesulphonic acid from Fisons plc.

2.6 Data analysis

1. Tissue levels of catecholamines

The calculated tissue content of each catecholamine (nmol/g tissue) for the individual treatments in individual animals was grouped and then compared with the equivalent catecholamine in control animals. The comparison was done using the Mann Whitney U test, where n = 11 individual animals for control and n = 6 individual animals in drug treated groups. A probability level of less than 5% was used to indicate significance.

2. Microdialysis data

(a) Drug controls

Total catecholamine efflux over a period of time was calculated for each individual animal. This was done by subtracting the mean basal efflux before the drug from each timepoint following the drug and then summing these values to give the total increase or decrease in efflux over the stated period of time. These values are then an indication of total increase or decrease in efflux compared to the control where no drug treatment was given. The two sets of data were compared using the unpaired t-test with a probability limit of $< 5\%$ for significance. The number of individual animals ranged from 6 to 18.

(b) Manipulated animals

These results were statistically assessed by comparing stimulated efflux over a certain time period in biochemically or pharmacologically manipulated animals with stimulated efflux over the equivalent time period in non-manipulated control animals. Catecholamine efflux in the first 20 min or total efflux in the 60 min following the releasing drug was calculated by subtracting the

mean efflux value immediately prior to the releasing drug from each individual timepoint following the releasing drug. These values were then summed for individual animals to give total efflux over 20 min or 60 min. This allowed for basal efflux under the conditions of the particular biochemical or pharmacological manipulation used in that experiment prior to the releasing drug. The same method was used for determination of control efflux in the absence of any treatment before the releasing drug.

These data were statistically analysed using either a Wilcoxon test for the paired data or a Mann Whitney U test for the unpaired data. A probability of $< 5\%$ was taken to indicate a significant result. The number of individual animals for each experiment is given in the text. Efflux data are presented as mean ± the standard error of the mean (mean ± S.E.M.).

CHAPTER 3

RESULTS

3.1 Effects of pharmacological and biochemical treatments upon tissue levels of catecholamines

3.1.1 Amphetamine

Amphetamine (4mg/kg i.p.) increased tissue levels of DA to 120% of control levels 30 min after injection (Fig. 3.1.1, control 19.6 ± 1.15nmole/g tissue [n = 11] AMPH 23.5 ± 1.72 nmoles/g tissue [n = 6], $p < 0.05$). Tissue levels of DOPAC decreased to 42% of control over 30 min (Fig. 3.1.1, control 1.97 ± 0.18nmole/g tissue [n = 11], AMPH 0.82 ± 0.11nmoles/g tissue [n = 6], $p < 0.001$). Tissue levels of HVA also decreased to 72% of control over 30 min (Fig. 3.1.2, control 1.37 ± 0.36nmole/g tissue [n = 11], AMPH 0.98 ± 0.11nmol/g tissue [n = 6], $p < 0.05$). Tissue levels of 5-HIAA were not affected; (control 1.06 ± 0.09nmole/g tissue [n = 11[, AMPH 1.05 ± 0.10nmole/g tissue [n = 6]. Tissue levels of 3-MT were also not affected (control 0.25 ± 0.01nmole/g tissue [n = 11], AMPH 0.24 ± 0.02nmole/g tissue [n = 6].

3.1.2 α-Methyl-p-tyrosine

AMT (250mg/kg i.p.) did not affect tissue levels of DA, DOPAC, HVA or 5-HIAA up to 20 min after injection (Figs. 3.1.1. and 3.1.2). In contrast to this, over a time period of 120 min AMT decreased the tissue levels of DA to 61% of control (Fig. 3.1.1, control 19.6 ± 1.15nmole/g tissue [n = 11], AMT 12.0 ± 1.0nmole/g tissue [n = 6], $p < 0.05$). Similarly, tissue levels of DOPAC were decreased to 31% of control over 120 min (Fig. 3.1.1, control 1.97 ± 0.18nmole/g tissue [n = 11], AMT 0.62 ± 0.13nmoles/g tissue [n = 6], $p < 0.001$). Tissue levels of HVA were reduced to 18% of control over 120 min (Fig. 3.1.2, control 1.37 ± 0.36nmole/g tissue [n = 11]

AMT 0.24 ± 0.02nmole/g tissue [n = 6], $p < 0.001$). Tissue levels of 5-HIAA were not affected by AMT (Fig. 3.1.2).

3.1.3 Reserpine

Injection of reserpine vehicle alone had no effect upon tissue levels of DA or DOPAC (Fig. 3.1.1). However, tissue levels of HVA increased to 197% of control over 180 min (Fig. 3.1.2, control 1.37 ± 0.36nmole/g tissue [n = 11], reserpine vehicle 2.70 ± 0.12nmoles/g tissue [n = 6], $p < 0.001$). Reserpine vehicle also increased tissue levels of 5-HIAA to 142% of control over 180 min (Fig. 3.1.2, control 1.06 ± 0.09nmole/g tissue [n = 11] reserpine vehicle 1.50 ± 0.19nmoles/g tissue [n = 6], $p < 0.05$).

Reserpine (5mg/kg i.p.) decreased tissue levels of DA to 27% of the reserpine vehicle control over 180 min (Fig. 3.1.1, control 20.1 ± 0.67nmole/g tissue [n = 6] reserpine 5.48 ± 2.10nmoles/g tissue [n = 6], $p < 0.01$). Tissue levels of DOPAC were increased by reserpine when compared to the reserpine vehicle control (Fig. 3.1.1, control 2.33 ± 0.14nmole/g tissue [n = 6[, reserpine 2.67 ± 0.34nmole/g tissue [n = 6[, $p < 0.05$). Tissue levels of HVA were increased by reserpine to 123% of vehicle control over 180 min (Fig. 3.1.2, control 2.20 ± 0.12nmole/g tissue [n = 6] reserpine 3.33 ± 0.61nmoles/g tissue [n = 6], $p < 0.05$). Reserpine also increased tissue levels of 5-HIAA to 203% of vehicle control over 180 min (Fig. 3.1.2, control 1.50 ± 0.19nmole/g tissue [n = 6], reserpine 3.04 ± 0.53nmoles/g tissue [n = 6], $p < 0.01$).

3.1.4 Pargyline

Pargyline (75mg/kg i.p.) induced a striking increase in tissue DA levels to 151% of control over 120 min (Fig. 3.1.1, control 19.6

± 1.15nmole/g tissue [n = 11] pargyline 29.6 ± 2.18nmoles/g tissue [n = 6], $p < 0.001$). Equally striking was the decrease in DOPAC tissue levels to 1% of control over 120 min. In some individual animals tissue DOPAC was undetectable (Fig. 3.1.1, control 1.97 ± 0.18nmole/g tissue [n = 11], pargyline 0.02 ± 0.02nmoles/g tissue [n = 6], $p < 0.001$). Pargyline also reduced tissue levels of HVA which decreased to 34% of control over 120 min (Fig. 3.1.2, control 1.37 ± 0.36nmole/g tissue [n = 11], pargyline 0.47 ± 0.36nmoles/g tissue [n = 6], $p < 0.05$). Tissue levels of 5-HIAA were reduced to 36% of control over 120 min (Fig. 3.1.2, control 1.06 ± 0.09nmole/g tissue [n = 11] pargyline 0.38 ± 0.04nmoles/g tissue [n = 6], $p < 0.01$). A good demonstration of pargyline upon MAO activity is the large increase in tissue 3MT levels to 876% of control over 120 min (Fig. 3.1.3, control 0.25 ± 0.01nmole/g tissue [n = 11] pargyline 2.19 ± 0.20nmoles/g tissue [n = 6], $p < 0.001$).

3.1.5 Selegeline

In selegeline (10mg/kg i.p.) treated animals, tissue levels of DA were increased to 123% of control over 60 min (Fig. 3.1.1, control 19.6 ± 1.15nmole/g tissue [n = 11], selegeline 24.2 ± 1.92 nmoles/g tissue [n = 6], $p < 0.05$). Tissue levels of DOPAC were not affected by selegeline (Fig. 3.1.1) whereas HVA increased to 120% of control over 60 min (Fig. 3.1.2, control 1.37 ± 0.36nmole/g tissue [n = 11] selegeline 1.65 ± 0.14nmole/g tissue [n = 6], $p < 0.05$). 5-HIAA tissue levels were also increased to 123% of control over 60 min (Fig. 3.1.2, control 1.06 ± 0.09nmole/g tissue [n = 11], selegeline 1.30 ± 0.12nmole/g tissue [n = 6], $p < 0.05$). Tissue levels of 3-MT were not affected by selegeline over 60 min when

compared to control Fig. 3.1.3).

3.1.6 Nomifensine

Nomifensine (20mg/kg i.p.) had no effect on tissue levels of DA, DOPAC, HVA or 5-HIAA when compared to control over 20 min (Fig. 3.1.1 and 3.1.2).

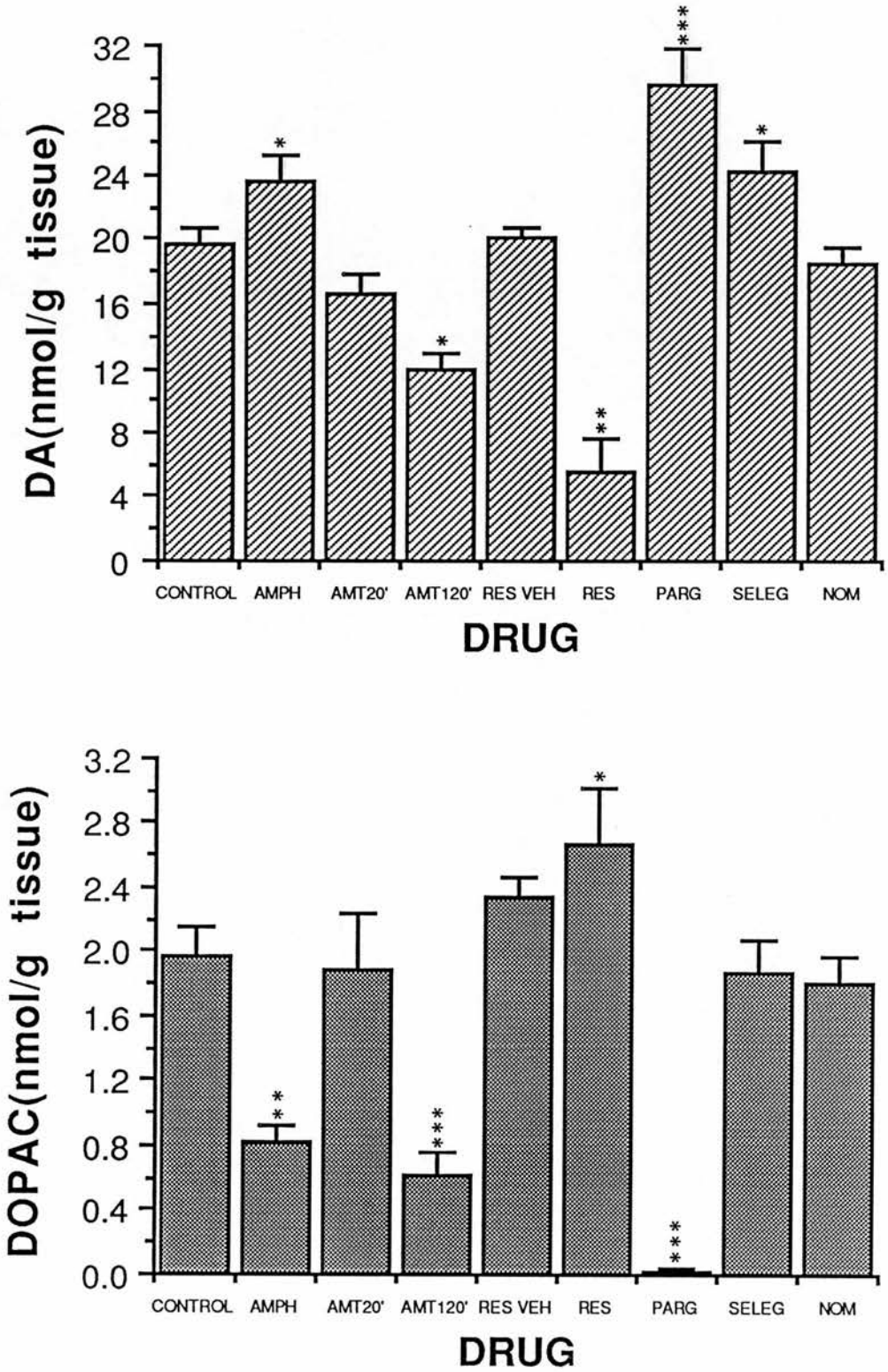


Fig. 3.1.1 Effects of drugs on the total striatal tissue content of DA (upper panel) and DOPAC (lower panel). Drugs were administered for the lengths of time indicated in the text. Results (nmole/g tissue) are the mean ± s.e.m. of eleven animals for control and six animals for drug treated.
*p < 0.05; **p < 0.01; ***p < 0.001

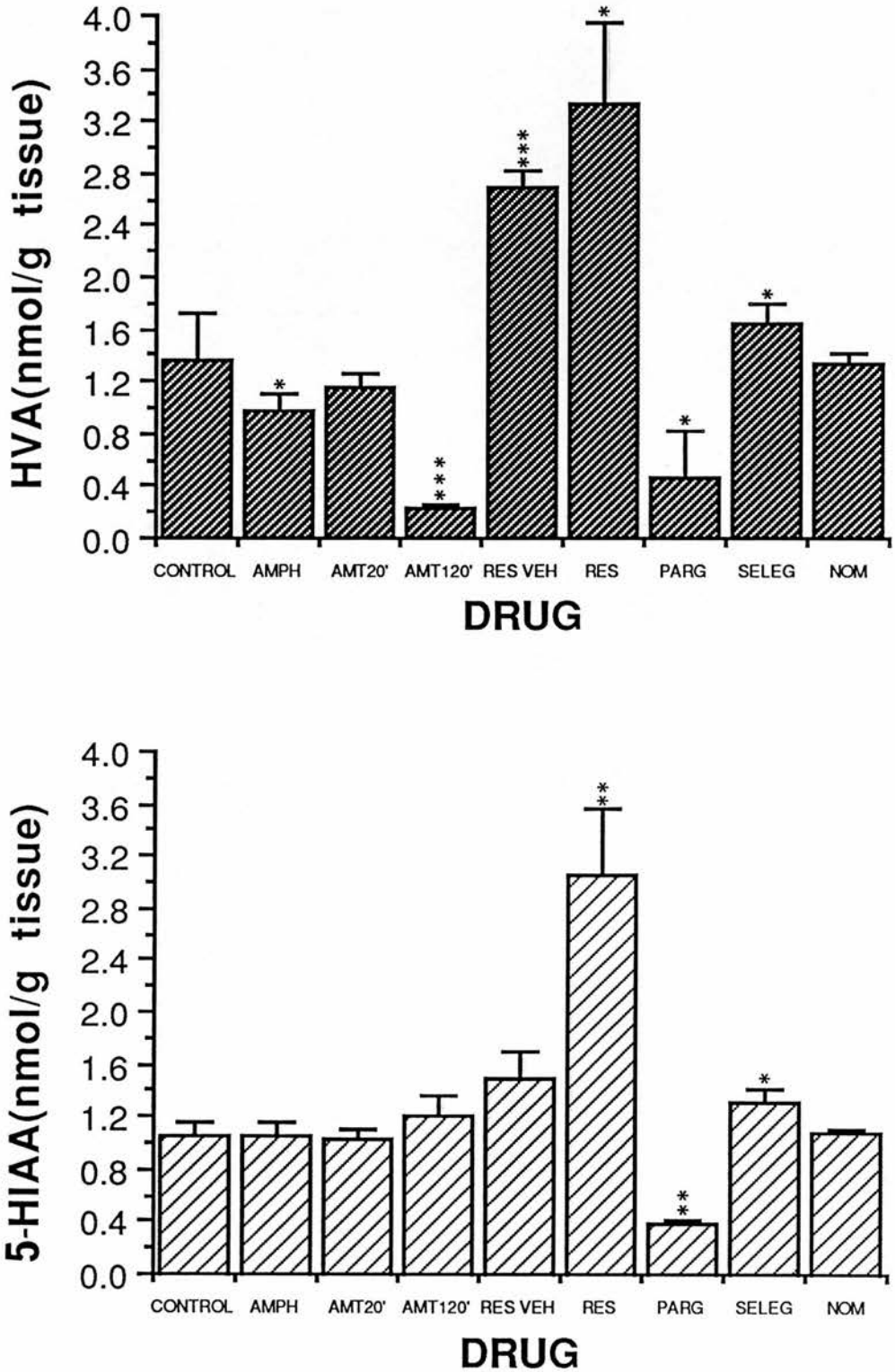


Fig. 3.1.2 Effects of drugs on the total striatal tissue content of HVA (upper panel) and 5-HIAA (lower panel). Drugs were administered for the lengths of time indicated in the text. Results (nmole/g tissue) are the mean ± s.e.m. of eleven animals for control and six animals for drug treated.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

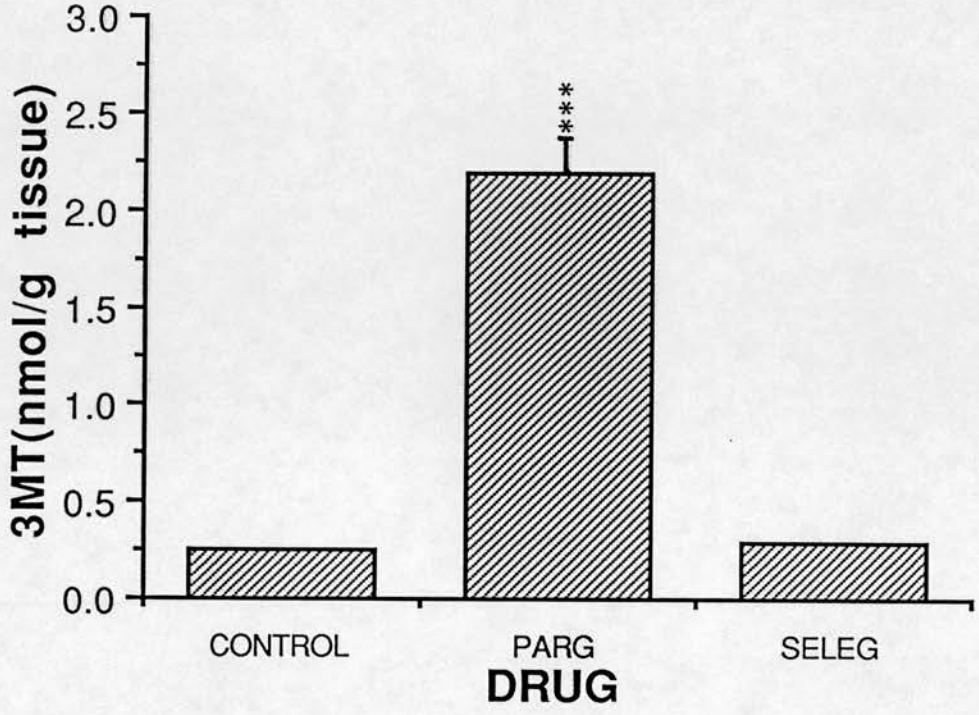


Fig. 3.1.3 Effects of pargyline (75mg/kg i.p.) and selegeline (10mg/kg i.p.) on the total striatal tissue content of 3-MT. Pargyline and selegeline were administered for the lengths of time indicated in the text. Results (nmole/g tissue) are the mean ± s.e.m. of eleven animals for control and six animals for drug treated. ***$p < 0.001$

3.2 Effects of pharmacological and biochemical manipulations upon the efflux of DA and metabolites

3.2.1 Control

Striatal perfusates were collected over a period of 4h without the addition of any drugs. Dialysate levels of DA remained constant over the whole time course with no apparent tendency to decline. Basal DA efflux varied between 0.06 and 0.10pmoles/20 min (n = 4) (Fig. 3.2.1).

Both DOPAC and the other metabolites, HVA and 5-HIAA maintained constant basal efflux levels over the time course of 4h (Fig. 3.2.1). DOPAC efflux levels were approximately 200 times greater than DA averaging between 18.3 and 23.9 pmoles/20 min n = 4. HVA efflux levels averaged between 7.4 and 9.0pmoles/20 min, n = 4. 5-HIAA efflux levels were the lowest of the metabolites averaging between 3.3 and 4.0pmoles/20 min, n = 4. Basal 3MT efflux levels could not be readily measured in all experiments. However, those peaks that were quantifiable produced mean values between 0.04 and 0.07pmoles/20 min (data not shown).

3.2.2 Amphetamine

DL-Amphetamine (4mg/kg i.p.) induced a rapid rise in DA efflux levels to 789% of control 40 min after injection (Fig. 3.2.2). DA efflux levels rose from a pre-stimulation level of 0.09 ± 0.02 to a maximal 0.71 ± 0.11pmoles/20 min ($n = 6$, $p < 0.05$). DA efflux subsequently declined over the following 60 min towards pre-stimulation levels.

The same dose of amphetamine induced a decrease in DOPAC to approximately 20% of control 100 min after injection from 14.6 ± 2.1

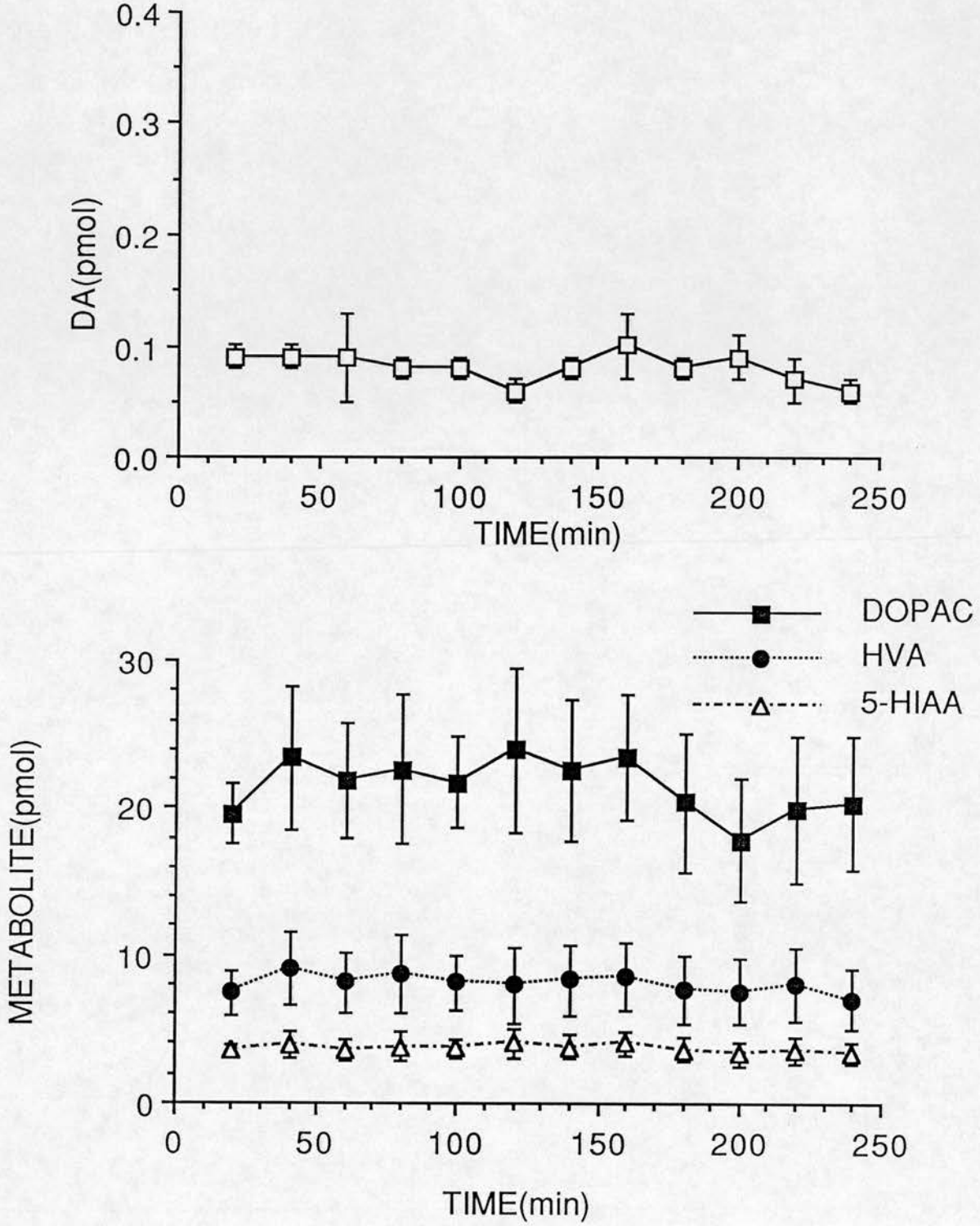


Fig. 3.2.1 The efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel) in striatal dialysates over 240 min in the absence of any pharmacological or biochemical intervention. Results (pmoles/20 min) are mean ± s.e.m. of four independent experiments.

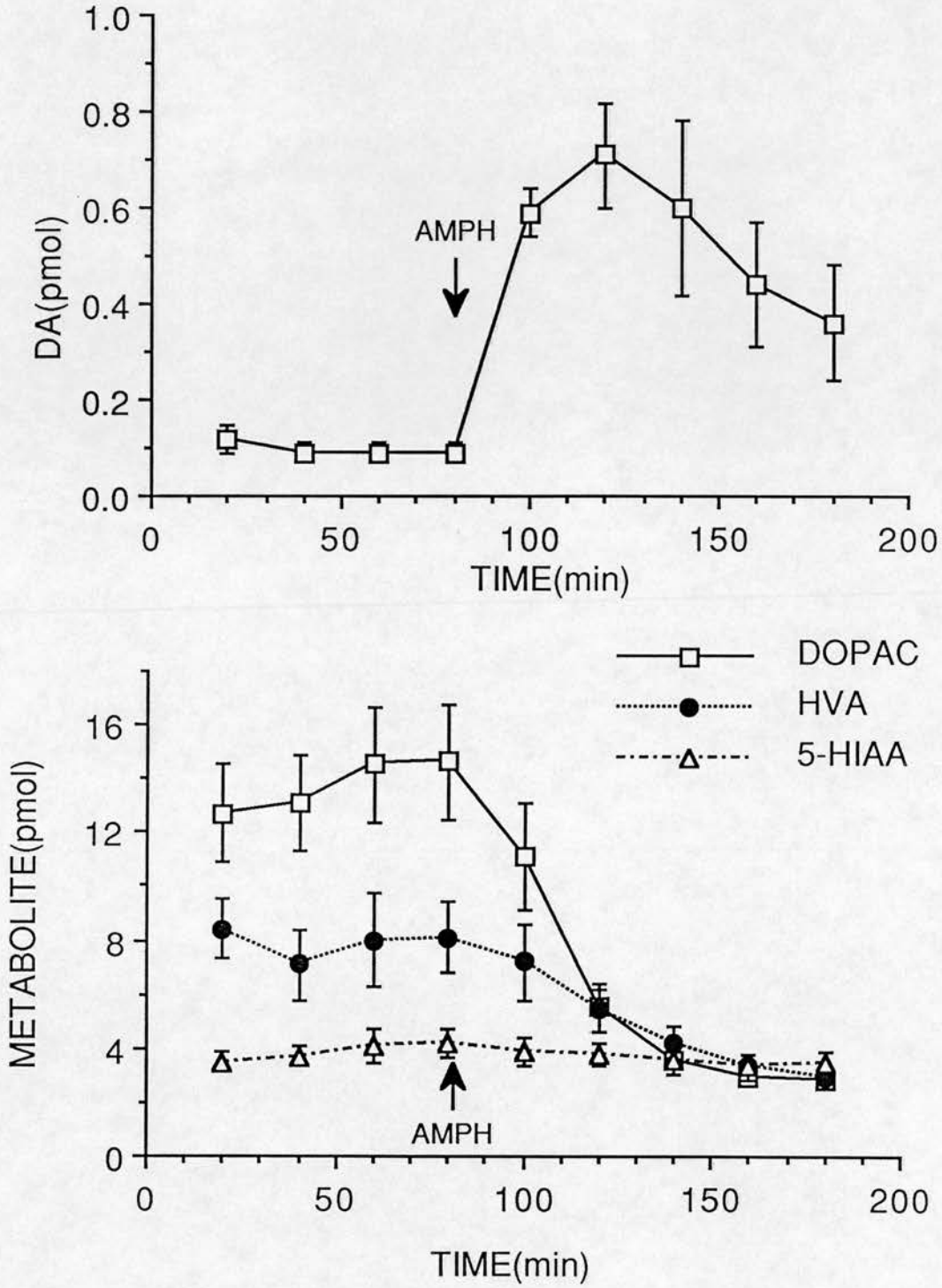


Fig. 3.2.2 Effects of AMPH (4mg/kg i.p.) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

to 3.6 ± 0.6pmoles/20 min (n = 6, p < 0.01) and HVA efflux to approximately 36% of control 100 min after injection from 8.1 ± 1.3 to 4.2 ± 0.6pmoles/20 min (n = 6, p < 0.05) (Fig. 3.2.2). 5-HIAA efflux was unaffected by amphetamine.

3.2.3 Potassium chloride

The inclusion of KCl (90mM) for 20 min in the perfusing buffer, induced a rapid increase in DA efflux to 1255% of control 20 min after infusion, from 0.11 ± 0.02 to 1.38 ± 0.32pmoles/20 min (Fig. 3.2.3, n = 14, p < 0.001). This was followed by a rapid return to pre-stimulation levels when KCl was removed.

DOPAC efflux declined following KCl stimulation (Fig. 3.2.3), from 33.0 ± 3.8 to 24.4 ± 3.1pmoles/20 min to 74% of control 40 min after infusion (n = 14, p < 0.05). After the removal of the stimulating dose of KCl, DOPAC efflux levels increased but remained below control levels. HVA efflux was not significantly affected by KCl despite an apparent tendency to decrease.

3.2.4 Tyramine

Administration of tyramine (5×10^{-5}M) via the dialysis probe for 20 min increased DA efflux to 1800% of control 20 min after infusion from a pre-stimulation level of 0.11 ± 0.01pmoles to a level of 1.80 ± 0.17pmoles/20 min (Fig. 3.2.4, n = 11, p < 0.001). Following removal of tyramine, DA efflux levels declined to control levels over the following 60 min.

In sharp contrast to other DA releasers, tyramine had no effect upon efflux levels of DOPAC or HVA (Fig. 3.2.4).

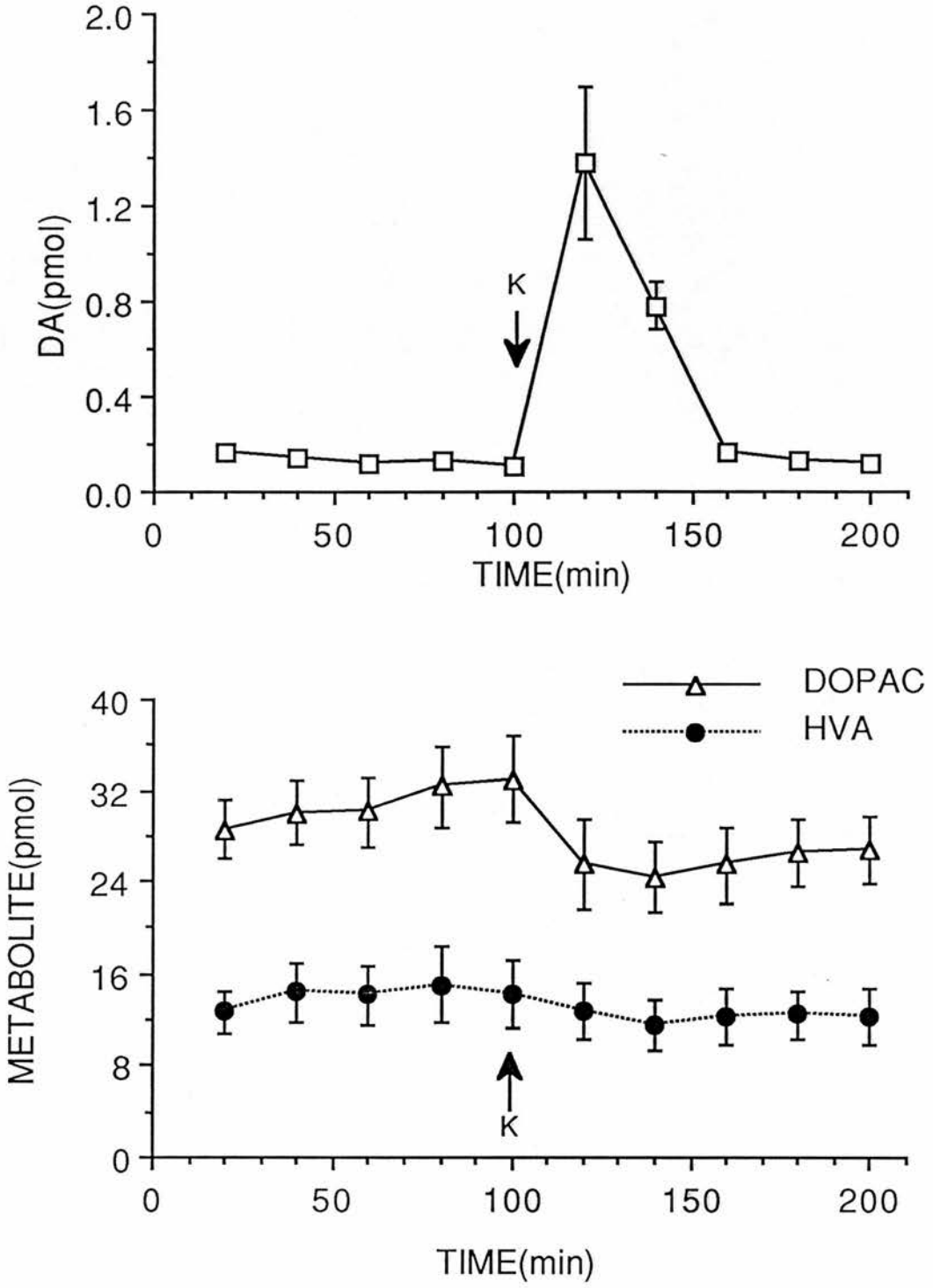


Fig. 3.2.3 Effects of KCl (90mM) perfused intrastriatally for 20 min from the time indicated on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of fourteen independent experiments.

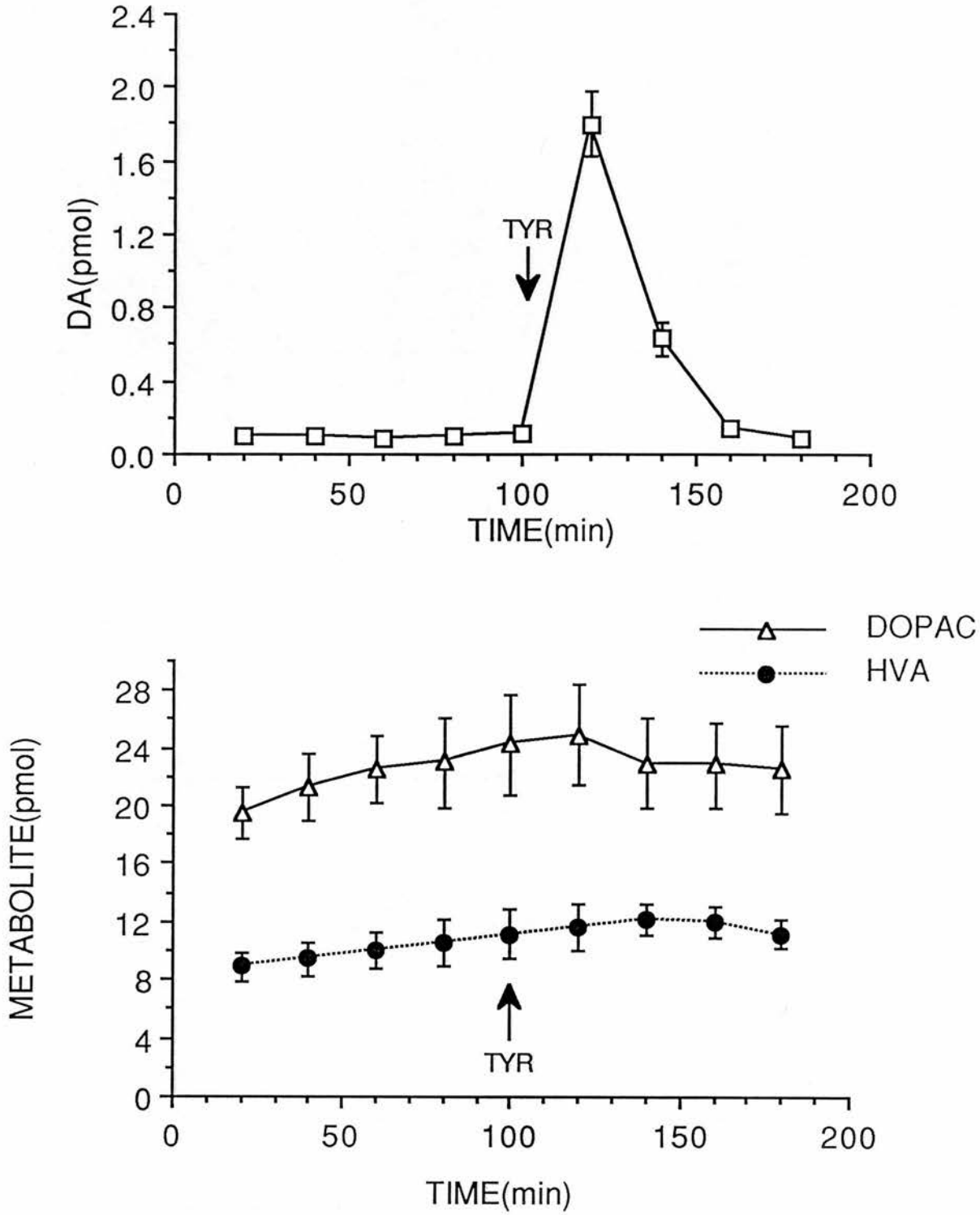


Fig. 3.2.4 Effects of tyramine (50μM) perfused intrastriatally for 20 min from the time indicated on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of eleven independent experiments.

3.2.5 Ouabain

Ouabain (100μM) perfused intrastriatally, increased DA efflux to 2358% of control 40 min after infusion from 0.12 ± 0.02 to 2.83 ± 0.56 pmoles/20 min (Fig. 3.2.5, n = 15, $p < 0.001$). As for amphetamine, maximal efflux occurred between 20 and 40 min following drug administration and subsequently declined.

DOPAC efflux decreased to 72% of control over 100 min from 24.6 ± 5.3 to 17.8 ± 4.2pmoles/20 min (Fig. 3.2.5, n = 15, $p < 0.01$). However, HVA efflux levels were not significantly decreased by ouabain.

3.2.6 Veratrine

Veratrine (100μg/ml) induced an increase in DA efflux to 3222% of control 20 min after infusion from 0.09 ± 0.01 to 2.9 ± 0.56pmoles/20 min (Fig. 3.2.6, n = 13, $p < 0.001$). DA efflux was maximal after 20 min.

Both DOPAC and HVA efflux declined following veratrine stimulation to 43% and 46% of control respectively over 100 min. DOPAC declined from 22.6 ± 3.1 to 9.7 ± 1.7pmoles/20 min and HVA from 15.0 ± 2.0 to 6.9 ± 0.9pmoles/20 min (Fig. 3.2.6, n = 13 for both, $p < 0.001$, $p < 0.05$ respectively).

3.2.7 Clorgyline

Clorgyline (10mg/kg i.p.) induced a gradual rise in DA efflux to 192% of control from 0.12 ± 0.02 to 0.23 ± 0.05pmoles/20 min (Fig. 3.2.7, n = 6, $p < 0.05$). Similarly, clorgyline induced an increase in 3MT efflux to 442% of control over 120 min from 0.12 ± 0.06 to 0.53 ± 0.15pmoles/20 min (Fig. 3.2.7, n = 6, $p < 0.01$). Both DOPAC and HVA efflux decreased during the 120 min of clorgyline treatment

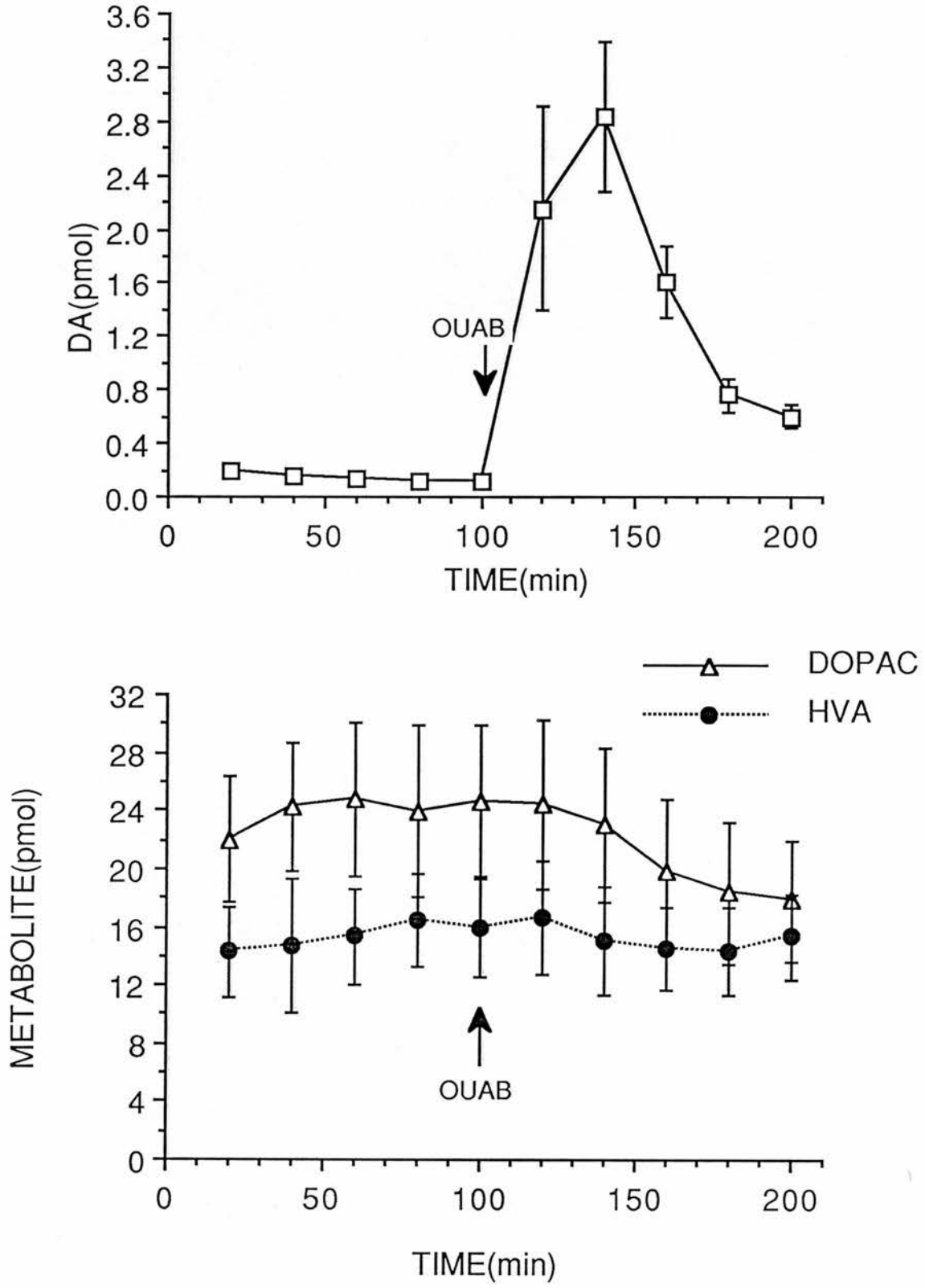


Fig. 3.2.5 Effects of ouabain (100μM) perfused continuously intrastriatally from the time indicated on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of fifteen independent experiments.

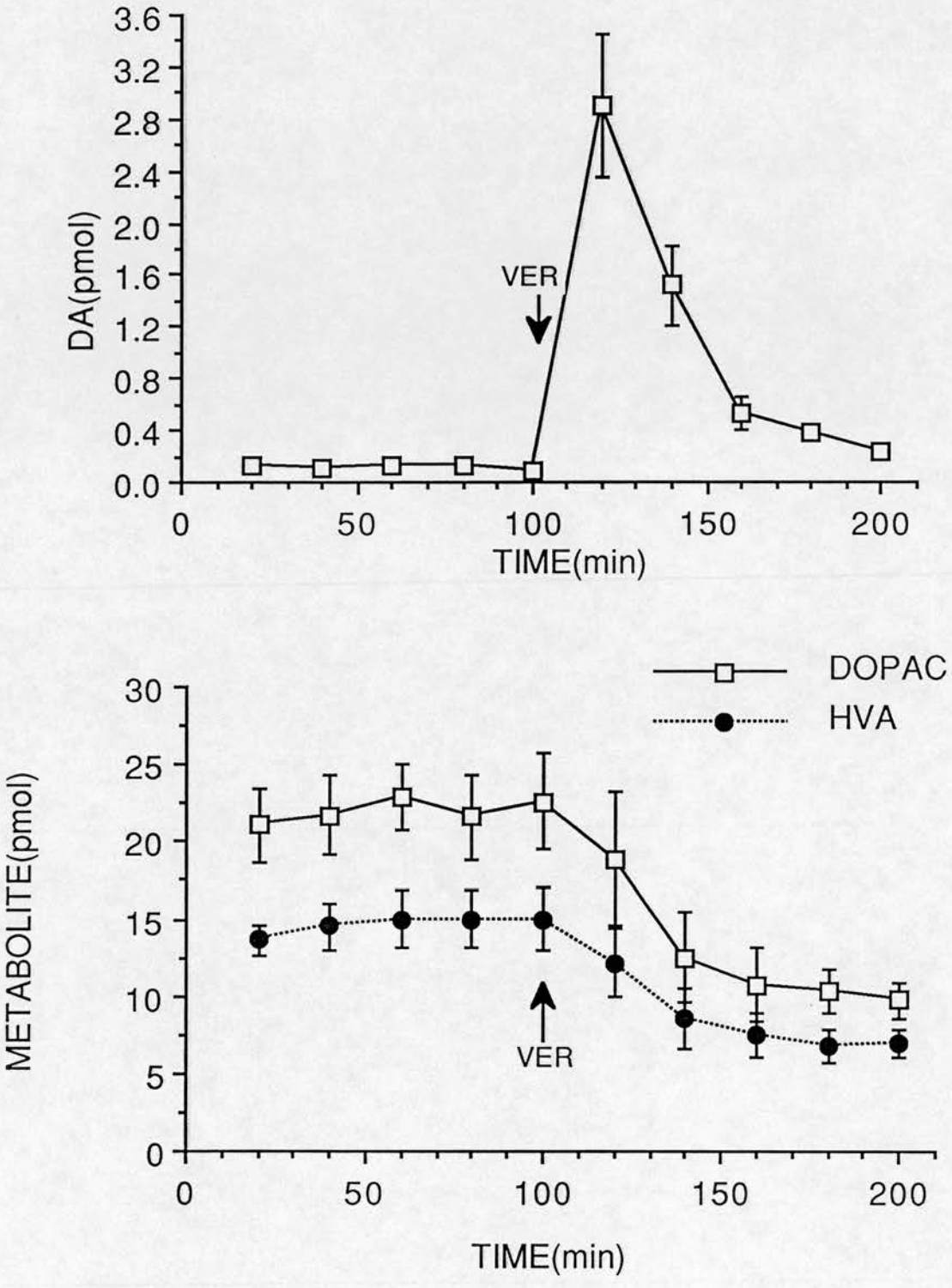


Fig. 3.2.6 Effects of veratrine (100µg/ml) perfused continuously intrastriatally from the time indicated on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of thirteen independent experiments.

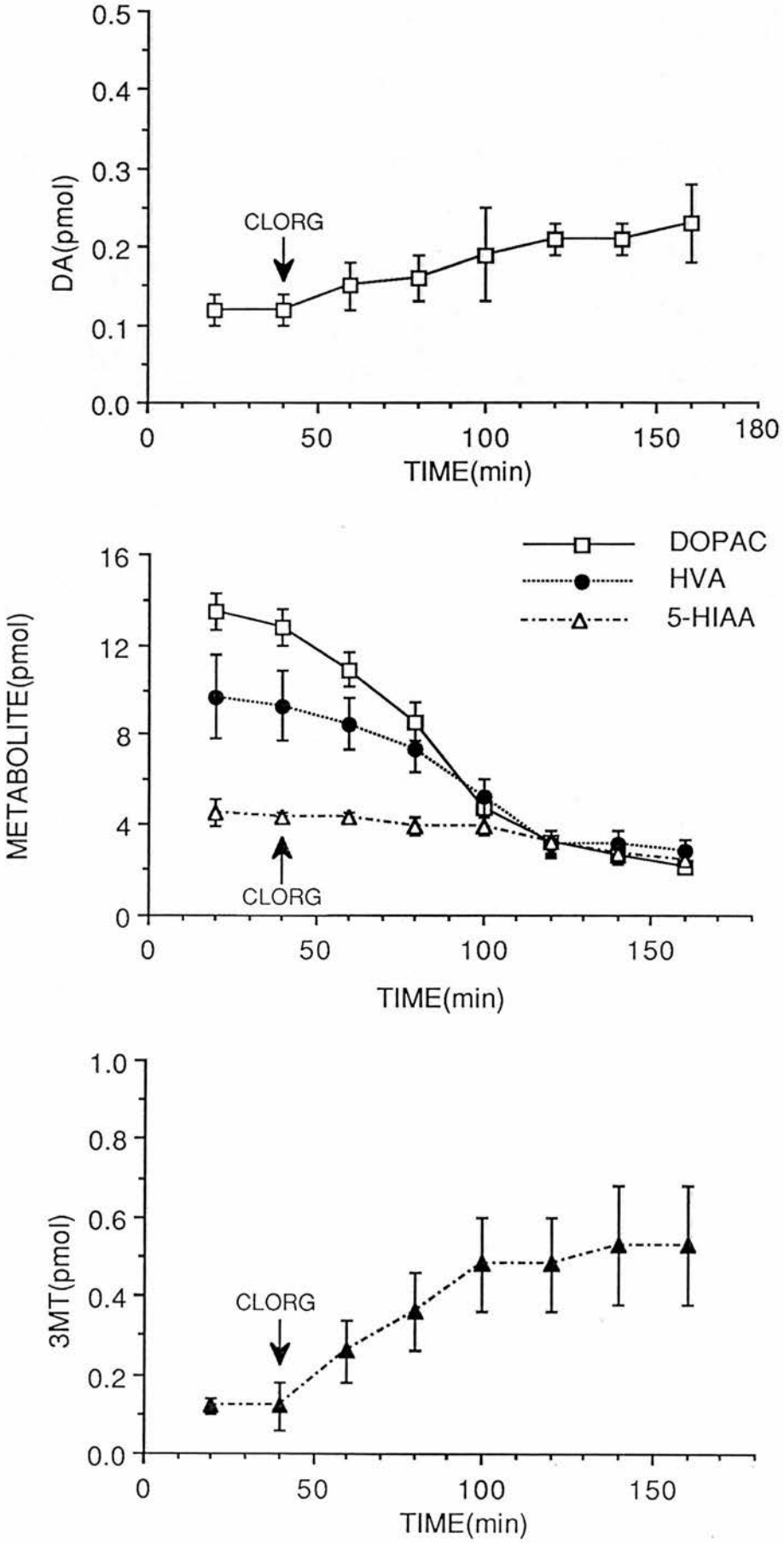


Fig. 3.2.7 Effects of clorgyline (10mg/kg i.p.) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

to 19% and 30% of control respectively. DOPAC decreased from 12.8 ± 0.81 to 2.4 ± 0.32pmoles/20 min, (Fig. 3.2.7, n = 6, $p < 0.01$) and HVA from 9.3 ± 1.6 to 2.8 ± 0.5pmoles/20 min (Fig. 3.2.7, n = 6, $p < 0.05$).

5-HIAA efflux was also reduced by clorgyline to 50% of control from 4.3 ± 0.18 to 2.4 ± 0.18pmoles/20 min (Fig. 3.2.7, n = 6, $p < 0.01$).

