Regulatory Effects of Nitric Oxide on Amino Acid and Monoamine Transmitter Release in the Brain
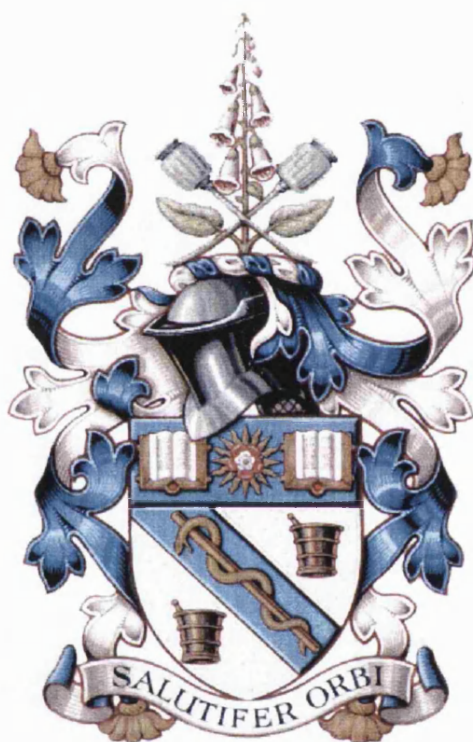
by

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Thesis submitted for the degree of Doctor of Philosophy
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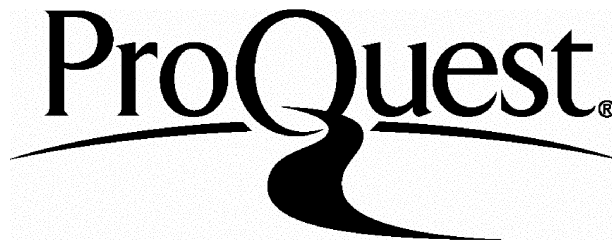
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Abstract

The effects of nitric oxide (NO) on neurotransmitter release in the hippocampus were investigated using in vivo microdialysis in conscious rats in an attempt to clarify the regulatory role of this messenger over basal and NMDA-evoked monoamine and amino acid release in this brain structure.

1.Infusion of N-methyl-D-aspartate (NMDA) caused a concentration-dependent increase in amino acid release and a decrease in monoamine release.

2. Infusion of S-nitroso-acetylpenicillamine (SNAP) at 5mM enhanced glutamate (L-GLU), aspartate (L-ASP) and γ-aminobutyric acid (GABA) release whilst at 500μM the donor decreased the release of these amino acids. In contrast, the release of dopamine (DA) and 5-hydroxytryptamine (5-HT) was reduced at 5mM but increased at 500μM SNAP.

3. The nitric oxide synthase (NOS) inhibitors 7-nitroindazole-monosodium salt (7-NINA), a selective neuronal NOS inhibitor, and N^G-nitro-L-arginine-methyl-esther (L-NAME), a non-selective inhibitor, were used to investigate whether the source of NO production (endothelial or neuronal) is important in determination of the final response. L-NAME at 1mM increased whilst 7-NINA, with exception of DA, decreased the levels of neurotransmitters. Co-infusion of the NOS inhibitors with NMDA blocked the NMDA-induced increase in L-GLU and L-ASP release. Both inhibitors reversed the NMDA-evoked inhibition of monoamine release.

4.Infusion of both inhibitors led to a decrease in the extracellular levels of citrulline (L-CIT). In contrast, arginine (L-ARG) levels were decreased by 7-NINA and increased by L-NAME.

5. The possible involvement of NO in the modulation of L-ARG and L-CIT release from glial cells in vitro was also investigated. In cultured cerebellar glia, SNAP caused a concentration-and Ca^{2+}-dependent release of L-ARG whilst the release of L-CIT was found to be neither concentration-nor Ca^{2+}-dependent. Administration of 8-bromo-cyclic-guanosine-monophosphate enhanced L-ARG and decreased L-CIT release whilst L-NAME caused a decline in the release of both amino acids from these cells.

6. It is concluded that NO modulates neurotransmitter release in the ventral hippocampus in vivo. The prevailing extracellular concentration of NO and also its source are important in determining the final response. In addition, this study has provided evidence that glial cells release L-ARG and that this might be involved in the supply of substrate to neurones during periods of sustained NOS activity.

Acknowledgements

For My Parents

"Discovery consists of seeing what

everybody has seen and thinking what

nobody thought"

Albert von Szent Gyorgyi

(1893-1986)

Publications arising from this Thesis

The following publications have resulted from original work