3.2.8 Pargyline

Pargyline (75mg/kg i.p.) induced a greater increase in DA efflux than clorgyline to 280% of control over 120 min from 0.10 ± 0.02 to 0.28 ± 0.06pmoles/20 min (Fig. 3.2.8, n = 18, $p < 0.05$). 3MT efflux also increased to a greater extent to 813% of control over 120 min from 0.08 ± 0.01 to 0.65 ± 0.17pmoles (Fig. 3.2.8, n = 18, $p < 0.05$).

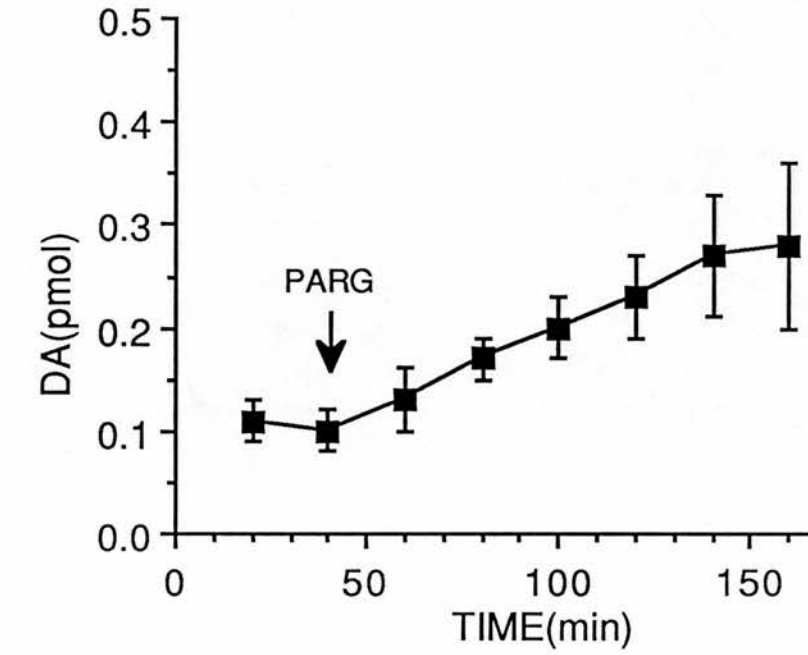
DOPAC and HVA efflux levels were both decreased by pargyline to 10% and 15% of control respectively over 120 min. DOPAC decreased from 18.6 ± 2.8 to 1.8 ± 0.5pmoles/20 min (Fig. 3.2.8, n = 18, $p < 0.01$ and HVA from 10.3 ± 1.5 to 1.5 ± 0.45pmoles/20 min (Fig. 3.2.8, n = 18, $p < 0.001$).

5-HIAA efflux was also reduced by pargyline to 60% of control over 120 min from 4.8 ± 0.80 to 2.9 ± 0.36pmoles/20 min (Fig. 3.2.8, n = 18, $p < 0.05$).

3.2.9 Selegeline

Selegeline (10mg/kg i.p.) had no effect upon DA efflux levels (Fig. 3.2.9) which averaged between 0.14 ± 0.02 to 0.17 ± 0.03pmoles/20 min. DOPAC efflux was reduced to 67% of control over 160 min from 20.1 ± 2.1 to 13.4 ± 3.0pmoles/20 min (Fig. 3.2.9,

Fig. 3.2.8 Effects of pargyline (75mg/kg i.p.) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel), and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of eighteen independent experiments.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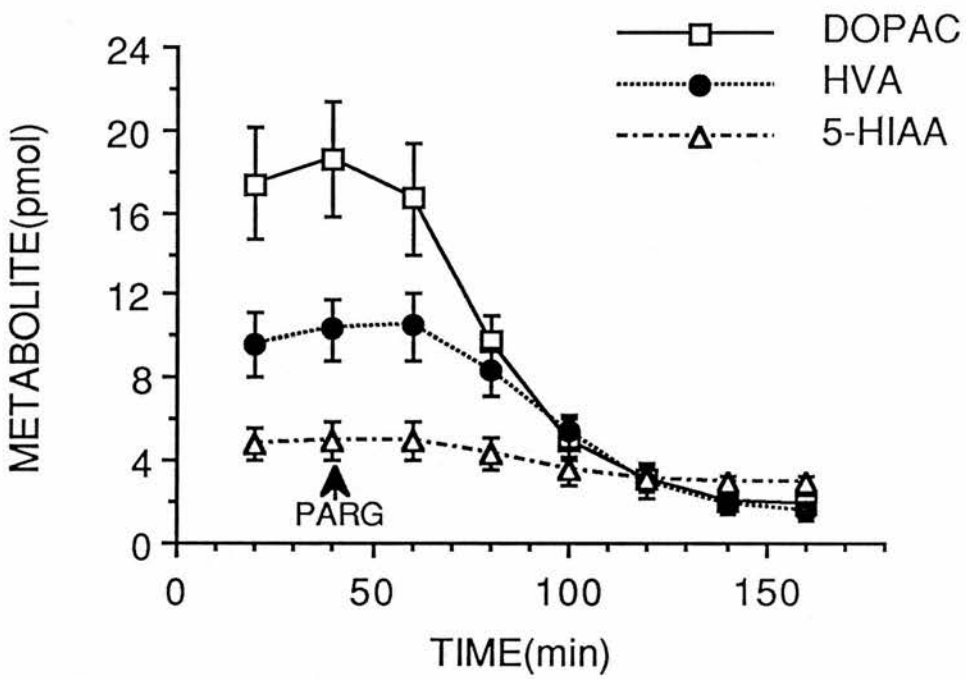
0.4
0.3
0.2
0.1
0.0
DA(pmol)
PARG
0
50
100
150
TIME(min)


DOPAC
HVA
5-HIAA
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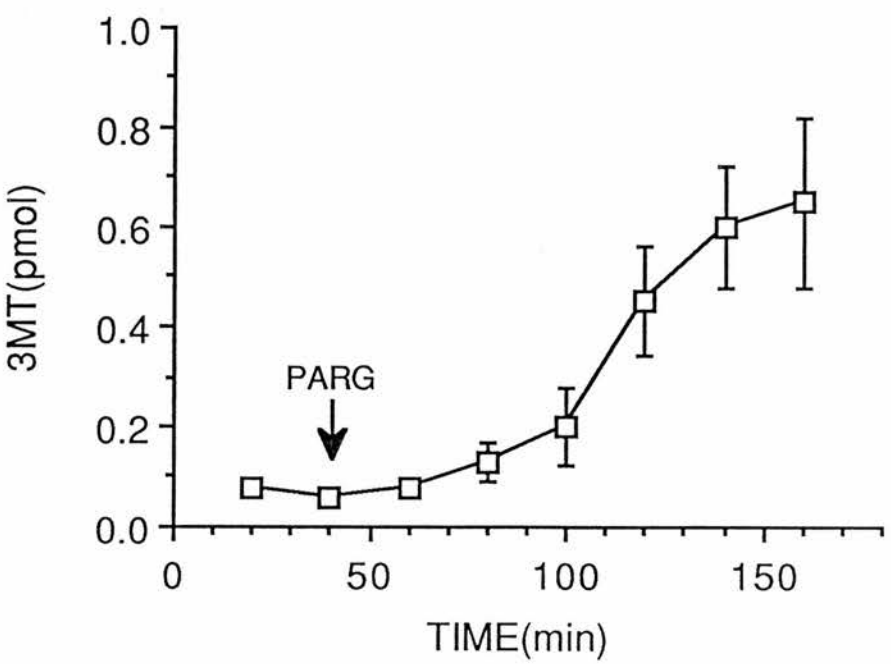
20
16
12
8
4
0
METABOLITE(pmol)
PARG
0
50
100
150
TIME(min)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0.8
0.6
0.4
0.2
0.0
3MT(pmol)
PARG
0
50
100
150
TIME(min)

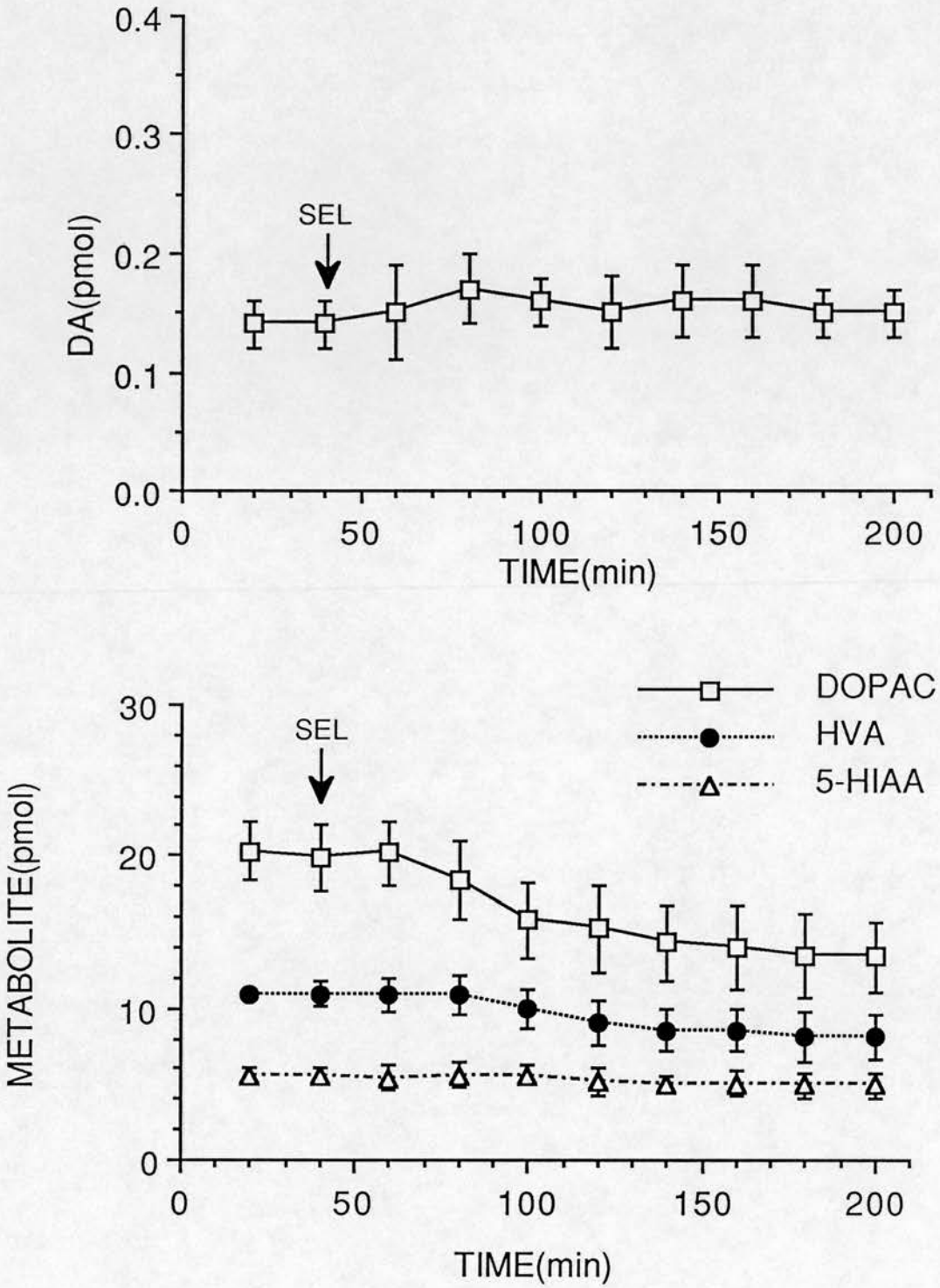


Fig. 3.2.9 Effects of selegeline (10mg/kg i.p.) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of seven independent experiments.

n = 7, $p < 0.01$).

HVA efflux was also reduced to 74% of control over 160 min from 10.9 ± 0.8 to 8.1 ± 1.5pmoles/20 min (Fig. 3.2.9, n = 7, $p < 0.001$). 5-HIAA efflux was consistently reduced in all experiments to 89% of control over 160 min from 5.5 ± 0.6 to 4.9 ± 0.76pmoles (Fig. 3.2.9, n = 7, $p < 0.001$).

3.2.10 Reserpine

Reserpine (5mg/kg i.p.) induced a progressive decline in DA efflux levels to 18% of control over 180 min from an average of 0.17 ± 0.04 to 0.03 ± 0.01pmoles/20 min (Fig. 3.2.10, n = 18, $p < 0.01$). Reserpine had no significant effect upon DOPAC, HVA or 5-HIAA efflux levels (Fig. 3.2.10).

3.2.11 α-Methyl-P-Tyrosine

AMT (250mg/kg i.p.) administration led to a progressive decline in DA efflux to 33% of control levels over 120 min from 0.14 ± 0.02 to 0.05 ± 0.01pmoles/20 min (Fig. 3.2.11, n = 6, $p < 0.05$). Both DOPAC and HVA efflux declined to 31% and 39% of control respectively over 120 min from 18.1 ± 3.2 to 5.7 ± 1.1pmoles/20 min and 10.3 ± 1.3 to 4.0 ± 0.6pmoles/20 min respectively (Fig. 3.2.11, n = 6, $p < 0.01$ for both), whereas 5-HIAA efflux was unaffected.

3.2.12 Nomifensine

Nomifensine induced an increase in DA efflux when given intraperitoneally (20mg/kg) or via the dialysis probe (10μM) to 308% and 225% of control respectively over 100 min. In the former case, efflux levels increased from 0.12 ± 0.05 to 0.37 ± 0.08pmoles/20 min (Fig. 3.2.12a, n = 6, $p < 0.05$) and in the latter case, efflux

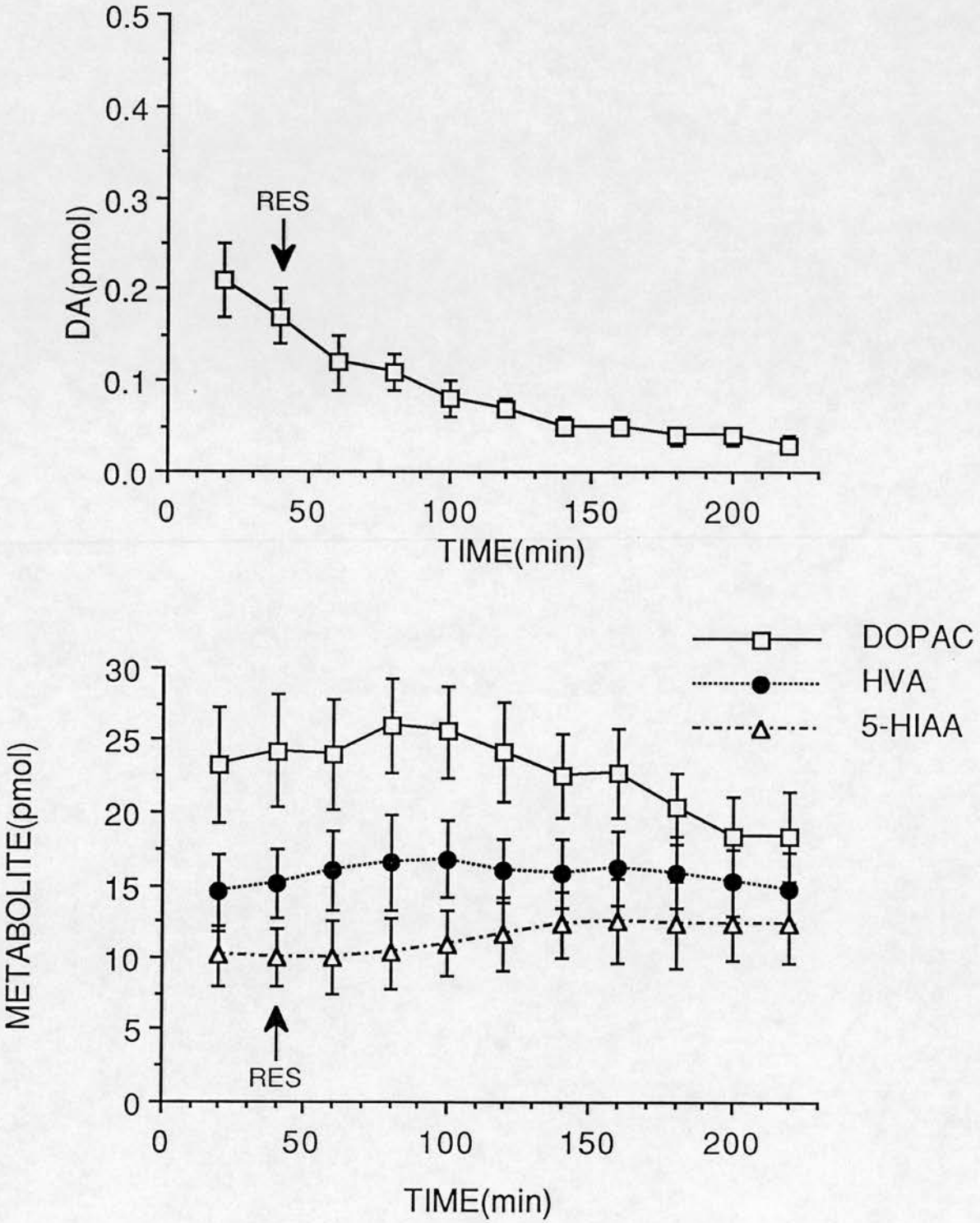


Fig. 3.2.10 Effects of reserpine (5mg/kg i.p.) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of eighteen independent experiments.

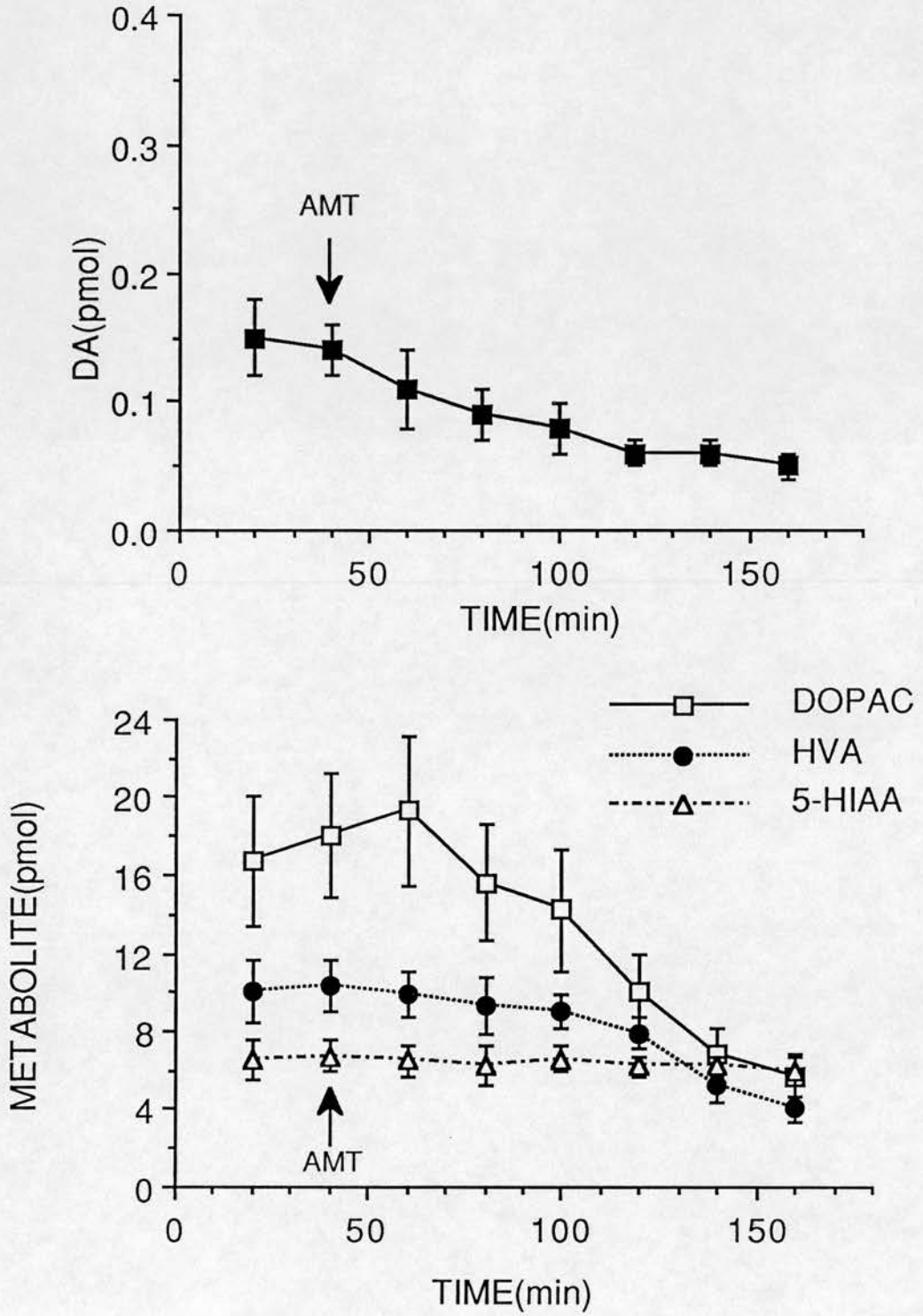


Fig. 3.2.11 Effects of AMT (250mg/kg i.p.) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

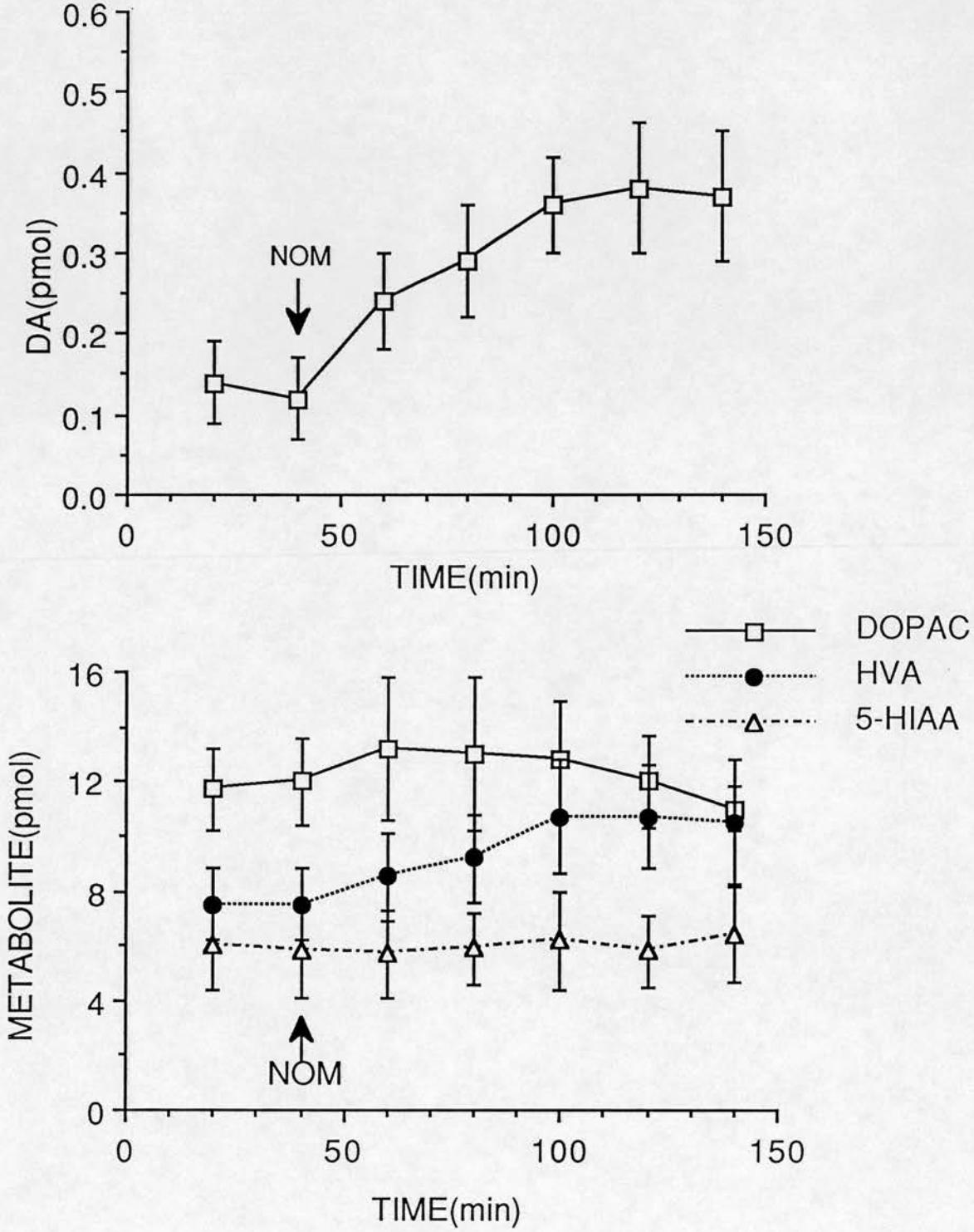


Fig. 3.2.12a Effects of nomifensine (20mg/kg i.p.) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

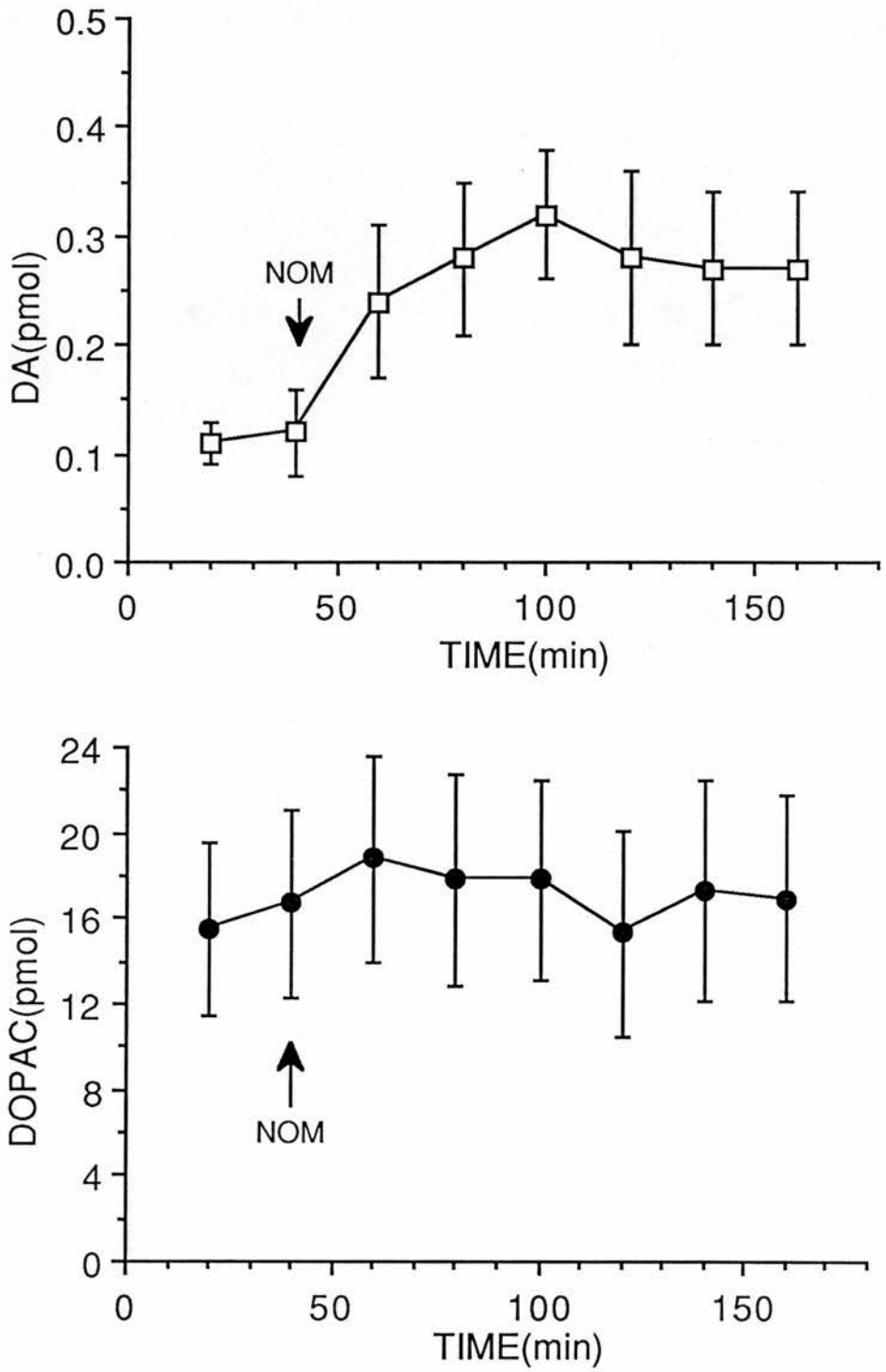


Fig. 3.2.12b Effects of nomifensine (10μM) perfused intrastriatally through the dialysis probe on the efflux of DA (upper panel) and DOPAC (lower panel). Nomifensine was perfused continuously from the time indicated. Results (pmoles/20 min) are the mean ± s.e.m. of twelve independent experiments.

levels increased from 0.12 ± 0.04 to 0.27 ± 0.07pmoles/20 min (Fig. 3.2.12b, n = 12, $p < 0.01$). Neither route of administration affected DOPAC efflux levels whereas HVA efflux was increased by i.p. nomifensine to 143% of control over 100 min, from an average of 7.5 ± 1.3 to a maximal 10.7 ± 1.9pmoles/20 min (Fig. 3.2.12a, n = 6 animals, $p < 0.05$). 5-HIAA efflux was not affected by i.p. nomifensine (Fig. 3.2.12a).

3.2.13 Tetrodotoxin

TTX (2μM) induced a steady decline in DA efflux levels to 20% of control over 180 min from 0.14 ± 0.03 to 0.03 ± 0.02pmoles/20 min (Fig. 3.2.13, n = 6, $p < 0.01$). Similarly, DOPAC efflux also decreased to 46% of control over 180 min from 24.3 ± 3.4 to 11.2 ± 2.2pmoles/20 min (Fig. 3.2.13, n = 6, $p < 0.01$). HVA and 5-HIAA efflux levels were not affected by TTX.

3.2.14 EGTA

EGTA (20mM) caused an overall decline in DA efflux levels to 0% of control over 160 min. The results were variable due to sometimes undetectable levels of DA. DA efflux levels decreased from 0.12 ± 0.03 to 0 ± 0.03pmoles/20 min (Fig. 3.2.14, n = 6, $p < 0.01$). DOPAC efflux also decreased to 56% of control over 160 min from a control value of 19.3 ± 4.2 to 10.9 ± 1.5pmoles/20 min (Fig. 3.2.14, n = 6, $p < 0.01$). HVA and 5-HIAA efflux were not affected by EGTA.

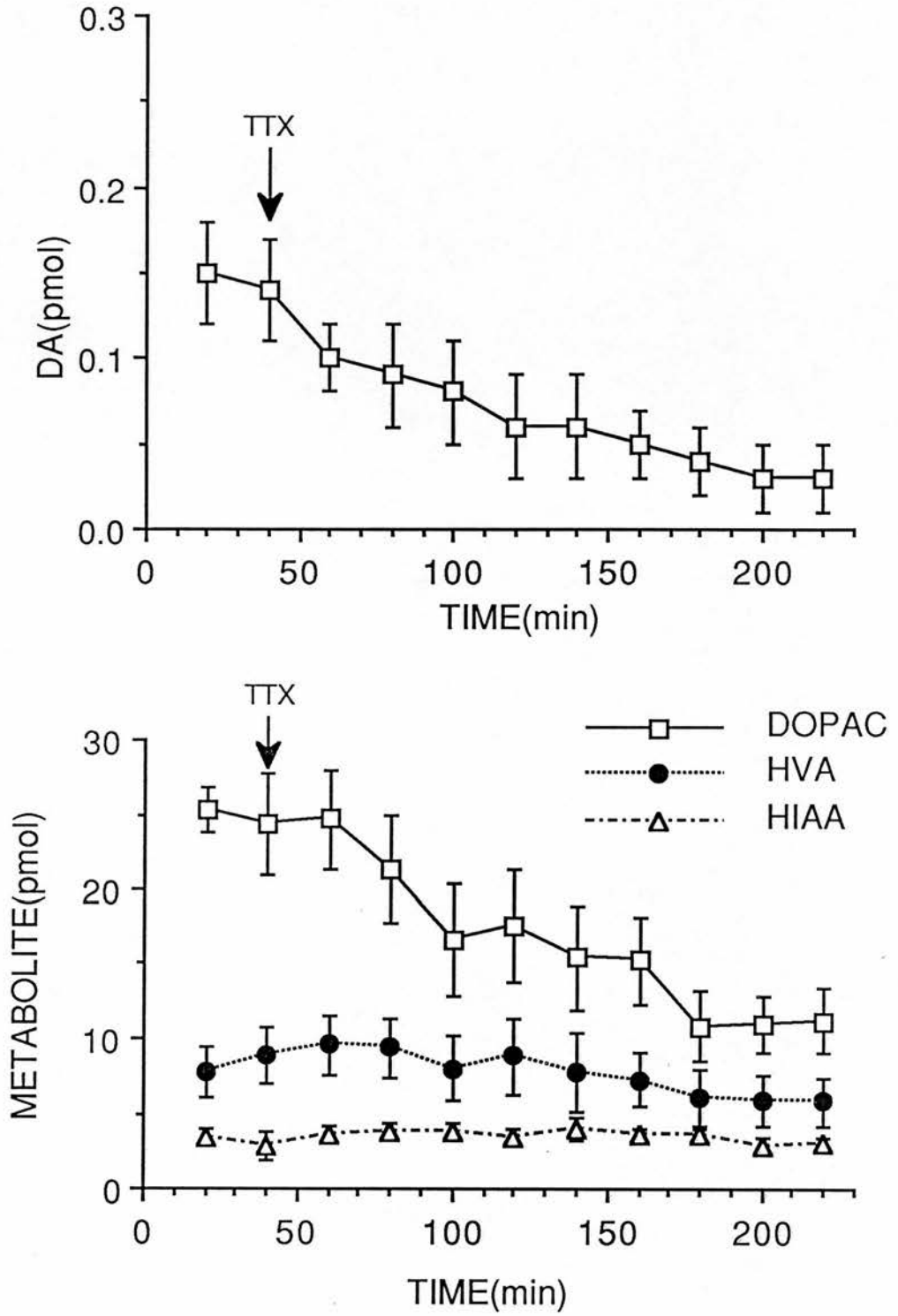


Fig. 3.2.13 Effects of TTX (2μM) perfused intrastriatally through the dialysis probe from the time indicated, on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

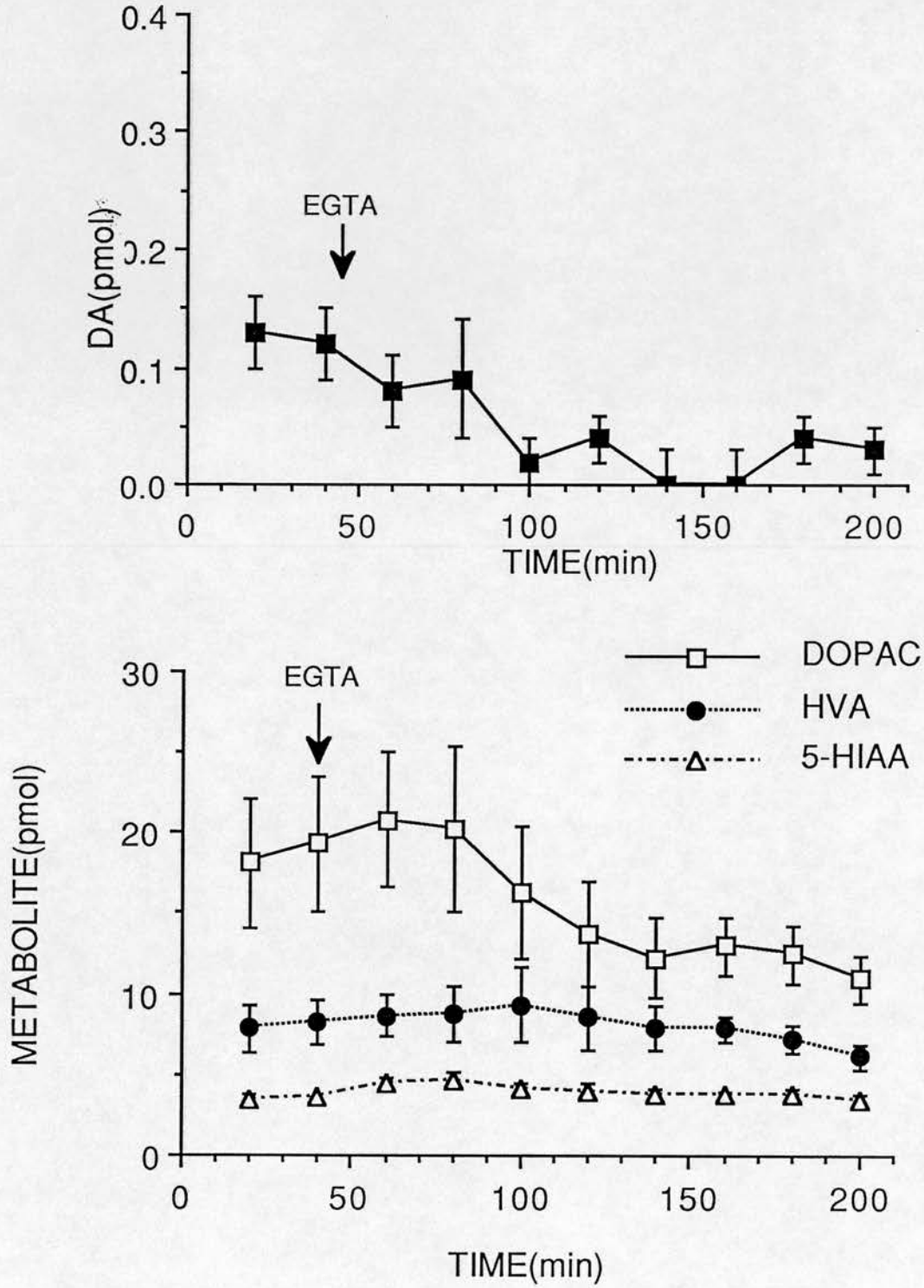


Fig. 3.2.14 Effects of calcium removal and EGTA (20mM) perfused intrastriatally through the dialysis probe on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

3.3 Effects of biochemical and pharmacological manipulations upon amphetamine induced efflux of DA, DOPAC, HVA, 5-HIAA and 3MT

3.3.1 Dose dependence of the amphetamine response

A dose of 2mg/kg AMPH induced an increase in DA efflux (maximal response = 350% of control) and decrease in DOPAC (20-30% of control) and HVA efflux (40-50% of control) (Fig. 3.3.1a and b). Increasing the dose of AMPH (4,8 and 16mg/kg), led to a graded increase in maximal DA efflux (720%, 1100% and 1400% of control respectively). In contrast, DOPAC and HVA efflux were not reduced further as the AMPH dose increased. Low doses of AMPH (2 and 4mg/kg) had no effect upon 5-HIAA efflux, whereas higher doses (8 and 16 mg/kg) induced a small dose related decrease (Fig. 3.3.1b). 3MT efflux was also unaffected at the two lower doses, but increased in a dose related manner at 8 and 16mg/kg AMPH (Fig. 3.3.1b). A 4mg/kg dose of AMPH was chosen for use in further experiments since this dose permits increases and decreases in AMPH induced DA efflux following drug pretreatment to be determined. This dose also allows behavioural correlations to be made with AMPH induced DA efflux.

3.3.2 α-Methyl-p-tyrosine

In AMT treated animals (250mg/kg simultaneous), AMPH maximally increased DA efflux to 256% of basal efflux 40 min after injection (Fig. 3.3.2a, 0.16 ± 0.03 to 0.41 ± 0.04pmoles/20 min, n = 6). Total DA efflux over a 60 min period was reduced by 70% when compared with control animals (Table 3.3.1; control 2.0 ± 0.4pmoles; AMT 0.6 ± 0.2pmoles; $p < 0.01$). When AMT was given 120 min prior to AMPH, DA efflux was maximally increased to 479% of

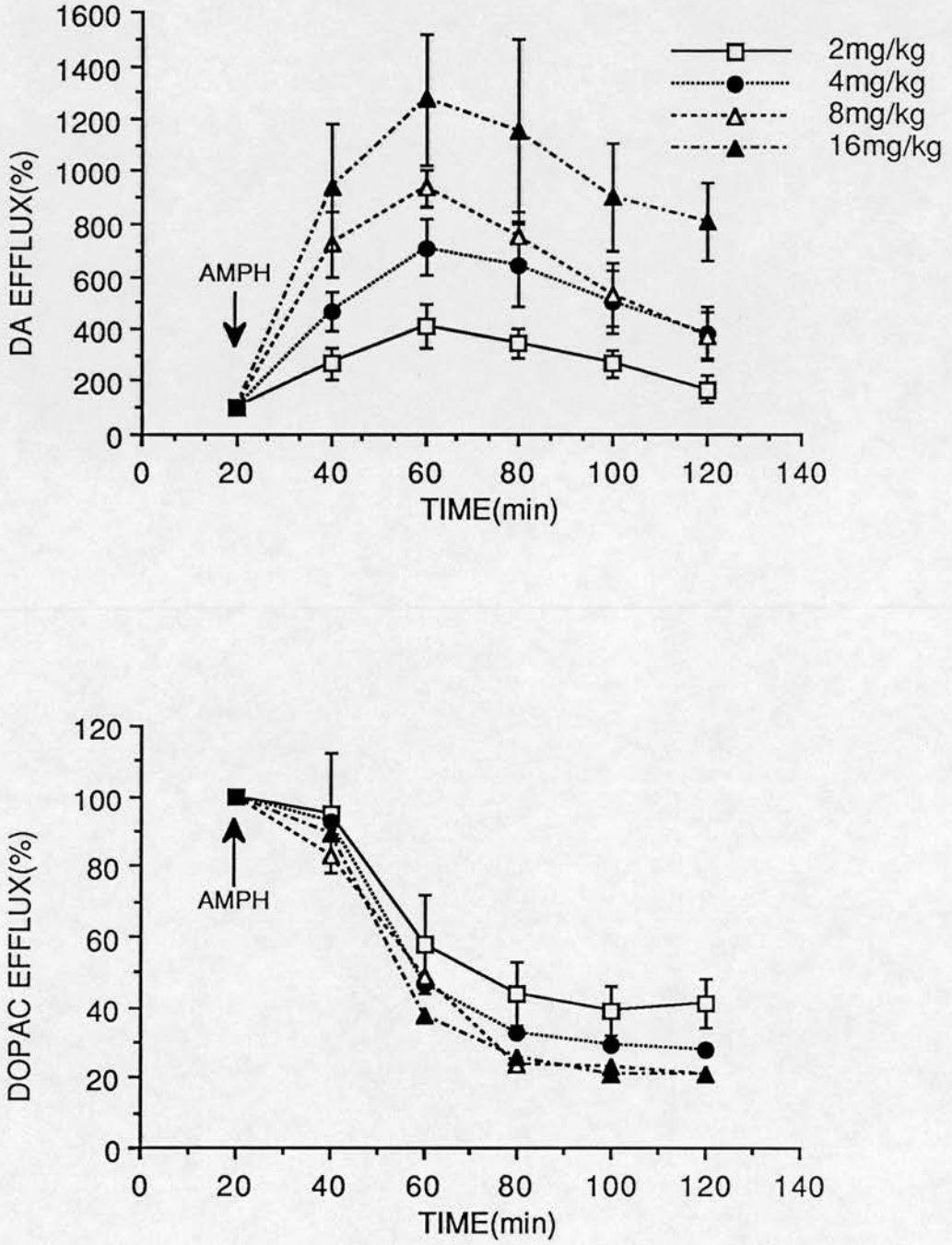
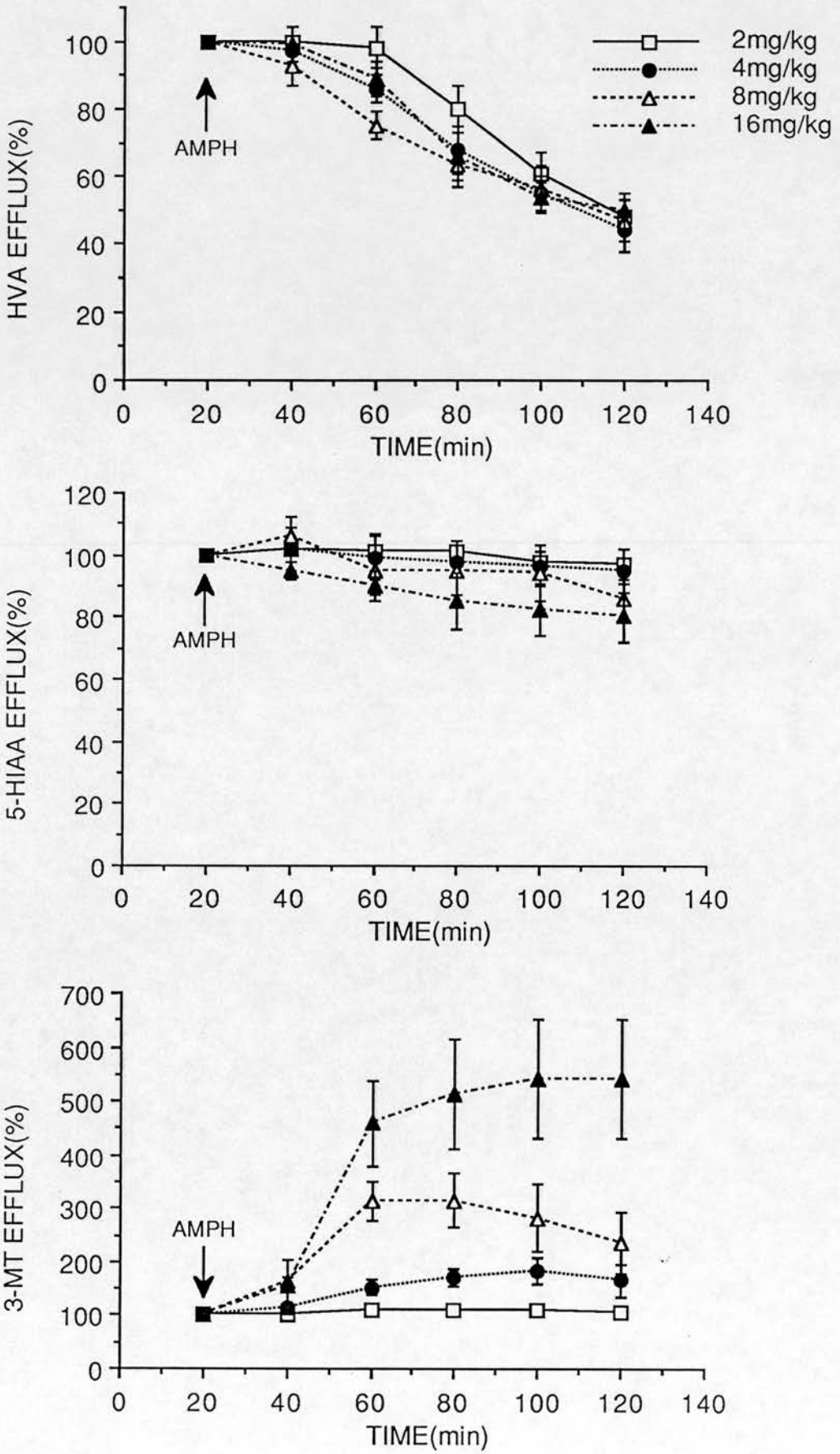


Fig. 3.3.1a Effects of increasing doses of AMPH on DA efflux (upper panel) and DOPAC efflux (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

Fig. 3.3.1b Effects of increasing doses of AMPH on HVA (upper panel), 5-HIAA (middle panel) and 3-MT efflux (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.


HVA EFFLUX(%)
100
80
60
40
20
0
AMPH
2mg/kg
4mg/kg
8mg/kg
16mg/kg
0
20
40
60
80
100
120
140
TIME(min)
5-HIAA EFFLUX(%)
120
100
80
60
40
20
0
AMPH
0
20
40
60
80
100
120
140
TIME(min)
3-MT EFFLUX(%)
700
600
500
400
300
200
100
0
AMPH
0
20
40
60
80
100
120
140
TIME(min)

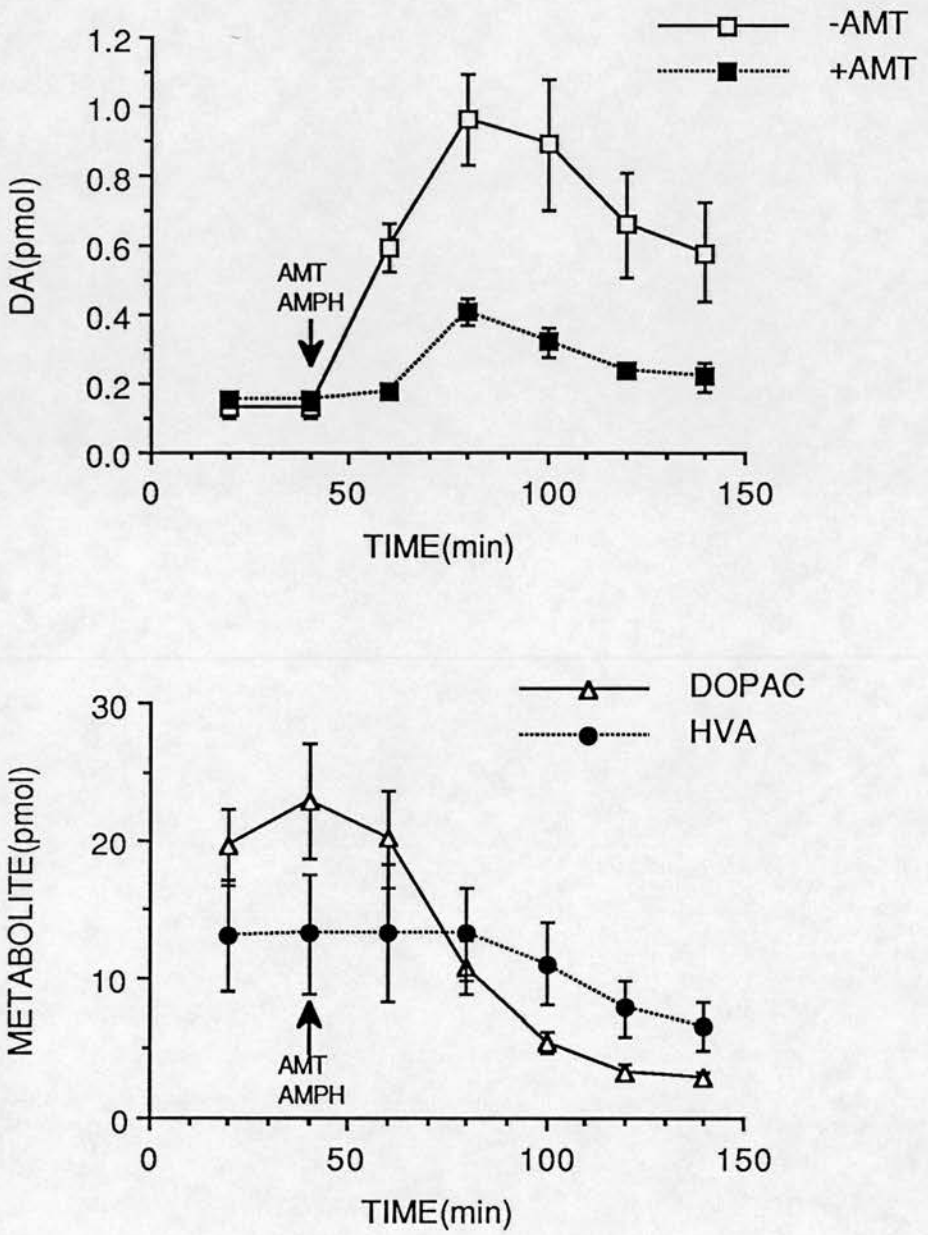


Fig. 3.3.2a Effects of AMT (250mg/kg i.p.) given simultaneously with AMPH (4mg/kg i.p.) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

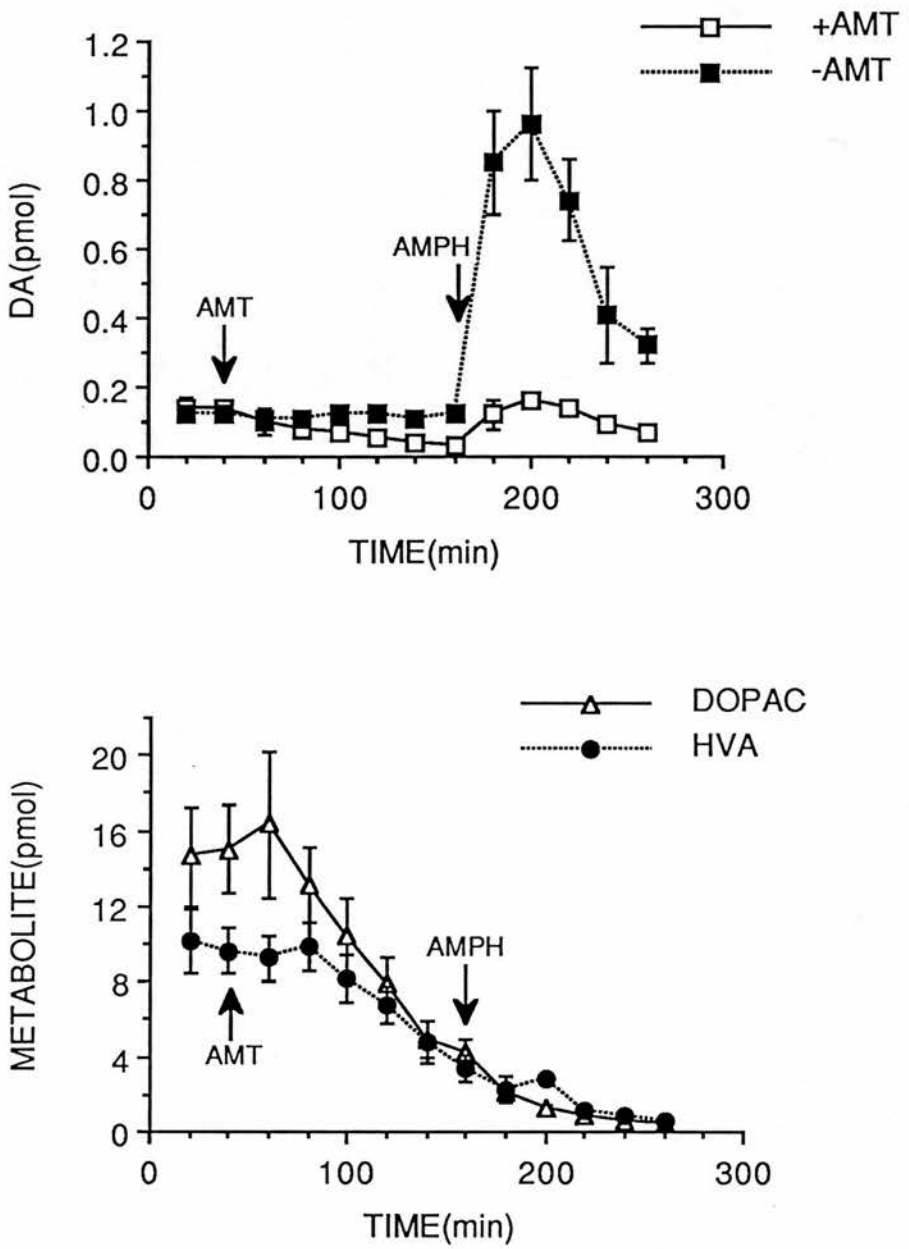


Fig.3.3.2b Effects of AMT (250mg/kg i.p.) given 120 min prior to AMPH (4mg/kg i.p.) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

basal efflux 40 min after injection of AMPH (Fig. 3.3.2b, 0.33 ± 0.10 to 1.58 ± 0.11pmoles/20 min, n = 6). Total DA efflux over a 60 min period was reduced by 85% when compared with control animals (Table 3.3.1, control 2.0 ± 0.4pmoles; AMT 0.3 ± 0.1pmoles; $p < 0.01$).

AMT induced a decline in DOPAC and HVA efflux (Fig. 3.3.2b) which appeared to be accelerated by AMPH.

3.3.3 Reserpine

In reserpine (5mg/kg i.p.) treated animals, AMPH maximally increased DA efflux to 545% of basal efflux 40 min after injection (Fig. 3.3.3, 0.11 ± 0.02 to 0.60 ± 0.08pmoles/20 min, n = 6). Total DA efflux over a 60 min period was not reduced by a significant amount (Table 3.3.1; control 1.8 ± 0.2pmoles; reserpine 1.3 ± 0.2pmoles). Reserpine induced a decline in DOPAC and HVA efflux which appeared to be accelerated by AMPH (Fig. 3.3.3).

3.3.4 Pargyline

In pargyline (75mg/kg i.p.) treated animals, AMPH maximally increased DA efflux to 627% of basal efflux 40 min after injection (Fig. 3.3.4, 0.33 ± 0.07 to 2.07 ± 0.22pmoles/20 min, n = 6). Total DA efflux over 60 min was increased by 239% when compared with control animals (Table 3.3.1, control 1.8 ± 0.4pmoles; pargyline 4.3 ± 0.6pmoles; $p < 0.01$). The rapid decline in DOPAC and HVA efflux following the pargyline injection (Fig. 3.3.4) prevented any evaluation of the effects of AMPH upon efflux of these metaolites.

Pargyline induced a rapid rise in 3-MT efflux to 722% of basal efflux over 100 min (Fig. 3.3.4). AMPH induced a further more rapid rise in 3-MT efflux to 246% of pre-AMPH levels, 1778% of

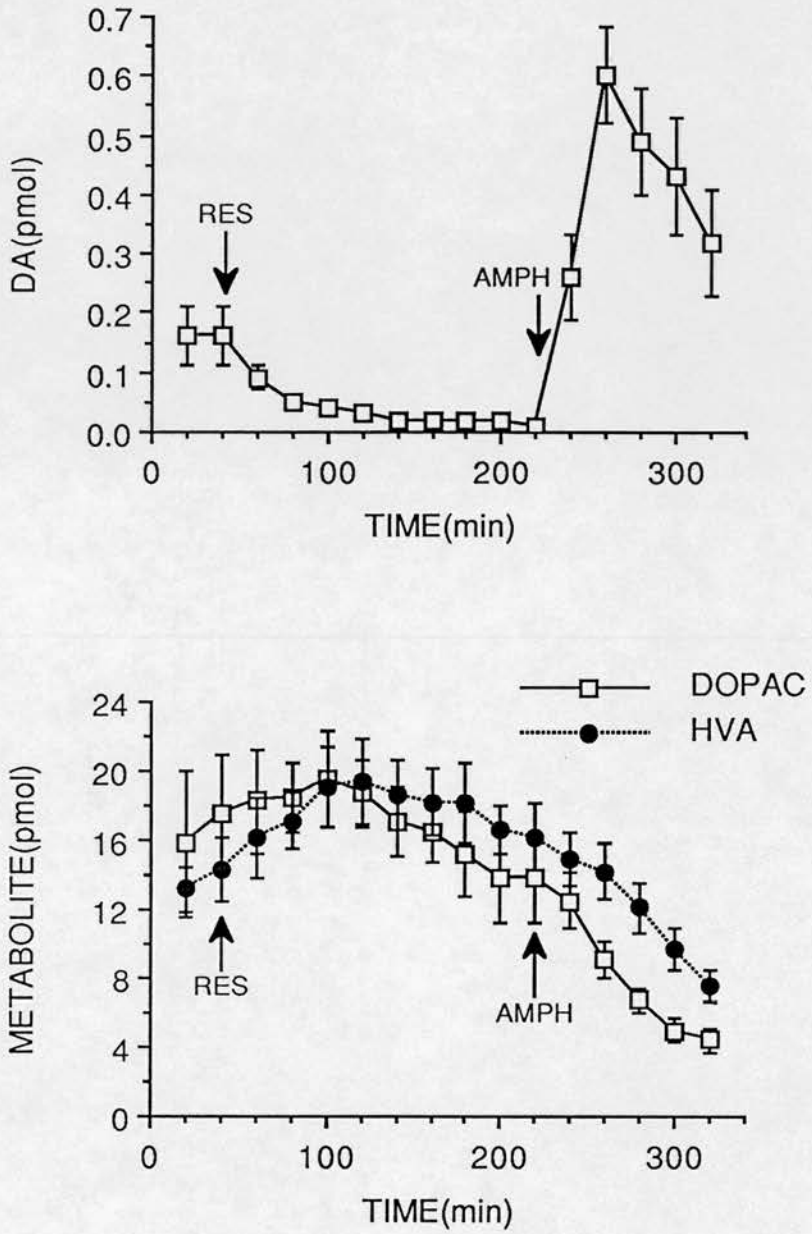
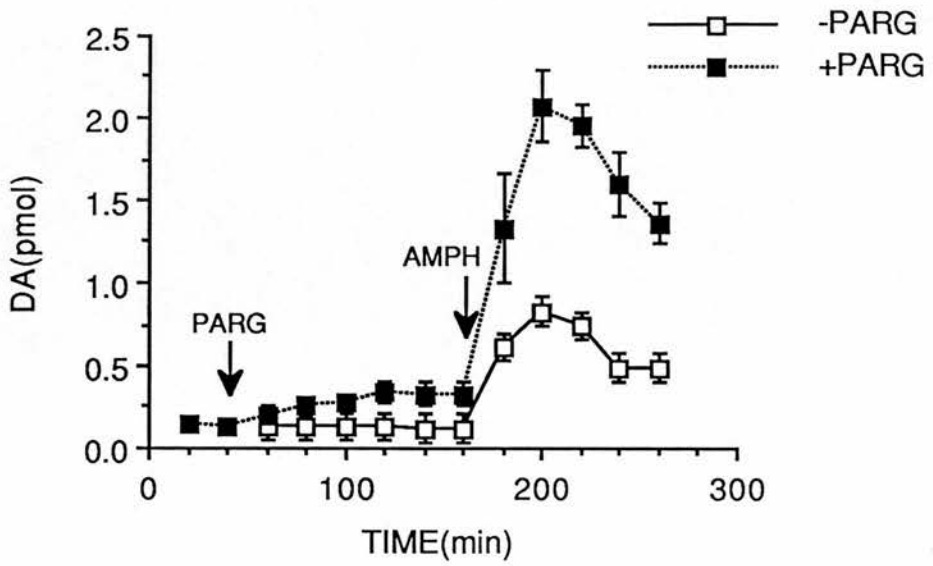
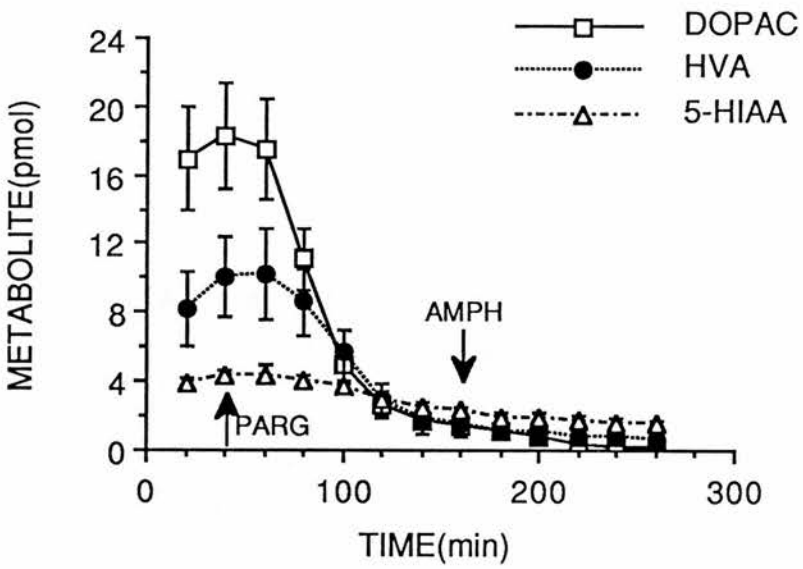
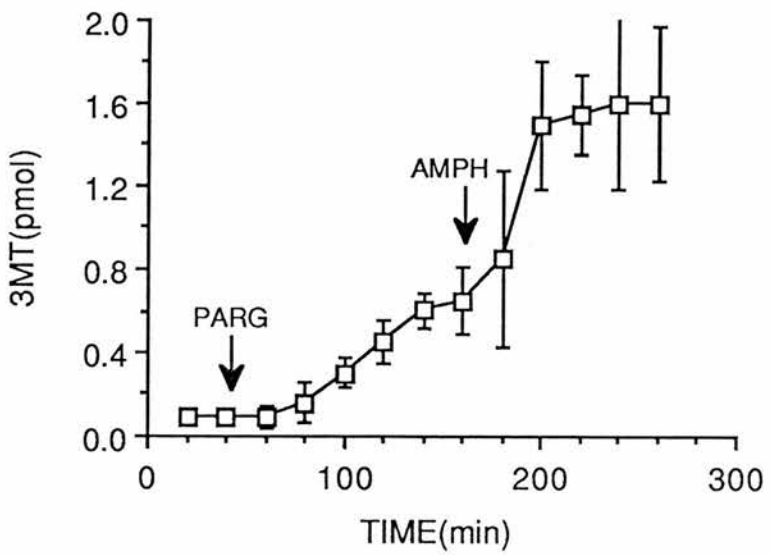


Fig. 3.3.3 Effects of reserpine (5mg/kg i.p.) given 180 min prior to AMPH (4mg/kg i.p.) on DA efflux (upper panel), DOPAC and HVA efflux (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent animals.

Fig. 3.3.4 Effects of pargyline (75mg/kg i.p.) given 120 min prior to AMPH (4mg/kg i.p.) on DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel) and 3-MT efflux (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


-PARG
+PARG
DA(pmol)
2.5
2.0
1.5
1.0
0.5
0.0
PARG
AMPH
0
100
200
300
TIME(min)


DOPAC
HVA
5-HIAA
METABOLITE(pmol)
24
20
16
12
8
4
0
AMPH
PARG
0
100
200
300
TIME(min)


3MT(pmol)
2.0
1.6
1.2
0.8
0.4
0.0
AMPH
PARG
0
100
200
300
TIME(min)

pre-pargyline levels. 5-HIAA efflux was not affected.

3.3.5 Selegeline

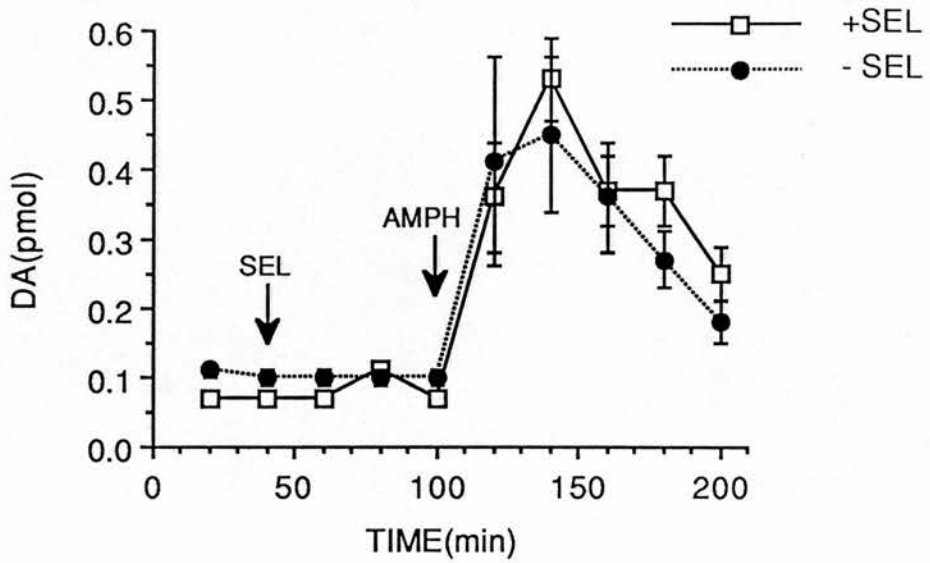
In selegeline (10mg/kg i.p.) treated animals, AMPH maximally increased DA efflux to 757% of basal efflux 40 min after injection (Fig. 3.3.5; 0.07 ± 0.01 to 0.53 ± 0.06pmoles/20 min, n = 6). Total DA efflux over 60 min was not increased by a significant amount (Table 3.3.1).

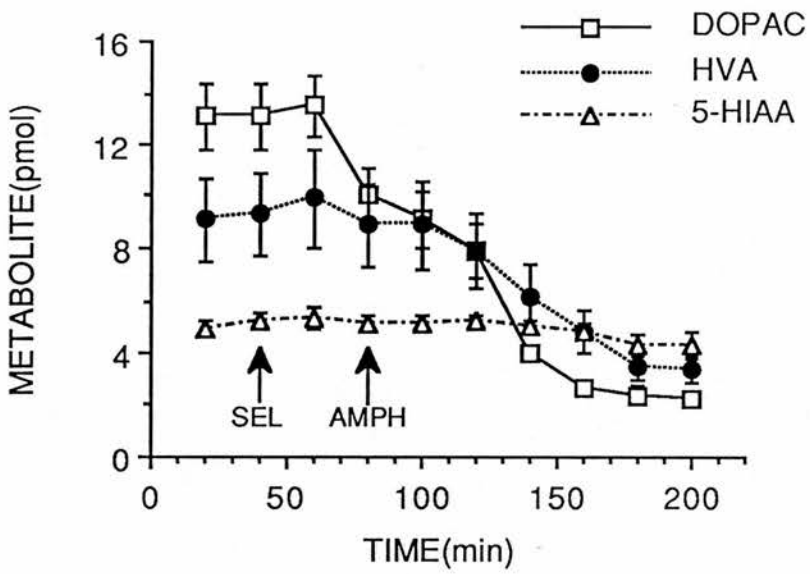
AMPH appeared to accelerate DOPAC and HVA efflux in selegeline treated animals (Fig. 3.3.5). 5-HIAA efflux was not affected whilst 3-MT efflux was maximally increased to 260% of basal efflux 40 min after AMPH injection (Fig. 3.3.5, 0.05 ± 0.01 to 0.13 ± 0.02pmoles/20 min, n = 6). A quantitative assessment of this was not possible due to the lack of control values for 3-MT.

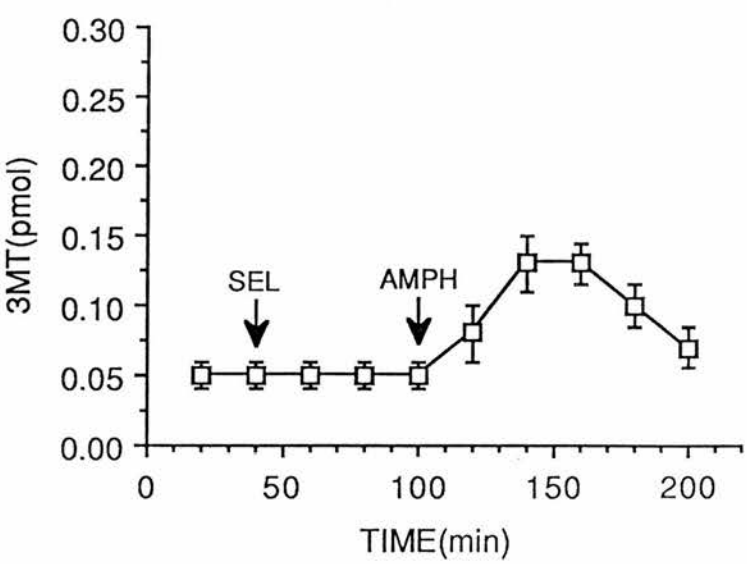
3.3.6 Nomifensine

In animals treated with an intraperitoneal dose of nomifensine (20mg/kg i.p.), AMPH maximally increased DA efflux to 344% of basal efflux 40 min after injection (Fig. 3.3.6a; 0.16 ± 0.06 to 0.55 ± 0.08pmoles/20 min, n = 6). Total DA efflux over 60 min was reduced by 78% when compared with control animals (Table 3.3.1; control 1.8 ± 0.3pmoles; nomifensine 0.4 ± 0.1pmoles; $p < 0.01$). Similarly, when nomifensine was perfused intrastriatally (10μM) through the dialysis probe, AMPH maximally increased DA efflux to 141% of basal efflux over 60 min (Fig. 3.3.6b; 0.27 ± 0.07 to 0.38 ± 0.16pmoles/20 min, n = 6). Total DA efflux over 60 min was reduced by 99% when compared with control animals (Table 3.3.1; control 1.9 ± 0.3pmoles; nomifensine 0.01 ± 0.01pmoles; $p < 0.01$). In animals treated with intraperitoneal nomifensine, AMPH maximally

Fig. 3.3.5 Effects of selegeline (10mg/kg i.p.) given 60 min prior to AMPH (4mg/kg i.p.) on DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel) and 3-MT efflux (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


+SEL
- SEL
DA(pmol)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SEL
AMPH
0
50
100
150
200
TIME(min)


DOPAC
HVA
5-HIAA
METABOLITE(pmol)
16
12
8
4
0
SEL
AMPH
0
50
100
150
200
TIME(min)


3MT(pmol)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
SEL
AMPH
0
50
100
150
200
TIME(min)

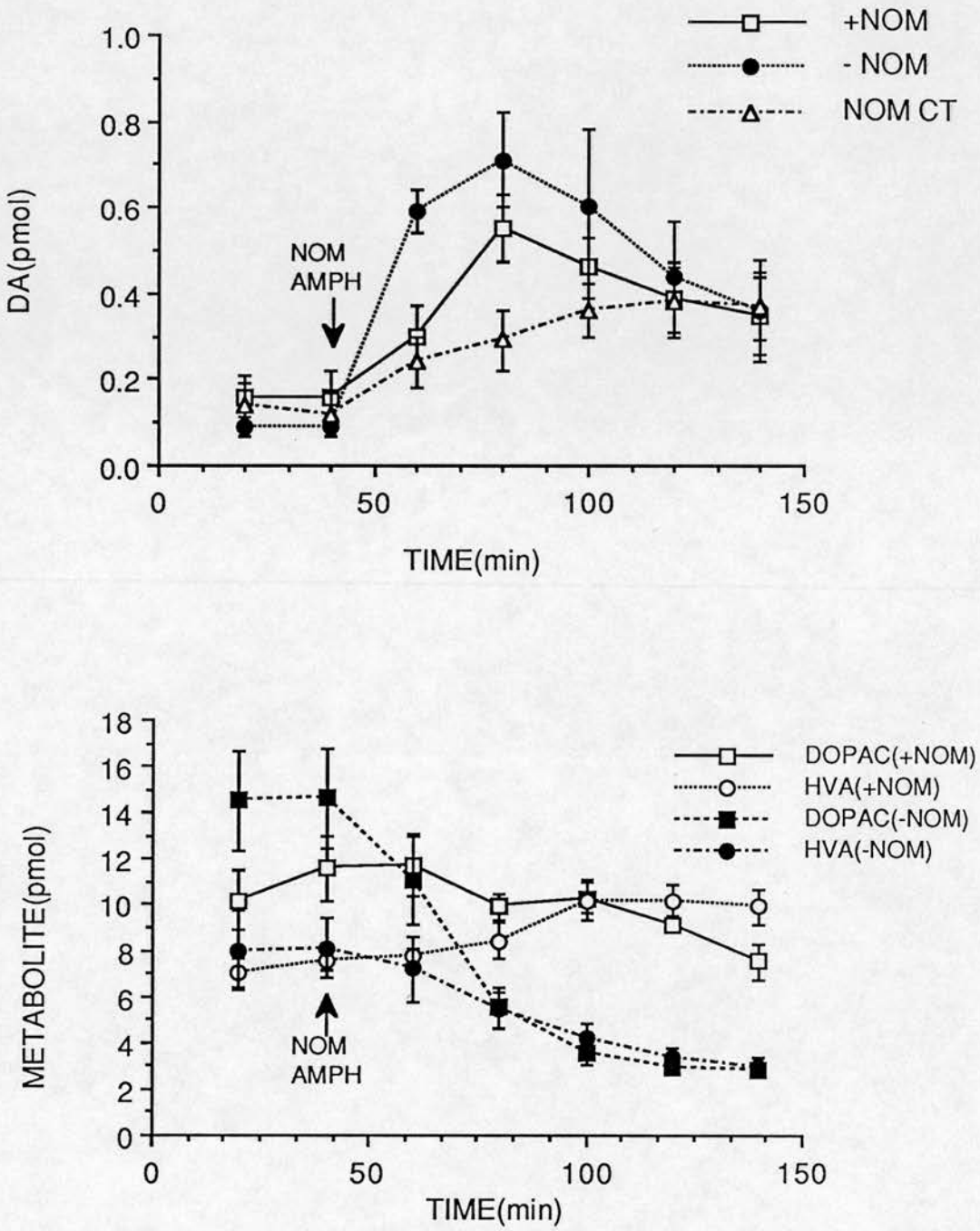


Fig. 3.3.6a Effects of nomifensine (20mg/kg i.p.) given simultaneously with AMPH (4mg/kg i.p.) on DA (upper panel), DOPAC and HVA efflux (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments. NOM CT = nomifensine control.

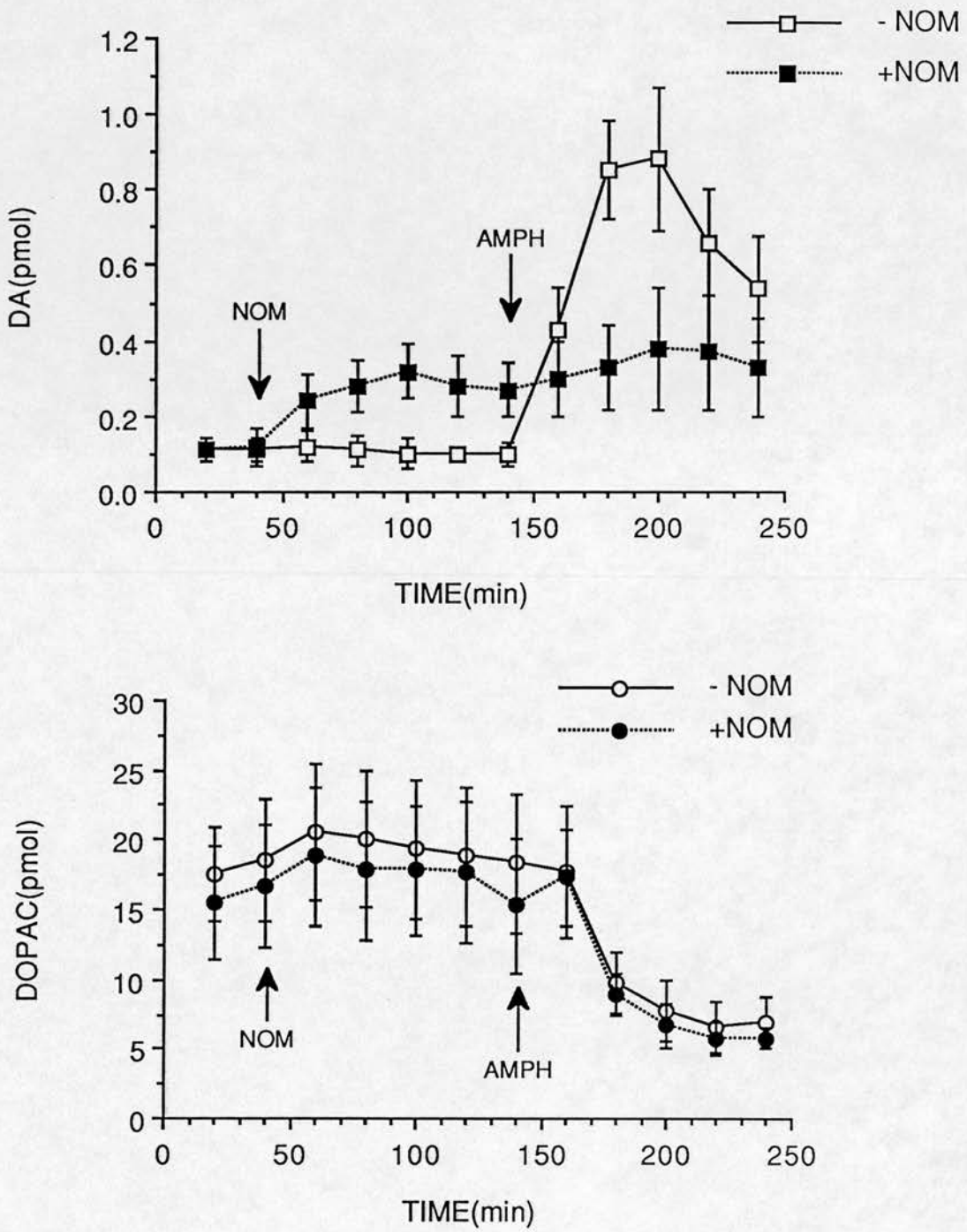


Fig. 3.3.6b Effects of nomifensine (10μM) perfused intrastriatally 120 min prior to AMPH (4mg/kg i.p.) on DA (upper panel) and DOPAC efflux (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

reduced DOPAC efflux to 65% of basal efflux over 100 min (Fig. 3.3.6a; 11.6 ± 1.4 to 7.5 ± 0.8pmoles/20 min, n = 6). The decline in total DOPAC efflux over 60 min was inhibited when compared with control animals (control 34.8 ± 4.9pmoles below basal efflux, nomifensine 3.0 ± 2.0pmoles below basal efflux, $p < 0.05$). When perfused via the dialysis probe however, nomifensine had no inhibitory effect upon the AMPH induced decline in DOPAC efflux (Fig. 3.3.6b). HVA efflux increased maximally to 136% of control over 80 min when nomifensine was given intraperitoneally with AMPH (Fig. 3.3.6a, 7.5 ± 0.4 to 10.2 ± 0.7pmoles/20 min, n = 6). The decline in total HVA efflux over 60 min was inhibited when compared with control animals (control 11.1 ± 1.3pmoles below basal efflux, nomifensine 3.7 ± 0.9pmoles above basal efflux, $p < 0.05$).

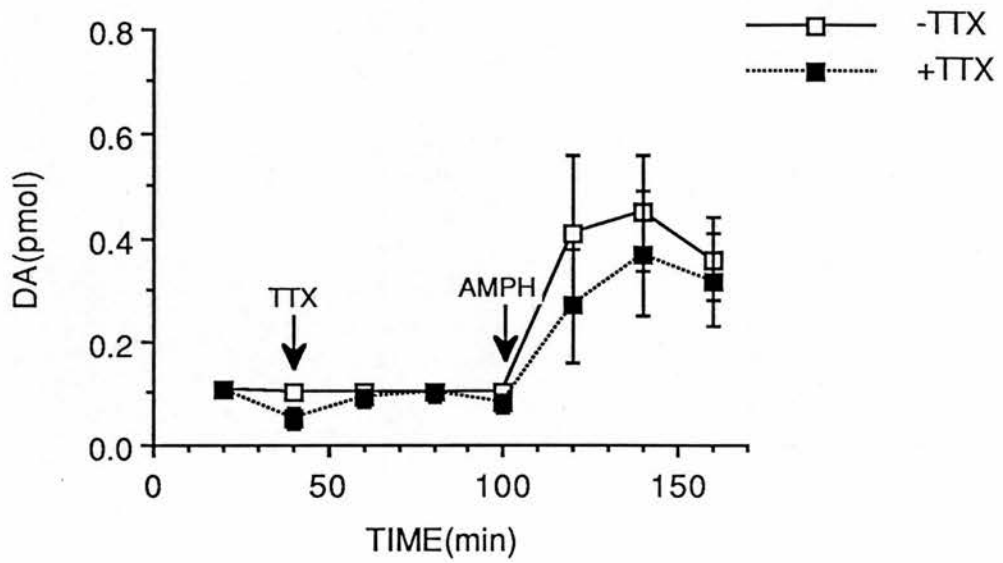
3.3.7 Tetrodotoxin

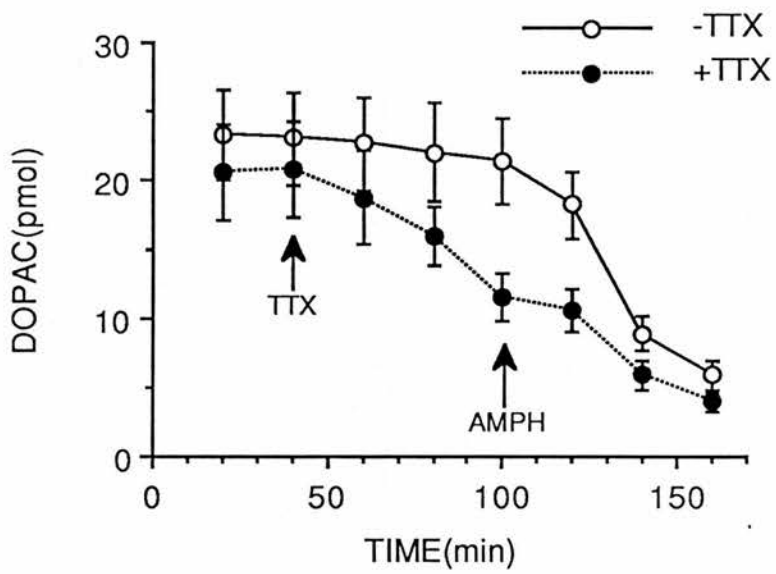
In TTX (2μM) treated animals, AMPH maximally increased DA efflux to 463% of basal levels 40 min after injection (Fig. 3.3.7, 0.08 ± 0.02 to 0.37 ± 0.12pmoles/20 min, n = 6). Total DA efflux over 60 min was not reduced by TTX (Table 3.3.1, control 0.9 ± 0.3pmoles, TTX 0.8 ± 0.4pmoles). TTX had no effect upon AMPH induced efflux of DOPAC or HVA (Fig. 3.3.7).

3.3.8 EGTA

When EGTA (20mM) was perfused through the dialysis probe in a Ca^{2+} free buffer, AMPH maximally increased DA efflux to 1288% of basal efflux 20 min after injection (Fig. 3.3.8, 0.09 ± 0.02 to 1.16 ± 0.52pmoles/20 min, n = 6). Total DA efflux over 60 min was not reduced by EGTA (Table 3.3.1, control 2.7 ± 0.4pmoles, EGTA 2.4 ± 0.3pmoles). AMPH induced an almost identical decline in DOPAC and HVA efflux in the presence or absence of EGTA (Fig. 3.3.8).

Fig. 3.3.7 Effects of TTX (2μM) perfused intrastriatally for 60 min prior to AMPH (4mg/kg i.p.) on DA (upper panel), DOPAC (middle panel) and HVA efflux (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


-TTX
+TTX
DA(pmol)
0.8
0.6
0.4
0.2
0.0
TTX
AMPH
0
50
100
150
TIME(min)


-TTX
+TTX
DOPAC(pmol)
30
20
10
0
TTX
AMPH
0
50
100
150
TIME(min)

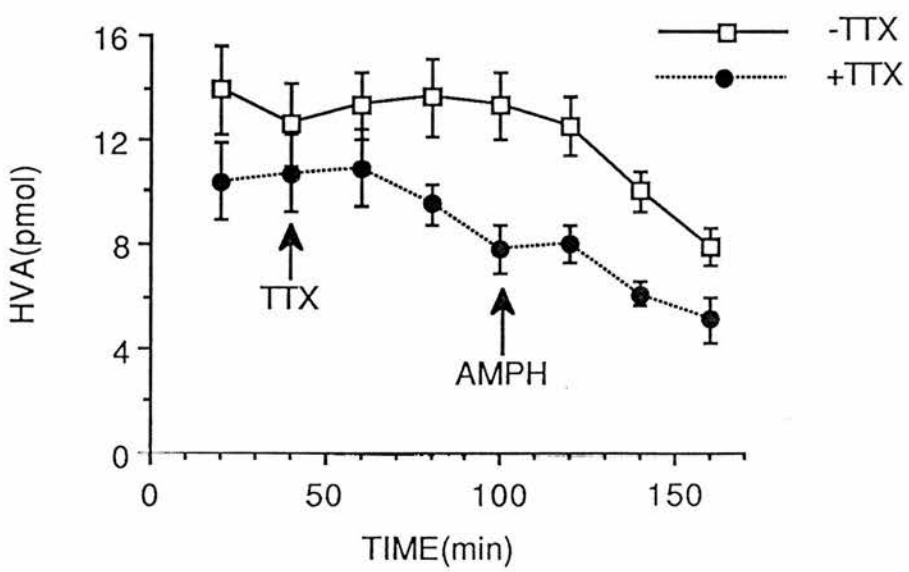
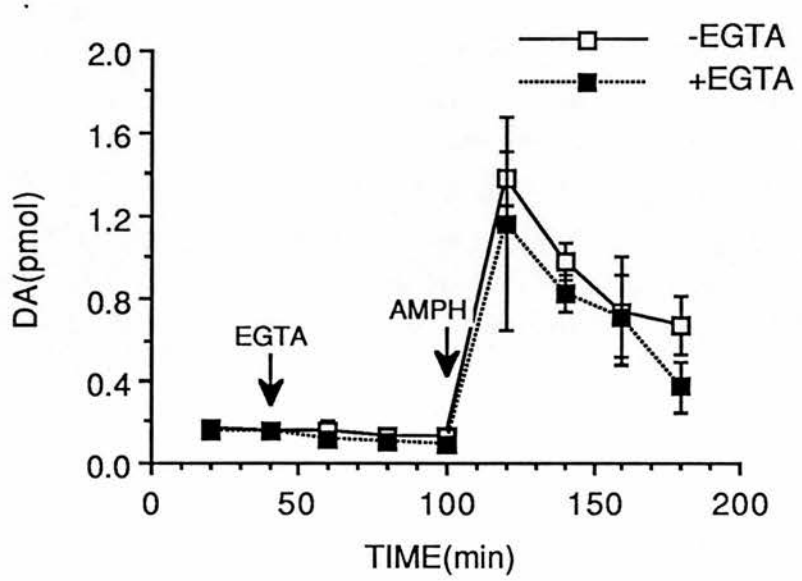
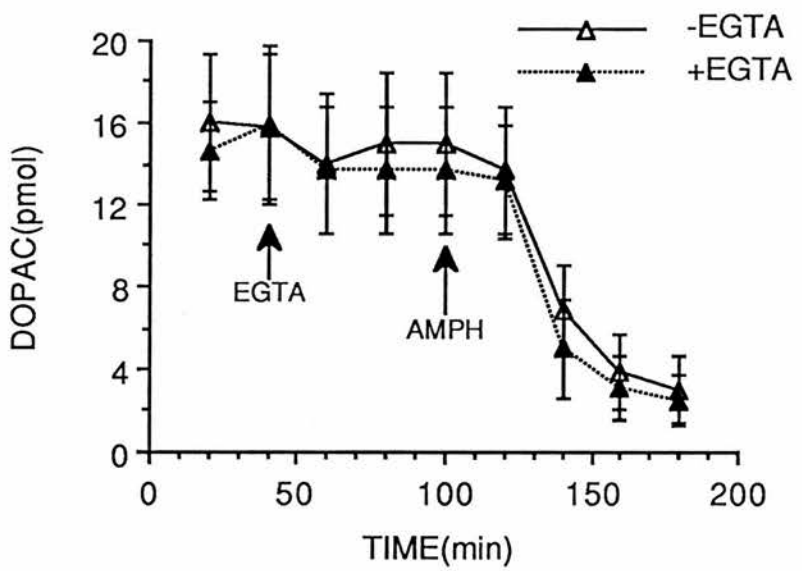

-TTX
+TTX
HVA(pmol)
16
12
8
4
0
TTX
AMPH
0
50
100
150
TIME(min)

Fig. 3.3.8 Effects of EGTA (20mM) and Ca^{2+} removal from the perfusing medium for 60 min prior to AMPH (4mg/kg i.p.) on DA (upper panel), DOPAC (middle panel) and HVA efflux (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


-EGTA
+EGTA
DA(pmol)
2.0
1.6
1.2
0.8
0.4
0.0
EGTA
AMPH
0
50
100
150
200
TIME(min)


-EGTA
+EGTA
DOPAC(pmol)
20
16
12
8
4
0
EGTA
AMPH
0
50
100
150
200
TIME(min)

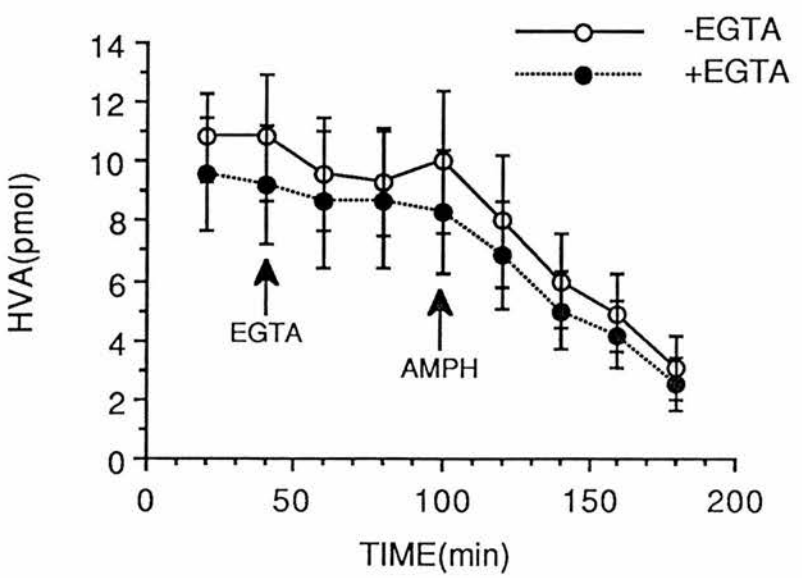

-EGTA
+EGTA
HVA(pmol)
14
12
10
8
6
4
2
0
EGTA
AMPH
0
50
100
150
200
TIME(min)

TABLE 3.3.1

EFFECTS OF BIOCHEMICAL AND PHARMACOLOGICAL MANIPULATIONS ON AMPH (4mg/kg i.p.) INDUCED DA EFFLUX

	n	DA EFFLUX (pmol/60 min)
Control	6	2.0 ± 0.4
AMT (simultaneous)	6	0.6 ± 0.2**
Control	6	2.0 ± 0.4
AMT (120 min pretreatment)	6	0.3 ± 0.1**
Control	6	1.8 ± 0.2
Reserpine	6	1.3 ± 0.2
Control	6	1.8 ± 0.4
Pargyline	6	4.3 ± 0.6**
Control	6	0.9 ± 0.3
Selegeline	6	1.1 ± 0.2
Control	6	1.8 ± 0.3
Nomifensine (i.p.)	6	0.4 ± 0.1**
Control	6	1.9 ± 0.3
Nomifensine (perfused)	6	0.01 ± 0.01**
Control	6	0.9 ± 0.3
TTX	6	0.8 ± 0.4
Control	6	2.7 ± 0.4
EGTA	6	2.4 ± 0.3

* $p < 0.05$, ** $p < 0.01$

Mean and standard errors are given for guidance only. Statistical differences in amount released were assessed using the non-parametric Mann-Whitney 'U' test. The data were corrected for basal release prior to comparison.

3.4 Effects of biochemical and pharmacological manipulations upon KCl induced efflux of DA, DOPAC, HVA, 5-HIAA and 3MT

3.4.1 Dose dependence of the KCl response

A dose of 30mM KCl had little effect upon DA efflux, maximal efflux was 137% of control (Fig. 3.4.1a, n = 4). Increasing the dose of KCl (60, 90 and 120mM) led to a graded increase in maximal DA efflux (242, 1017 and 1521% of control respectively). The same was true for the metabolites DOPAC (Fig. 3.4.1a), HVA and 5-HIAA (Fig. 3.4.1b) which exhibited a dose dependent decrease in efflux levels. Following the removal of the stimulating dose of KCl, DA, DOPAC, HVA and 5-HIAA efflux levels returned towards control levels. A dose of 90mM KCl was chosen for future experiments since these results demonstrate that this dose is neither maximal or minimal for an elicited response.

3.4.2 α-Methyl-p-tyrosine

In AMT (250mg/kg i.p., 20 min pretreatment) treated animals, KCl maximally increased DA efflux to 420% of basal efflux over 20 min (Fig. 3.4.2a, 0.25 ± 0.08 to 1.05 ± 0.17pmoles/20 min, n = 6). Total DA efflux over 60 min was reduced by 42% when compared with control animals (Table 3.4.1, control 1.91 ± 0.26pmoles, AMT 1.10 ± 0.27pmoles, $p < 0.05$). When the same dose of AMT was given 120 min prior to KCl, DA efflux was maximally increased to 433% of basal efflux 20 min after KCl infusion (Fig. 3.4.2b, 0.12 ± 0.04 to 0.52 ± 0.11pmoles/20 min, n = 6). Total DA efflux over 60 min was reduced to a much greater extent by 74% when compared with control animals (Table 3.4.1, control 1.91 ± 0.26pmoles, AMT 0.50 ± 0.14pmoles, $p < 0.001$). AMT caused a decline in DOPAC and HVA efflux which did not

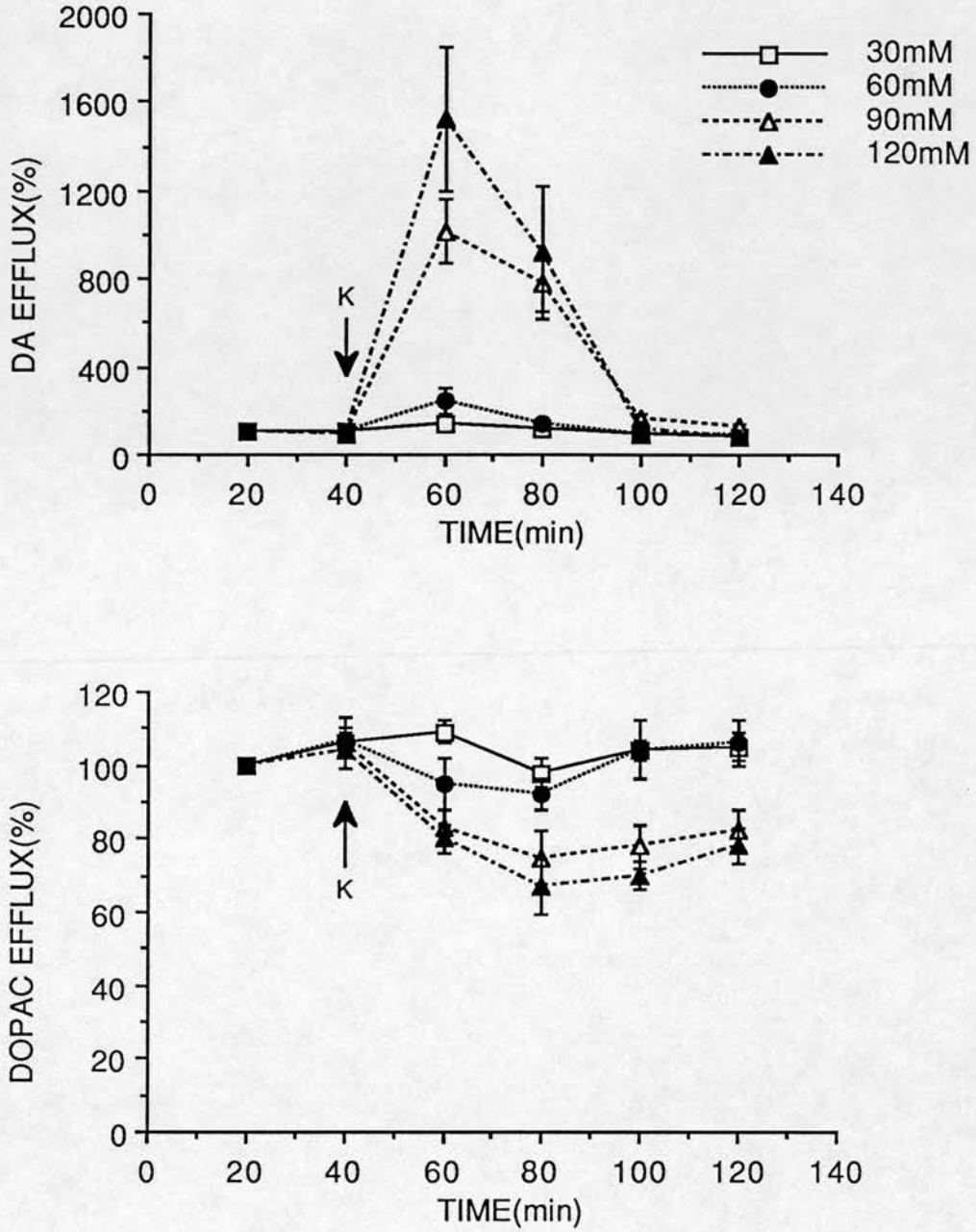


Fig. 3.4.1a Effects of increasing doses of KCl included in the perfusing medium on DA (upper panel) and DOPAC efflux (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

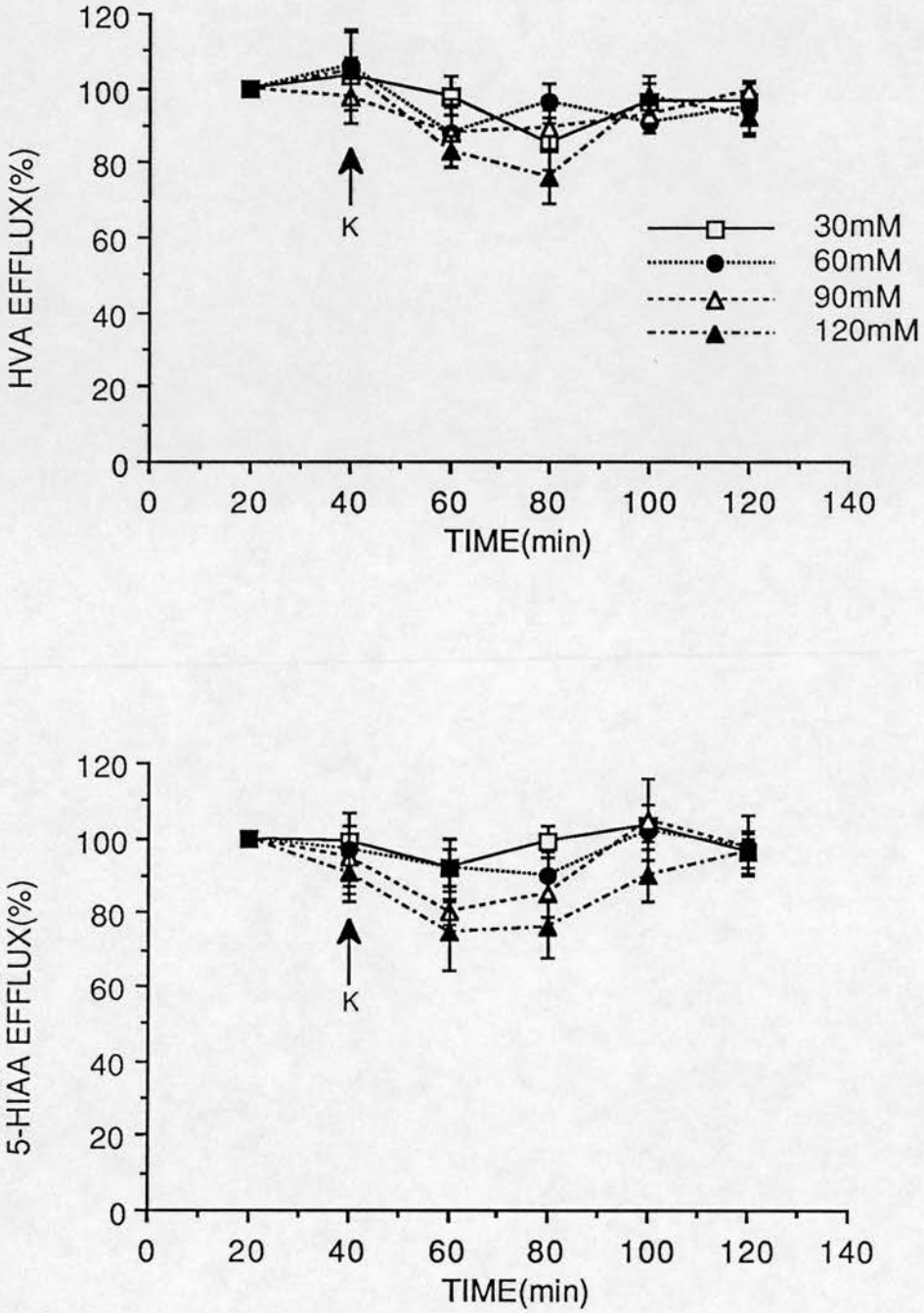


Fig. 3.4.1b Effects of increasing doses of KCl included in the perfusing medium on HVA (upper panel) and 5-HIAA efflux (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

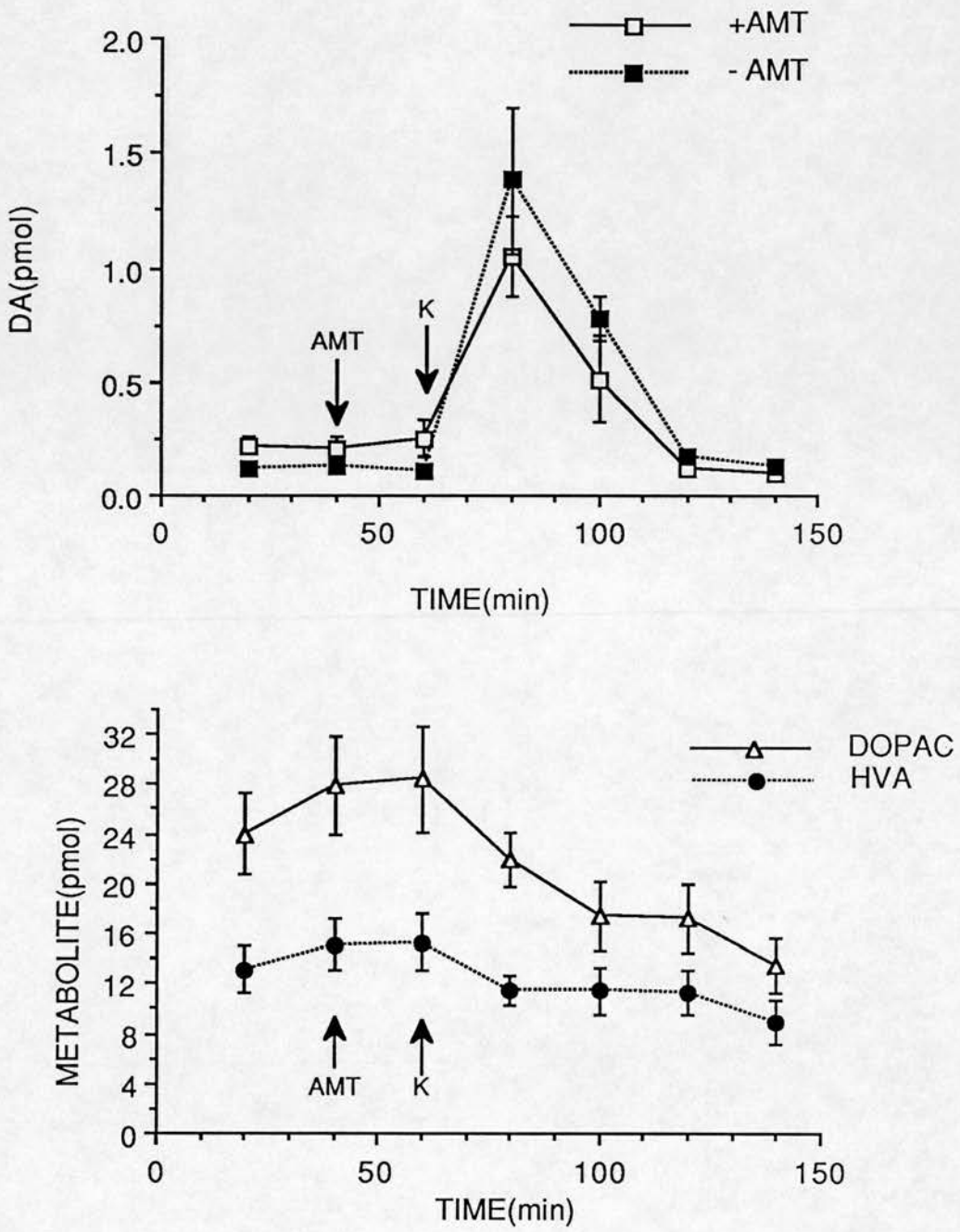


Fig. 3.4.2a Effects of AMT (250mg/kg i.p.) given 20 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

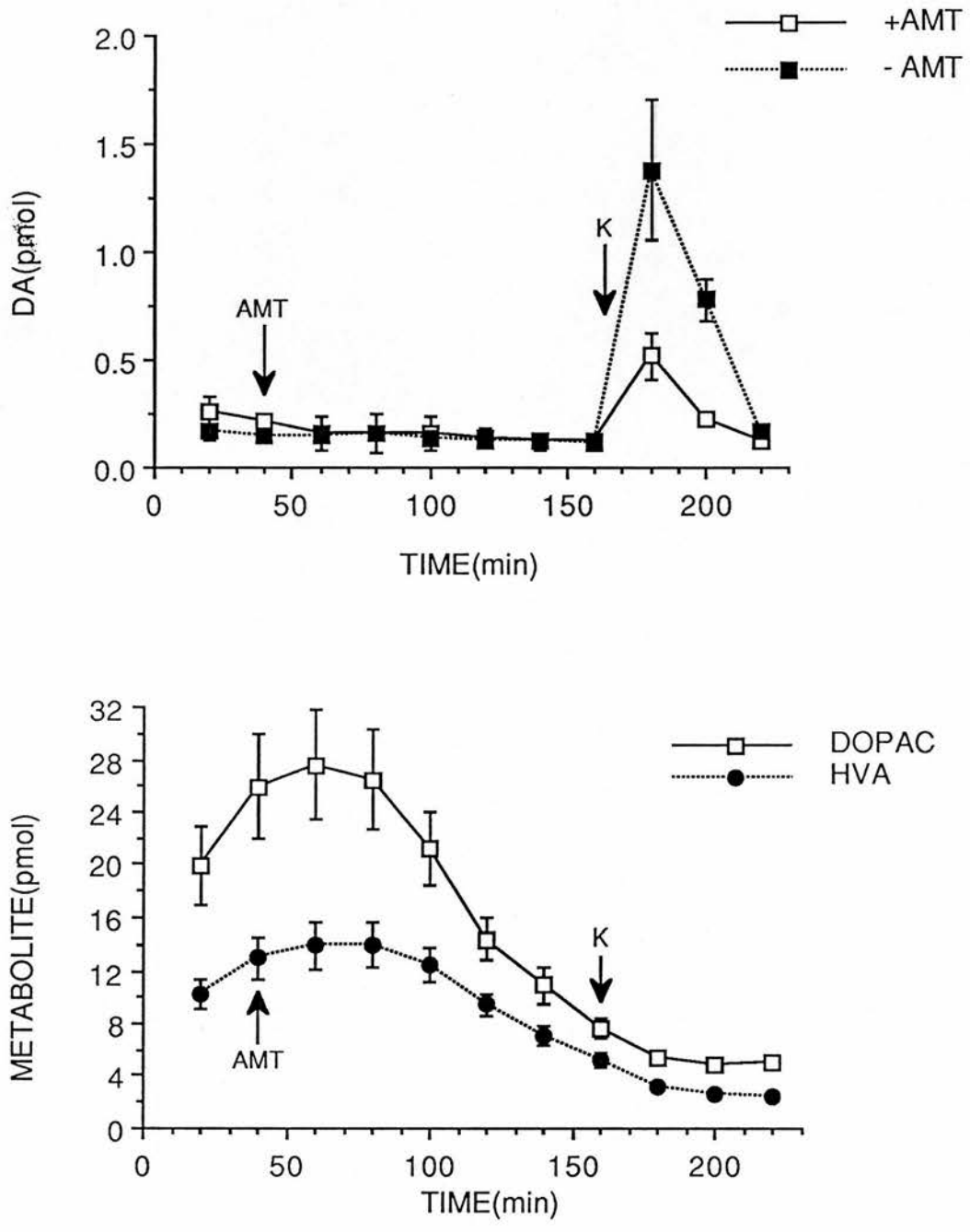


Fig. 3.4.2b Effects of AMT (250mg/kg i.p.) given 120 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

seem to be accelerated by KCl (Fig. 3.4.2b).

3.4.3 Reserpine

In reserpine (5mg/kg i.p.) pretreated animals, KCl maximally increased DA efflux to 4400% of basal efflux over 20 min (Fig. 3.4.3, 0.02 ± 0.02 to 0.88 ± 0.3pmoles/20 min, n = 6). Total DA efflux over 60 min was not reduced by reserpine (Table 3.4.1, control 1.91 ± 0.26pmoles, reserpine 1.16 ± 0.35pmoles) despite an apparent reduction. This was probably due to the high variation of the results.

Reserpine induced a rising followed by a decreasing efflux for both DOPAC and HVA (Fig. 3.4.3). KCl infusion seemed to accelerate the decline in both DOPAC and HVA.

3.4.4 Pargyline

In pargyline (75mg/kg i.p.) treated animals, KCl maximally increased DA efflux to 1241% of basal efflux over 20 min (Fig. 3.4.4, 0.29 ± 0.05 to 3.60 ± 0.82pmoles/20 min, n = 6). Total KCl induced DA efflux over 60 min was increased by pargyline (Table 3.4.1, control 1.91 ± 0.26pmoles, pargyline 4.45 ± 1.19pmoles, $p < 0.05$).

Pargyline caused a rapid decline in DOPAC and HVA efflux over 120 min to low levels (Fig. 3.4.4., 23.9 ± 3.5 to 2.8 ± 0.7pmoles/20 min, 12.8 ± 1.1 to 1.6 ± 0.7pmoles/20 min respectively). Consequently, no additional effect of KCl was apparent following this decline.

3.4.5 Selegeline

In selegeline (10mg/kg i.p.) treated animals, KCl maximally

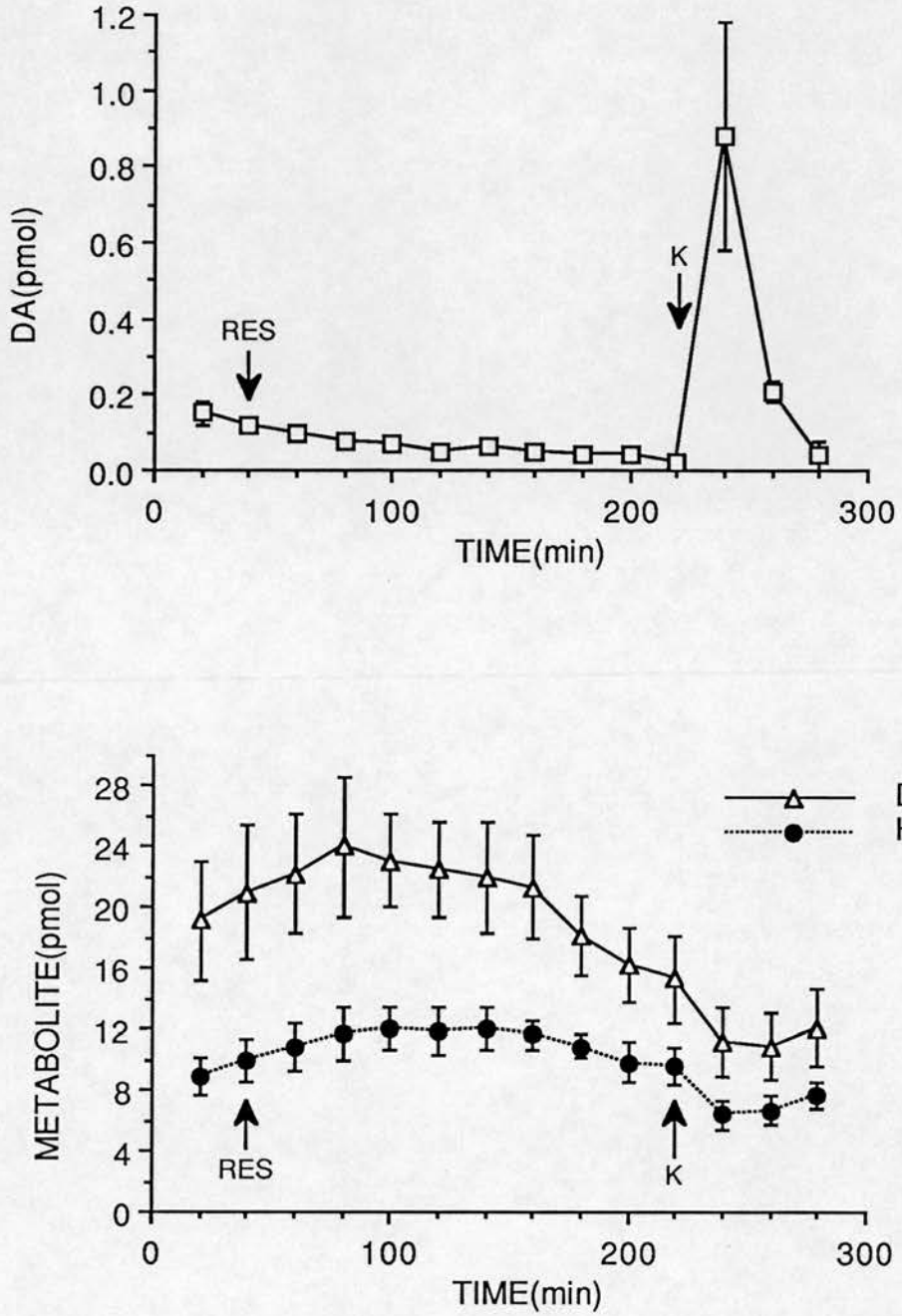
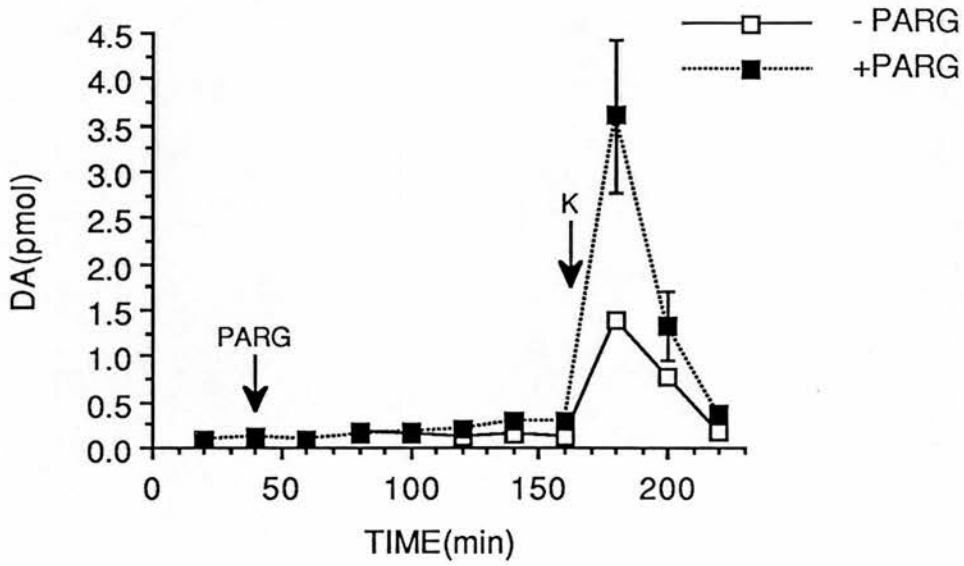
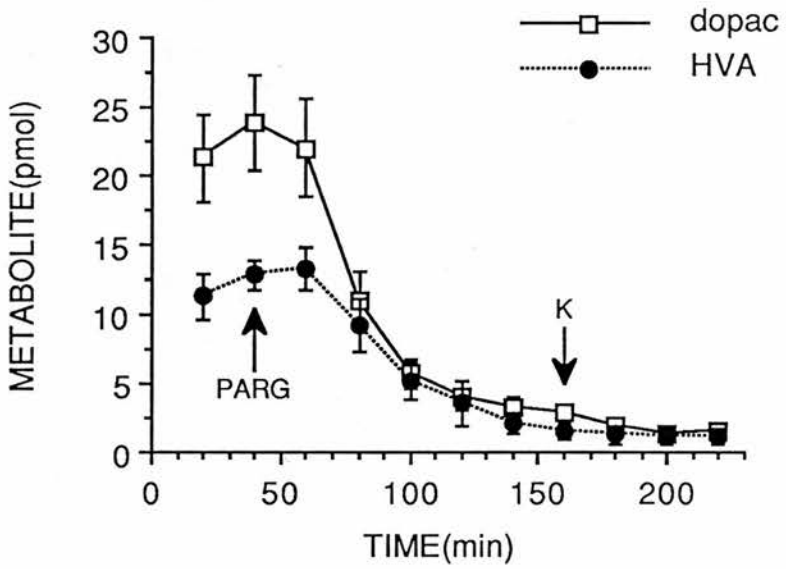


Fig. 3.4.3 Effects of reserpine (5mg/kg i.p.) given 180 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

Fig. 3.4.4 Effects of pargyline (75mg/kg i.p.) given 120 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC and HVA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


- PARG
+PARG
DA(pmol)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
K
PARG
0
50
100
150
200
TIME(min)


dopac
HVA
METABOLITE(pmol)
30
25
20
15
10
5
0
K
PARG
0
50
100
150
200
TIME(min)

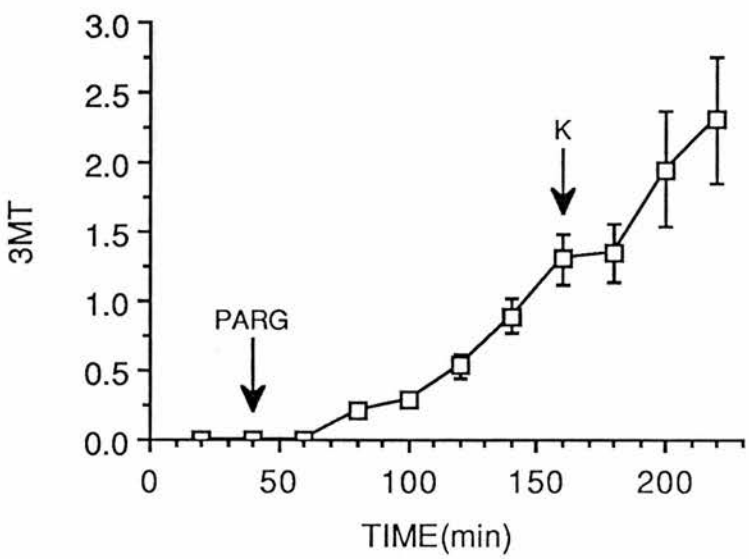
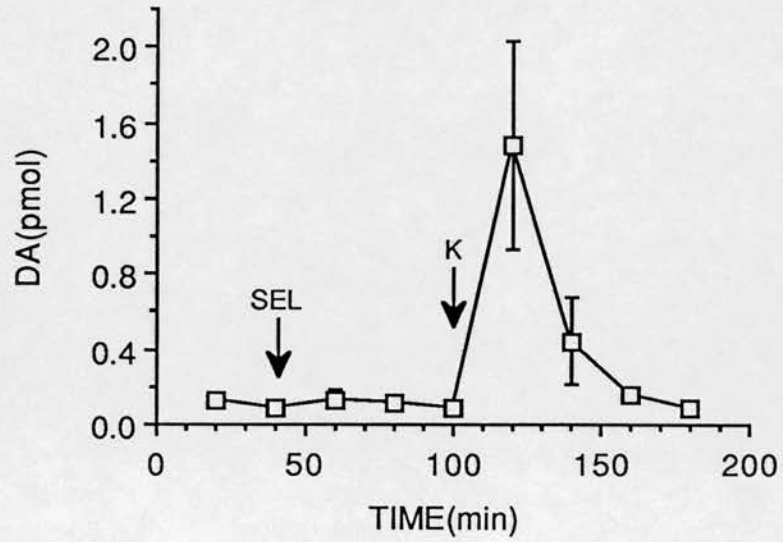
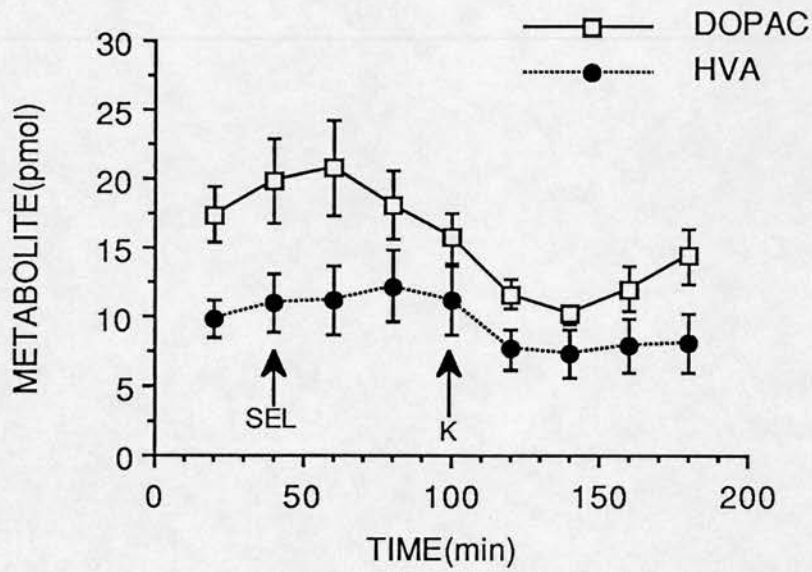
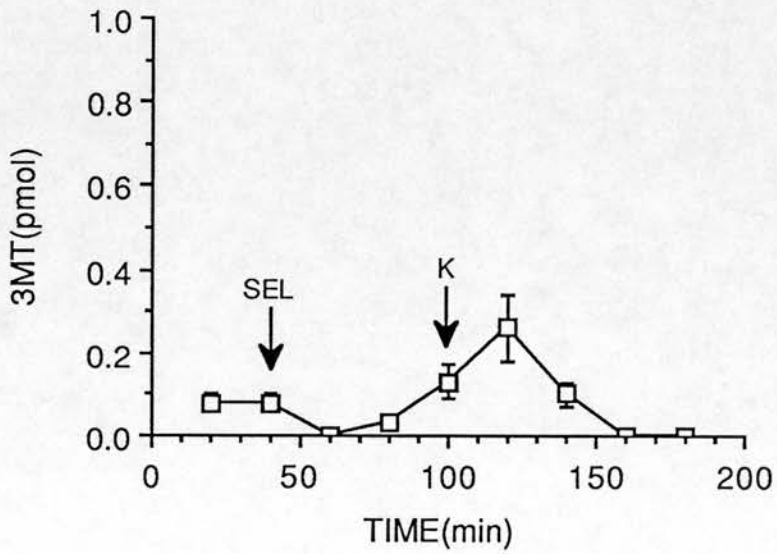

3MT
3.0
2.5
2.0
1.5
1.0
0.5
0.0
K
PARG
0
50
100
150
200
TIME(min)

Fig. 3.4.5 Effects of selegeline (10mg/kg i.p.) given 60 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC and HVA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


DA(pmol)
2.0
1.6
1.2
0.8
0.4
0.0
SEL
K
0
50
100
150
200
TIME(min)


METABOLITE(pmol)
30
25
20
15
10
5
0
DOPAC
HVA
SEL
K
0
50
100
150
200
TIME(min)


3MT(pmol)
1.0
0.8
0.6
0.4
0.2
0.0
SEL
K
0
50
100
150
200
TIME(min)

increased DA efflux to 1644% of basal efflux over 20 min (Fig. 3.4.5, 0.09 ± 0.02 to 1.48 ± 0.55pmoles/20 min, n = 6). Total KCl induced DA efflux over 60 min was not affected by selegeline (Table 3.4.1, control 1.91 ± 0.26pmoles, selegeline 1.72 ± 0.69pmoles).

The declining efflux of DOPAC and HVA induced by selegeline appeared to be accelerated by KCl (Fig. 3.4.5).

3.4.6 Nomifensine

In nomifensine (20mg/kg i.p.) treated animals, KCl maximally increased DA efflux to 850% of basal efflux over 20 min (Fig. 3.4.6, 0.24 ± 0.05 to 2.04 ± 0.2pmoles/20 min, n = 6). Total DA efflux over 60 min was increased by 34% when compared with control animals (Table 3.4.1, control 1.91 ± 0.26pmoles, nomifensine 2.57 ± 0.34pmoles, $p < 0.05$). Nomifensine had no effect on DOPAC and HVA efflux (Fig. 3.4.6).

3.4.7 Tetrodotoxin

In TTX (2μM) treated animals, KCl maximally increased DA efflux to 1417% of basal efflux over 20 min (Fig. 3.4.7, 0.12 ± 0.03 to 1.7 ± 0.53pmoles/20 min, n = 6). Total DA efflux over 60 min was not affected by TTX (Table 3.4.1, control 2.19 ± 0.65pmoles, TTX 1.19 0.50pmoles).

TTX caused a decline in DOPAC and HVA efflux which continued at the same rate for DOPAC and at a slightly faster rate for HVA when KCl was infused (Fig. 3.4.7).

3.4.8 EGTA

In animals perfused with EGTA (20mM) in a Ca^{2+} free buffer, KCl maximally increased DA efflux to 1633% of basal efflux over 20

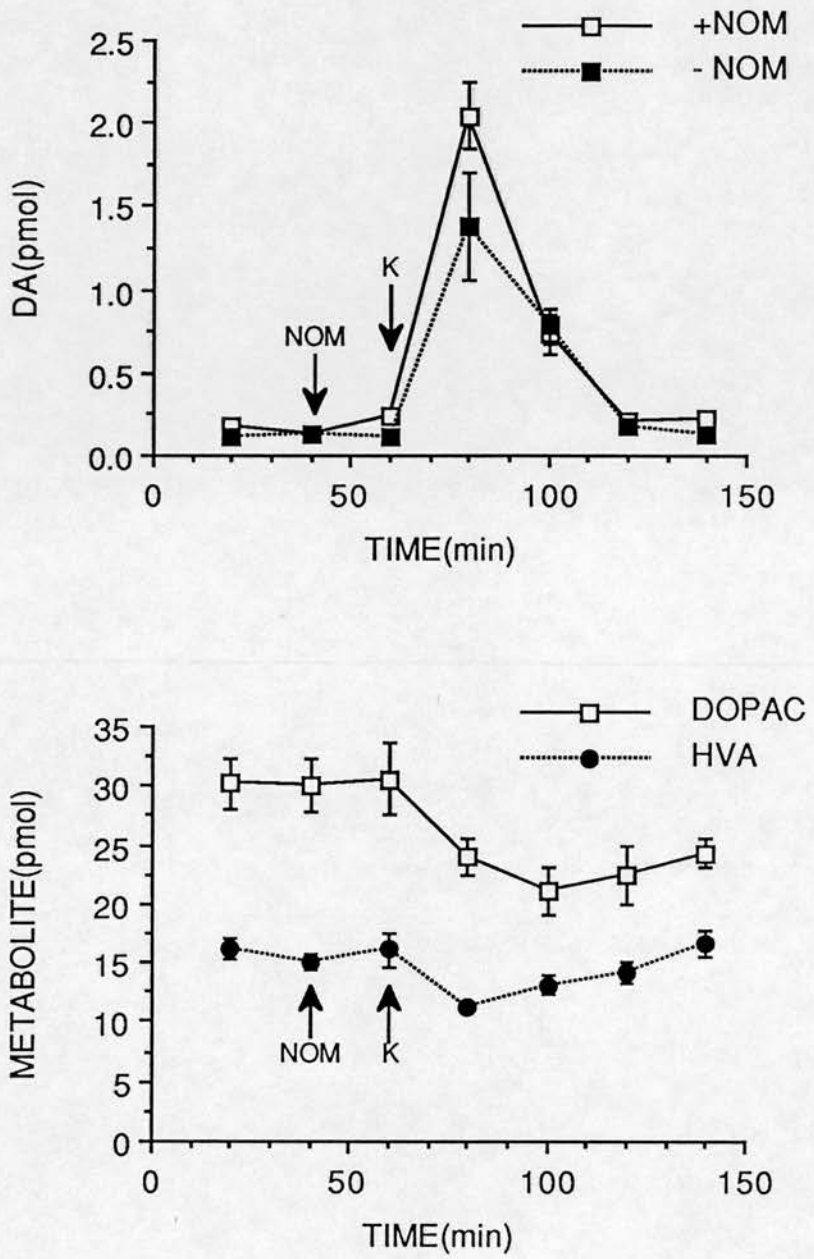
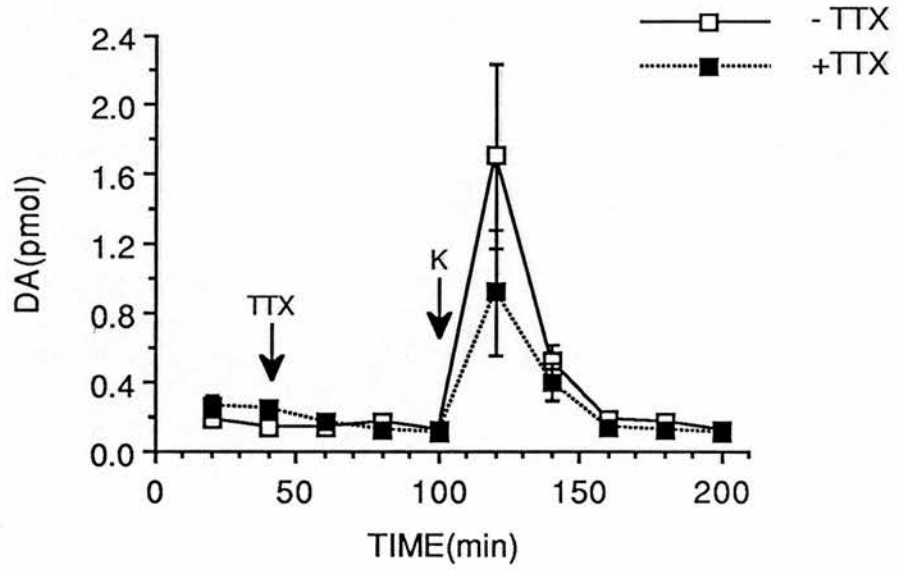
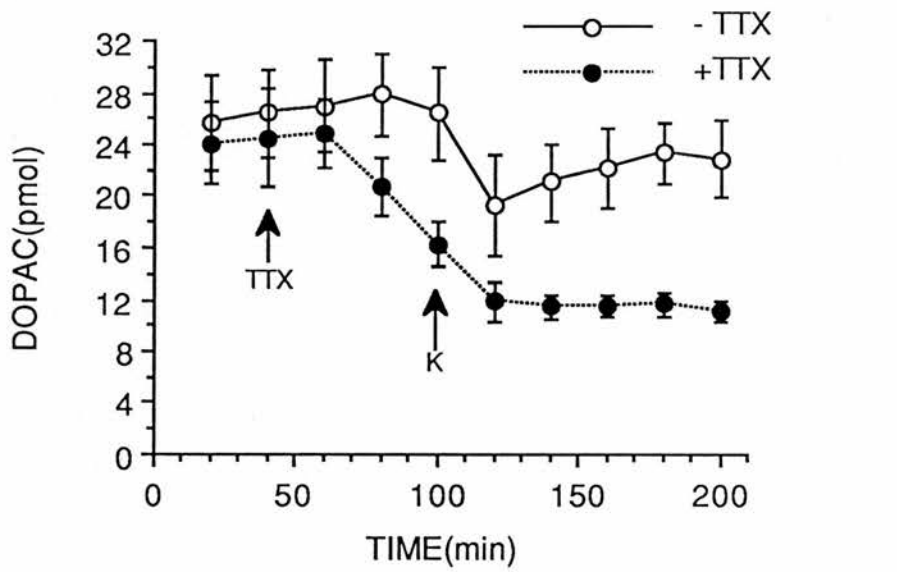
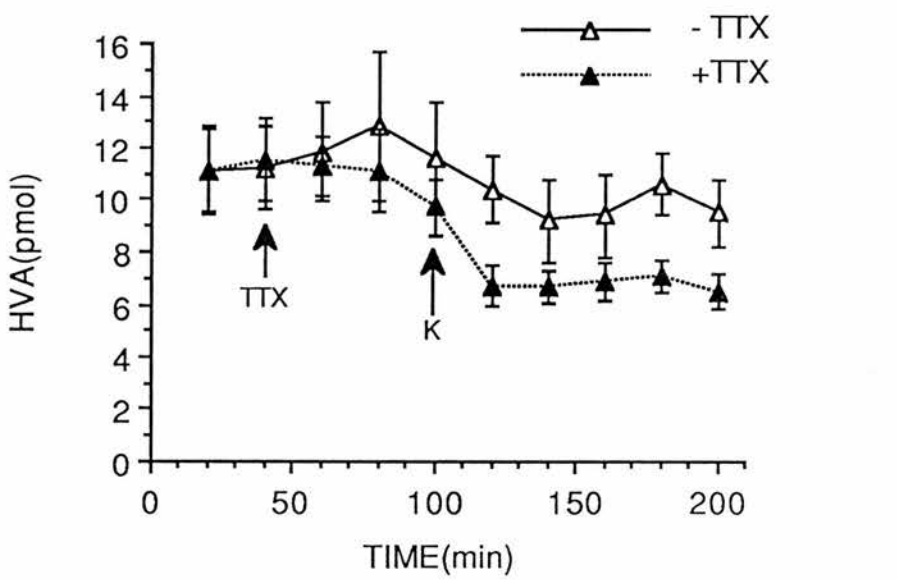


Fig. 3.4.6 Effects of nomifensine (10mg/kg i.p.) given 20 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

Fig. 3.4.7 Effects of TTX (2μM) perfused intrastriatally for 60 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC (middle panel) and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


- TTX
+TTX
DA(pmol)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
TTX
K
0
50
100
150
200
TIME(min)


- TTX
+TTX
DOPAC(pmol)
32
28
24
20
16
12
8
4
0
TTX
K
0
50
100
150
200
TIME(min)


- TTX
+TTX
HVA(pmol)
16
14
12
10
8
6
4
2
0
TTX
K
0
50
100
150
200
TIME(min)

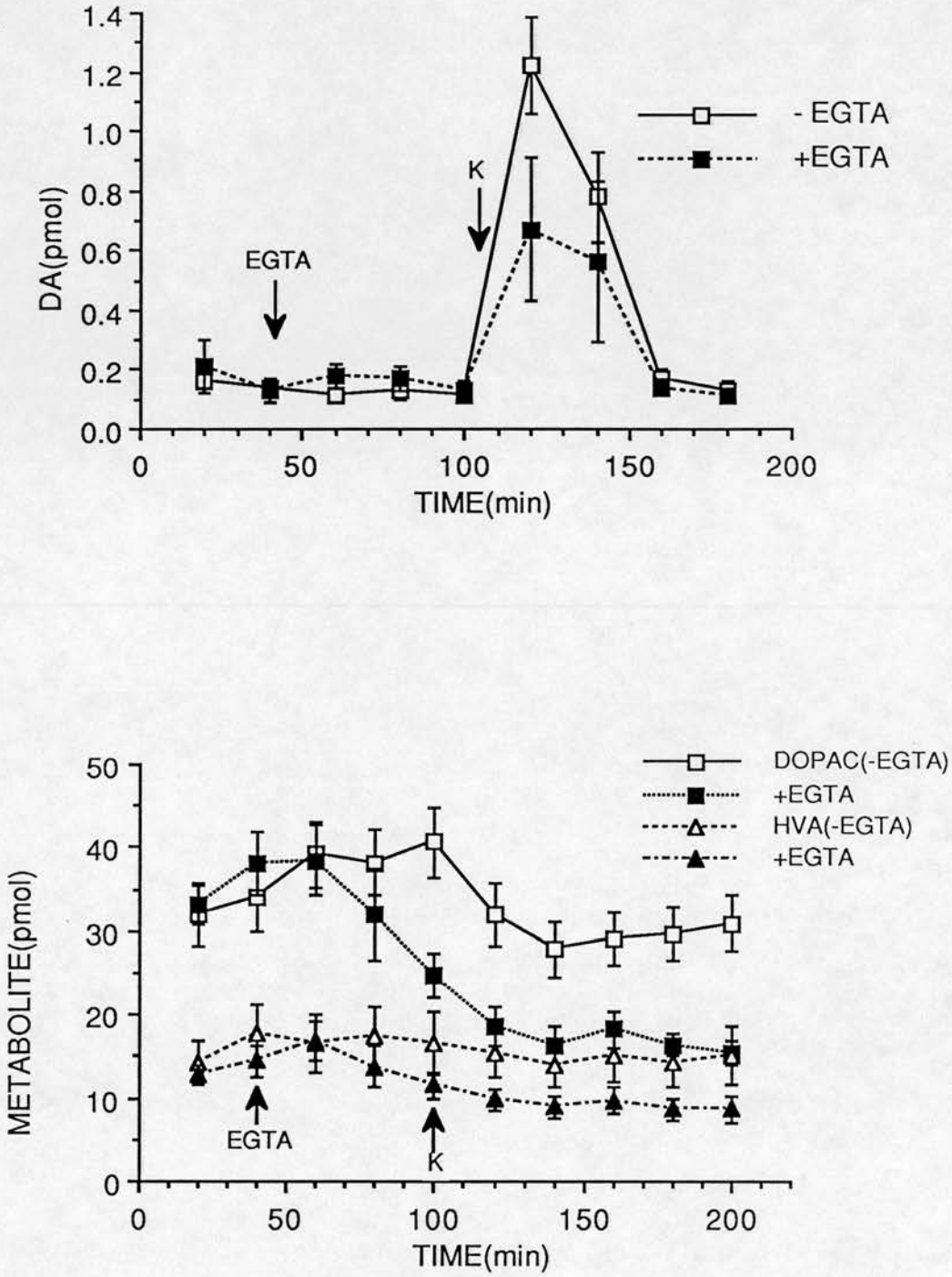


Fig. 3.4 8 Effects of calcium removal and EGTA (20mM) perfused intrastriatally for 60 min prior to KCl (90mM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.

min (Fig. 3.4.8, 0.09 ± 0.01 to 1.47 ± 0.29pmoles/20 min, n = 6). Total DA efflux over 60 min was reduced by 61% when compared with control animals (Table 3.4.1, control 2.09 ± 0.52pmoles, EGTA 0.82 ± 0.33pmoles, $p < 0.05$).

EGTA caused a decline in DOPAC and HVA efflux, but no additional effect upon KCl induced DOPAC and HVA efflux was apparent (Fig. 3.4.8).

TABLE 3.4.1

EFFECTS OF BIOCHEMICAL AND PHARMACOLOGICAL MANIPULATIONS ON KCl (90mM) INDUCED DA EFFLUX

	n	DA EFFLUX (pmol/60 min)
Control	16	1.91 ± 0.26
AMT (20 min pretreatment)	6	1.10 ± 0.27*
Control	16	1.91 ± 0.26
AMT (120 min pretreatment)	6	0.50 ± 0.14***
Control	16	1.91 ± 0.26
Reserpine	6	1.16 ± 0.35
Control	16	1.91 ± 0.26
Pargyline	6	4.45 ± 1.19*
Control	16	1.91 ± 0.26
Selegeline	6	1.72 ± 0.69
Control	16	1.91 ± 0.26
Nomifensine	6	2.57 ± 0.34*
Control	6	2.19 ± 0.65
TTX	6	1.19 ± 0.50
Control	6	2.09 ± 0.52
EGTA	6	0.82 ± 0.33*

* $p < 0.05$, *** $p < 0.001$

Mean and standard errors are given for guidance only. Statistical differences in amount released were assessed using the non-parametric Mann-Whitney 'U' test. The data were corrected for basal release prior to comparison.

3.5 Effects of biochemical and pharmacological manipulations upon tyramine induced efflux of DA, DOPAC, HVA, 5-HIAA and 3MT

3.5.1 Dose dependence of the tyramine response

A concentration of 1μM tyramine perfused through the dialysis probe had no effect upon DA efflux levels (Fig. 3.5.1a). Increasing the dose of tyramine (10, 50 and 100μM) led to a graded increase in maximal DA efflux (360, 1780 and 2270% of control respectively). The maximal DA response occurred during the 20 min perfusion of tyramine and subsequently declined to control levels 40 min after the removal of tyramine. A dose of 50μM tyramine was chosen for future experiments since these results indicate this to be neither a saturating dose or a minimal dose for stimulating DA efflux.

In striking contrast to other DA releasers, tyramine had no effect upon DOPAC, HVA or 5-HIAA efflux levels at any of the doses used (Figs. 3.5.1a and 3.5.1b).

3.5.2 α-Methyl-p-tyrosine

In animals with a 20 min pretreatment of AMT (250mg/kg i.p.), tyramine maximally increased DA efflux to 1342% of basal efflux (Fig. 3.5.2a, 0.12 ± 0.02 to 1.61 ± 0.34pmoles/20 min, n = 6). Total tyramine induced DA efflux over 60 min was not affected by AMT (Table 3.5.1, control 2.4 ± 0.28pmoles, AMT 2.01 ± 0.39pmoles). When AMT was injected 120 min prior to tyramine infusion, DA efflux was maximally increased to 930% of basal efflux over 20 min (Fig. 3.5.2b, 0.10 ± 0.03 to 0.93 ± 0.09pmoles/20 min, n = 6). Total tyramine induced DA efflux over 60 min was reduced by 54% when compared with control animals (Table 3.5.1, control 2.4 ±

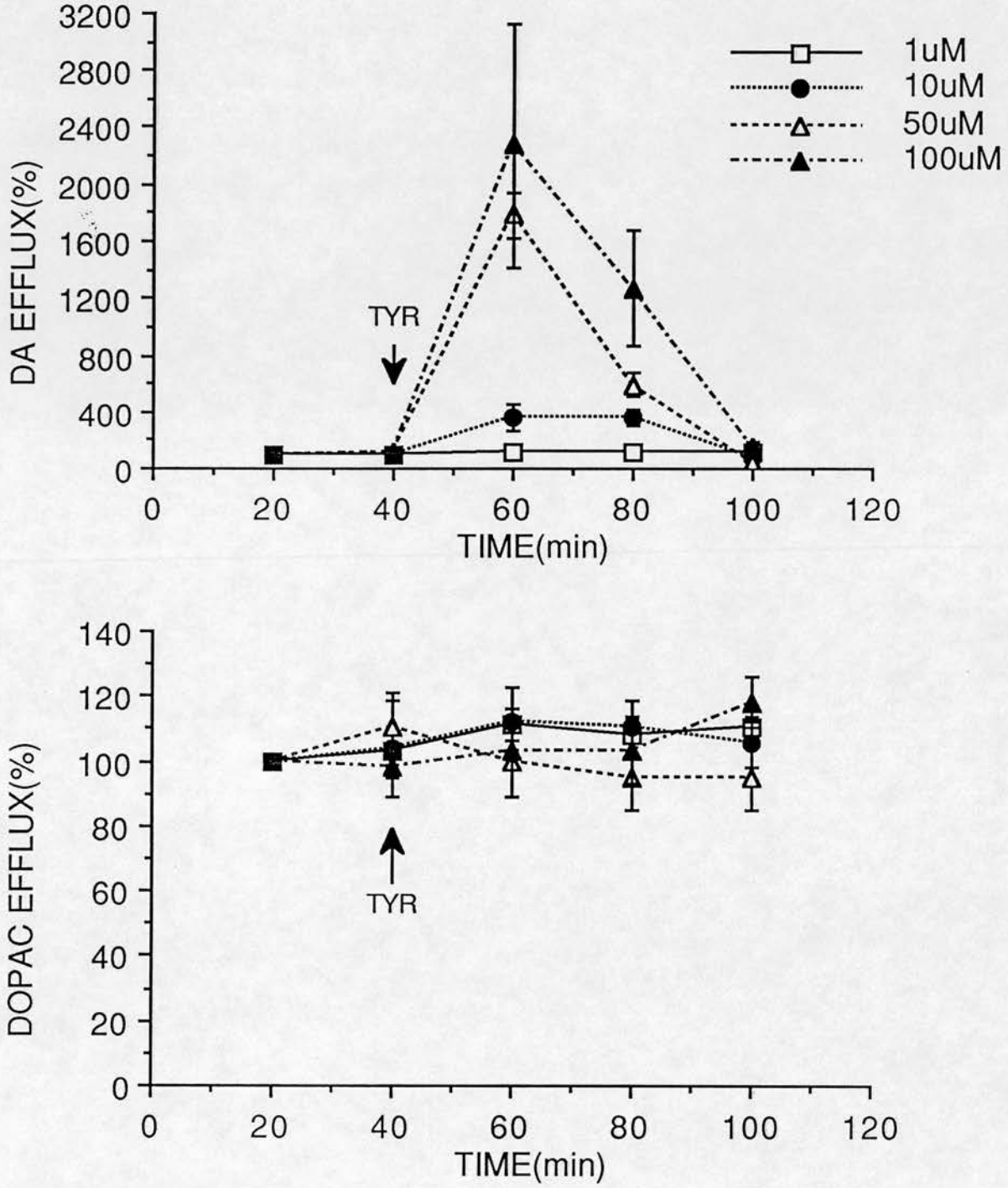


Fig. 3.5.1a Effects of increasing doses of tyramine (perfused intrastriatally) on the efflux of DA (upper panel) and DOPAC (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

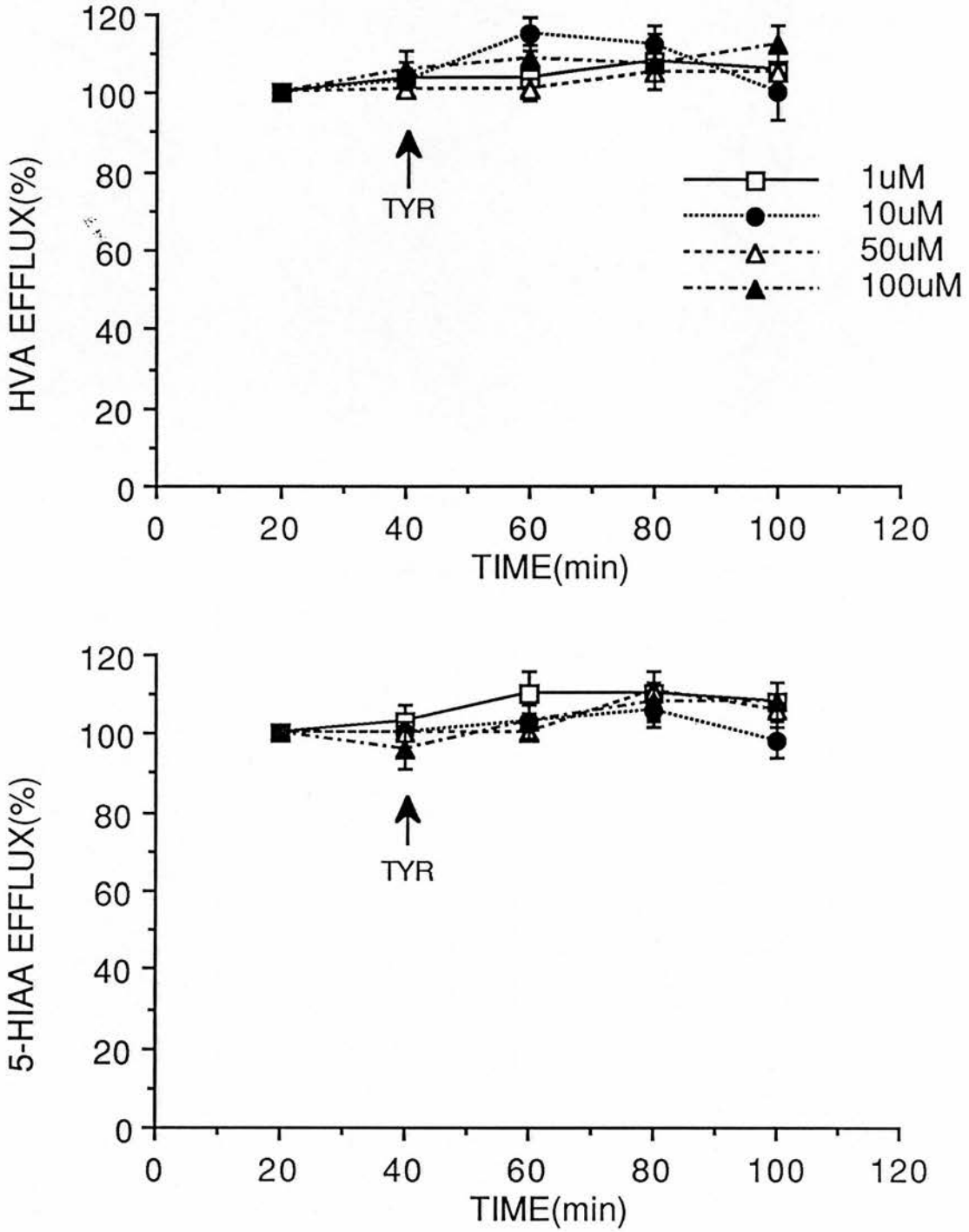


Fig. 3.5.1b Effects of increasing doses of tyramine (perfused intrastriatally) on the efflux of HVA (upper panel) and 5-HIAA (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

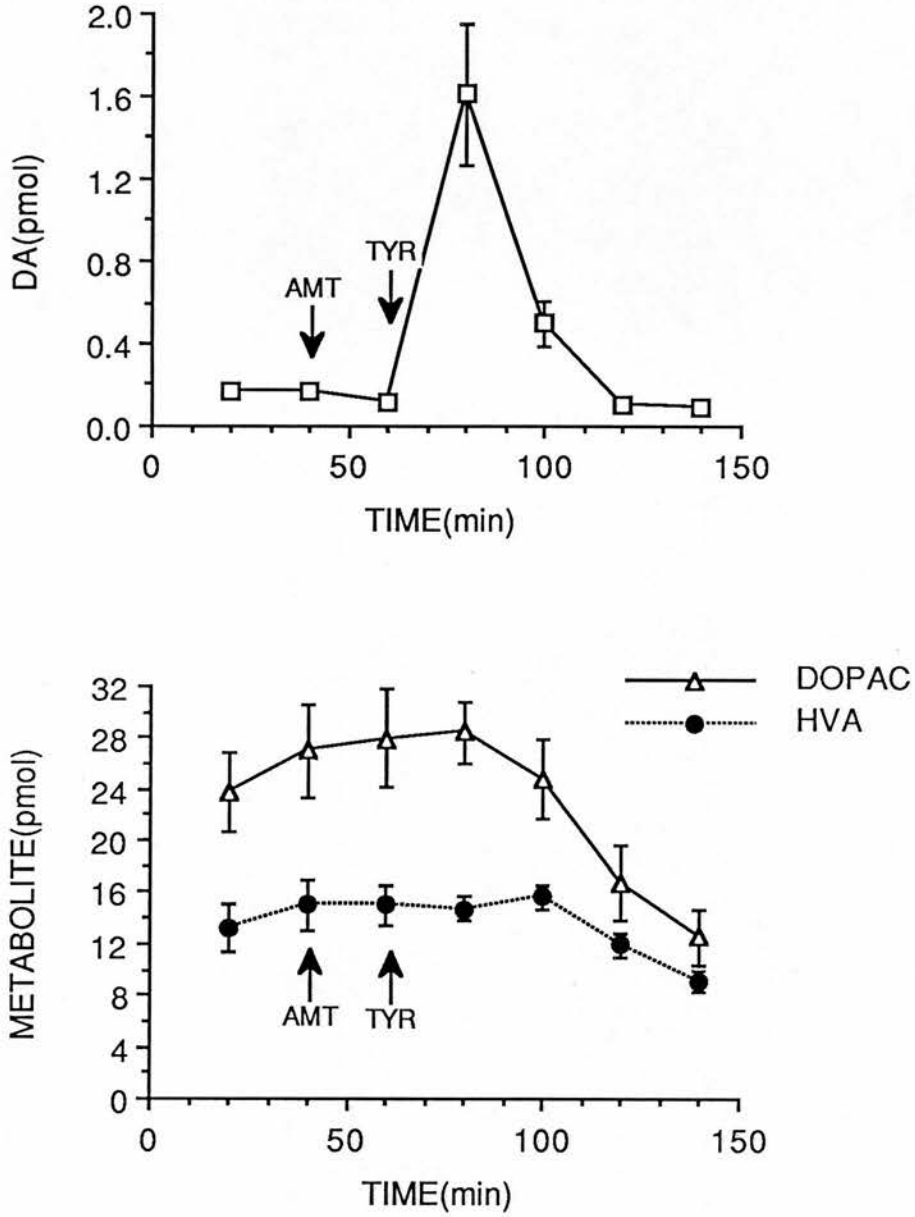


Fig. 3.5.2a Effects of AMT (250mg/kg i.p.) given 20 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

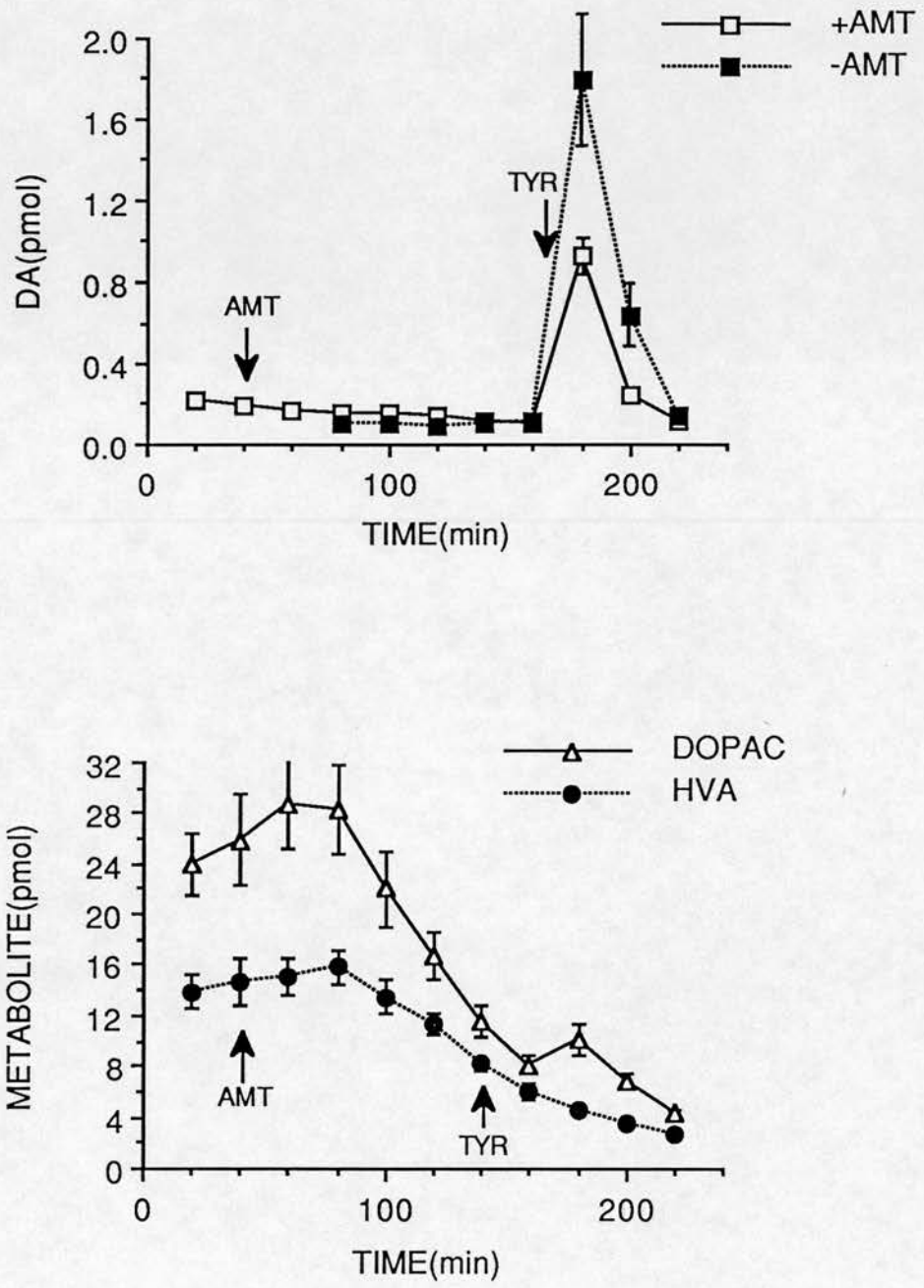


Fig. 3.5.2b Effects of AMT (250mg/kg i.p.) given 120 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

0.28pmoles, AMT 1.11 ± 0.16pmoles, $p < 0.001$). This is in striking contrast to the other result for AMT.

AMT caused a decline in DOPAC and HVA efflux which became apparent 60 min after injection (Figs. 3.5.2a, 3.5.2b). Tyramine did not appear to alter the pattern of this decline.

3.5.3 Reserpine

In reserpine (5mg/kg i.p.) pretreated animals, tyramine maximally increased DA efflux to 3500% of basal efflux over 20 min (Fig. 3.5.3, 0.02 ± 0.01 to 0.70 ± 0.27pmoles/20 min, $n = 6$). Total DA efflux over 60 min was reduced by 60% when compared with control animals (Table 3.5.1, control 2.4 ± 0.28pmoles, reserpine 0.97 ± 0.22pmoles, $p < 0.001$).

Tyramine did not appear to have any effect on DOPAC and HVA efflux in reserpinised animals (Fig. 3.5.3).

3.5.4 Pargyline

In pargyline (75mg/kg i.p.) treated animals, tyramine maximally increased DA efflux to 689% of basal efflux over 20 min (Fig. 3.5.4, 0.35 ± 0.13 to 2.41 ± 0.22pmoles/20min, $n = 6$). Total DA efflux over 60 min was increased by 20% when compared to control animals (Table 3.5.1, control 2.4 ± 0.28pmoles, pargyline 3.0 ± 0.29pmoles, $p < 0.05$).

Pargyline induced a rapid decline in DOPAC and HVA efflux which did not appear to alter after tyramine (Fig. 3.5.4). The pargyline induced rise in 3-MT efflux appeared to be accelerated and prolonged by tyramine (Fig. 3.5.4).

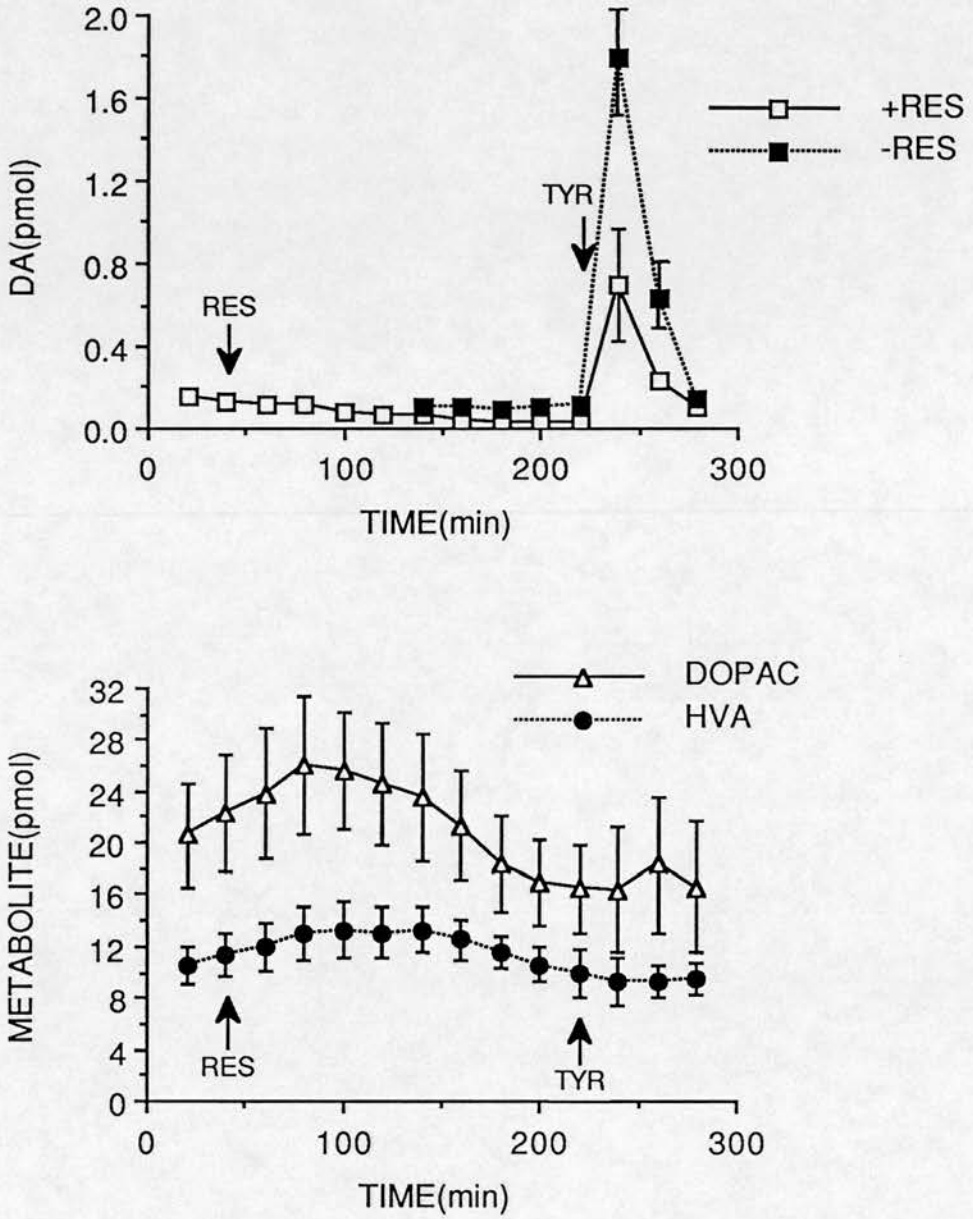
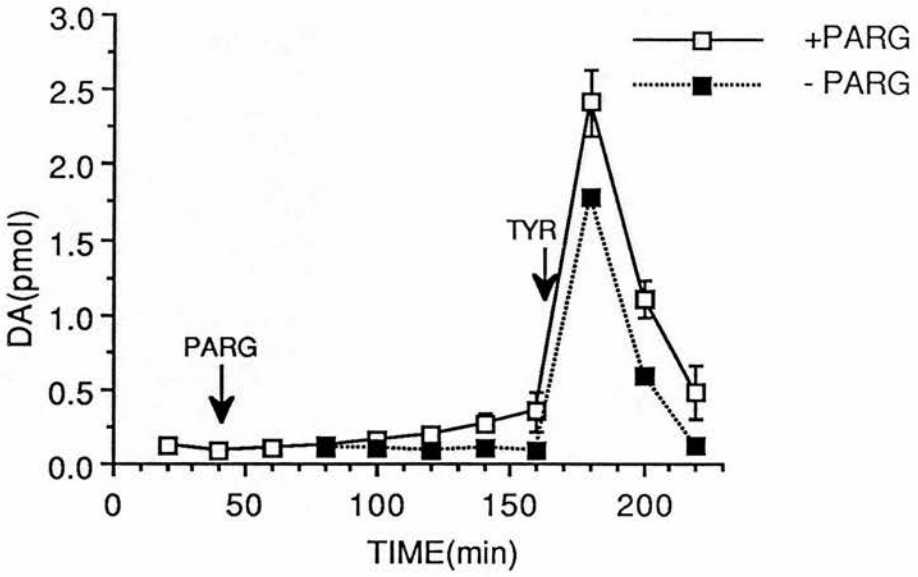
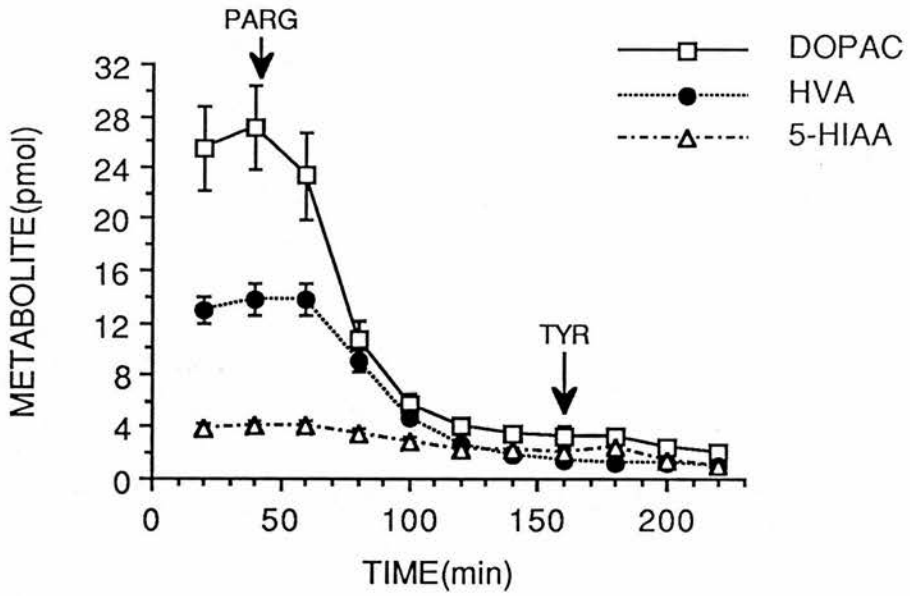
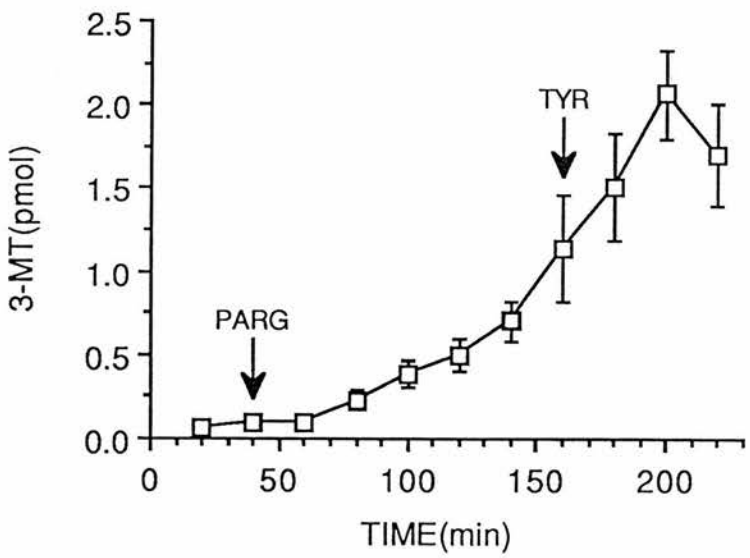


Fig. 3.5.3 Effects of reserpine (5mg/kg i.p.) given 180 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

Fig. 3.5.4 Effects of pargyline (75mg/kg i.p.) given 120 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


3.0
2.5
2.0
1.5
1.0
0.5
0.0
DA(pmol)
PARG
TYR
+PARG
- PARG
0
50
100
150
200
TIME(min)


PARG
32
28
24
20
16
12
8
4
0
METABOLITE(pmol)
TYR
DOPAC
HVA
5-HIAA
0
50
100
150
200
TIME(min)


2.5
2.0
1.5
1.0
0.5
0.0
3-MT(pmol)
TYR
PARG
0
50
100
150
200
TIME(min)

3.5.5 Selegeline

In selegeline (10mg/kg i.p.) treated animals, tyramine maximally increased DA efflux to 1922% of basal efflux over 20 min (Fig. 3.5.5, 0.10 ± 0.01 to 1.73 ± 0.3pmoles/20 min, n = 6). Total DA efflux over 60 min was not affected by selegeline (Table 3.5.1, control 2.4 ± 0.28pmoles, selegeline 2.1 ± 0.34pmoles).

DOPAC and HVA efflux appeared to be unaffected whereas 3-MT efflux was maximally increased to 500% of basal efflux over 20 min (Fig. 3.5.5, 0.05 to 0.25pmoles/20 min, n = 3). A quantitative assessment of this was not possible due to the small value for n and the lack of a 3-MT control.

3.5.6 Nomifensine

In nomifensine (20mg/kg i.p.) treated animals, tyramine maximally increased DA efflux to 629% of basal efflux over 20 min (Fig. 3.5.6, 0.14 ± 0.04 to 0.88 ± 0.18pmoles/20 min, n = 6). Total DA efflux over 60 min was markedly reduced by 73% when compared to control animals (Table 3.5.1, control 2.4 ± 0.28pmoles, nomifensine 0.89 ± 0.19pmoles, $p < 0.001$).

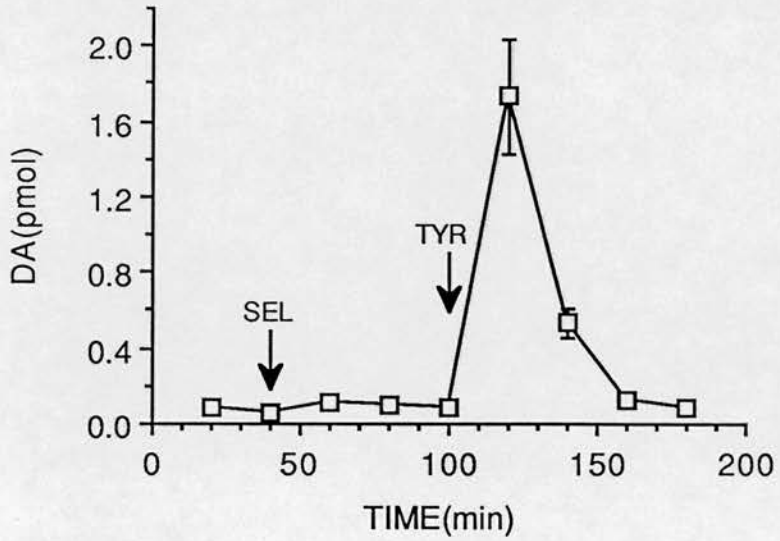
DOPAC and HVA efflux levels remained constant (Fig. 3.5.6).

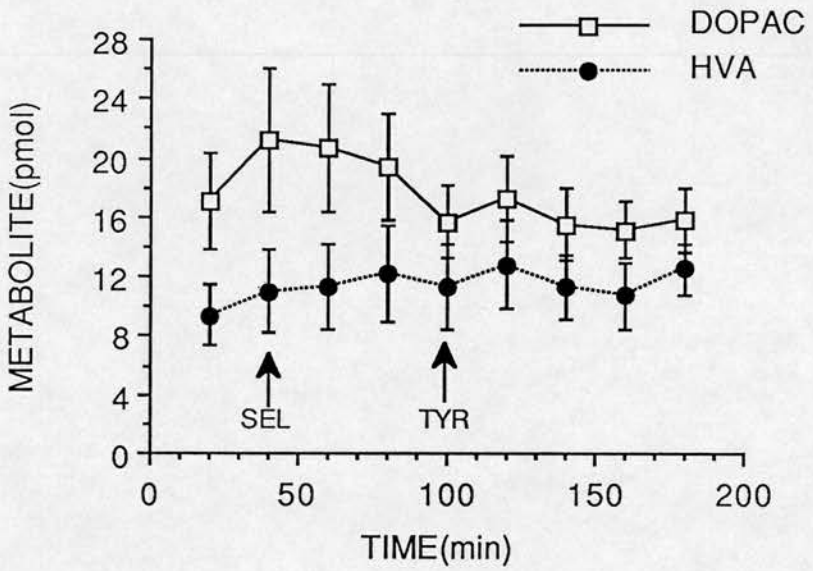
3.5.7 Tetrodotoxin

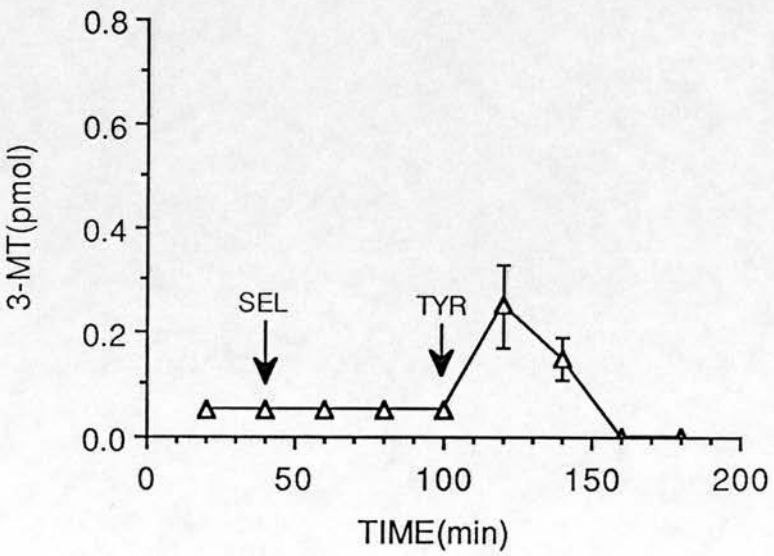
In TTX (2μM) treated animals, tyramine maximally increased DA efflux to 5900% of basal efflux over 20 min (Fig. 3.5.7, 0.02 ± 0.02 to 1.18 ± 0.12pmoles/20 min, n = 6). Total tyramine induced DA efflux over 60 min was not affected when compared to control animals (Table 3.5.1, control 1.89 ± 0.48pmoles, TTX 1.50 ± 0.9pmoles).

TTX caused a rapid decline in DOPAC efflux and a less rapid decline in HVA efflux which did not appear to be affected by

Fig. 3.5.5 Effects of selegeline (10mg/kg i.p.) given 60 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC and HVA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


DA(pmol)
2.0
1.6
1.2
0.8
0.4
0.0
SEL
TYR
0
50
100
150
200
TIME(min)


METABOLITE(pmol)
28
24
20
16
12
8
4
0
DOPAC
HVA
SEL
TYR
0
50
100
150
200
TIME(min)


3-MT(pmol)
0.8
0.6
0.4
0.2
0.0
SEL
TYR
0
50
100
150
200
TIME(min)

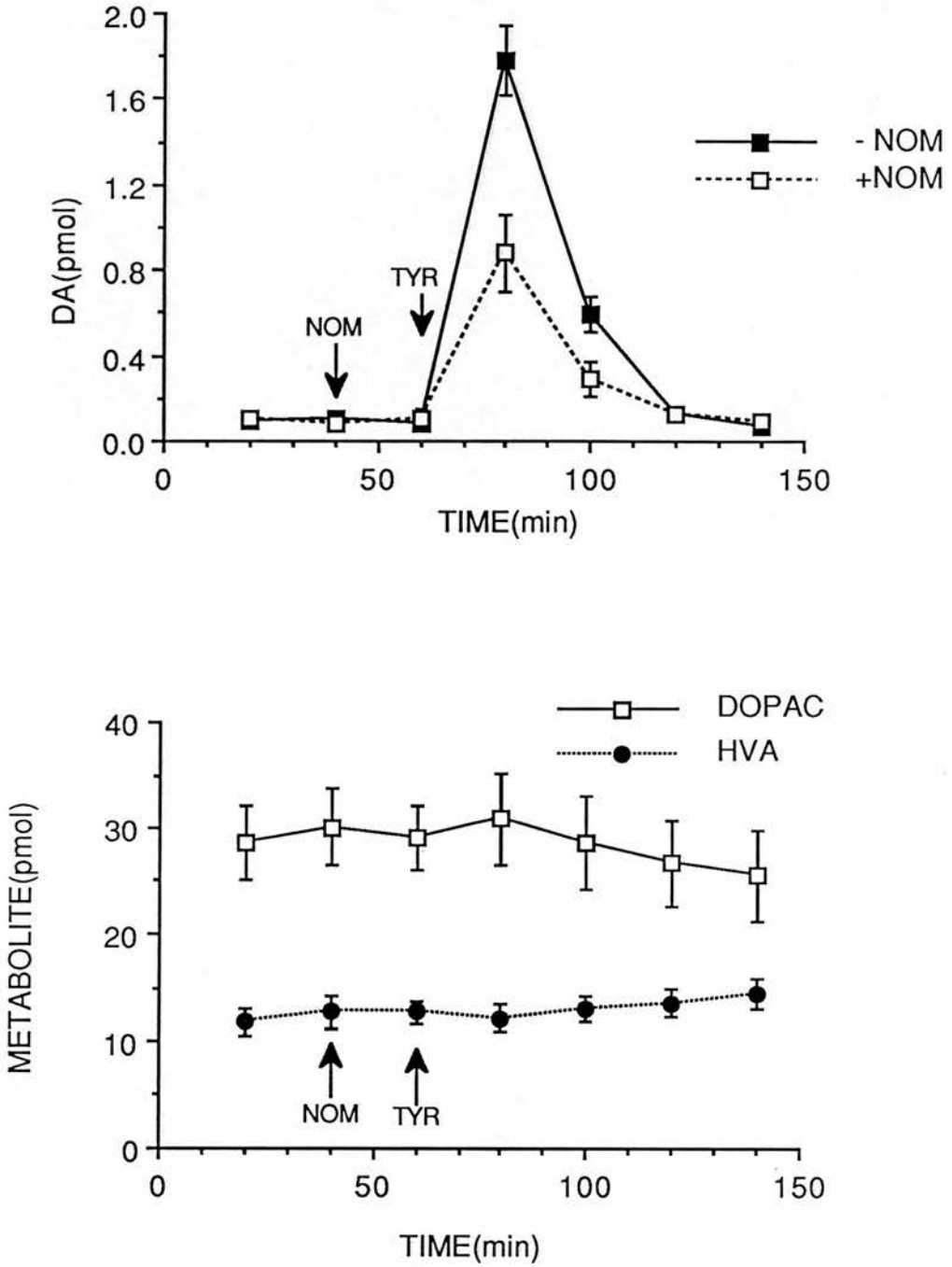
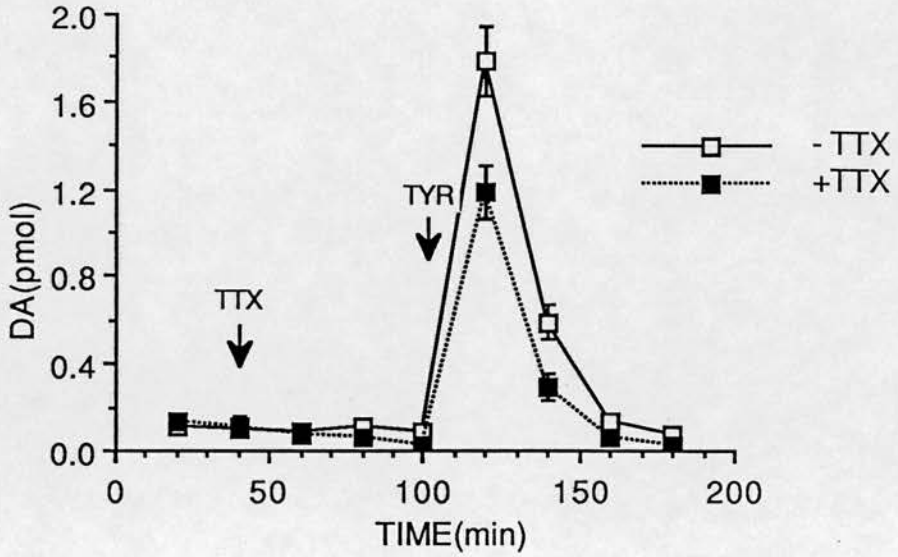
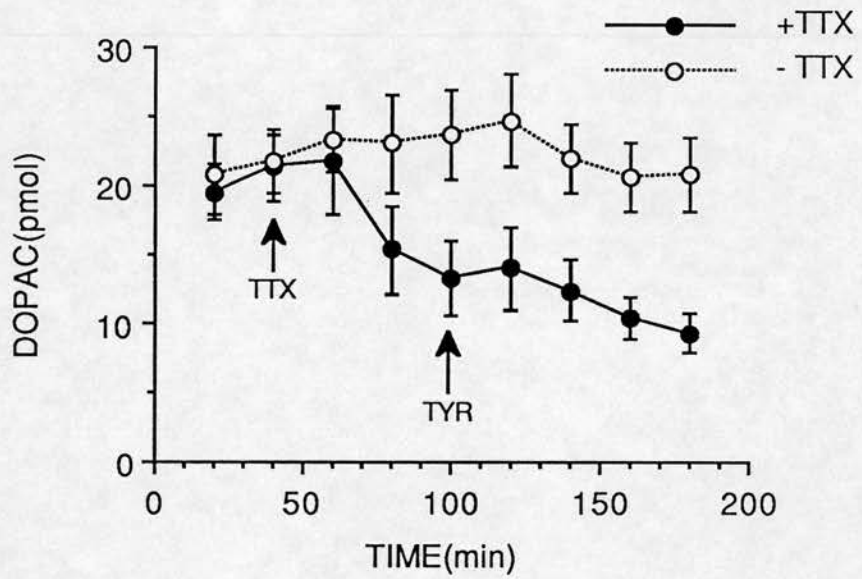
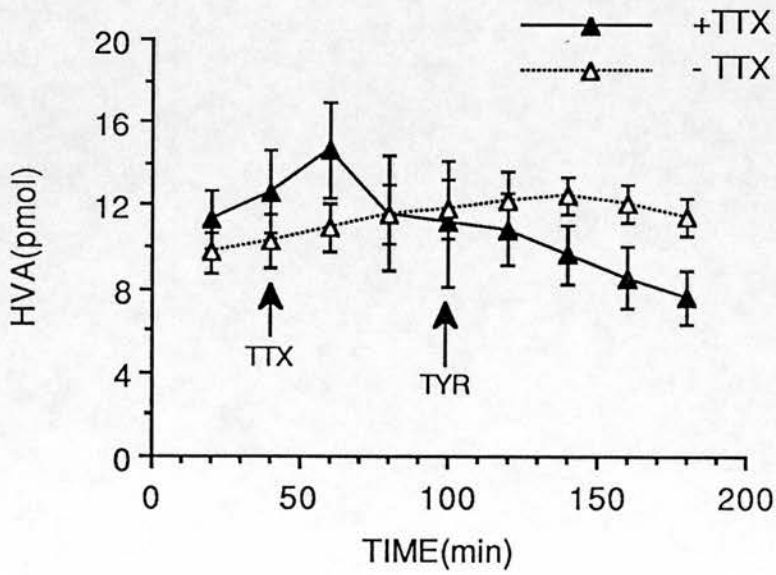


Fig. 3.5.6 Effects of nomifensine (20mg/kg i.p.) given 20 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

Fig. 3.5.7 Effects of TTX (2μM) perfused intrastriatally for 60 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC (middle panel) and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


2.0
1.6
1.2
0.8
0.4
0.0
DA(pmol)
TTX
TYR
- TTX
+TTX
0
50
100
150
200
TIME(min)


30
20
10
0
DOPAC(pmol)
+TTX
- TTX
TTX
TYR
0
50
100
150
200
TIME(min)


20
16
12
8
4
0
HVA(pmol)
+TTX
- TTX
TTX
TYR
0
50
100
150
200
TIME(min)

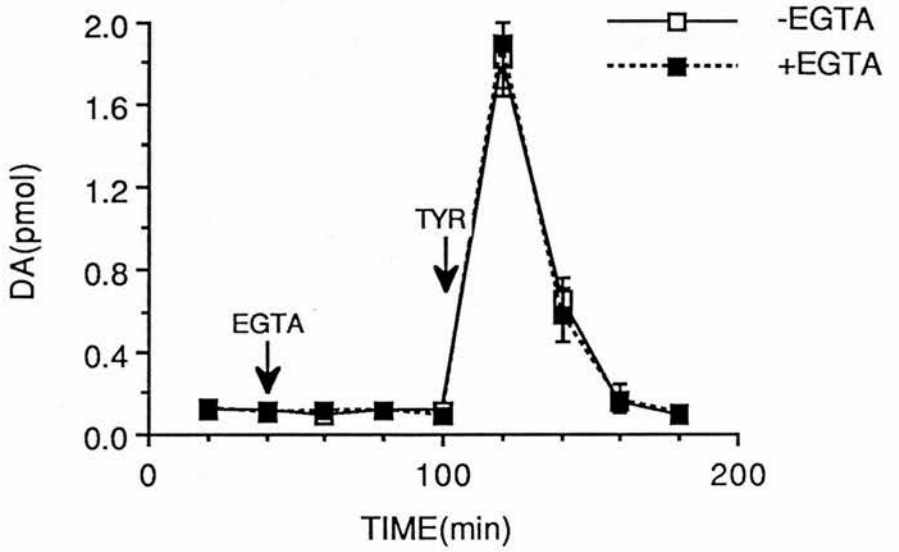
tyramine (Fig. 3.5.7).

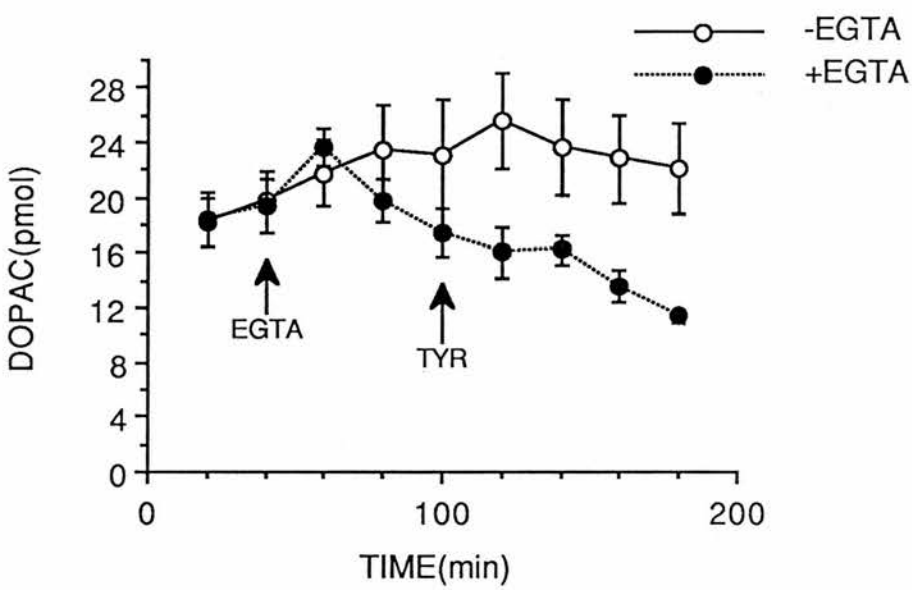
3.5.8 EGTA

In animals perfused with EGTA (20mM) in a Ca^{2+} free buffer, tyramine maximally increased DA efflux to 211% of basal efflux over 20 min (Fig. 3.5.8, 0.09 ± 0.01 to 1.90 ± 0.22pmoles/20 min, n = 6). Total tyramine induced DA efflux over 60 min was not affected when compared to control animals, indeed the patterns of release were about identical for control and EGTA treated animals (Table 3.5.1, control 2.48 ± 0.22pmoles, EGTA 2.32 ± 0.33pmoles).

EGTA caused a lesser rate of efflux of DOPAC and HVA which did not appear to alter following tyramine infusion (Fig. 3.5.8).

Fig. 3.5.8 Effects of calcium removal and EGTA (20mM) perfused intrastriatally for 60 min prior to tyramine (50μM) on the efflux of DA (upper panel), DOPAC (middle panel) and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


-EGTA
+EGTA
DA(pmol)
2.0
1.6
1.2
0.8
0.4
0.0
TYR
EGTA
0
100
200
TIME(min)


-EGTA
+EGTA
DOPAC(pmol)
28
24
20
16
12
8
4
0
EGTA
TYR
0
100
200
TIME(min)

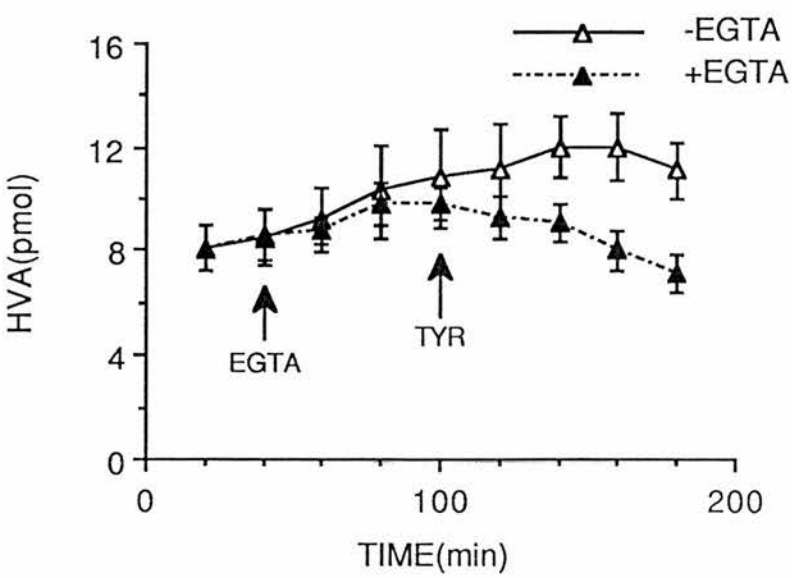

-EGTA
+EGTA
HVA(pmol)
16
12
8
4
0
EGTA
TYR
0
100
200
TIME(min)

TABLE 3.5.1

EFFECTS OF BIOCHEMICAL AND PHARMACOLOGICAL MANIPULATIONS ON TYRAMINE (5×10^{-5}M) INDUCED DA EFFLUX

	n	DA EFFLUX (pmol/60 min)
Control	12	2.4 ± 0.28
AMT (20 min pretreatment)	6	2.01 ± 0.39
Control	12	2.4 ± 0.28
AMT (120 min pretreatment)	6	1.11 ± 0.16***
Control	12	2.4 ± 0.28
Reserpine	6	0.97 ± 0.22***
Control	12	2.4 ± 0.28
Pargyline	6	3.0 ± 0.29*
Control	12	2.4 ± 0.28
Selegeline	6	2.1 ± 0.34
Control	12	2.4 ± 0.28
Nomifensine	6	0.89 ± 0.19***
Control	6	1.89 ± 0.48
TTX	6	1.50 ± 0.9
Control	6	2.48 ± 0.22
EGTA	6	2.32 ± 0.33

* $p < 0.05$, *** $p < 0.001$

Mean and standard errors are given for guidance only. Statistical differences in amount released were assessed using the non-parametric Mann-Whitney 'U' test. The data were corrected for basal release prior to comparison.

3.6 Effects of biochemical and pharmacological manipulations upon ouabain induced efflux of DA, DOPAC, HVA, 5-HIAA and 3MT.

3.6.1 Dose dependence of the ouabain response

Ouabain was perfused through the dialysis probes at concentrations of 10,100 and 1000μM. The lowest concentration of 10μM had no effect upon DA, DOPAC, HVA or 5-HIAA efflux levels. However, increasing the concentration to 100 and 1000μM caused a graded increase in DA efflux levels (Fig. 3.6.1a, maximal DA efflux = 2241% and 6604% of control respectively) and a graded decrease in DOPAC, HVA and 5-HIAA efflux levels (Figs. 3.6.1a and 3.6.1b). A dose of 100μM ouabain was chosen for all further experiments as it was apparent from these results that this dose was neither minimal or maximal for stimulating DA efflux.

3.6.2 α-Methyl-p-tyrosine

In animals given a 20 min pretreatment of AMT (250mg/kg i.p.) ouabain maximally increased DA efflux to 1113% of basal efflux over 40 min (Fig. 3.6.2a, 0.15 ± 0.02 to 1.67 ± 0.44pmoles/20 min, n = 6). Ouabain induced DA efflux over 20 min was not affected when compared with control values (Table 3.6.1, control 2.0 ± 0.49pmoles, AMT 1.0 ± 0.36pmole) whereas DA efflux over 60 min was reduced by 43% when compared with control animals (Table 3.6.1, control 5.87 ± 0.80pmoles, AMT 3.37 ± 0.86pmoles, $p < 0.05$).

When AMT was injected 120 min prior to ouabain, maximal DA efflux was 1150% of basal efflux over 40 min (Fig. 3.6.2b, 0.02 ± 0.01 to 0.23 ± 0.05pmoles/20 min, n = 6). Ouabain induced DA efflux over 20 min was reduced by 90% when compared with control animals (Table 3.6.1, control 2.0 ± 0.49pmoles, AMT 0.19 ±

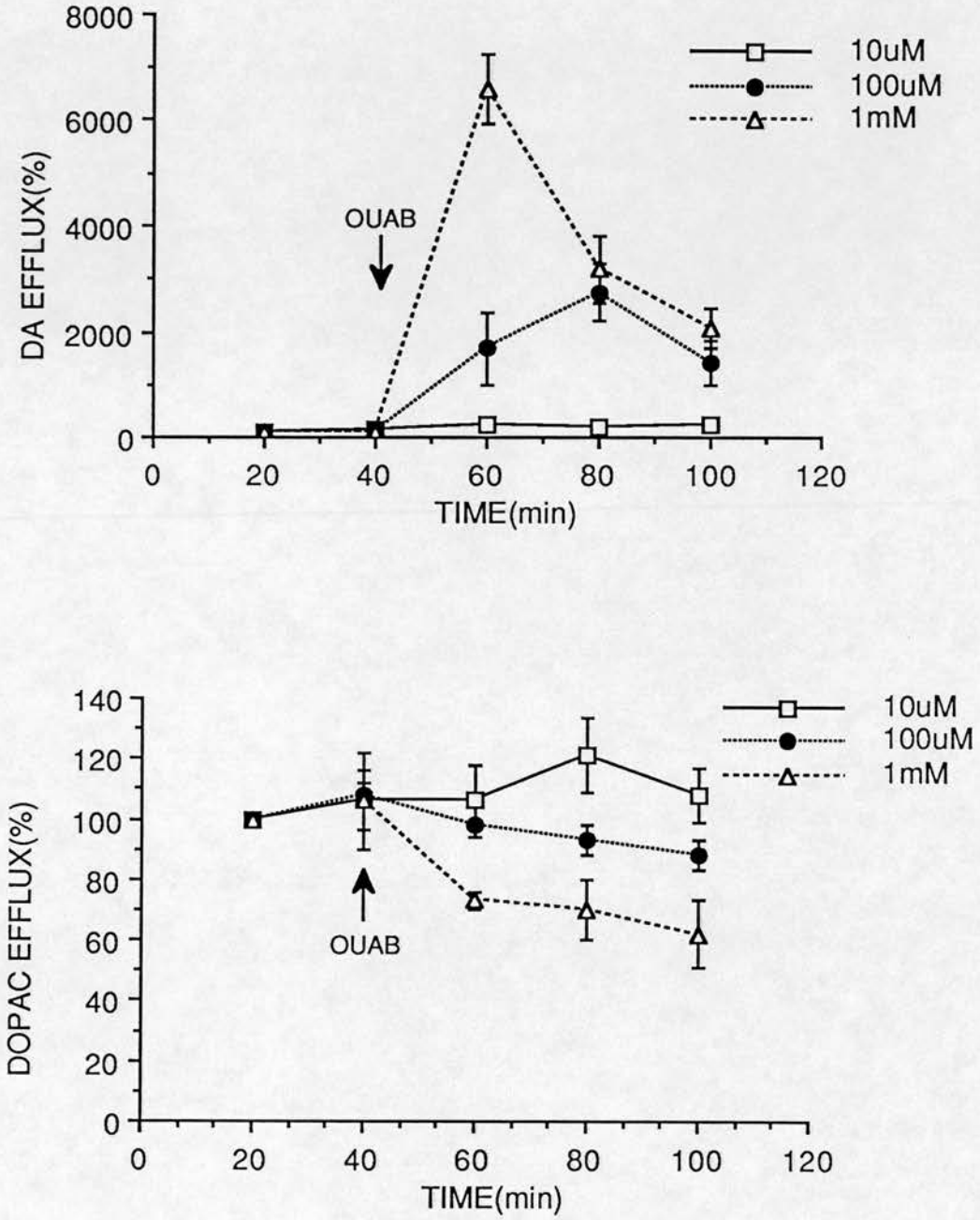


Fig. 3.6.1a Effects of increasing doses of ouabain (perfused intrastriatally) on the efflux of DA (upper panel) and DOPAC (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

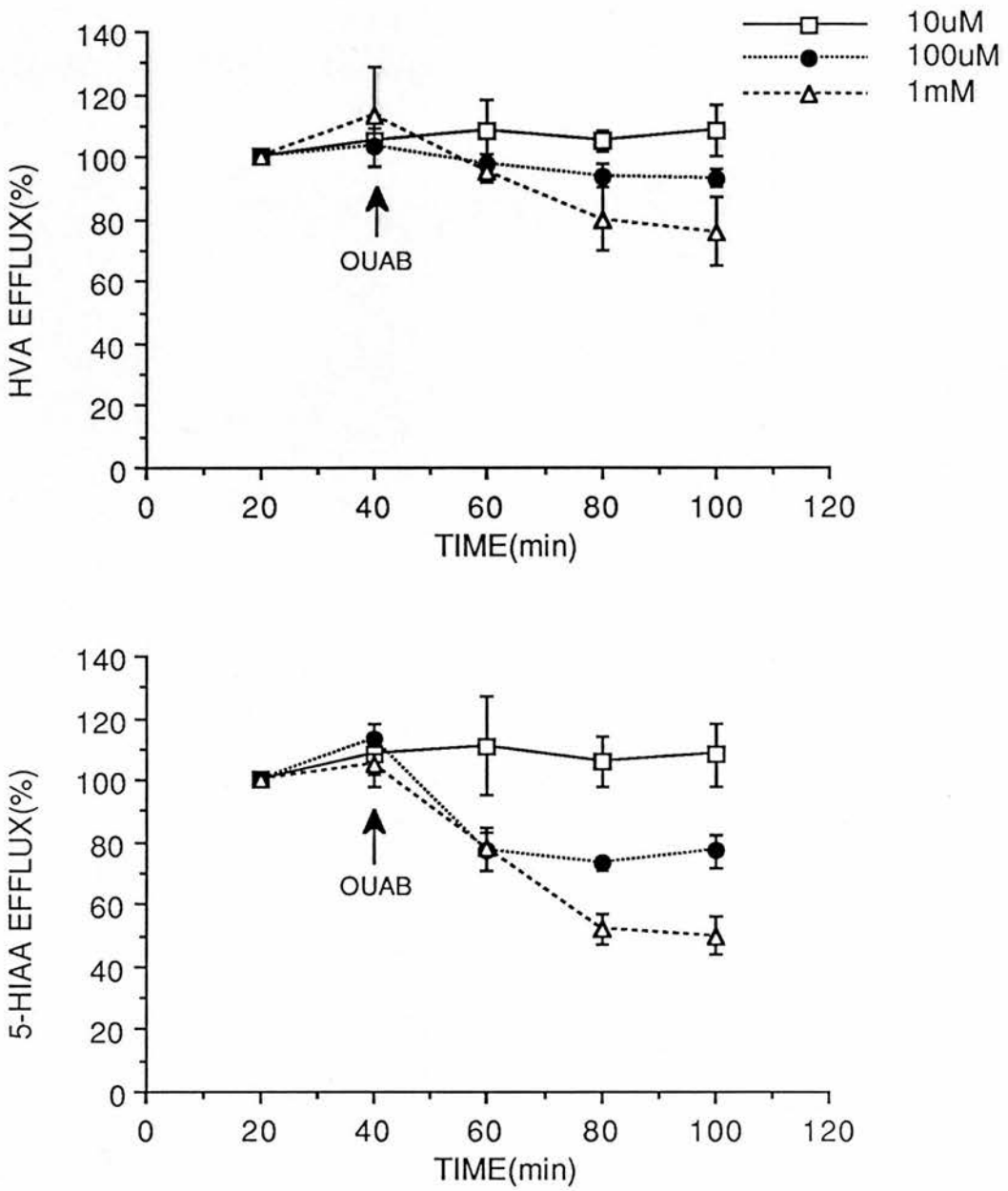


Fig. 3.6.1b Effects of increasing doses of ouabain (perfused intrastriatally) on the efflux of HVA (upper panel) and 5-HIAA (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

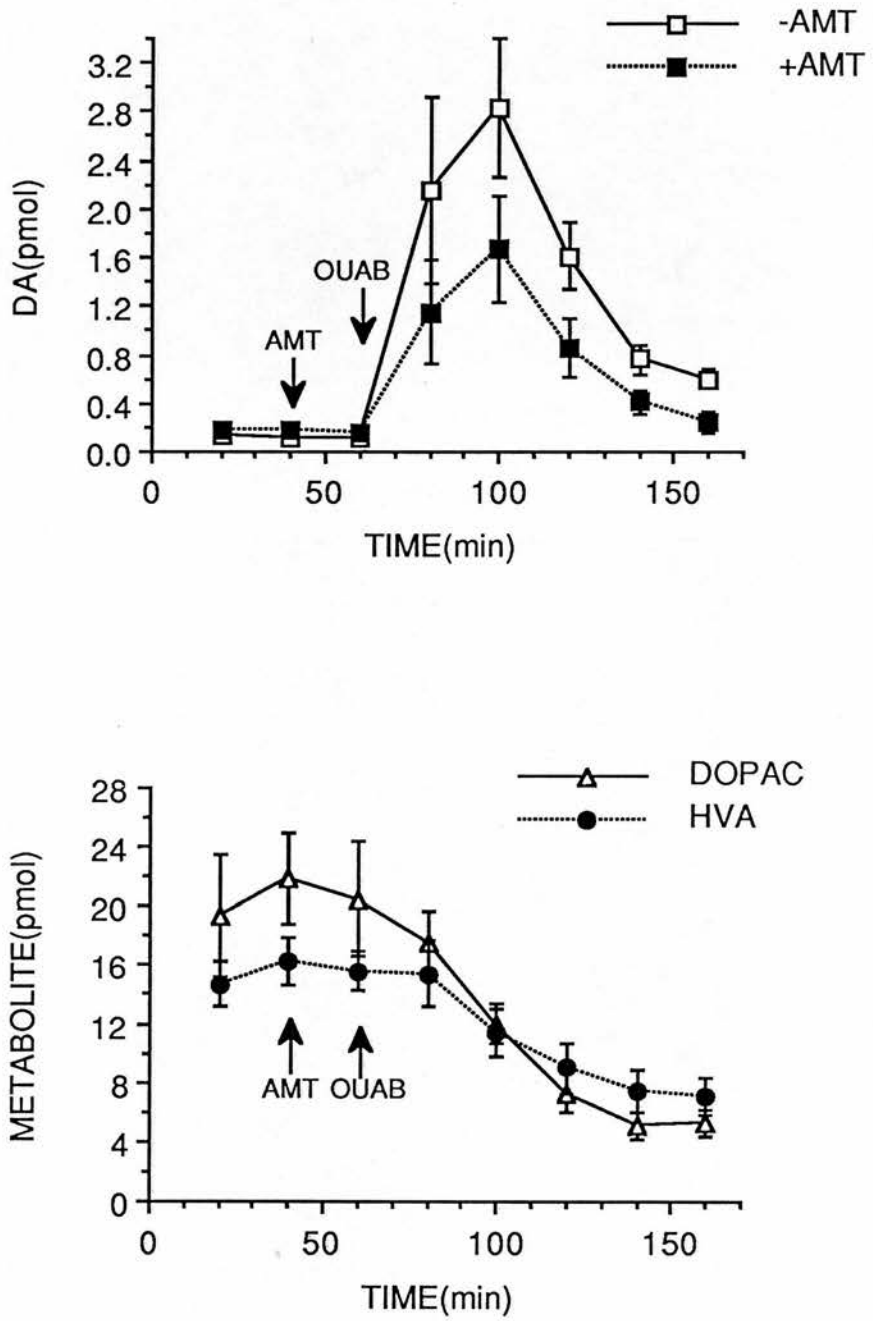


Fig. 3.6.2a Effects of AMT (250mg/kg i.p.) given 20 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

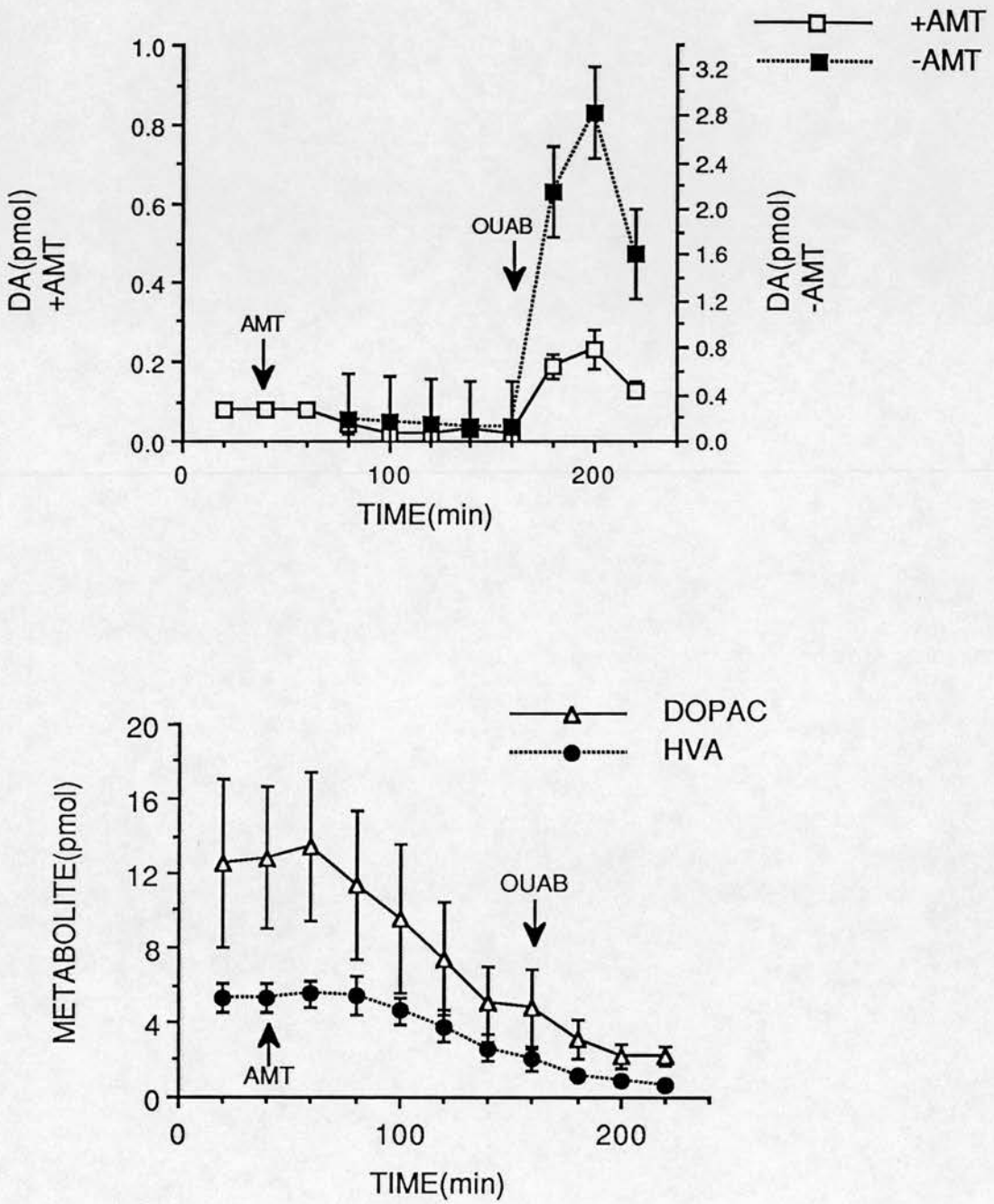


Fig. 3.6.2b Effects of AMT (250mg/kg i.p.) given 120 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

0.16pmoles, $p < 0.01$) and also by 90% over a 60 min period (Table 3.6.1, control 5.87 ± 0.80pmoles, AMT 0.58 ± 0.08pmoles, $p < 0.001$).

Qualitatively, ouabain appeared to accelerate the decline in DOPAC and HVA efflux caused by AMT (Fig. 3.6.2b).

3.6.3 Reserpine

In reserpine (5mg/kg i.p.) treated animals, ouabain maximally increased DA efflux to 2350% of basal efflux over 40 min (Fig. 3.6.3, 0.08 ± 0.02 to 1.88 ± 0.56pmoles/20 min, $n = 6$). Ouabain induced DA efflux over 20 min and over 60 min was not affected by reserpine (Table 3.6.1).

In qualitative terms, ouabain appeared to accelerate the decline in DOPAC and HVA efflux caused by reserpine (Fig. 3.6.3).

3.6.4 Pargyline

In pargyline (75mg/kg i.p.) treated animals, ouabain maximally increased DA efflux to 2136% of basal efflux over 20 min (Fig. 3.6.4, 0.22 ± 0.05 to 4.7 ± 2.0pmoles/20 min, $n = 6$). Ouabain induced DA efflux over 20 min was not affected by pargyline despite the higher value (Table 3.6.1, control 2.0 ± 0.49pmoles, pargyline 4.5 ± 2.1pmoles) whereas over a 60 min period, DA efflux was increased by 227% when compared to control animals (Table 3.6.1, control 5.87 ± 0.80pmoles, pargyline 13.3 4.53pmoles, $p < 0.05$).

Pargyline induced a rapid decline in DOPAC and HVA efflux and a less rapid decline in 5-HIAA efflux (Fig. 3.6.4). Ouabain did not seem to have any additional effect on the efflux of any of these metabolites.

Ouabain appeared to substantially increase the rate of 3-MT efflux above that elicited by pargyline (Fig. 3.6.4).

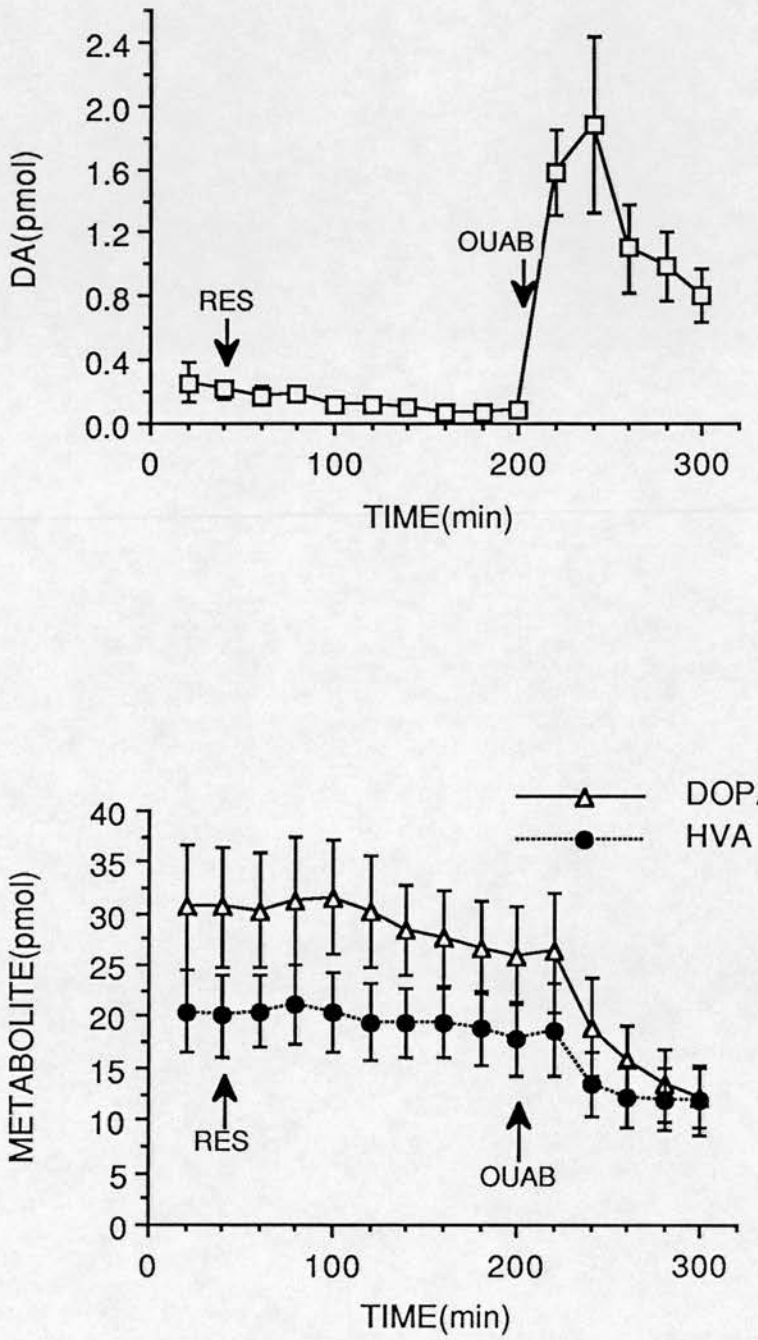
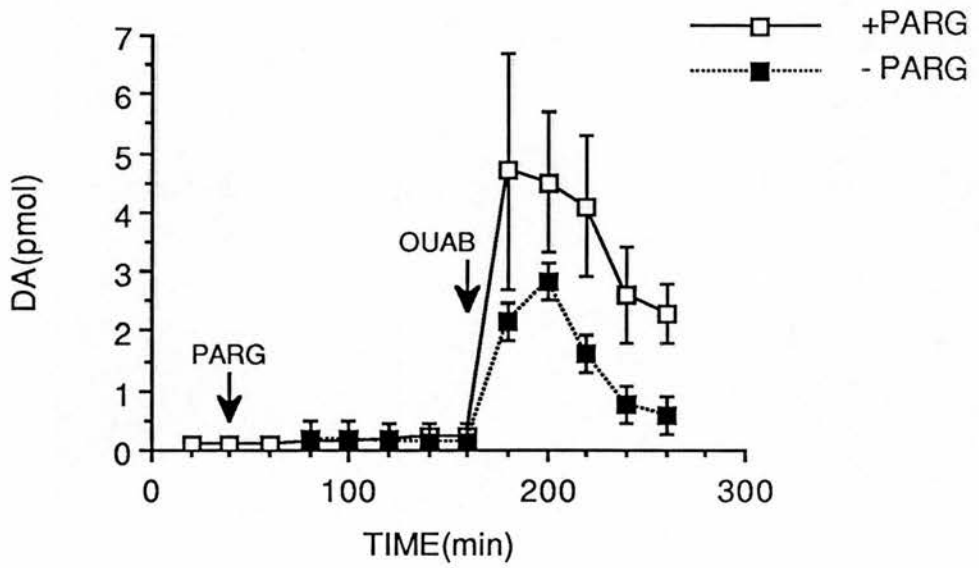
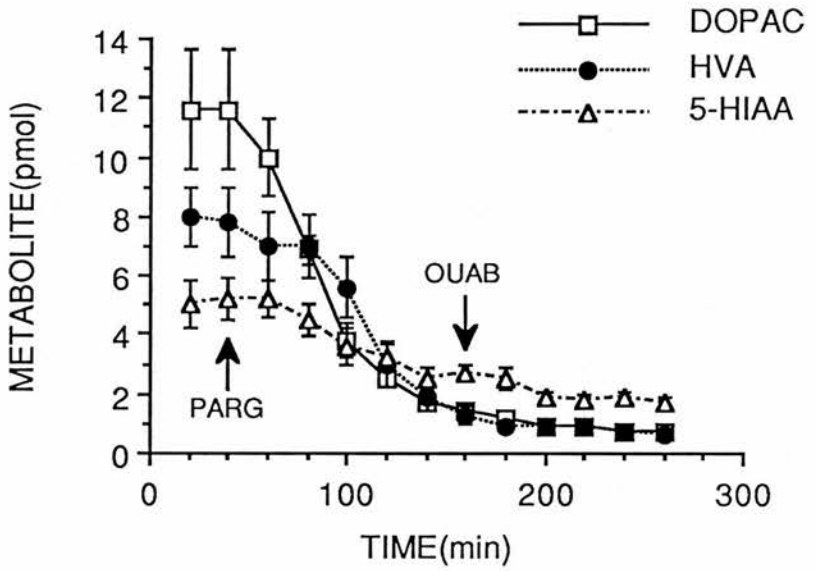
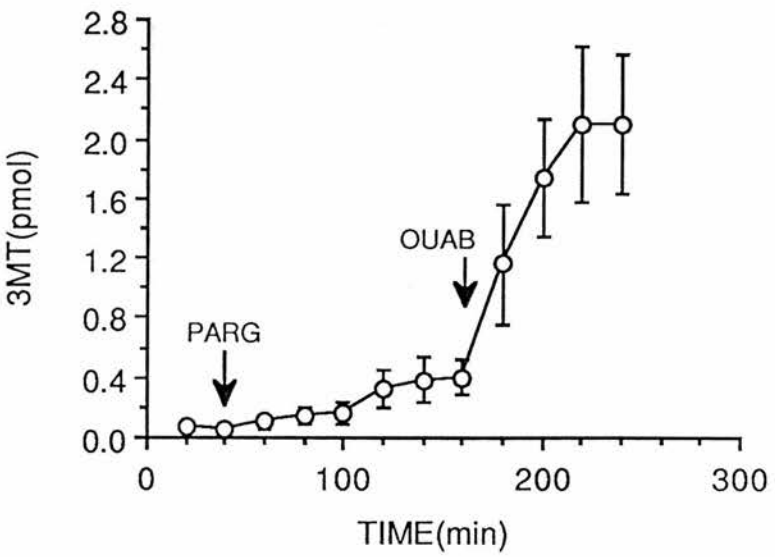


Fig. 3.6.3 Effects of reserpine (5mg/kg i.p.) given 180 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

Fig. 3.6.4 Effects of pargyline (75mg/kg i.p.) given 120 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


+PARG
- PARG
DA(pmol)
OUAB
PARG
TIME(min)


DOPAC
HVA
5-HIAA
METABOLITE(pmol)
OUAB
PARG
TIME(min)


3MT(pmol)
OUAB
PARG
TIME(min)

3.6.5 Selegeline

In selegeline (10mg/kg i.p.) treated animals, ouabain maximally increased DA efflux to 2692% of basal efflux over 40 min (Fig. 3.6.5. 0.13 ± 0.02 to 3.5 ± 1.3pmoles/20 min, n = 4). the efflux of DA over 20 and 60 min was not affected when compared to control animals (Table 3.6.1).

The efflux of DOPAC, HVA and 5-HIAA did not appear to be affected by selegeline whereas 3-MT efflux on the other hand was maximally increased by ouabain to 222% of basal efflux over 40 min (Fig. 3.6.5, 0.09 ± 0.02 to 0.20 ± 0.12pmoles/20 min, n = 4). The significance of this was not determined due to the lack of a 3-MT control.

3.6.6 Nomifensine

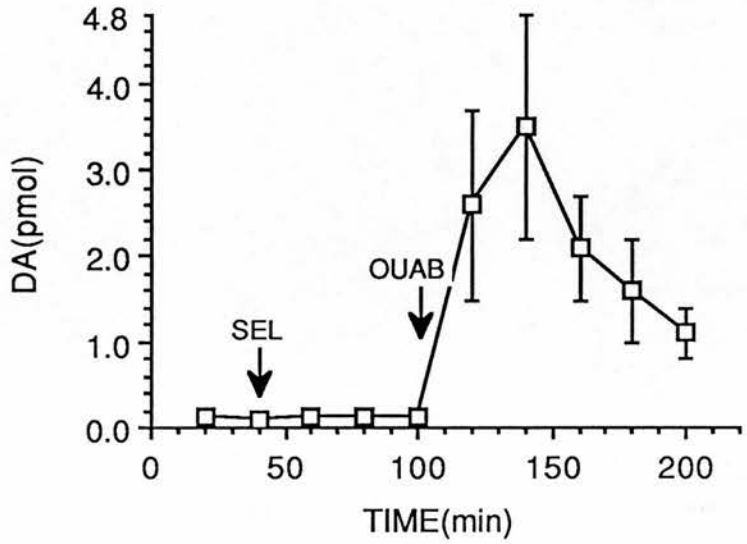
In nomifensine (20mg/kg i.p.) treated animals, ouabain maximally increased DA efflux to 1167% of basal efflux over 60 min (Fig 3.6.6, 0.12 ± 0.02 to 1.4 ± 0.50 pmoles/20 min, n = 6). Interestingly, DA efflux over a 20 min period was reduced by 86% when compared with control animals (Table 3.6.1, control 2.0 ± 0.49pmoles, nomifensine 0.28 ± 0.06pmoles, $p < 0.001$), whereas over a 60 min period, DA efflux was reduced to a lesser extent by 56% when compared with control animals (Table 3.6.1; control 5.87 ± 0.80pmoles, nomifensine 2.6 ± 0.83pmoles, $p < 0.01$).

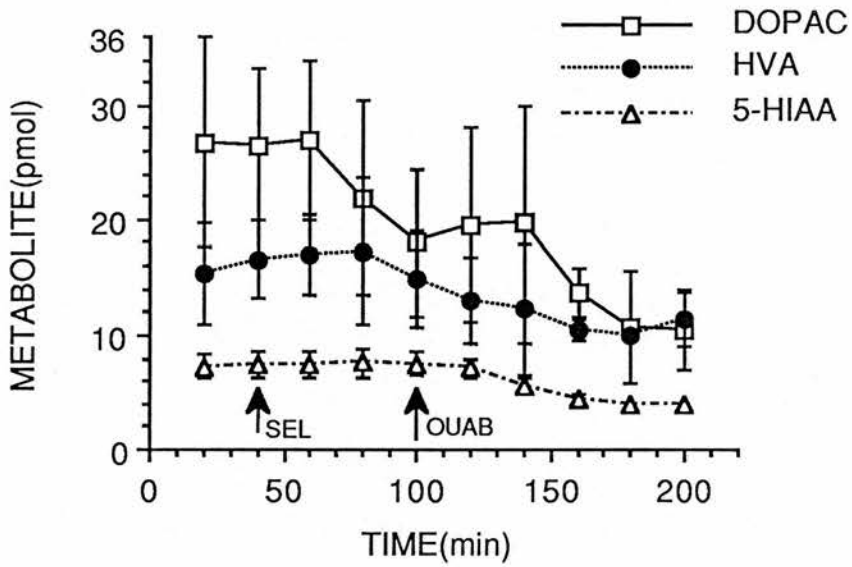
Ouabain induced efflux of DOPAC and HVA did not appear to be affected by nomifensine (Fig. 3.6.6).

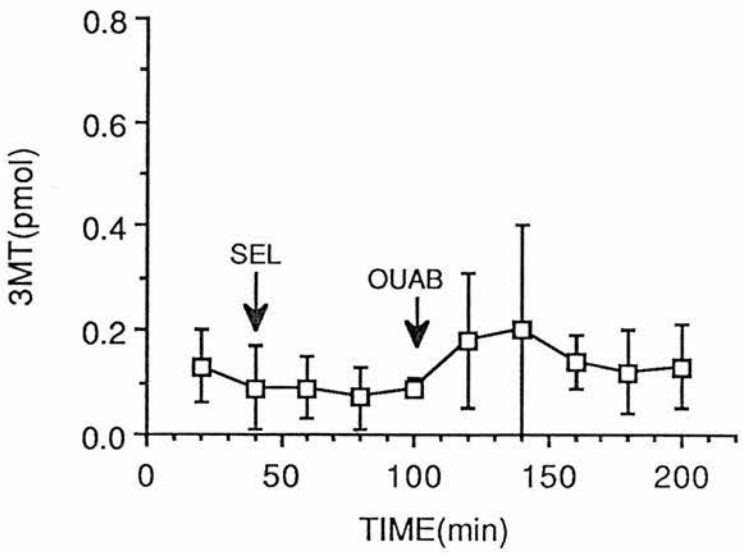
3.6.7 Tetrodotoxin

In TTX (2μM) treated animals, ouabain maximally increased DA efflux to 3333% of basal efflux over 40 min (Fig. 3.6.7, 0.06 ± 0.02

Fig. 3.6.5 Effects of selegeline (10mg/kg i.p.) given 60 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of four independent experiments.


4.8
4.0
3.0
2.0
1.0
0.0
DA(pmol)
SEL
OUAB
0
50
100
150
200
TIME(min)


36
30
20
10
0
METABOLITE(pmol)
DOPAC
HVA
5-HIAA
SEL
OUAB
0
50
100
150
200
TIME(min)


0.8
0.6
0.4
0.2
0.0
3MT(pmol)
SEL
OUAB
0
50
100
150
200
TIME(min)

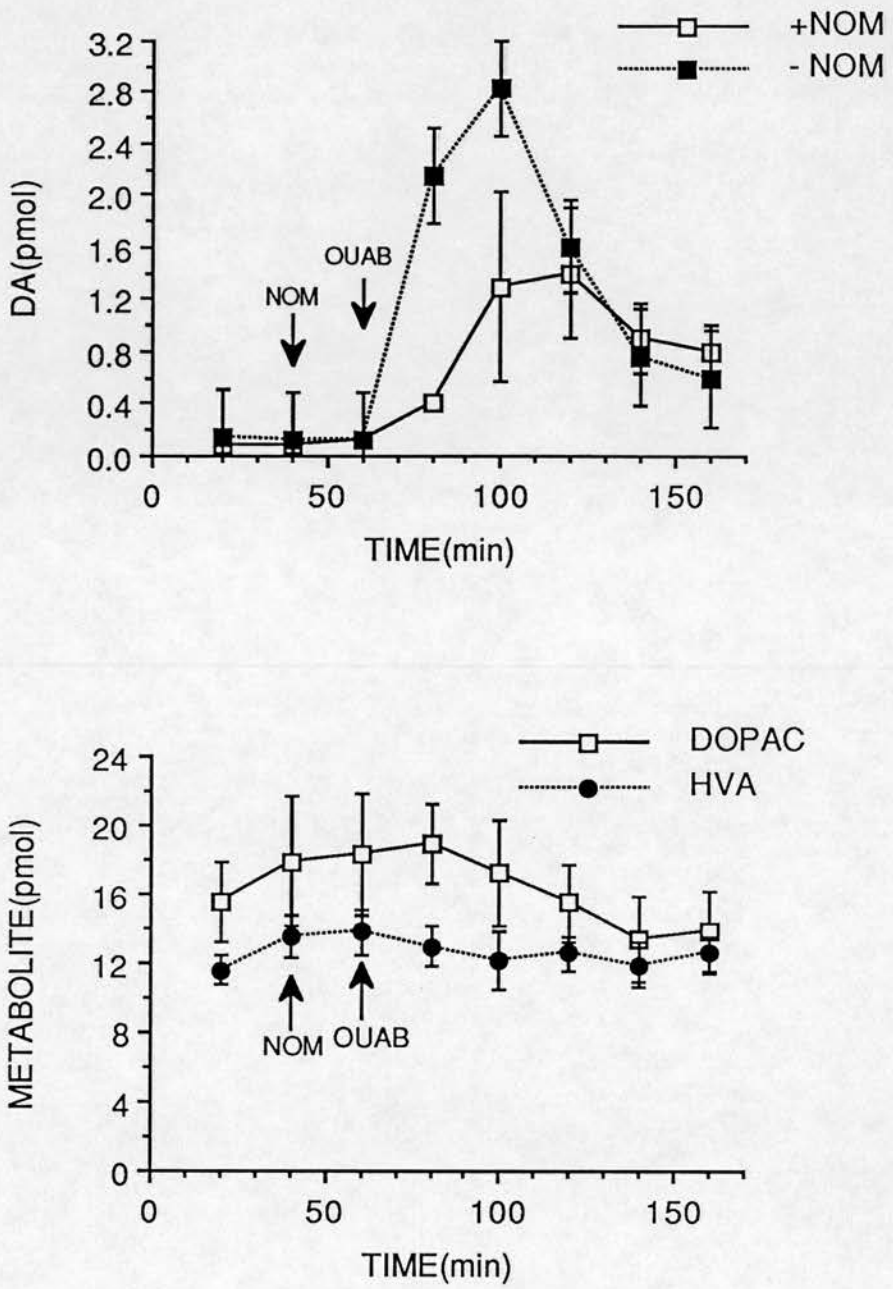
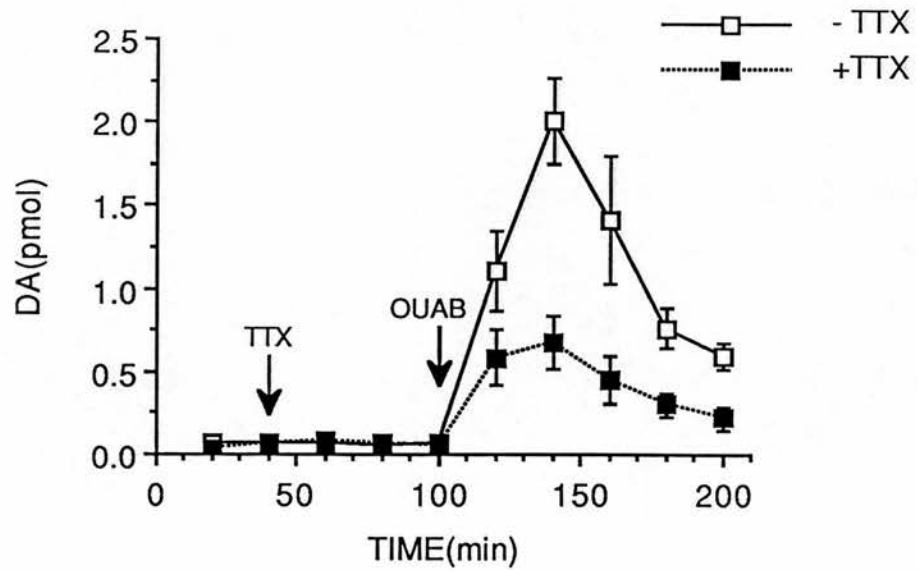
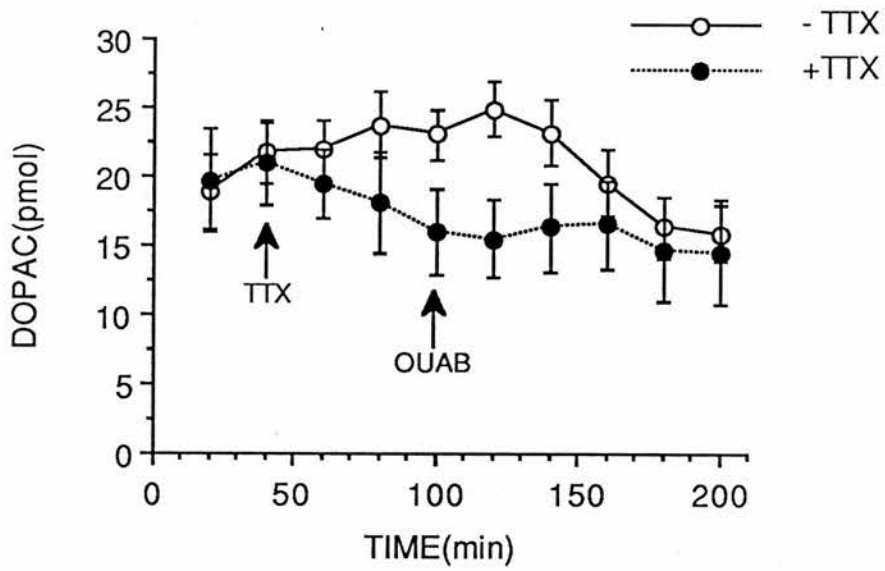
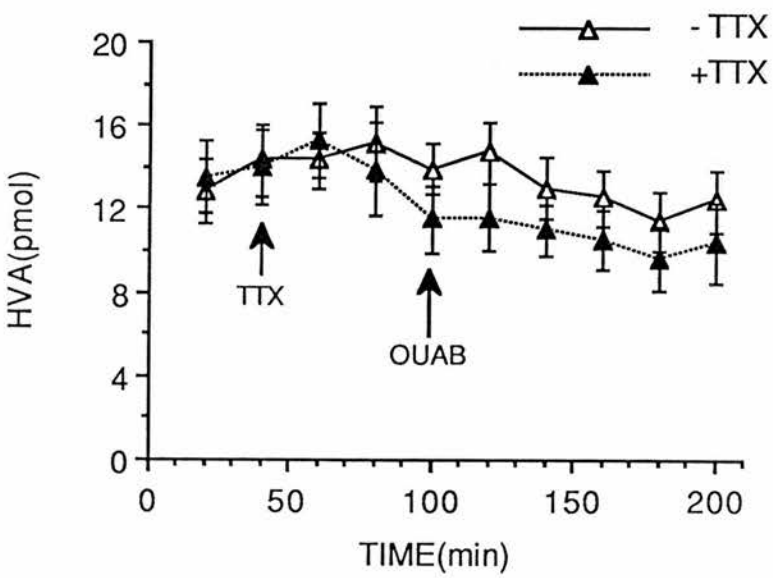


Fig. 3.6.6 Effects of nomifensine (20mg/kg i.p.) given 20 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

Fig. 3.6.7 Effects of TTX (2μM) perfused intrastriatally for 60 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC (middle panel) and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


- TTX
+TTX
DA(pmol)
2.5
2.0
1.5
1.0
0.5
0.0
TTX
OUAB
0
50
100
150
200
TIME(min)


- TTX
+TTX
DOPAC(pmol)
30
25
20
15
10
5
0
TTX
OUAB
0
50
100
150
200
TIME(min)


- TTX
+TTX
HVA(pmol)
20
16
12
8
4
0
TTX
OUAB
0
50
100
150
200
TIME(min)

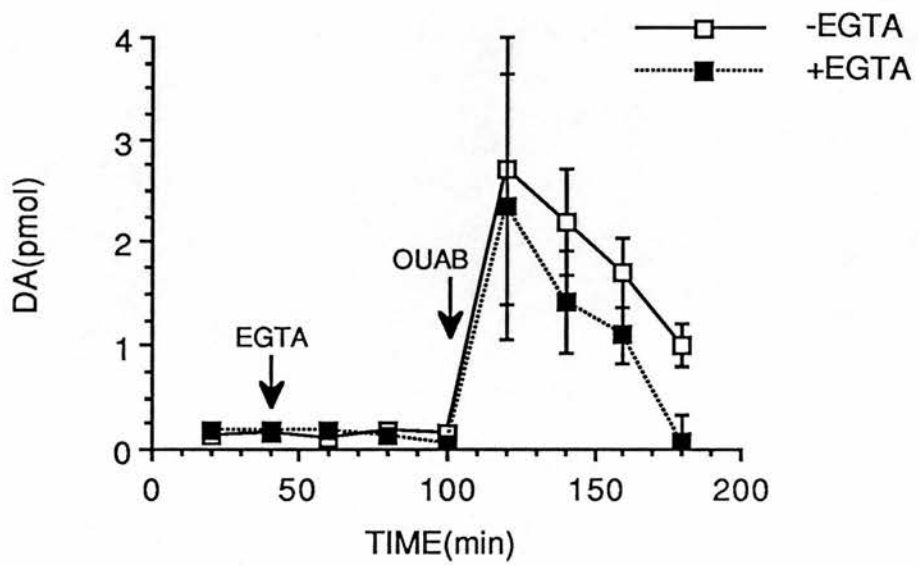
to 2.0 ± 0.26pmoles/20 min, n = 6). Total DA efflux over a 20 min period was reduced by 50% when compared with control animals (Table 3.6.1, control 1.02 ± 0.17pmoles, TTX 0.51 ± 0.18pmoles, $p < 0.05$) and by 74% over 60 min when compared with control animals (Table 3.6.1, control 4.30 ± 0.63pmoles, TTX 1.55 ± 0.42pmoles, $p < 0.05$).

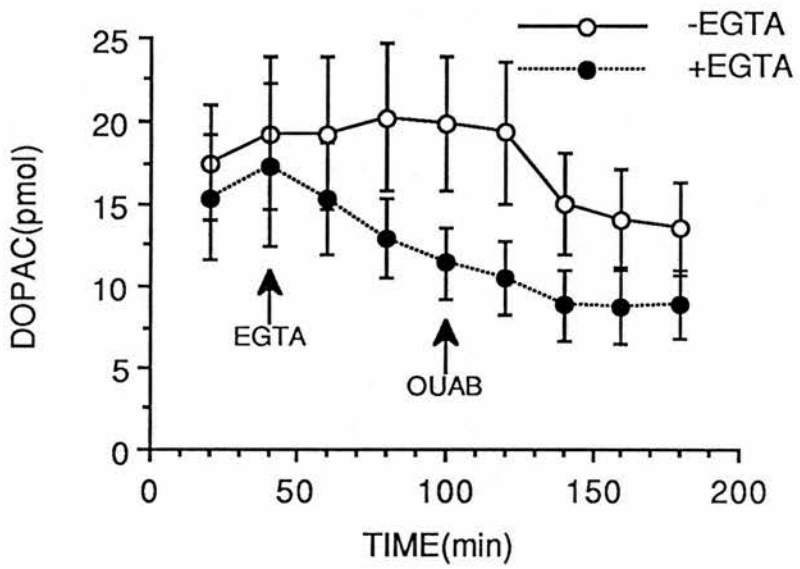
TTX caused a decline in the efflux of DOPAC and HVA which did not appear to be affected by ouabain (Fig. 3.6.7).

3.6.8 EGTA

In animals perfused with EGTA (20mM) in a Ca^{2+} free buffer, ouabain maximally increased DA efflux to 3917% of basal efflux over 20 min (Fig. 3.6.8, 0.06 ± 0.02 to 2.35 ± 1.3pmoles/20 min, n = 6). Ouabain induced DA efflux over 20 min was not affected (Table 3.6.1) whereas over 60 min DA efflux was decreased by 26% when compared to control animals (Table 3.6.1, control 6.2 ± 1.75pmoles, EGTA 4.6 ± 1.49pmoles, $p < 0.05$). EGTA caused a declining efflux of DOPAC and HVA which appeared to be prolonged by ouabain.

Fig. 3.6.8 Effects of calcium removal and EGTA (20mM) perfused intrastriatally for 60 min prior to ouabain (100μM) on the efflux of DA (upper panel), DOPAC (middle panel) and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


-EGTA
+EGTA
DA(pmol)
OUAB
EGTA
TIME(min)


-EGTA
+EGTA
DOPAC(pmol)
EGTA
OUAB
TIME(min)

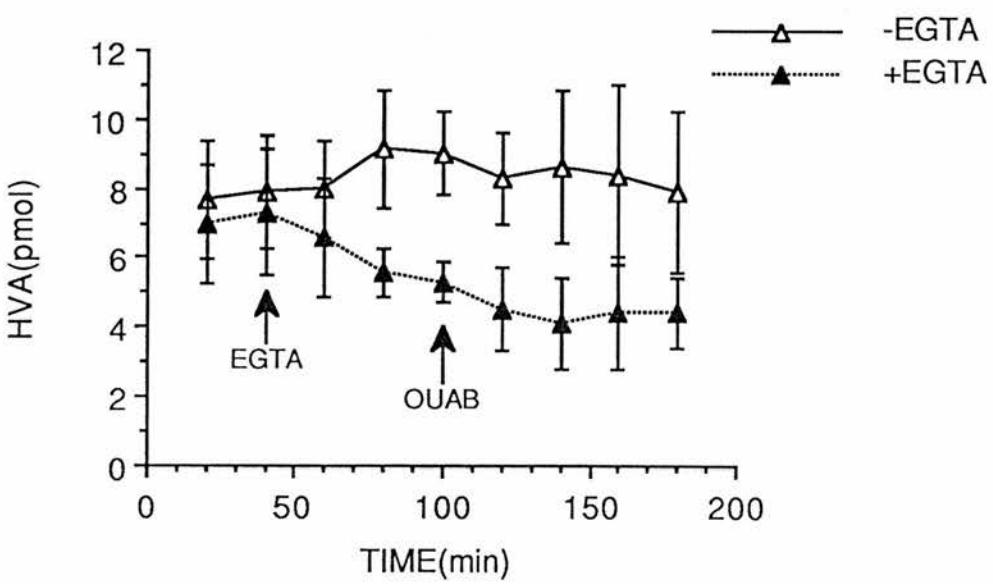

-EGTA
+EGTA
HVA(pmol)
EGTA
OUAB
TIME(min)

TABLE 3.6.1

EFFECTS OF BIOCHEMICAL AND PHARMACOLOGICAL MANIPULATIONS ON OUABAIN (100μM) INDUCED DA EFFLUX

	n	DA EFFLUX (pmole/20 min)	DA EFFLUX (pmole/60 min)
Control	14	2.0 ± 0.49	5.87 ± 0.80
AMT (20 min pretreatment)	6	1.0 ± 0.36	3.37 ± 0.86*
Control	14	2.0 ± 0.49	5.87 ± 0.80
AMT (120 min pretreatment)	6	0.19 ± 0.16**	0.58 ± 0.08***
Control	14	2.0 ± 0.49	5.87 ± 0.80
Reserpine	6	1.52 ± 0.29	4.20 ± 0.80
Control	14	2.0 ± 0.49	5.87 ± 0.80
Pargyline	6	4.5 ± 2.1	13.3 ± 4.53*
Control	14	2.0 ± 0.49	5.87 ± 0.80
Selegeline	4	2.38 ± 0.31	7.18 ± 0.61
Control	14	2.0 ± 0.49	5.87 ± 0.80
Nomifensine	6	0.28 ± 0.06***	2.60 ± 0.83**
Control	6	1.02 ± 0.17	4.30 ± 0.63
TTX	6	0.51 ± 0.18*	1.55 ± 0.42*
Control	6	2.55 ± 1.31	6.2 ± 1.75
EGTA	6	2.30 ± 0.93	4.6 ± 1.49*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Mean and standard errors are given for guidance only. Statistical differences in amount released were assessed using the non-parametric Mann-Whitney 'U' test. The data were corrected for basal release prior to comparison.

3.7 Effects of biochemical and pharmacological manipulations upon veratrine induced efflux of DA, DOPAC, HVA, 5-HIAA and 3MT

3.7.1 Dose dependence of the veratrine response

Increasing doses of veratrine were given (10,100 and 1000µg/ml) via the dialysis probe. DA efflux was unaffected by the lowest dose of 10µg/ml but exhibited a graded response to the two higher doses (Fig. 3.7.1a) reaching 2813% (100µg/ml) and 6500% (1000µg/ml). DOPAC, HVA and 5-HIAA efflux levels decreased in a graded fashion in response to the two higher doses of veratrine (Figs. 3.7.1a and 3.7.1b). A dose of 100µg/ml veratrine was used for all further experiments due to its submaximal effects upon DA efflux.

3.7.2 α-Methyl-p-tyrosine

In animals injected with AMT (250mg/kg i.p.) 20 min prior to the infusion of veratrine, DA efflux was maximally increased to 1760% of basal efflux over 20 min (Fig. 3.7.2a, 0.10 ± 0.02 to 1.76 ± 0.47pmoles/20 min, n = 6). DA efflux over 20 min was not reduced when compared to control animals (Table 3.7.1, control 2.65 ± 0.26pmoles, AMT 1.71 ± 0.30pmoles) whereas over 60 min DA efflux was reduced by 39% when compared to control animals (Table 3.7.1, control 4.48 ± 0.46pmoles, AMT 2.75 ± 0.43pmoles, $p < 0.05$). When AMT was injected 120 min prior to veratrine infusion, DA efflux was maximally increased to 1850% of basal efflux over 20 min (Fig. 3.7.2b, 0.02 ± 0.01 to 0.37 ± 0.09pmoles/20 min, n = 6). In contrast to the above result, the efflux of DA over 20 min was reduced by 86% when compared with control animals (Table 3.7.1, control 2.65 ± 0.26pmoles, AMT 0.37 ± 0.06pmoles, $p < 0.001$) whereas

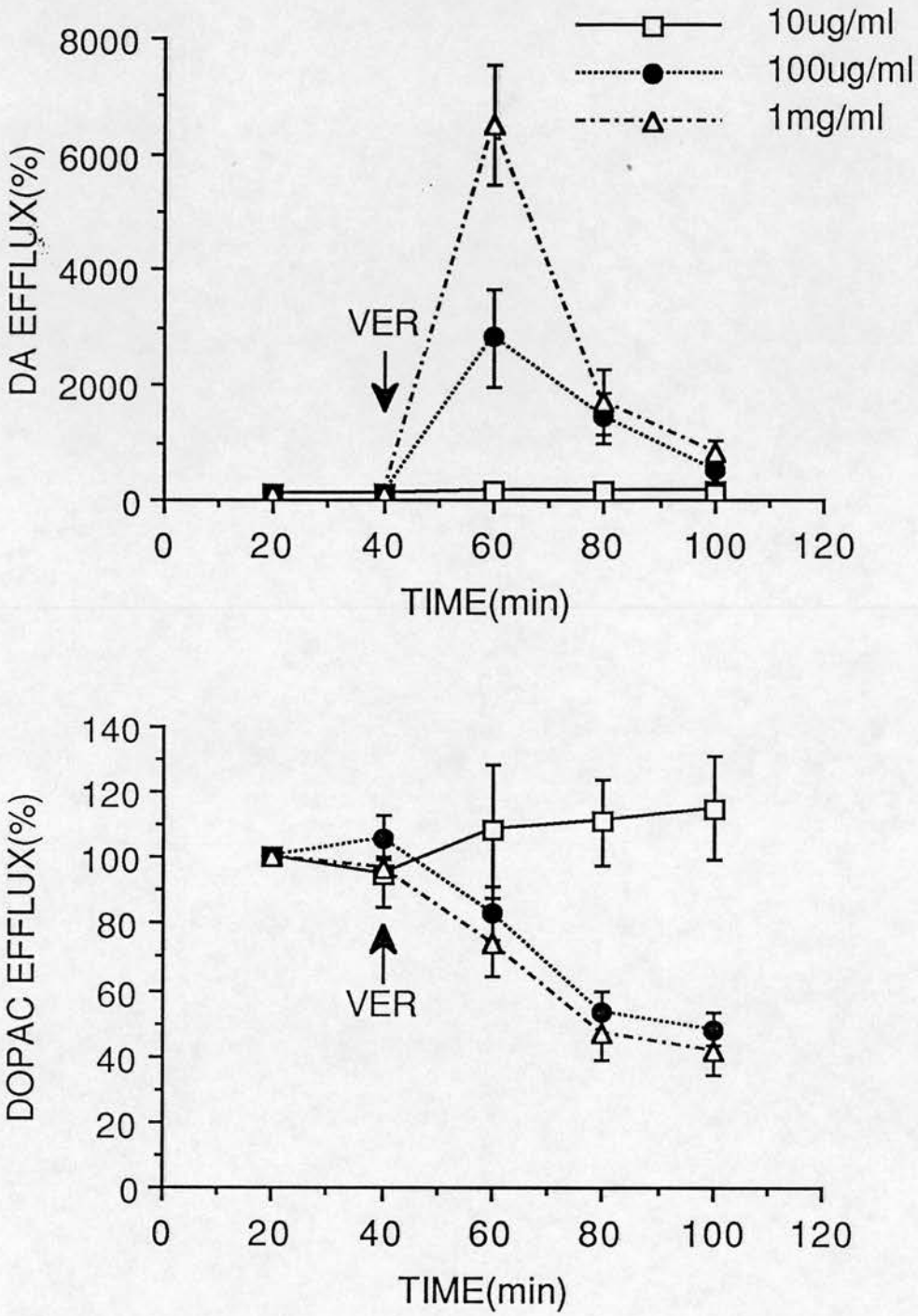


Fig. 3.7.1a Effects of increasing doses of veratrine, perfused intrastriatally for 20 min from the time indicated, on the efflux of DA (upper panel) and DOPAC (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

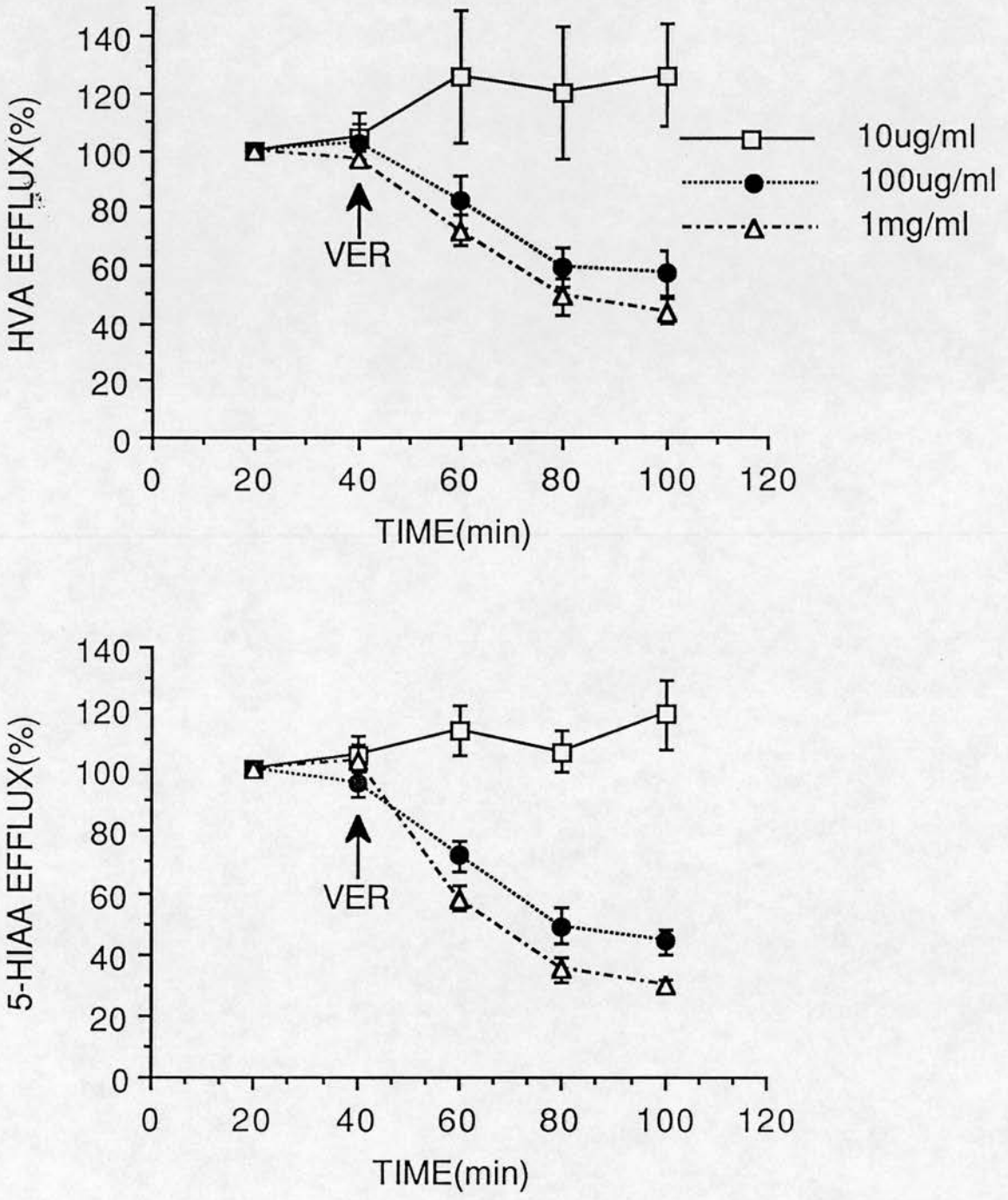


Fig. 3.7.1b Effects of increasing doses of veratrine, perfused intrastriatally for 20 min from the time indicated, on the efflux of HVA (upper panel) and 5-HIAA (lower panel). Results are expressed as a percentage ± s.e.m. of basal efflux of four independent experiments.

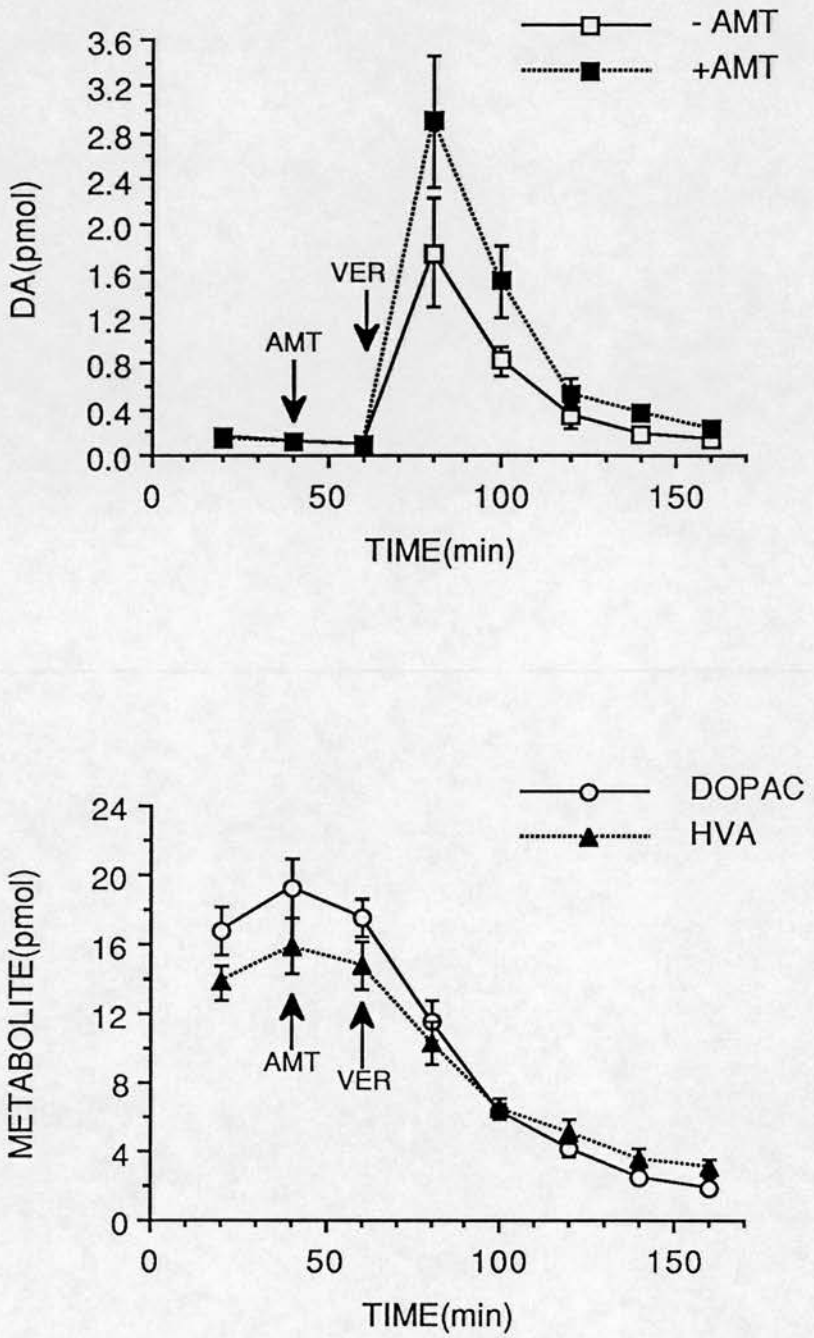


Fig. 3.7.2a Effects of AMT (250mg/kg i.p.) given 20 min prior to veratrine (100µg/ml) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

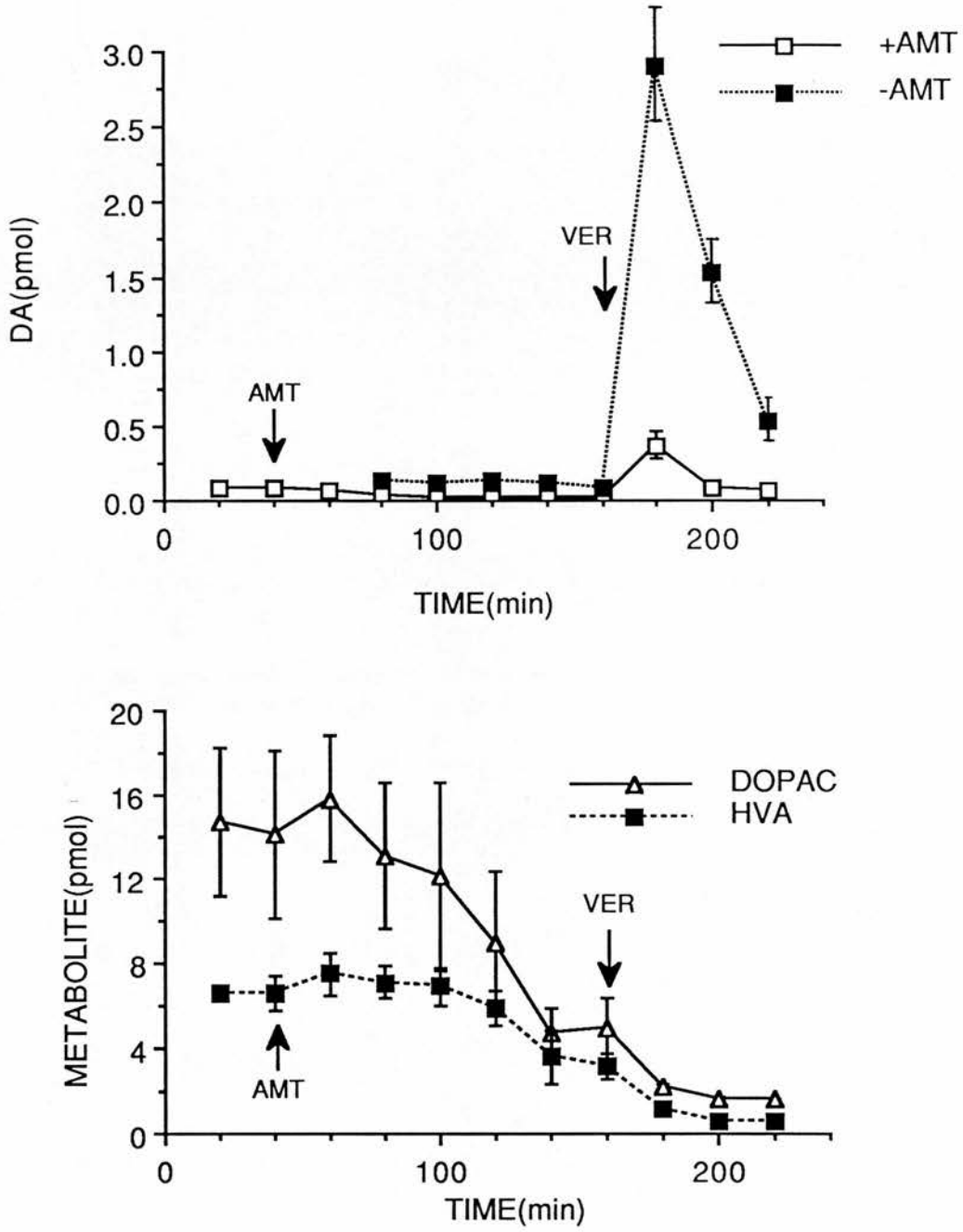


Fig. 3.7.2b Effects of AMT (250mg/kg i.p.) given 120 min prior to veratrine (100µg/ml) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

DA efflux over 60 min was reduced by 88%, a statistically significantly decrease when compared to control animals (Table 3.7.1, Control 4.48 ± 0.46pmoles, AMT 0.55 ± 0.08pmole, $p < 0.01$).

No qualitative effect of the 20 min pretreatment of AMT on veratrine induced efflux of DOPAC and HVA was apparent (Fig. 3.7.2a). When AMT was injected 120 min before veratrine infusion, veratrine appeared to accelerate the efflux of DOPAC and HVA the decrease of which was tailing off before veratrine (Fig. 3.7.2b).

3.7.3 Reserpine

In reserpine (5mg/kg i.p.) treated animals, veratrine maximally increased DA efflux to 2714% of basal efflux over 20 min (Fig. 3.7.3, 0.07 ± 0.02 to 1.9 ± 0.37pmoles/20 min, $n = 6$). DA efflux over 20 min was reduced by 31% when compared to control animals (Table 3.7.1, control 2.65 ± 0.26pmoles, reserpine 1.83 ± 0.24pmoles, $p < 0.05$). However, surprisingly, DA efflux over 60 min was not reduced by reserpine (Table 3.7.1, control 4.48 ± 0.46pmoles, reserpine 3.23 ± 0.38pmoles).

No qualitative differences in veratrine induced DOPAC and HVA efflux were apparent in reserpine treated animals.

3.7.4 Pargyline

In pargyline (75mg/kg i.p.) treated animals, veratrine maximally increased DA efflux to 2033% of basal efflux over 20 min (Fig. 3.7.4, 0.30 ± 0.05 to 6.1 ± 1.2pmoles/20 min, $n = 6$). DA efflux over 20 min was increased by 234% when compared to control animals (Table 3.7.1, control 2.65 ± 0.26pmoles, pargyline 6.2 ± 1.3pmoles, $p < 0.01$) and by 248% over a 60 min period (Table 3.7.1, control 4.48 ± 0.46pmoles, pargyline 11.10 ± 2.4pmoles, $p < 0.01$).

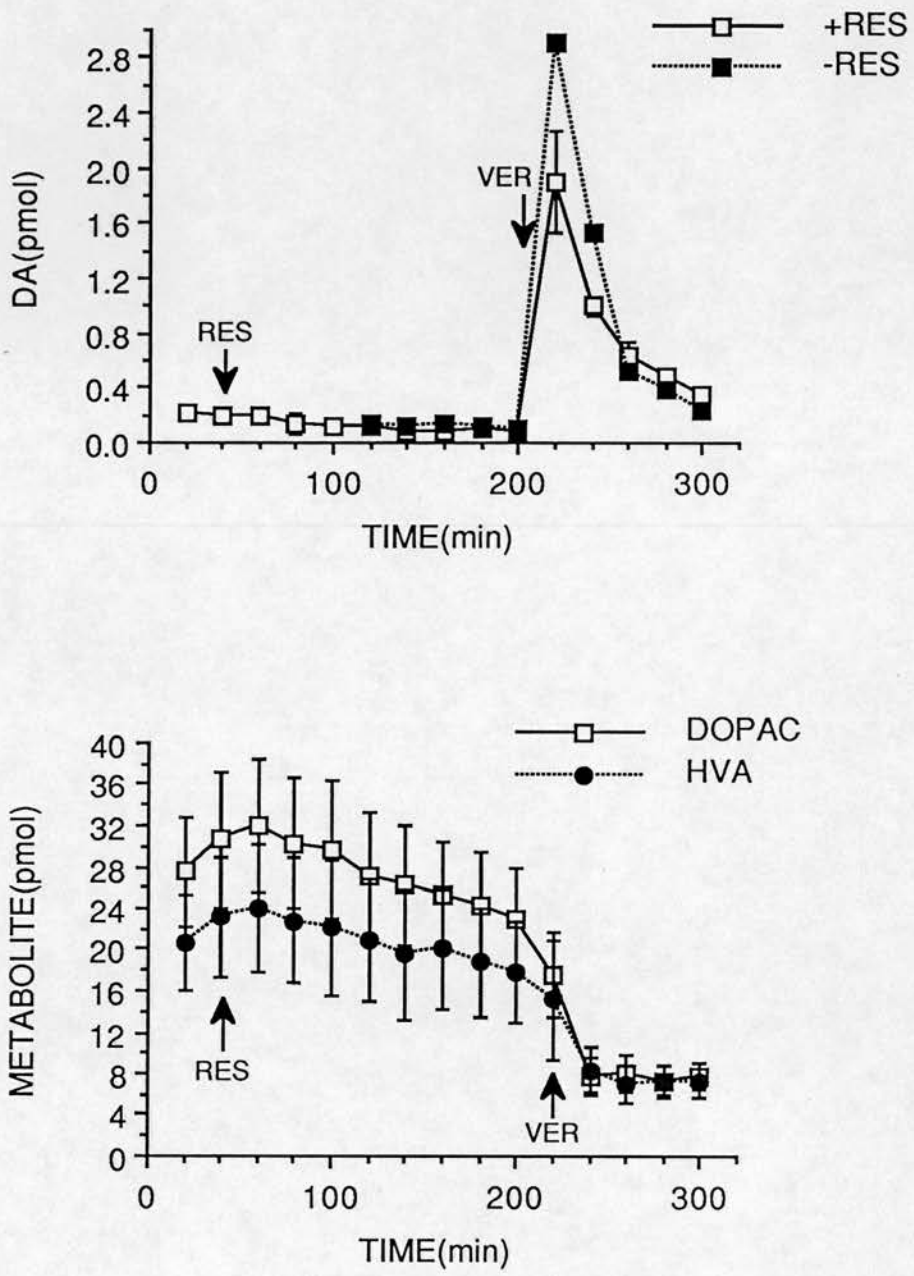
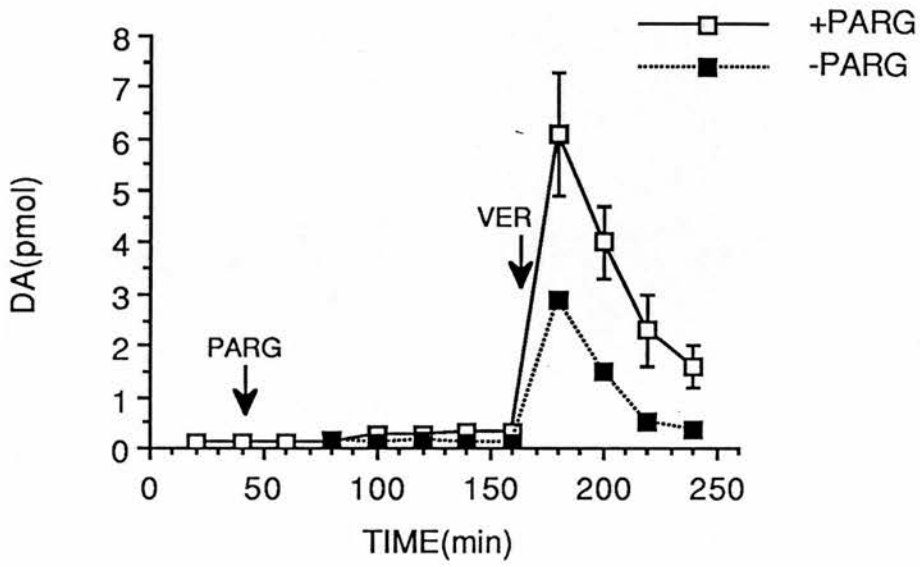
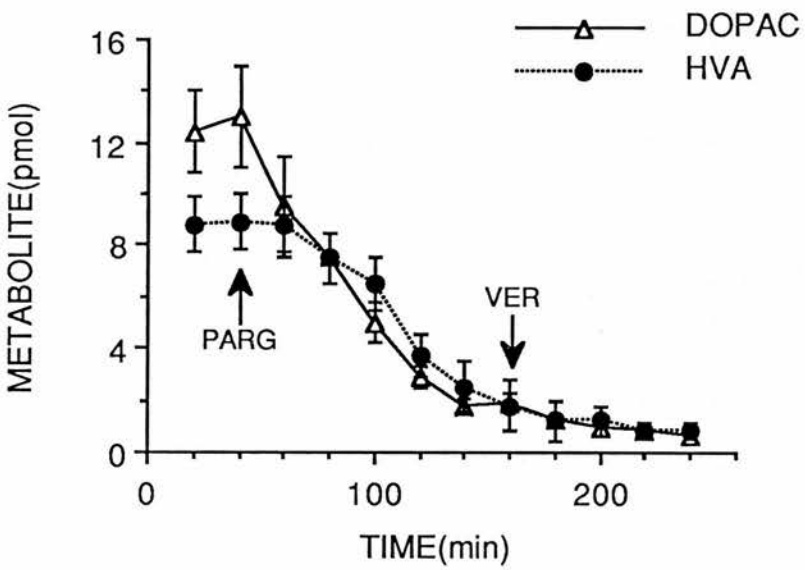
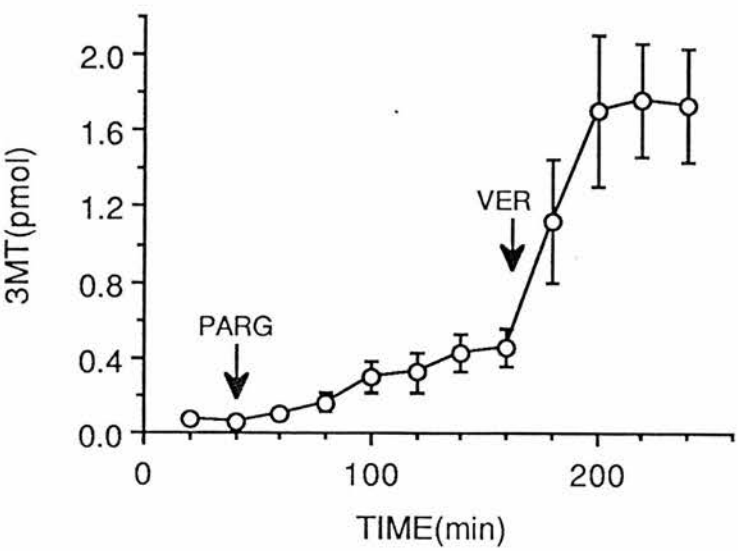


Fig. 3.7.3 Effects of reserpine (5mg/kg i.p.) given 180 min prior to veratrine (100µg/ml) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

Fig. 3.7.4 Effects of pargyline (75mg/kg i.p.) given 120 min prior to veratrine (100µg/ml) on the efflux of DA (upper panel), DOPAC and HVA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.


+PARG
-PARG
DA(pmol)
VER
PARG
TIME(min)


DOPAC
HVA
METABOLITE(pmol)
PARG
VER
TIME(min)


3MT(pmol)
VER
PARG
TIME(min)

Pargyline induced a rapid decline in DOPAC and HVA efflux which appeared to be unaffected by the subsequent infusion of veratrine (Fig. 3.7.4).

Pargyline induced a rise in 3-MT efflux to 750% of basal efflux over 120 min (Fig. 3.7.4). The infusion of veratrine accelerated this rise to 384% of pre-veratrine efflux and 2883% of pre-pargyline efflux.

3.7.5 Selegeline

In selegeline (10mg/kg i.p.) treated animals, veratrine maximally increased DA efflux to 2357% of basal efflux over 20 min (Fig. 3.7.5, 0.14 ± 0.02 to 3.3 ± 0.6pmoles/20 min, n = 4). DA efflux over 20 min and 60 min was not different to that for control animals (Table 3.7.1).

In qualitative terms, veratrine appeared to prolong the decrease in DOPAC and HVA efflux in selegeline treated animals but had no effect on 5-HIAA efflux (Fig. 3.7.5).

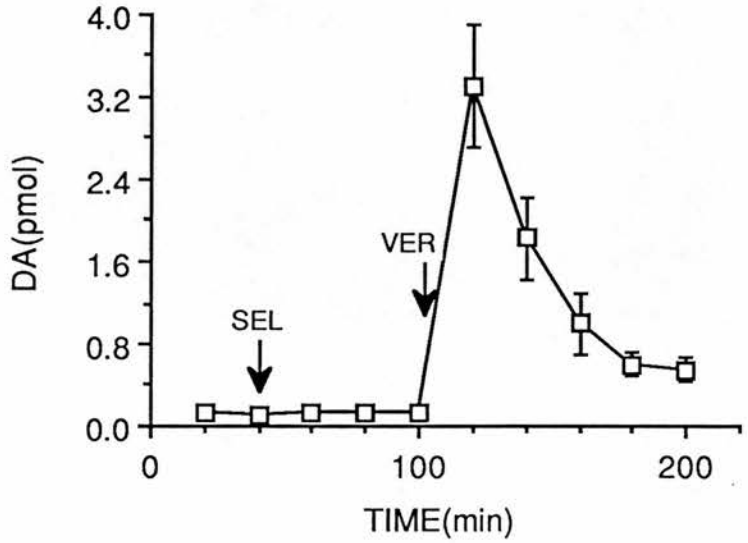
A rise in 3-MT efflux was detected after veratrine infusion (Fig. 3.7.5), however, the effect was not statistically tested due to the lack of adequate control values.

3.7.6 Nomifensine

In nomifensine (20mg/kg i.p.) treated animals, veratrine maximally increased DA efflux to 1476% of basal efflux over 20 min (Fig. 3.7.6, 0.21 ± 0.08 to 3.1 ± 1.1pmoles/20 min, n = 6). DA efflux over 20 min and 60 min was not affected by nomifensine (Table 3.7.1).

No qualitative differences in veratrine induced DOPAC and HVA efflux were apparent in nomifensine treated animals (Fig. 3.7.6).

Fig. 3.7.5 Effects of selegeline (10mg/kg i.p.) given 60 min prior to veratrine (100µg/ml) on the efflux of DA (upper panel), DOPAC, HVA and 5-HIAA (middle panel) and 3-MT (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of four independent experiments.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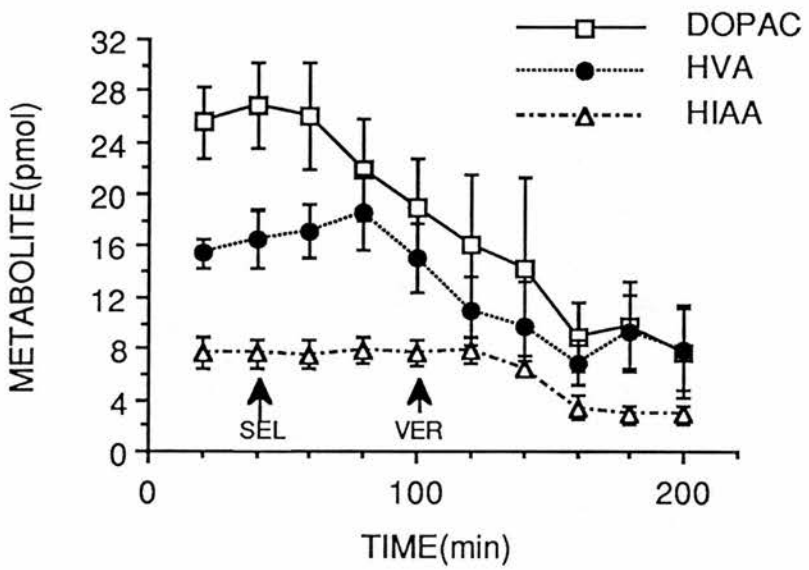
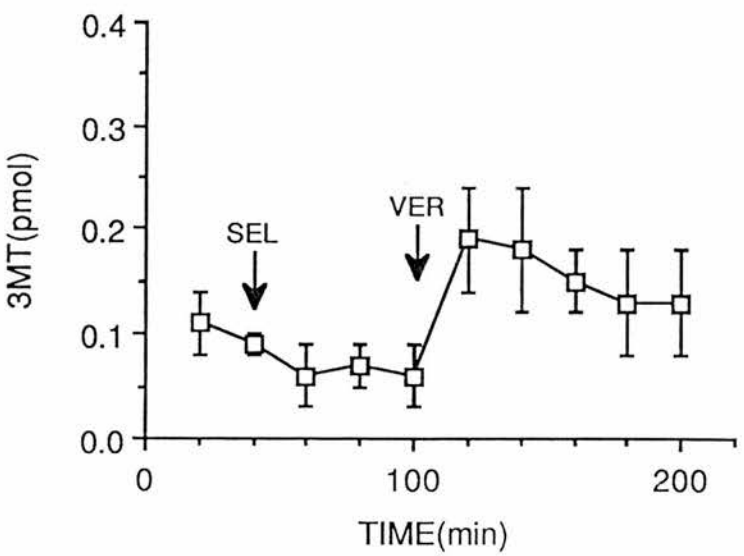
SEL
VER
0
100
200
TIME(min)


32
28
24
20
16
12
8
4
0
METABOLITE(pmol)
DOPAC
HVA
HIAA
SEL
VER
0
100
200
TIME(min)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0.3
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0.1
0.0
3MT(pmol)
SEL
VER
0
100
200
TIME(min)

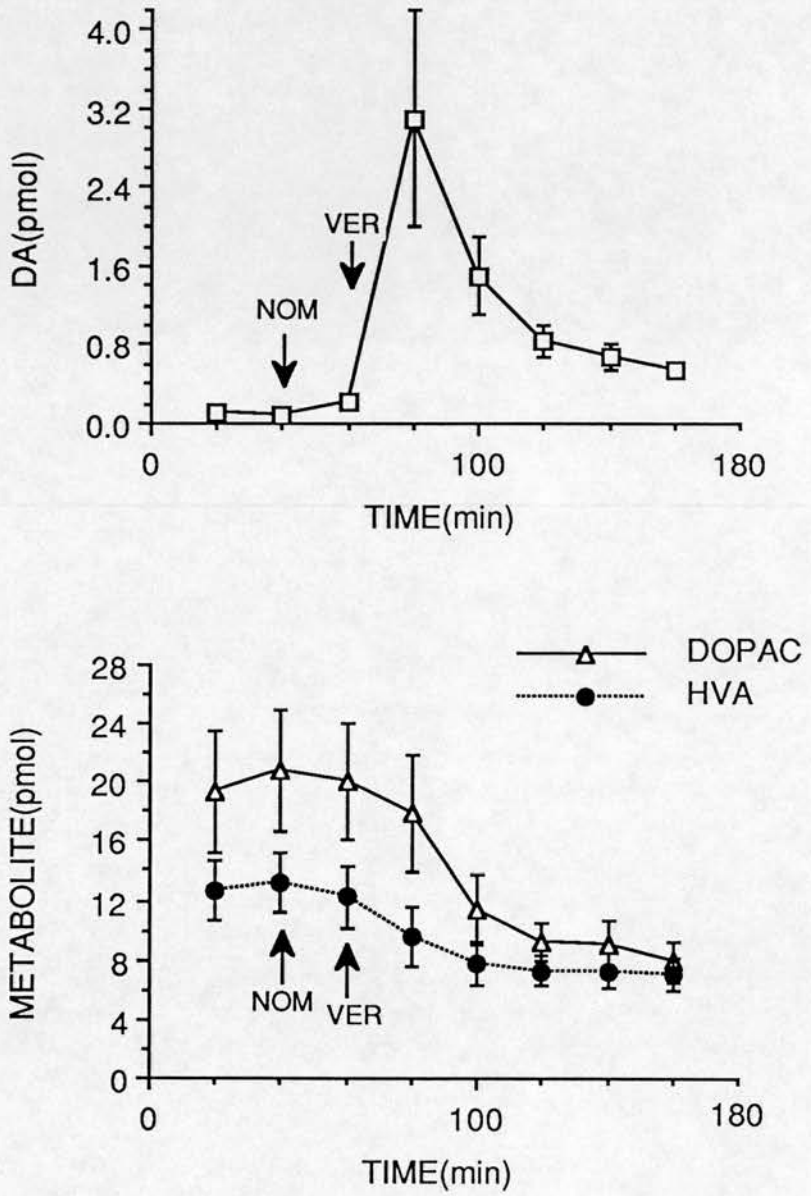


Fig. 3.7.6 Effects of nomifensine (20mg/kg i.p.) given 20 min prior to veratrine (100µg/ml) on the efflux of DA (upper panel), DOPAC and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six independent experiments.

3.7.7 Tetrodotoxin

In TTX (2μM) treated animals, veratrine maximally increased DA efflux to 250% of basal efflux over 20 min (Fig. 3.7.7, 0.08 ± 0.01 to 0.20 ± 0.04pmoles/20 min, n = 4). DA efflux over 20 min was dramatically reduced by 92% when compared with control animals (Table 3.7.1, control 2.08 ± 0.37pmoles, TTX 0.16 ± 0.06pmoles, $p < 0.01$) and similarly over 60 min, DA efflux was reduced by 90% when compared with control animals (Table 3.7.1, control 3.6 ± 0.51pmoles, TTX 0.35 ± 0.06pmoles, $p < 0.01$).

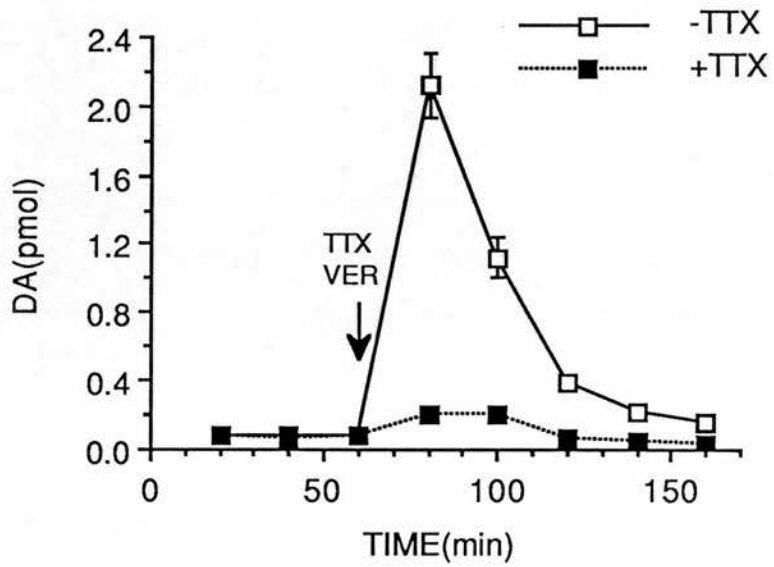
No qualitatively different effects of veratrine upon DOPAC and HVA efflux were apparent.

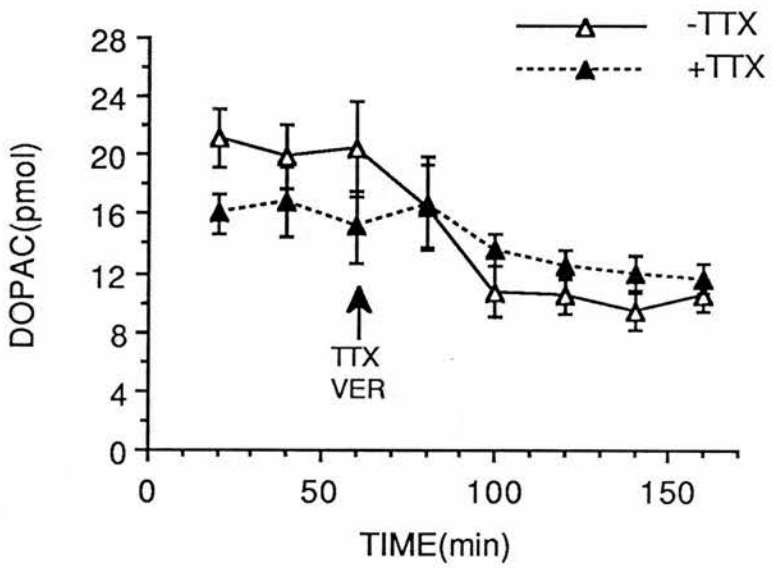
3.7.8 EGTA

In animals perfused with EGTA (20mM) in a Ca^{2+} free buffer, veratrine maximally increased DA efflux to 2270% of basal efflux over 20 min (Fig. 3.7.8, 0.10 ± 0.02 to 2.27 ± 1.28pmoles/20 min, n = 6). DA efflux over a 20 min period was decreased by 55% when compared with control animals (Table 3.7.1, control 2.17 ± 1.2pmoles, EGTA 0.98 ± 0.43pmoles, $p < 0.05$) and by 49% over a 60 min period (Table 3.7.1, control 3.9 ± 1.3pmoles, EGTA 2.0 ± 0.61pmoles, $p < 0.05$).

In qualitative terms, EGTA appeared to inhibit the veratrine induced decrease in DOPAC and HVA efflux (Fig. 3.7.8).

Fig. 3.7.7 Effects of TTX (2μM) perfused intrastriatally simultaneously with veratrine (100μg/ml) on the efflux of DA (upper panel), DOPAC (middle panel) and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of four paired experiments.


-TTX
+TTX
DA(pmol)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
TTX
VER
0
50
100
150
TIME(min)


-TTX
+TTX
DOPAC(pmol)
28
24
20
16
12
8
4
0
TTX
VER
0
50
100
150
TIME(min)

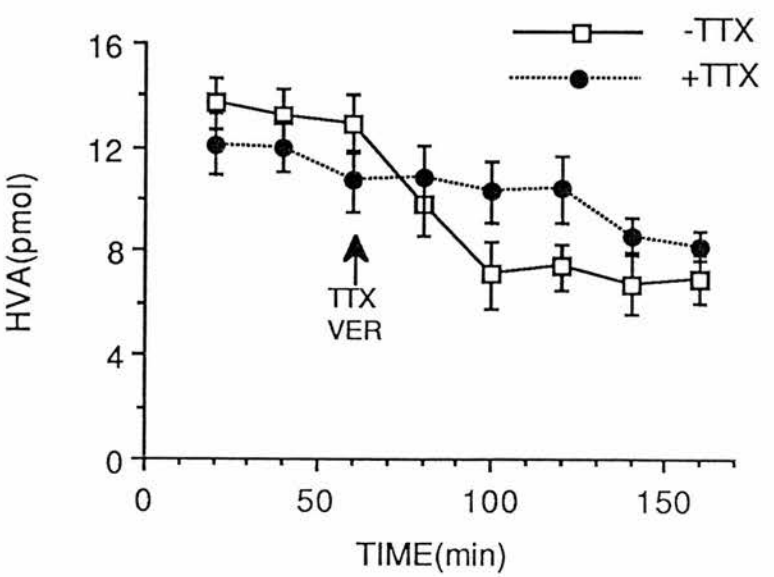
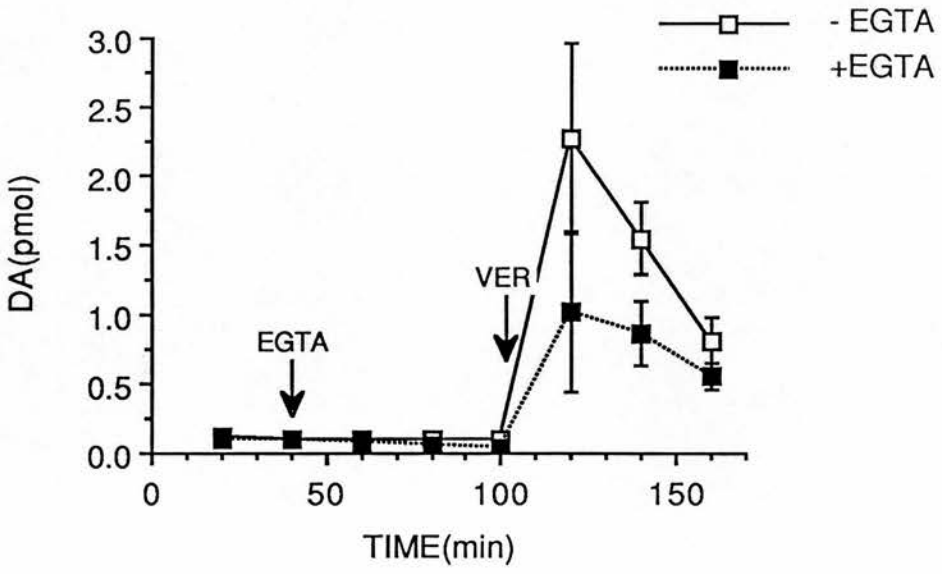
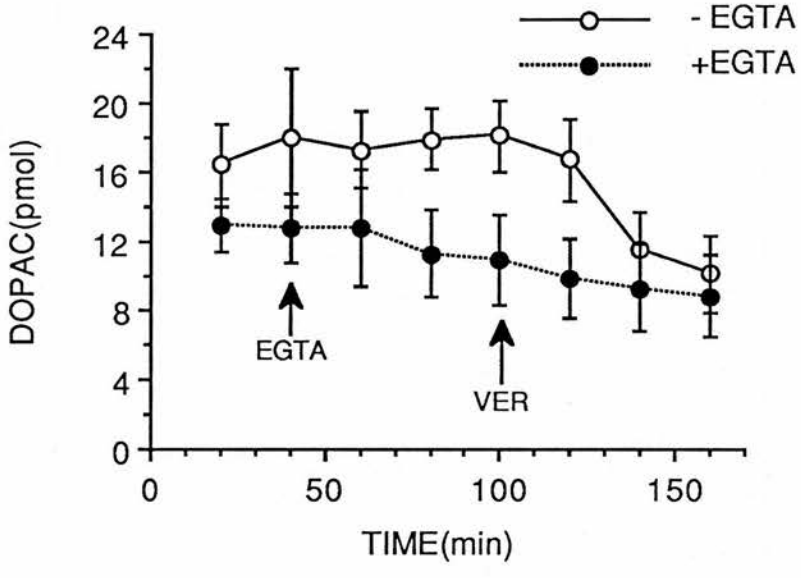

-TTX
+TTX
HVA(pmol)
16
12
8
4
0
TTX
VER
0
50
100
150
TIME(min)

Fig. 3.7.8 Effects of calcium removal and EGTA (20mM) perfused intrastriatally for 60 min prior to veratrine (100µg/ml) on the efflux of DA (upper panel) DOPAC (middle panel) and HVA (lower panel). Results (pmoles/20 min) are the mean ± s.e.m. of six paired experiments.


- EGTA
+EGTA
DA(pmol)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EGTA
VER
0
50
100
150
TIME(min)


- EGTA
+EGTA
DOPAC(pmol)
24
20
16
12
8
4
0
EGTA
VER
0
50
100
150
TIME(min)

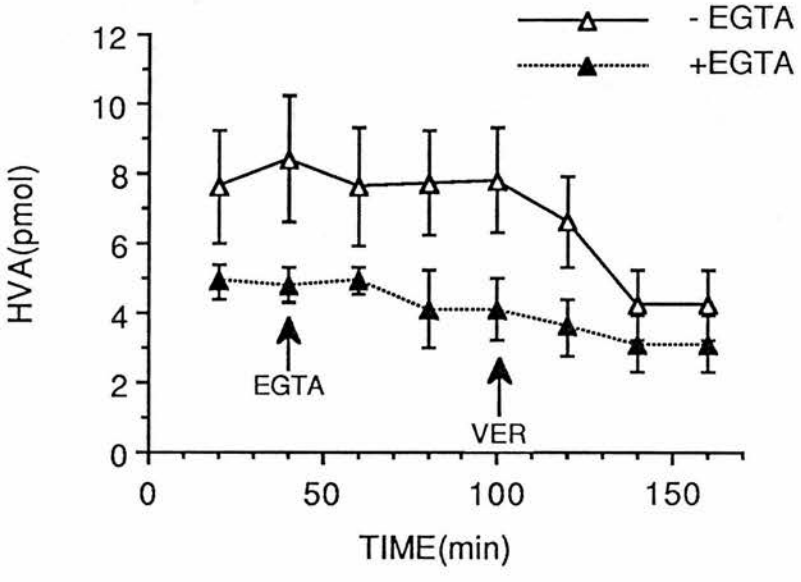

- EGTA
+EGTA
HVA(pmol)
12
10
8
6
4
2
0
EGTA
VER
0
50
100
150
TIME(min)

TABLE 3.7.1

EFFECTS OF BIOCHEMICAL AND PHARMACOLOGICAL MANIPULATIONS ON VERATRINE (100μg/ml) INDUCED DA EFFLUX

	n	DA EFFLUX (pmole/20 min)	DA EFFLUX (pmole/60 min)
Control	14	2.65 ± 0.26	4.48 ± 0.46
AMT (20 min pretreatment)	6	1.71 ± 0.30	2.75 ± 0.43*
Control	14	2.65 ± 0.26	4.48 ± 0.46
AMT (120 min pretreatment)	6	0.37 ± 0.06***	0.55 ± 0.08**
Control	14	2.65 ± 0.26	4.48 ± 0.46
Reserpine	6	1.83 ± 0.24*	3.23 ± 0.38
Control	14	2.65 ± 0.26	4.48 ± 0.46
Pargyline	6	6.2 ± 1.3**	11.10 ± 2.40**
Control	14	2.65 ± 0.26	4.48 ± 0.46
Selegeline	4	2.89 ± 0.72	4.61 ± 1.1
Control	14	2.65 ± 0.26	4.48 ± 0.46
Nomifensine	6	2.82 ± 1.1	4.51 ± 1.51
Control	4	2.08 ± 0.37	3.60 ± 0.51
TTX	4	0.16 ± 0.06**	0.35 ± 0.06**
Control	6	2.17 ± 1.2	3.9 ± 1.30
EGTA	6	0.98 ± 0.43*	2.0 ± 0.61*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Mean and standard errors are given for guidance only. Statistical differences in amount released were assessed using the non-parametric Mann-Whitney 'U' test. The data were corrected for basal release prior to comparison.

CHAPTER 4

DISCUSSION

4.1 In vivo microdialysis

In vivo brain microdialysis is a technique which has progressed considerably since its inception in the early 1970s. The present work demonstrates that this technique is suitable for the simultaneous in vivo measurement of catecholamines and their metabolites in brain tissue. Moreover, pharmacologically and biochemically induced changes in DA efflux and metabolism have been studied. The technique has allowed correlations to be made between DA efflux and the efflux of DA metabolites thereby increasing the amount of information conveyed by the results.

The limitations of in vivo microdialysis must be borne in mind when interpreting the results. Synaptic release of DA occurs on a sub-second timescale into a space of only several nanometres in width. The dialysis probes used in this work measured approximately 300μM in diameter. It is immediately apparent that any alteration in the level of synaptic DA release will only be apparent if it is measured collectively. No existing technique is able to measure the release of a transmitter at a single synapse. Consequently, the stimulation or inhibition of DA release measured in these experiments is the collective release from many nerve terminals. Moreover, the DA that is collected into the perfusate reflects the DA that escapes reuptake back into the nerve terminal. For this reason, the release of DA into the extracellular space has been referred to as DA efflux since it represents the net release of DA and not the absolute release.

It may be argued that the DA efflux measured in these experiments is derived from non-synaptic sources on the basis that the majority of DA released into a synapse is probably captured on

the DA uptake carrier. Therefore, little if any DA will reach the extrasynaptic space. This may be so during normal nerve cell activity when the quantitites of DA released are much less than those stimulated biochemically or pharmacologically. However, lesions of the nigro-striatal pathway have been reported to reduce basal DA efflux by > 95% (Zetterstrom et al., 1986). This suggests that the basal extracellular level of DA is derived from synaptic sources released by normal physiological nerve activity. Under artificial stimulating conditions, it seems likely that the synaptic DA uptake carrier will rapidly be saturated especially if it is remembered that some of the stimulating conditions used here apparently depleted the vesicular or cytosolic pools of DA quite rapidly. This situation probably does not normally occur during normal physiological activity. The results illustrate the fact that a majority of the DA efflux measured is synaptically derived, since any interference with the DA uptake carrier affected the efflux of DA. Thus nomifensine, by inhibiting the action of the DA uptake carrier, increased the extracellular level of DA (Fig. 3.2.6). This discounts the argument that the DA efflux measured in these experiments was non-synaptic. Raiteri et al. (1979) demonstrated that the action of nomifensine is solely that of uptake inhibition when nomifensine failed to release [^{3}H] DA in their superfused striatal slice preparation. Thus, nomifensine does not increase the extracellular level of DA by stimulating release.

The previously mentioned limitations of <u>in vivo</u> microdialysis relating to spatial and temporal resolution, prevent its use for following electrically induced changes in DA release that occur over a millisecond timescale. This is not surprising if it is

remembered that stimulation of the medial forebrain bundle (MFB) for example, releases DA from dopaminergic nerve endings in the striatum in a fashion analogous to the physiological norm. Thus, the released DA will rapidly be taken back up into the nerve terminal and the nett DA efflux into the extracellular space over a sampling period of minutes will be minimal. This is illustrated by Kuhr & Wightman (1986) and Wightman et al. (1988) who used fast scan cyclic voltammetry to measure DA release in the striatum following stimulation of the MFB at various stimulation parameters. Their results demonstrated raised levels of DA in the extracellular fluid for periods up to 3 or 4 seconds. Such short lasting increases will not be detected by microdialysis which samples over a period of several minutes, unless electrically stimulated release can be maintained for the duration of the sampling time, which is not likely.

In vivo microdialysis has been used to follow behaviourally induced changes in extracellular DA. Thus, Church et al. (1987) used a sampling time of 5 min to measure feeding induced changes in DA levels in rats. The sensitivity of their technique was enhanced by the use of an automated smallbore chromatography system. This can handle small sample volumes without a loss in sensitivity and so allows for short sampling times, enhancing the temporal resolution of the technique.

4.2 The DA releasing effect of amphetamine

The results demonstrate that AMPH increases the efflux of endogenous DA into the extracellular space of the striatum (Fig. 3.2.2). The effect on DA efflux is maximal 40 min after the

injection of AMPH. DA efflux subsequently declines over the following 60 min. As well as increasing DA efflux, AMPH also increased the tissue content of DA to 120% of control (Fig. 3.1.1). This result may at first seem to be paradoxical since it seems logical that a releasing agent should deplete the tissue stores of DA. AMPH has been reported to decrease impulse flow in neurones (Roth et al., 1976). If impulse flow is reduced using γ-butyrolactone (GBL) or by electrolytic lesion of the dopaminergic pathway, tissue levels of DOPAC decrease (Roth et al., 1976). Amphetamine was found to produce a much greater decrease in DOPAC than either GBL or lesioning. Roth interpreted this as indicating that the DOPAC lowering effect of AMPH was not due solely to its impulse blocking properties but could also be attributed to its uptake blocking properties. In the present experiments, AMPH caused a 60% reduction in tissue DOPAC levels (Fig. 3.1.1) and an 80% reduction in DOPAC efflux levels (Fig. 3.2.2). AMPH is known to inhibit the neuronal uptake of DA (Green, 1971; Mantle et al., 1976; Green & El Hait, 1978). It is thus possible that AMPH could indirectly increase DA efflux by blocking the re-uptake of transmitter that is spontaneously released from the neurone. However, in reserpinised striatal slices, the DA uptake inhibitor nomifensine produced only a slight acceleration of DA efflux (Parker & Cubeddu, 1986). Moreover, under the conditions of blockade of DA reuptake by nomifensine in the present experiments, there was only a slight increase in DA efflux. Additionally, unlike AMPH nomifensine did not cause tissue levels of DOPAC (Fig. 3.1.1) or DOPAC efflux (Fig. 3.2.12) to decrease. This is in agreement with Westerink et al. (1987) who reported that the DA re-uptake inhibitor

GBR 12909 had no effect on the efflux level of DOPAC when measured by intracerebral dialysis. Consequently, it is unlikely that the sole action of AMPH is to inhibit the reuptake of DA. The question still remains as to why AMPH should increase tissue levels of DA if its action is to release DA. The possibility exists that AMPH is inhibiting MAO so reducing the intraneuronal metabolism of DA to DOPAC. This action of AMPH has been reported by several groups (Green, 1971; Mantle et al., 1976; Green & Elhait, 1978) and would explain both the AMPH induced decrease in DOPAC concentration in tissue and perfusates as well as the increase in DA levels in tissue and perfusates. However, there are several results that refute this possibility. Firstly, the MAO inhibitor pargyline was much more effective than AMPH at increasing tissue DA and decreasing tissue DOPAC levels (Fig. 3.1.1). Secondly, pargyline induced only a slow rise in DA efflux levels to a maximal 280% of control over 120 min (Fig. 3.2.8) in contrast to the more rapid and greater effect of AMPH. Thirdly, pargyline induced an increase in 3-MT efflux to 813% of control over 120 min (Fig. 3.2.8), an effect that was not seen after the dose of AMPH used in these experiments. Finally, and most convincingly, in pargyline pretreated animals i.e., in animals where MAO activity was already inhibited, AMPH further increased the efflux of DA by 239% above control animals (Table 3.3.1). A similar effect was reported in vitro by Miller & Shore (1982) who observed an elevation of [^{3}H]-DA accumulation on top of that caused by pargyline. So whilst the inhibition of MAO by AMPH may partially explain the reduction in DOPAC efflux, such an action of AMPH does not completely account for the results. The present results suggest that AMPH has little, if any, MAO inhibitory

activity at doses below 8mg/kg. At 8mg/kg or higher, 3-MT efflux is increased which does suggest an inhibition of MAO (Fig. 3.3.1b). Similar results were reported for AMPH stimulated DA efflux from striatal slices (Parker & Cubeddu, 1986). Moreover, Parker & Cubeddu perfused the slices with both nomifensine and pargyline together. However, this combination still failed to elicit the same magnitude of DA efflux as that elicited by AMPH.

Thus, in the light of these results that do not support the hypothesis that the releasing action of AMPH is a result of an inhibition of DA reuptake and of MAO activity, AMPH must, therefore, have a direct DA releasing action as well as stimulating the accumulation of DA in tissue. The possibility exists that AMPH, as well as causing the release of DA into the synaptic cleft (Azzaro & Rutledge, 1973; Arnold et al., 1977; Kamal et al., 1981), makes available more DA for sequestration into storage sites. This could be achieved if AMPH were to stimulate DA synthesis. Such an effect of AMPH was demonstrated in vitro by Uretsky & Snodgrass (1977). A concentration of 1μM AMPH in the perfusing medium maximally stimulated the synthesis of [^{3}H]-DA from [^{3}H]-tyrosine. Such an effect could explain the accumulation of DA in tissue exposed to AMPH.

Reserpine, which depletes vesicular stores of DA (Carlsson, 1958), reduced striatal tissue content of DA by 73% (Fig. 3.1.1). Other workers have reported more than a 95% reduction in tissue levels of DA by reserpine (Umezu & Moore, 1979). Under these conditions, AMPH induced efflux of DA was not affected (Fig. 3.3.3) which indicates that the pool of DA that is sensitive to the releasing action of AMPH is not the vesicular storage pool. This

is in harmony with *in vitro* results obtained by other workers (e.g. Scheel-Kruger, 1971; Chiueh & Moore, 1975; see McMillen, 1983) and behavioural results (Scheel-Kruger, 1971). This is substantiated by the effects of the DA synthesis inhibitor AMT which led to a dramatic inhibition of the AMPH induced DA efflux (Table 3.3.1). When AMT was given 120 min before AMPH, DA efflux was reduced by 85%. However, at this timepoint, tissue levels of DA were reduced by 39% (Table 3.1.1) which suggests a concurrent depletion of both newly synthesised and vesicular stores of DA. This difficulty was overcome by administering AMT simultaneously with AMPH. AMT had no effect on tissue levels of DA after 20 min (Table 3.1.1), the time at which the effect of AMPH becomes maximal. However, the effect of AMPH on DA efflux was still reduced by 70% (Table 3.1.1). This suggests that the newly synthesised pool of DA is released by AMPH which explains the exquisite sensitivity of this release to AMT. Moreover, the location of the newly synthesised pool of DA could be in the cytosol of the neurone since this pool is not affected by reserpine which depletes vesicular pools. This is in agreement with results obtained by other groups (Moore, 1977, 1978; McMillen, 1983) who also proposed that AMPH releases newly synthesised DA. Moreover, this pool of DA has been reported to be responsible for AMPH stimulated behaviours which are blocked by AMT (Weissman et al., 1966; Rech & Stolk, 1970; Scheel-Kruger, 1971). In keeping with this suggestion, the effects of AMPH on DA efflux were facilitated in pargyline treated animals (Table 3.3.1). Pargyline inhibits the metabolism of free cytosolic DA that would normally be vulnerable to MAO. This is borne out by the pargyline induced increase in tissue levels of DA to 151% of control (Table 3.1.1).

The increase in cytosolic DA levels are reflected in an increased efflux of DA by AMPH. On the other hand, selegeline, an inhibitor of MAO B (see Houslay & Tipton, 1976) was without effect on AMPH stimulated DA efflux. MAO B has been reported to occur predominantly postsynaptically and extraneuronally in glial cells with only a small amount of activity in presynaptic terminals (Levitt et al., 1982; Francis et al., 1985). This is further evidence for the origin of the DA efflux stimulated by AMPH. Moreover, it implies that little if any DA is metabolised by MAO B in the striatum.

Nomifensine was very effective at inhibiting AMPH induced DA efflux (Fig. 3.3.6a,b, Table 3.3.1). This is in harmony with results obtained by Parker & Cubeddu (1986) in striatal slices and also with Liang & Rutledge (1982a) who used the DA uptake inhibitor benztropine in striatal slices. Both groups demonstrated that uptake inhibition virtually abolished AMPH elicited DA efflux. This was also demonstrated for a variety of other phenylethylamines as well as AMPH by Raiteri et al. (1979). Nomifensine blocked their ability to increase the efflux of [^{3}H]-DA from striatal synaptosomes. AMPH stimulated behavioural effects were also inhibited by a variety of uptake inhibitors (Ross, 1979). This suggests that the uptake carrier is essential for the action of AMPH.

Correlations appear to exist between these pharmacological effects on AMPH induced DA efflux and the effects of similar treatments on AMPH induced behavioural responses. Such direct correlations should be treated with caution but do suggest a consistency of events. Thus, reserpine pretreatment fails to affect AMPH stimulated DA efflux as well as AMPH stimulated

behaviours (Scheel-Kruger, 1971; McMillen, 1983). In contrast, AMT when administered 30 min or 120 min before AMPH causes either a partial or complete blockade of behavioural responses (Weissman et al., 1966; Scheel-Kruger, 1971; Papeschi, 1975; Widerlov & Lewander, 1978; see McMillen, 1983).

The effects of AMPH on DA metabolites also convey much information concerning the nature of DA release. What is immediately apparent is that although increasing doses of AMPH led to a graded response in DA efflux, the same was not true for DOPAC and HVA efflux (Figs. 3.3.1a, b). Such a result has been interpreted previously as evidence that the AMPH stimulated decline in DOPAC and HVA efflux is closely related to the depletion of the newly synthesised pool of DA (Zetterstrom et al., 1986). The possibility that uptake blockade of DA by AMPH is responsible for the decrease in DOPAC efflux is unlikely since nomifensine had no effect on DOPAC efflux (Fig. 3.2.12a,b) (See Westerink et al., 1987). The suggestion is that the pool supplying MAO is rapidly exhausted and at higher doses of AMPH (8 and 16mg/kg) DA is released from vesicular pools as well (Zetterstrom et al., 1986). The results presented here are in agreement with this. This is strengthened by the results obtained with nomifensine since this inhibited the AMPH stimulated decline in DOPAC and HVA efflux as well as the AMPH stimulated increase in DA efflux (Fig. 3.3.6a). Thus, protection of the newly synthesised pool by nomifensine, prevents the depletion of DA and so maintains a pool of substrate for MAO.

At this point, it seems pertinent to highlight the contrasting results obtained when nomifensine was administered via two different

routes. When administered intraperitoneally, it inhibited the effect of AMPH on DA, DOPAC and HVA (Fig. 3.3.6a). However, when nomifensine was infused through the dialysis probe, there was a lack of effect on the AMPH induced alterations in DOPAC and HVA efflux but not on DA efflux (Fig. 3.3.6b). The latter route was still effective for the inhibition of DA uptake in the immediate vicinity of the probe, as shown by the inhibition of AMPH stimulated DA efflux, but was not effective over a sufficient distance from the probe from where DOPAC was able to diffuse to the probe and enter the perfusate. The possible reason for this discrepancy between efficacy against DA and DOPAC may be that DOPAC has been reported to diffuse more readily through extracellular fluid than DA (Rice et al., 1985), thereby extending the distance over which DOPAC is collected by the dialysis probe. Intraperitoneally administered nomifensine overcomes this problem by universally affecting the whole striatum.

Nomifensine was ineffective against basal DOPAC efflux (Fig. 3.2.12) which suggests that the basal overflow of DOPAC is not derived from DA taken up into nerve terminals but rather solely from cytosolic DA within the terminals. This is in agreement with the results of Zetterstrom et al. (1988).

The effects of AMT and reserpine on DOPAC and HVA efflux substantiate their effects on DA efflux. Reserpine was without effect on DOPAC and HVA (Fig. 3.2.10) whereas AMT caused a decline in both DOPAC and HVA efflux (Fig. 3.2.11). This suggests that the extracellular pool of these compounds is formed by the metabolism of newly synthesised DA.

The increase in HVA efflux following nomifensine (Fig. 3.2.12a)

may reflect a shift towards a postsynaptic and/or glial metabolism of DA. This result is to be expected since the inhibition of DA reuptake by nomifensine is likely to increase the flux of extracellular DA into postsynaptic and glial cells.

The precise mechanism by which AMPH releases DA from the nerve terminal remains unclear. These results demonstrate that AMPH releases newly synthesised DA by a carrier dependent mechanism. The involvement of the carrier is beyond doubt since any interference with it resulted in a decreased efflux of DA. AMPH is a highly lipophilic compound and readily accumulates within the nerve terminal by diffusion (Fischer & Cho, 1979). Consequently, a functioning membrane carrier is not required for AMPH to enter the terminal, but it is required for the AMPH stimulated efflux of DA. This suggests that DA may exit the nerve terminal in a carrier dependent fashion. Such a mechanism has been proposed previously and has been called exchange diffusion (Levine et al., 1965; Paton, 1973; Fischer & Cho, 1979; Raiteri et al., 1979; Liang & Rutledge, 1982b; McMillen, 1983; Parker & Cubeddu, 1986). This process was originally described for glucose transport into erythrocytes (Levine et al., 1965) and later for NA release from nerve terminals (Paton, 1973). The data presented here are compatible with an exchange-diffusion model of AMPH-induced DA release for the reasons outlined below. For the process of exchange diffusion to be viable, the membrane uptake carrier is envisaged as a protein located within the neurolemma that is able to shuttle from one side of the neurolemma to the other. In the presence of high $[Na^+]$, the carrier has a high affinity for DA. In conditions of low $[Na^+]$, the carrier has a lower affinity for

DA. Consequently, DA present on the outside of a polarised cell membrane i.e., where [Na^+] is high, is bound by the carrier to form a DA-Na^+-carrier complex. This complex is then transported to the cytoplasmic side of the neurolemma where [Na^+] is low and the DA and Na^+ dissociate from the carrier which then recycles. It has been hypothesised that AMPH binds to the DA uptake carrier and is transported across the neurolemma in the same way as DA. The increased shuttling of the carrier as well as the increase in intraneuronal Na^+ brought about by the transport of the AMPH-Na^+-carrier complex might favour the outward transport of DA. In support of this, the reduction of extracellular [Na^+] has been used to stimulate DA release in a carrier dependent fashion (see Raiteri et al., 1978). In these conditions, the Na^+ gradient is reversed which causes the uptake carrier to operate in the reverse direction. Furthermore, if Na^+ is allowed to accumulate intracellularly by the ouabain induced inhibition of Na^+-K^+-ATPase, DA efflux is also stimulated in a carrier dependent fashion (Raiteri et al., 1978).

Under normal physiological conditions, if carrier mediated efflux of DA occurred, the process would not be saturated since intraneuronal metabolism probably holds DA at a low concentration. The Km of the carrier for DA is likely to be high on the cytoplasmic surface of the neurone due to the relatively low levels of Na^+.

It appears that Na^+ and more particularly the Na^+ gradient across the neurolemma determines the action of the DA uptake carrier. The hypothesis that this carrier is reversed by AMPH explains the results obtained in the present experiments.

1. Exchange diffusion provides a mechanism whereby the diffusional

barrier to DA is circumvented. Despite the fact that AMPH is able to accumulate intracellularly by diffusion across the neurolemma (Fischer & Cho, 1979), DA efflux is only stimulated if the membrane carrier is operative. The hypothesis predicts that inhibitors of the carrier mechanism should block the efflux of DA produced by AMPH. This explains the ability of nomifensine to inhibit the efflux of DA elicited by AMPH.

2. If DA is transported out on the uptake carrier, then only the cytosolic pool of DA should have access to the carrier. This is supported by the results for AMT and pargyline which decrease and increase the size of the cytosolic pool respectively and concurrently decrease and increase the AMPH stimulated efflux of DA.

3. The AMPH stimulated decline in DOPAC efflux may be partially explained by exchange diffusion. By facilitating the efflux of DA, AMPH would indirectly lower the amount of cytoplasmic DA available for deamination by MAO. This is supported by the effect of nomifensine which protected against the AMPH induced decline in DOPAC efflux.

To summarise, the results support the hypothesis that AMPH stimulates DA efflux by a process of exchange diffusion. The mechanism probably relies on alterations of the Na^+ gradient that act to reverse the direction of action of the DA uptake carrier. It is probable that other drugs that interfere with the Na^+ gradient have a similar action on the uptake carrier. This will be discussed with relation to ouabain, veratrine and increased K^+ levels in the following sections.

4.3 The DA releasing effect of raised extracelluar K^+

Calcium ions play a central role in the release of neurotransmitter substances from a nerve ending. Calcium entry into the nerve terminal is the trigger for the exocytotic release of neurotransmitter (Augustine, 1987; Nachshen & Sanchez-Armass, 1987). Potassium evoked release of neurotransmitter is closely allied to the influx of Ca^{2+} ions (Nachshen & Sanchez-Armass, 1987). This method of depolarisation induced release is widely used and has been demonstrated recently for NA release from peripheral tissue (El-Din & Malik, 1982) and from synaptosomes (Raiteri et al., 1977), for amino acids from the intact striatum (Tossman & Ungerstedt, 1984), and for DA from superfused striatal synaptosomes (Raiteri et al., 1979) and from the isolated rat striatum (Harsing & Vizi, 1984). When KCl was perfused intrastriatally through the dialysis probe, there was a large and rapid increase in DA efflux (Fig. 3.2.3). The DA response was maximal during the 20 min perfusion of KCl, subsequent to which it rapidly declined to pre-stimulation levels over 40 min. However, the origin of this DA in the presynaptic terminal is not exclusively vesicular as would be expected when stimulating release with a depolarising agent. This is evident from the sensitivity of KCl stimulated DA efflux to AMT (Fig. 3.4.2a,b). If the KCl sensitive pool of DA was exclusively vesicular, then inhibition of synthesis of extravesicular DA would not be expected to affect this pool. However, AMT administered 20 min and 120 min prior to KCl infusion was effective at reducing KCl stimulated DA efflux (Table 3.4.1). Moreover, KCl infusion also reduced the efflux of DOPAC (Fig. 3.2.3) which is evidence of an effect on the non-vesicular pool of DA.

This effect is in contrast to results obtained by other groups who reported that KCl released unmetabolised NA in an exocytotic Ca^{2+}-dependent fashion without affecting deaminated metabolites (Baldessarini & Kopin, 1967; Raiteri et al., 1975; Mulder et al., 1975; Holz, 1975). Another surprising result was that despite an apparently strong tendency of reserpine to inhibit KCl stimulated DA efflux (Fig. 3.4.3), the result was not statistically significant (Table 3.4.1). However, this may be attributed to the high variation of the results since reserpine, which is a potent depletor of the vesicular stores of DA, would be expected to affect the depolarisation induced efflux elicited by KCl. Overall, these results would seem to suggest that depolarisation induced by high extracellular $[K^+]$, does not exclusively release vesicular stores of DA. This is probably the case and can be explained as follows. Depolarisation by constant perfusion of 90mM KCl through the dialysis probe was found to sub-maximally stimulate DA efflux in the striatum (Fig. 3.4.1). However, constant exposure of the tissue to elevated extracelluar $[K^+]$ for 20 min grossly departs from the physiological norm where depolarisation over a timescale of milliseconds stimulates exocytotic release of DA. It seems likely and indeed probable from these results that a prolonged exposure of the striatal tissue to KCl rapidly depletes the small releaseable vesicular pool of DA, probably within seconds. Following this, the vesicles are replenished from the newly synthesised (cytosolic?) pool of DA which thereby contributes to the pool of DA sensitive to KCl stimulation. This would explain the sensitivity to synthesis inhibition by AMT. This would also explain the apparent lack of sensitivity to reserpine since the reserpine sensitive pool of DA

would contribute only a small proportion of the DA released during 20 min by KCl. The KCl induced decline in DOPAC efflux supports the involvement of a cytosolic component.

If it is to be accepted that depolarisation induced release occurs directly from synaptic vesicles through an exocytotic like process, then inhibition of the DA uptake carrier with nomifensine should have no effect. This was reported to be so by Raiteri et al. (1979) in superfused synaptosomes. Thus, co-perfusion of nomifensine with 56mM KCl had no inhibitory effect on the KCl-induced efflux of DA. In the present experiments, the injection of nomifensine 20 min before KCl infusion, facilitated KCl-stimulated DA efflux (Table 3.4.1). This is probably as a result of the inhibition of re-uptake of the DA released by KCl. This result was not obtained by Raiteri and co-workers because of the superfusion conditions used which effectively nullifies the effect of DA re-uptake. If part or all of the KCl stimulated DA efflux was via the membrane carrier in a fashion analogous to that of AMPH (i.e. by reversal of the carrier to transport DA outwards) then nomifensine would be expected to inhibit and not facilitate the effect. Therefore, this result substantiates the evidence that KCl stimulates DA efflux via exocytosis from a vesicular pool. Further credence is given to this mechanism by the inhibition of KCl stimulated DA efflux by EGTA in a Ca^{2+} free buffer (Table 3.4.1). The exocytotic release of DA is triggered by the influx of Ca^{2+} into the cell. Ca^{2+} influx is largely prevented in Ca^{2+} free EGTA containing buffer which would explain this inhibition. The operation of the plasma membrane reuptake carrier is Ca^{2} independent and consequently insensitive to conditions of low

extracellular [Ca^{2+}]. Similar results were obtained by Raiteri et al. (1979), by Harsing & Vizi (1984) and by El-Din & Malik (1982). Raiteri and co-workers demonstrated the Ca^{2+} dependency by progressively reducing the concentration of Ca^{2+} perfused over striatal synaptosomes. Even in conditions of no extracellular Ca^{2+}, KCl was still able to stimulate some DA efflux albeit substantially reduced. This was in contrast to KCl-stimulated NA and 5-HT efflux which was totally dependent on extracellular Ca^{2+} in these superfusion conditions (Raiteri et al., 1979).

Tetrodotoxin blocks Na^{+} channels in the plasma membrane and consequently inhibits depolarisation induced DA release by such depolarising agents as veratrine and electrical stimulation. However, it is not effective against KCl stimulated DA efflux (Table 3.4.1). This is because it is the high extracellular [K^{+}] that facilitates the influx of Ca^{2+} necessary for depolarisation induced DA release. Na^{+} entry into the cell is not an absolute pre-requisite in these conditions although some Na^{+} dependency is suggested by the apparent tendency of TTX to inhibit KCl stimulated DA efflux (Fig. 3.4.7). Statistical significance was probably not obtained due to the large standard error of the results.

4.4 The DA releasing effect of tyramine

Tyramine is classed as an indirectly acting sympathomimetic amine. Indirect because the effects of tyramine are brought about by the released amine transmitter and not by tyramine itself. The mechanism by which tyramine achieves this is a matter of speculation although it has been studied in depth in noradrenergic nerve endings (see Trendelenburg, 1987). The results of the present experiments

on dopaminergic neurones as well as those of Zetterstrom et al. (1988) and those of others on noradrenergic neurones (Langeloh & Trendelenburg, 1987; Trendelenburg et al., 1987), suggests that tyramine releases DA or NA from vesicular stores of the transmitter. Thus, in the present experiments, tyramine stimulated the efflux of DA in a dose dependent fashion but had no effect whatsoever on the efflux of DOPAC or HVA (Figs. 3.5.1a,b). This is in agreement with similar in vivo results obtained by Zetterstrom et al. (1988). It has already been suggested for AMPH that a decline in DOPAC efflux is indicative of effects on a newly synthesised possibly cytosolic pool of DA. The lack of such an effect in the presence of stimulated DA efflux is indicative of the release of solely non-cytosolic i.e., vesicular pools of DA.

AMT administered 20 min before tyramine, a timepoint at which tissue levels of DA are not affected (Fig. 3.1.1), had no effect on tyramine stimulated DA efflux (Table 3.5.1). However, when administered 120 min previously, a timepoint at which tissue levels and consequently vesicular stores of DA are depleted, tyramine stimulated DA efflux was substantially reduced (Table 3.5.1). Moreover, reserpine pretreatment dramatically reduced the effect of tyramine (Table 3.5.1). These results combine to suggest that the releasing effect of tyramine is on vesicular stores of DA in the absence of the release of cytosolic DA.

As an amine, tyramine is a substrate for MAO which has been reported to have a Km for tyramine around 600 to 800μM (Caramona, 1982). When MAO activity was inhibited by pargyline, the DA releasing effect of tyramine was facilitated by 25% (Table 3.5.1). It might be argued that the raising of cytosolic concentrations of

DA by pargyline should have no effect on the size of the tyramine releasable pool of DA which is distinct from the cytosolic pool. However, it may be that the inhibition of MAO by pargyline protects tyramine from the enzyme. This action would effectively raise the intracellular level of tyramine resulting in the increased releasing effect. In addition, the protection of DA degradation by pargyline will effectively protect the DA that is displaced from the vesicles into the cytoplasm by tyramine so increasing the size of the DA pool released by tyramine.

The mechansim by which tyramine enters the nerve terminal is probably via the membrane carrier as a substrate of high affinity DA uptake. Tyramine has a Km for this uptake process of 1.4μM which is lower than that quoted for DA of 2.0μM (Langeloh et al., 1987). The present experiments support this since nomifensine was very effective at inhibiting tyramine stimulated DA efflux (Table 3.5.1, Fig. 3.5.6). Once inside the terminal, tyramine displaces DA from the vesicles. This may be achieved in a combination of two ways. Firstly, tyramine has a high affinity for the vesicular uptake carrier (< 15μM, Langeloh & Trendelenburg, 1987) and so by saturating the carrier competes with DA for reuptake. In this way, DA re-uptake into the vesicles is inhibited with a consequent increase in the rate of net leakage from the vesicles. Secondly, the presence of high levels of tyramine in the nerve terminal cytoplasm raises the intravesicular pH. This has been reported to stimulate release of vesicular NA into the cytoplasm (Langeloh & Trendelenburg, 1987). The same may be true for DA although the authors express some doubt that this is a significant mechanism for the effect of tyramine on transmitter mobilisation from vesicles.

Whatever the precise mechanism, tyramine displaces DA from vesicles. This may explain the small but consistent apparent rise in DOPAC efflux in tyramine stimulated animals pretreated with AMT (Fig. 3.5.2b), reserpine (Fig. 3.5.3), selegeline (Fig. 3.5.5), nomifensine (Fig. 3.5.6) and TTX (Fig. 3.5.7). This was a small but consistent tendency and may reflect an increased metabolism of DA due to the influx of vesicular DA into the cytoplasm.

Subsequent to release from the vesicles, DA probably exits the nerve terminal via the reversed action of the membrane reuptake carrier. The mechanism by which this is achieved i.e., exchange diffusion, has already been discussed for AMPH. A similar process has been proposed for tyramine stimulated release of NA (see Langeloh & Trendelenburg, 1987). These workers reported that the increased operation of the uptake carrier in transporting tyramine, co-transports Na^+ and Cl^- inside the cell. This is proposed to lower the Km for the outward transport of NA as well as stimulating the reverse operation of the carrier. This mechanism would also explain the nomifensine sensitivity of the tyramine stimulated release process as well as the lack of sensitivity to TTX and Ca^{2+} free medium (Table 3.5.1). A lack of Ca^{2+} dependency for the tyramine releasing process has been described previously for NA (Starke & Montel, 1974) and for DA efflux from rabbit caudate slices (Kamal et al., 1981). Tyramine can be distinguished from reserpine since despite the fact that both agents displace DA from the vesicles, tyramine is a substrate for uptake on the membrane uptake carrier (Steinberg & Smith, 1970) whereas reserpine is not. Consequently, reserpine is unable to promote the release of DA by accelerated exchange-diffusion.

4.5 The DA releasing effect of ouabain

Depolarisation induced release of a transmitter from a nerve ending depends upon the opening of Ca^{2+} channels followed by the Ca^{2+} dependent efflux of transmitter (Katz, 1969). A role for Na^+-K^+-ATPase in depolarisation induced transmitter release has been proposed (Vizi, 1978). This enzyme derives energy from the splitting of ATP to drive the membrane Na^+ pump and requires internal Na^+ and external K^+ for activation (Skou, 1965). Thus, depolarisation, which causes an influx of Na^+ by opening Na^+ channels, activates Na^+-K^+-ATPase which then restores the ionic gradient by pumping Na^+ out of the cell.

Ouabain is an inhibitor of Na^+-K^+-ATPase (see Vizi, 1978) and in common with other inhibitors of this enzyme, has been shown to stimulate the efflux of transmitters (see Paris, 1983 for review). The mechanism by which this is achieved is quite controversial and there is much literature devoted to this. Inhibition of the Na^+-K^+-ATPase could lead to depolarisation of the neurone by causing the intracellular accumulation of Na^+. This has been proposed to stimulate the influx of Ca^{2+} and transmitter release by exocytosis (Banks, 1976). Alternatively, it could lead indirectly to a rise in intracellular Ca^{2+} through mobilisation of intracellular Ca^{2+} stores (Baker & Crawford, 1975), through Ca^{2+} exchange for intracellular Na^+ (Nakazato et al., 1978) or through inhibition of Ca^{2+} efflux (Pocock, 1983), all of which would permit transmitter release by exocytosis.

When perfused intrastriatally, ouabain stimulated an increase in DA efflux and a concurrent decrease in DOPAC efflux (Fig. 3.2.5). The maximal response was slower in onset than that for tyramine, KCl

and veratrine and occurred 40 min after administration. The DA releasing effect of ouabain over 60 min was inhibited when AMT was injected 20 min prior to ouabain (Table 3.6.1). This indicates that the newly synthesised pool of DA is sensitive to the effect of ouabain. However, it would appear that ouabain also released vesicular DA since AMT administration 120 min prior to ouabain, virtually abolished DA efflux (Table 3.6.1, Fig. 3.6.2b). This result agrees with those of others mentioned above in that ouabain appeared to release vesicular DA but contrasts in that newly synthesised possibly cytosolic DA was also released. Surprisingly, a vesicular releasing effect was not substantiated by reserpine. In reserpinised animals, ouabain stimulated DA efflux was not reduced although the tendency appeared to be a reduced efflux (Table 3.6.1). This may be explained by the evidence that ouabain appears to release DA not only by exocytosis from vesicles but also possibly by a carrier dependent mechanism. Such a mechanism will continue to release DA in the absence of vesicular pools of DA in reserpinised animals. The evidence for this mechanism is discussed below.

The effects of nomifensine on ouabain stimulated DA efflux are also of interest. Nomifensine inhibited the effect of ouabain more potently over the initial 20 min of ouabain stimulated DA efflux than over 60 min (Table 3.6.1). However, both timepoints were highly significant. Therefore, the results indicate that ouabain releases vesicular and newly synthesised DA via a mechanism that involves the DA uptake carrier. It would appear that DA is also released by exocytosis since both TTX and Ca^{2+} free buffer with EGTA, two conditions that block depolarisation induced release,

inhibit ouabain stimulated DA efflux (Table 3.6.1). This indicates that the effect of ouabain is a complex one that appears to release DA by two mechanisms, exocytosis and carrier dependent release.

The inhibition of Na^+K^+ATPase by ouabain blocks the activity of the Na^+ pump. This leads to the accumulation of Na^+ inside the cell and the loss of K^+ from inside the cell. Furthermore, these conditions stimulate the influx of Ca^{2+} and cause a fall in the resting membrane potential (Vizi, 1979). Conditions such as these mimic those occurring during depolarisation and may release transmitter in this fashion. However, in addition to this, the accumulation of intracellular sodium may cause a reversal of the membrane uptake carrier by a mechanism analogous to that for AMPH and tyramine. This was also proposed by Raiteri & Levi (1978) where an increase in intracellular Na^+ permits the nonvesicular efflux of transmitters by reversal of the uptake carrier in a fashion similar to that of exchange diffusion. A dual action such as this appears quite attractive since it is possible to conclude from these results that ouabain releases DA in an exocytotic, Na^+ induced, Ca^{2+} dependent fashion as well as a carrier dependent fashion. Much of the literature to date appears to have concentrated on the effect of Na^+-K^+-ATPase inhibition on depolarisation induced exocytotic transmitter release (see Vizi, 1978, 1979; Brosemer, 1985). Indeed, Brosemer (1985) discounted a carrier mediated efflux of transmitter during Na^+-K^+-ATPase inhibition mainly because of the lack of evidence in the literature. Other groups, however, have favoured a carrier-reversal mechanism caused by Na^+-K^+-ATPase inhibition for the release of NA (Sweadner, 1985; Artalejo & Garcia, 1986;

Raiteri & Levi, 1978) and for amino acids (Jacobson et al., 1986). The present results also support such a mechanism for the release of DA. It is possible that the initial effect of ouabain is to reverse the uptake carrier and release DA in this fashion. This would explain the early exquisite sensitivity to nomifensine as well as AMT. As the effect of ouabain upon the Na^+, K^+ gradients progresses, the release of DA may be from vesicular pools via an exocytotic process as well as via the carrier. This would explain the decreasing sensitivity to nomifensine and the increasing sensitivity to Ca^{2+} free conditions (which were ineffective on the initial efflux of DA over 20 min but effective over 60 min). In support of this, a delay in sensitivity of transmitter release by ouabain was reported by Sweadner (1985) and by Vizi & Vyskocil (1979). Moreover, Vizi & Vyskocil (1979) reported that ouabain stimulated both quantal and non-quantal release of ACh from the mouse diaphragm. Quantal release was sensitive to calcium whilst non-quantal release was insensitive. This was proposed to represent exocytotic vesicular release of ACh (quantal) and also non-vesicular release of ACh stimulated by ouabain.

4.6 The DA releasing effect of veratrine

Veratrine is a mixture of five alkaloids including veratridine and cevadine whose action is to depolarise nerve terminals (Minchin, 1980). This action has been demonstrated in a variety of excitable tissues including squid and crayfish axons, single frog node of Ranvier and amphibian muscle (see Minchin, 1980 for review). The mechanism of action of veratrine involves an increase of membrane permeability to Na^+. This effect was demonstrated in

synaptosomes by Li & White (1977). Veratridine increased the influx of $^{24}Na^+$ into synaptosomes, increased the total Na^+ content and decreased K^+ content within 10 seconds of exposure. This effect was prevented by TTX and by a reduction of external $[Na^+]$ in the superfusing medium. The present results support this mechanism of action for veratrine but also suggest that veratrine has additional effects on DA release from the nerve terminals that are not consistent with a sole depolarising action of the drug.

As for ouabain, veratrine stimulated DA efflux was determined over 20 min as well as over 60 min. This is because there are certain anomalies in the efflux data that depart from a sole releasing action of veratrine on vesicular pools. As might be expected, AMT administered 120 min before veratrine infusion (conditions that deplete vesicular pools) dramatically inhibited veratrine stimulated DA efflux (Table 3.7.1). DA efflux over 20 min was more significantly reduced than over 60 min although it is doubtful that any mechanistic significance can be attached to this result since the extent of inhibition was about identical over the two time periods (14% and 12% of control respectively). AMT administered 20 min before veratrine was also effective at inhibiting DA efflux but only over 60 min (Table 3.7.1). Under these conditions tissue levels and consequently vesicular pools of DA are not depleted. On the other hand, reserpine was only effective against veratrine over 20 min (Table 3.7.1) despite an apparent inhibition over 60 min. Thus, the results have departed from a pure release of DA from vesicular pools as would be expected with a depolarising agent. This would also explain the reduction

in DOPAC and HVA efflux following veratrine (Fig. 3.2.6) and the sensitivity of the veratrine response to pargyline (Table 3.7.1) both of which are indicators of DA efflux from the cytosolic pool of DA.

If the mechanism of action of veratrine is to release vesicular pools of DA by depolarisation-induced exocytosis, then the process should be sensitive to inhibition by TTX and by Ca^{2+} removal. Calcium dependency was demonstrated for veratridine stimulated efflux of endogenous and exogenous DA by Mulder et al. (1975) and by Patrick & Barchas (1976). The TTX sensitivity of the process has been demonstrated for veratrine stimulated efflux of GABA (Bowery et al., 1979) amino acids (De Belleroche & Bradford, 1977) and for NA and DA efflux from synaptosomes (Blaustein, 1975; Mulder et al., 1975; Patrick & Barchas, 1976). Conversely, veratrine-stimulated DA efflux should be unaffected by nomifensine if the drug is releasing DA solely by exocytosis. The present results support this since nomifensine had no effect whatsover on veratrine stimulated DA efflux (Table 3.7.1). Moreover, in contrast to KCl induced DA efflux, nomifensine did not enhance veratrine induced DA efflux. This may be due to the action of veratrine on the Na^+ gradient across the neurolemma which would disrupt the activity of the Na^+, K^+-ATPase thereby blocking DA uptake. The addition of nomifensine in these conditions would not be expected to have any further effect. However, veratrine does appear to have similarities to K^+ stimulated DA efflux in that both cytosolic and vesicular DA is released probably by exocytosis and not involving carrier mediated efflux. In agreement with this are the results for TTX and EGTA. TTX virtually abolished veratrine stimulated DA

efflux (Table 3.7.1 and Fig. 3.7.7) as might be expected if the action of veratrine is solely to open Na^+ channels. The Ca^{2+} dependency of veratrine stimulated DA efflux is demonstrated in Fig. 3.7.8. The results suggest that veratrine releases vesicular DA via exocytosis. The prolonged exposure of the tissue to veratrine rapidly depletes the vesicular pool of DA. This pool which is in a dynamic equilibrium with the cytosolic pool, is replenished constantly. Both this result and that for KCl stimulated DA efflux illustrate a limitation of the present *in vivo* dialysis technique in that the temporal resolution is poor when attempting to follow physiological events.

4.7 General discussion

The results presented here are an attempt to further elucidate the nature of the DA pools in striatal dopaminergic nerve endings. In addition, information has been obtained concerning the mechanisms underlying DA release from these nerve endings. The results will be collectively discussed in relation to DA pools and DA release.

These results support the hypothesis that DA can be released by two different mechanisms. The first is by exocytosis which is thought to be the mechanism of depolarisation induced release (Holz, 1975; Mulder & Snyder, 1976; Raiteri et al., 1978). The second is by a reversal of the membrane uptake carrier, the so called chemical release of DA (Raiteri et al., 1978; McMillen, 1983; Parker & Cubeddu, 1986; Butcher et al., 1988). Drugs known to be chemical releasers (AMPH), or known to affect the action of the membrane uptake carrier (ouabain) or to mimic depolarisation induced DA release (high $[K^+]$, veratrine and tyramine to some extent) have

been used to distinguish between these two mechanisms. Thus, amphetamine is a chemical releaser of DA that probably causes release by a reversal of the membrane uptake carrier. The results are summarised in Fig. 4.1. This figure enables a 'profile' to be constructed of each releasing agent. These can then be compared and characterised for chemical release and/or depolarisation induced release. Thus, it would be expected that only cytosolic DA would be a substrate for carrier mediated release. So an agent that stimulates release via the carrier should be very sensitive to alterations in the size of the cytosolic pool of DA. AMT given 20 min before AMPH greatly reduced the efflux of DA. On the other hand, the efflux of DA stimulated by tyramine or veratrine was inhibited to a much lesser degree by AMT given over the short term. This is the expected result if tyramine and veratrine release DA from a vesicular pool. Furthermore, AMPH stimulated DA efflux was insensitive to reserpine as would be expected. The most distinguishing feature of the AMPH profile is the exquisite sensitivity to inhibition by nomifensine which was ineffective against the depolarising agents KCl and veratrine. Tyramine stimulated efflux was inhibited for reasons already described. Moreover, the Na^+ and Ca^{2+} independency of the AMPH effect are demonstrated by the lack of effect of TTX and Ca^{2+} removal plus EGTA respectively. The depolarising agents KCl and veratrine were exquisitely sensitive to both TTX and EGTA. Thus, these profiles suggest the nature of DA release stimulated by these agents and allow the categorisation of depolarising agents such as veratrine and KCl, which release DA from vesicular stores by a process of exocytosis. AMPH on the other hand, with its contrasting profile,

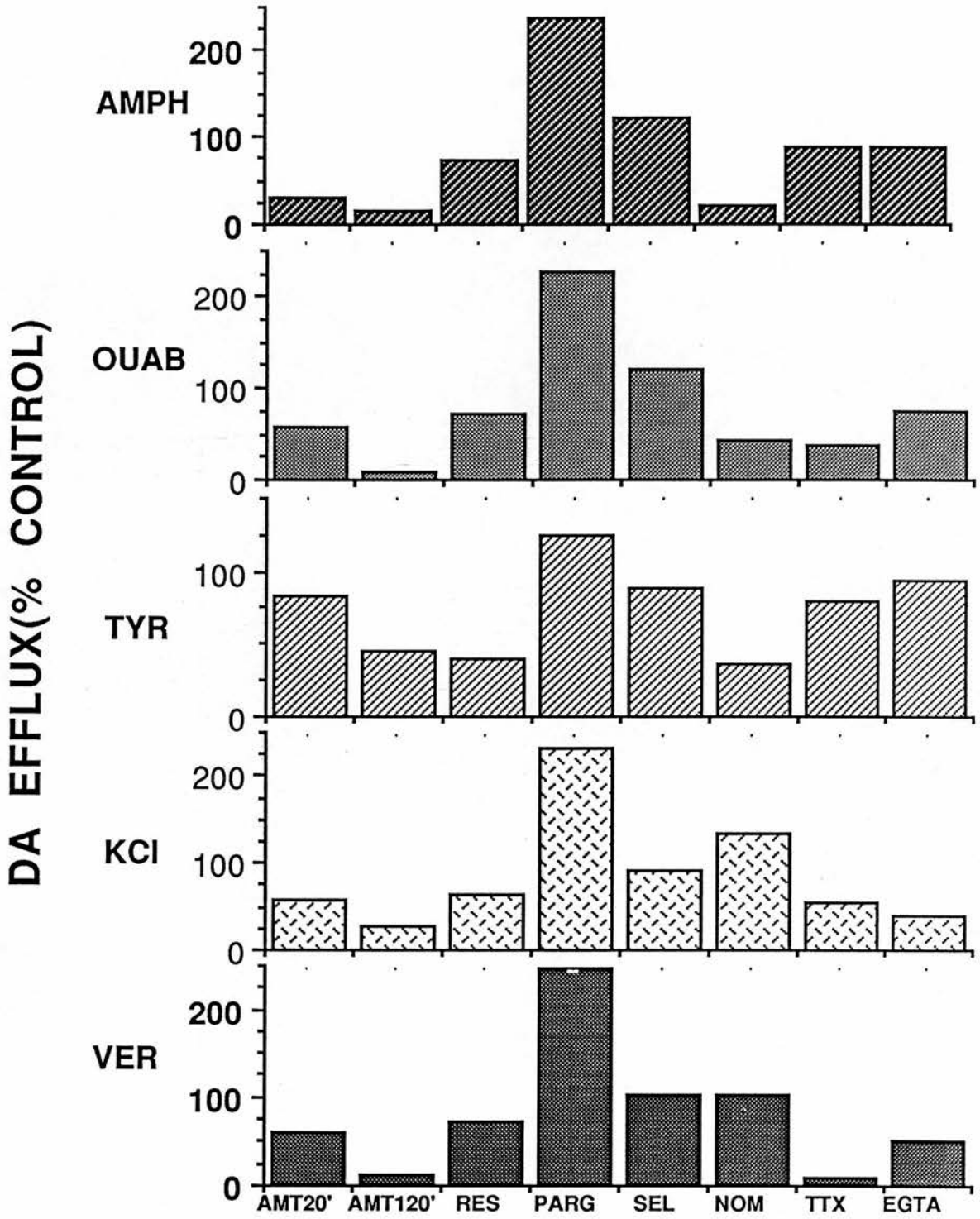


Fig.4.1 Summary of the effects of pharmacological and biochemical interventions on the DA releasing agents. Results are the % change in DA efflux over 60 min. when compared to control.

releases DA exclusively via a carrier dependent mechanism with cytosolic DA as the substrate. Tyramine releases vesicular DA not by exocytosis but most probably by a carrier dependent mechanism. Thus, after displacing DA from the vesicles into the cytosol, this pool of DA becomes a substrate for carrier mediated efflux. Such a mechanism would be facilitated by the sudden increase in concentration of DA in the cytosol induced by tyramine. This would have the effect of raising the concentration of DA above the Km for the carrier and so facilitate the reversal of the carrier. If this is the actual mechanism by which tyramine releases DA, then it could be construed that tyramine is releasing from a vesicular pool indirectly via the cytosolic pool. Other cytosolic releasing agents such as AMPH and ouabain decrease the efflux of DOPAC whilst tyramine does not. This is illustrated in Fig. 4.2 which shows the effects of the releasing agents on DOPAC efflux. AMPH, by releasing DA from a cytosolic pool, rapidly and significantly reduces DOPAC efflux probably by depriving MAO of substrate. Tyramine, on the other hand, does not affect DOPAC efflux. This may be due to the fact that in displacing DA into the cytosol, the pool of DA substrate for MAO is maintained. Indeed, it is probably increased and this may be reflected in the slight rise in DOPAC efflux immediately after tyramine. AMPH and ouabain are not known to displace DA from vesicular storage pools and so deplete the cytosolic pool and consequently the pool of DA exposed to MAO.

The results do not demonstrate a 100% response one way or the other i.e., an exclusively chemical releaser that only releases cytosolic DA or an exclusively vesicular releaser that only releases vesicular DA. The reasons for this are two fold; firstly, the

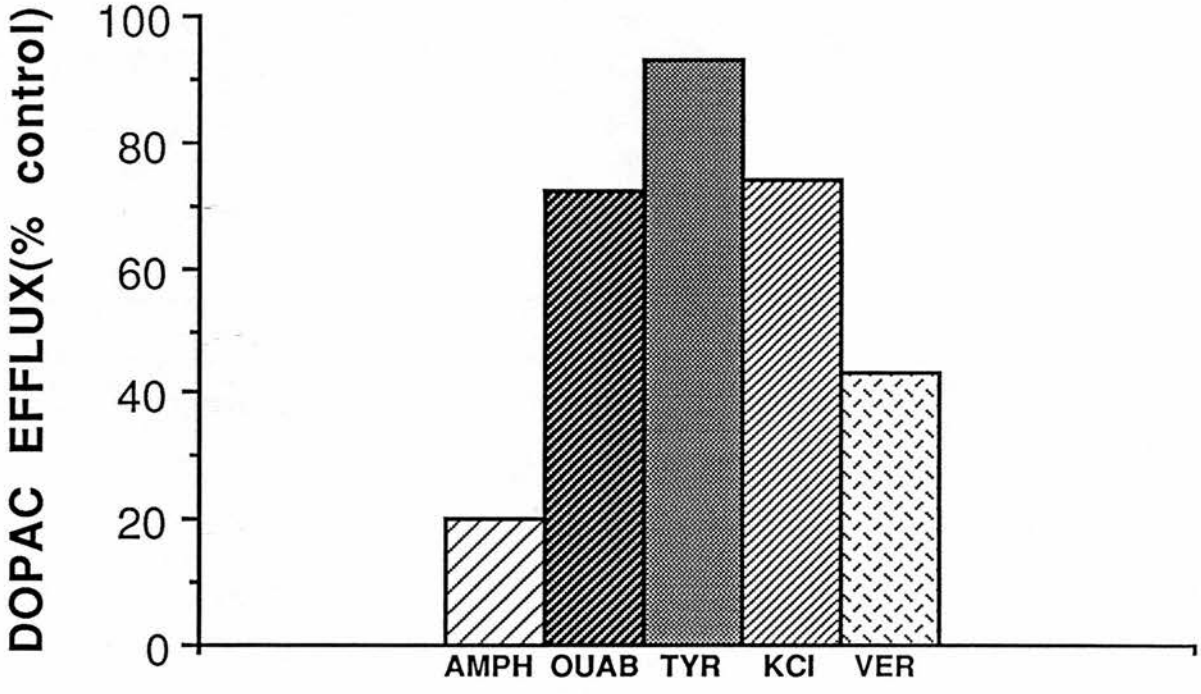


Fig.4.2 Summary of the effects of the releasing agents on DOPAC efflux. Results are the % maximal decrease in DOPAC efflux seen following the releasing agent.

cytosolic and vesicular pools are in a rapidly exchanging dynamic equilibrium with each other (Parker & Cubeddu, 1986). Consequently, if one pool is depleted, it is rapidly replenished by the other. Secondly, the methodology demands that events are measured over a time period of 20 min in these experiments which reduces the power of resolution between measuring exclusively cytosolic releasing events or exclusively vesicular releasing events. This is not to say that these results suggest that AMPH and depolarising agents all release DA by two mechanisms, but rather that it is not possible to resolve a 'clean' response.

It is apparent from Fig. 4.1 that ouabain does not appear to fit a profile for a chemical or a vesicular releaser. The same is also true to some extent for KCl. Thus ouabain was affected by both TTX and EGTA. As mentioned already, it seems reasonable to infer that ouabain acts both as a chemical releaser and as a depolarising agent under these conditions. Ouabain has been demonstrated previously to not only inhibit $Na\text{-}K^{+}$-ATPase and so induce a chemical release of transmitter (Raiteri & Levi, 1978) but also to depolarise neurones and consequently stimulate the exocytotic release of transmitter (Banks, 1967). This is borne out by Fig. 4.1.

What is also apparent is that different releasing agents stimulate DA release by different effects upon the same two mechanisms. Thus, carrier mediated release of DA is directly stimulated by AMPH which is a substrate for the carrier and indirectly by ouabain and probably by veratrine which reverse the action of the carrier by affecting the ionic gradient that controls the carrier. On the other hand, tyramine releases vesicular DA by displacing it from vesicles into the cytoplasm. Veratrine and KCl

release vesicular DA by depolarisation induced exocytotic release. These results support the existence of two mechanisms of DA release and convey novel information on the mechanism of action of the releasing drugs in vivo.

In addition to conveying information on the mechanism of action of the releasing drugs and chemicals used in this study, there is much that may be inferred from the results concerning the heterogeneity of DA pools in nerve terminals. The existence of multiple pools of DA within the nerve terminal as described in the introduction seems to be beyond doubt. The present results support such a heterogeneity of intraterminal pools of DA. The evidence for this is derived from the differing sensitivites of the releasing drugs/chemicals to pharmacological or biochemical interventions.

Numerous groups have proposed the existence of multiple pools of DA within nerve terminals (Glowinski, 1975; Groppetti et al., 1977; Schoemaker & Nickolson, 1983; Leviel & Guibert, 1987; Justice et al., 1987). From these studies has emerged various models of the distribution of DA within the terminal. Radioactive tracer studies using [^{3}H]-tyrosine were able to distinguish two pools of DA according to the rate at which they became labelled (Schoemaker & Nickolson, 1983; Leviel & Guibert, 1987). These workers concluded that DA was contained in a cytosolic pool that contained newly synthesised DA and a storage pool contained within vesicles. An alternative model was proposed by Justice et al. (1987). This group proposed that DA was contained in three intraterminal pools, a cytosolic pool, a releasable bound pool and an inactive bound pool. Moreover, Justice and co-workers proposed that the newly synthesised pool of DA is represented by the

releasable bound pool rather than the free cytosolic pool. In other words, DA synthesis directly replenishes the releasable vesicular pool of DA as well as the cytosolic pool which would then route DA into the vesicles. This proposal was made for their model to avoid competition between DA uptake into vesicles and DA metabolism. It was also based on results obtained by this group when DA release induced by electrical stimulation was followed. The level of DA release measured could not be accounted for by the kinetic parameters used in the model if DA synthesis contributed directly to the cytosolic pool and not the vesicular pool. However, this model does not agree with the results of others (Raiteri et al., 1979; Fischer & Cho, 1979; Parker & Cubeddu, 1986a,b), which demonstrate that the newly synthesised pool of DA and the cytosolic pool of DA may be the same pool. Thus, if DA synthesis is acutely inhibited with AMT simultaneous to giving AMPH which releases newly synthesised (cytosolic?) DA, then DA efflux is markedly reduced (Fig. 3.3.2). The contrasting results may be due to the very different methdologies between these in vivo microdialysis data and the mathematical model used by Justice and co-workers. However, Glowinski et al. (1975) reported the newly synthesised pool to be contained within vesicles. This conclusion was based on the fact that both electrical stimulation and AMPH stimulated the release of [^{3}H]-DA synthesised from [^{3}H]-tyrosine. So the situation remains unresolved since the evidence suggests that the pool of newly synthesised DA can be released by exocytosis or by chemical release.

The present results also convey other evidence for multiple pools of DA. Thus, AMT and reserpine have contrasting effects in

that they appear to deplete two different pools of DA. AMT reduced the effects of AMPH, ouabain and KCl all of which appear to release cytosolic DA whereas the effect of tyramine, a vesicular releaser, was not inhibited. Reserpine reduced the effects of the vesicular releasers tyramine and veratrine but not of AMPH.

The effects on the efflux of DA metabolites also differentiate between different pools of DA. Pargyline protects cytosolic DA from metabolism by MAO. The fact that pargyline failed to enhance tyramine stimulated DA efflux to the same extent as the non-vesicular releasers is evidence for an exclusive action of tyramine on vesicular pools of DA. In agreement with this any drug or chemical manipulation that releases cytosolic DA would be expected to reduce the size of the substrate pool of DA for MAO and consequently reduce DOPAC efflux. This was the case for DA efflux stimulated by AMPH, ouabain, veratrine and KCl but not by tyramine. This suggests the existence of at least two pools of DA, one cytosolic and one vesicular.

Justice et al. (1987) went further than this and proposed a third pool of DA, namely an inactive bound pool which represents an inactive vesicular pool. The basis for this was formed on their kinetic data which best fitted a model with this third pool. The present results do not contain any kinetic evaluation of DA release and consequently no definitive evidence for a third pool. However, it is tempting to postulate the presence of such a storage pool based on the fact that both the cytosolic and the releasable vesicular pool appear to be rapidly depleted. Thus, the depolarising agents KCl and veratrine were sensitive to synthesis inhibition by short-term AMT suggesting that the releasable

vesicular pool is quickly depleted and then replenished from the cytosolic pool. However, AMT over the short term did not deplete total tissue levels of DA (Fig. 3.1.1a) suggestsing that a larger inactive storage pool is present that is not susceptible to depolarisation induced release. Similarly, the fact that AMPH maximally decreased DOPAC efflux at a dose of 4mg/kg whilst DA efflux was further increased at higher doses suggests that the cytosolic pool is also small and that at higher doses AMPH also releases vesicular DA.

These results have demonstrated a heterogeneity of intraterminal DA pools. By using various combinations of biochemical and pharmacological agents, different pools of DA have been selectively affected. This has been reflected in the stimulated efflux of DA and its metabolites which have allowed inferences to be made concerning the nature of the DA pools. Furthermore, the results support the contention that DA release may occur by two mechanisms, exocytotic and carrier mediated release. The actions of the releasing agents are not 'clean'. They appear to release DA either via exocytosis of vesicular pools or via carrier mediated release of cytosolic pools. However, the prolonged depletion of either of these pools appears to 'pull' DA from the remaining pools possibly in an attempt to maintain the equilibrium between the pools. This is reflected in a sensitivity of either release mechanism to drugs not directly affecting that mechanism.

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APPENDIX

PUBLISHED PAPERS

878P

IN VIVO MICRODIALYSIS STUDIES ON DOPAMINE RELEASE FROM THE STRIATUM EVOKED BY NIGROSTRIATAL STIMULATION

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A considerable body of evidence supports the candidacy of dopamine as the neurotransmitter utilised by a majority of neurones in the nigrostriatal pathway (Graybiel and Ragsdale, 1983). For example, researchers using both push-pull cannulae and voltametric methods have demonstrated that stimulation of this pathway in vivo increases dopamine release from the striatum (Glowinski et al., 1978; Ewing et al, 1983). In the present study we have further examined the in vivo release of endogenous dopamine in response to nigrostriatal stimulation using brain microdialysis.

Wistar rats (260-300 g; either sex) were anaesthetised and tracheotomised. Anaesthesia was then maintained using halothane (< 1%). Burr holes were drilled in the skull directly above the striatum (AP + 0.5; ML ± 2.5) and the medial forebrain bundle (AP - 3.8, ML ± 1.0). A cannula type dialysis probe was positioned into the striatum, and a steel concentric electrode placed in the medial forebrain bundle. The nigrostriatal pathway was stimulated at 0.5 mA intensity at 100 H_Z for 0.5 msec at 1 second intervals for 10 mins. The dialysis probe was perfused continuously (2.5 µl/min) with oxygenated Krebs bicarbonate buffer and dialysate samples (25 µl) were collected at 10 min. intervals. Dopamine (DA), 3,4 dihydroxyphenylacetate (DOPAC) and homovanillic acid (HVA) were measured by high performance liquid chromatography and electrochemical detection.

The basal dialysate concentration of each compound was: DA 6.5 ± 0.7 nM; DOPAC, 0.5 ± 0.1 µM; HVA 0.2 ± 0.1 µM). Electrical stimulation did not affect the release of these compounds. Inclusion of the dopamine uptake inhibitor, nomifensine (1 µM) in the perfusion medium did not affect either basal or stimulated levels of DA, DOPAC and HVA. These parameters were also unaffected when 4-aminopyridine (1 mM; a dose which facilitates Ca^{2+} entry into nerve terminals) was added to the perfusion medium. In a final series of experiments, ouabain (an inhibitor of Na^+/K^+ ATPase) was perfused at a dose which did not influence basal DA or metabolite levels. In the presence of 10 µM ouabain electrical stimulation induced a rapid and reliable increase (180 % of control) in DA release, without affecting metabolite levels.

These data demonstrate that the microdialysis technique can be used to evaluate DA efflux in response to afferent stimulation under appropriate conditions.

S.P.B. is supported by an M.R.C. Training Fellowship and I.S.F. by an M.R.C. Scholarship.

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ON THE MECHANISMS UNDERLYING AMPHETAMINE INDUCED DOPAMINE RELEASE: IN VIVO MICRODIALYSIS STUDIES.

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The psychostimulant, amphetamine (AMPH) is believed to interact with dopaminergic neurones in the brain. AMPH facilitates dopaminergic responses via a number of mechanisms, including a stimulation of dopamine (DA) efflux (see Zetterstrom et al., 1983; Parker and Cubeddu, 1986). Although the precise mechanism by which AMPH influences DA release is unclear, the finding that this response is inhibited by the dopamine uptake inhibitor, nomifensine, is clearly of great importance (Arbuthnott et al., 1985; Parker and Cubeddu, 1986). In the present study we examined the effects of other agents which influence DA uptake and release on basal and AMPH stimulated DA efflux using the in vivo brain microdialysis method.

Wistar rats (250-280g; either sex) were anaesthetised, tracheotomised and placed in the stereotaxic frame. Anaesthesia was then maintained using halothane (< 1.5%). Cannula type dialysis probes were positioned into both striata (AP + 0.5; ML ± 2.5) and perfused with oxygenated Krebs bicarbonate buffer at 1.25 µl/min. Dialysis samples were analysed for DA and related metabolites (3,4 dihydroxyphenylacetate; DOPAC and homovanillic acid; HVA) by high performance liquid chromatography and electrochemical detection. Ouabain (10-1000 µM) or veratridine (10-500 µM) were included in the perfusion buffer as indicated. AMPH 4mg/kg) was administered by a single intraperitoneal injection.

The basal dialysis concentrations of DA and related compounds were: DA, 18.3 ± 0.5 nM; DOPAC, 1.3 ± 0.2 µM; HVA, 0.6 ± 0.2 µM). AMPH induced rapid increase (450 %) in DA, and a parallel drop in DOPAC and HVA. Although ouabain (10 µM) did not influence basal levels of any compound, a striking facilitation of the AMPH response was noted. Moreover, increasing concentrations of ouabain induced a massive increase in dialysis DA concentration (20-40 fold), which was not affected when nomifensine (1µM) was coperfused. Veratridine also provoked a dose related increase in the extracellular concentration of DA, and a decrease in DOPAC and HVA.

These data demonstrate that AMPH, ouabain and veratridine all increase DA efflux from the striatum. However, it is unlikely that these actions are mediated by a common mechanism.

S.P.B. is supported by an M.R.C. Training Fellowship and I.S.F by an M.R.C. Scholarship.

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IN VIVO COMPARTMENTATION STUDIES USING BRAIN MICRODIALYSIS : FOCUS ON DOPAMINE

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Experimental data obtained using a variety of experimental approaches suggest that brain dopamine is compartmentalised in a number of discrete pools within nerve terminals (see McMillan, 1983). In vitro release studies have been used previously to distinguish the various pools and to assess their physiological significance.

In the present study, we have utilised the brain microdialysis method, to study the relevance of these pools in vivo. A number of widely used chemical releasing agents were used to stimulate the efflux of dopamine into the synaptic cleft.

For example, amphetamine was found to elevate dialysate dopamine levels, and depress levels of its metabolites. The releasing action was blocked by αMPT pretreatment, but not by reserpine, suggesting a preferential release of a newly synthesised (cytosolic ?) pool of dopamine. The mechanism of amphetamine action was also examined using a number of agents which interfere with the operation of the membrane dopamine carrier. Our data suggests that active accumulation of amphetamine is a prerequisite for dopamine release.

These studies demonstrate the potential of the microdialysis method for studying the compartmentilisation of dopamine in vivo.

McMillan, B.A. (1983). Trends Pharmacol.Sci. 4, 429-432.

612P

A PHYSIOLOGICAL ROLE FOR MONOAMINE OXIDASE-B IN BRAIN DOPAMINE METABOLISM?

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The catecholamine degrading enzyme, monoamine oxidase (MAO) exists as two distinct isoenzymes within the brain. These are differentially localised within neurones and glia, with MAO-A being found predominantly within neurones, and MAO-B mainly in glia (see Francis et al, 1985). The major route of dopamine (DA) inactivation in the brain is believed to involve high affinity uptake followed by enzymatic breakdown by MAO-A. However, recent microdialysis data have questioned this assumption (Fairbrother et al, 1987), since DA uptake inhibitors do not influence extracellular levels of the primary DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). The mode of DA inactivation is therefore unclear, and the present study is designed to examine whether MAO-B plays a physiological role in this process.

Cannula design dialysis probes (Sandberg et al, 1986) were implanted into rat striata (AP ± 0.5, ML ± 2; from Paxinos and Watson, 1982) under halothane anaesthesia. These were perfused with oxygenated Krebs bicarbonate buffer at 1.25µl/min. After a 60 min washout period, 20 min fractions were collected throughout the experiment and DA and related metabolites were detected by high performance liquid chromatography with electrochemical detection. Selegiline (10mg/kg), an inhibitor of MAO-B (Housley et al, 1976) was administered as an intraperitoneal injection 60 min later. Amphetamine (4mg/kg; i.p. injection), veratrine (100µg/ml; via dialysis probe) and ouabain (100µM; via dialysis probe) were administered 60 min after selegiline injection.

Basal dialysate concentrations of DA and related metabolites were: DA, 7nM; DOPAC, 0.8µM; homovanillic acid (HVA), 0.4µM; 3-methoxytyramine (3-MT), 2nM. Selegiline did not influence basal efflux of DA, HVA or 3-MT. However, basal DOPAC efflux was consistently decreased by selegiline (80-85% of control). Amphetamine induced an identical increase in DA efflux (700-800% of control) and decline in DOPAC (20-30% of control) and HVA (35-40% of control) in the presence and absence of selegiline. 3-MT efflux was consistently increased by amphetamine (150% of control) only in the presence of selegiline. Veratrine and ouabain also evoked similar patterns of DA overflow in the presence and absence of selegiline. 3-MT efflux induced by veratrine and ouabain was slightly greater in selegiline treated animals (250-300% of control) than in control animals (200-250% of control).

These data suggest that MAO-B plays a minor role in dopamine degradation both in unstimulated and chemically stimulated striata. The reduction in basal DOPAC efflux induced by selegiline may reflect a MAO-B mediated breakdown of extracellular DA to DOPAC in glia. However, a partial inhibition of MAO-A could also be responsible for these effects. The chemically induced stimulation of 3-MT efflux noted in the presence of selegiline most probably results from a MAO-B mediated degradation of released dopamine in glia. However, this relatively small response suggests that MAO-B does not play a major role in the degradation of extracellular DA.

Selegiline was a gift from Britannia Pharmaceuticals, Ltd.

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99.2

IN VIVO ASSESSMENT OF THE COMPARTMENTALIZATION OF DOPAMINE IN STRIATAL NERVE TERMINALS G.W. Arbuthnott, I.S. Fairbrother, S. Butcher. (Spon: European Neurosciences Association) MRC Brain Metabolism Unit and Department of Pharmacology 1 George Square, Edinburgh EH8 9JZ, UK.

In rats anaesthetised with Halothane we have examined the release of dopamine (DA) into the perfusate passing through a dialysis fibre implanted into the neostriatum. The actions of various releasing agents including K^+, amphetamine, veratrine, ouabain and tyramine have been studied. The amount of DA release is altered to different extents by pretreatment of the animals with reserpine, or α-methyl-p-tyrosine (αmpt), according to the releaser administered. Similarly the drugs show markedly different sensitivities to EGTA or to TTX applied through the probe. Veratrine and K^+ cause release from a pool which is exquisitely sensitive to TTX, EGTA and αmpt. Tyramine results in release which is little affected by TTX, or by EGTA. Amphetamine and ouabain share an intermediate position.

The amount of DOPAC in the dialysate is also differently altered by the various releasing agents. At doses that cause the release of DA, K^+ causes a marked reduction of DOPAC, whilst tyramine does not.

Thus release in vivo seems to derive from different nerve terminal pools with differing sensitivities to pharmacological manipulations.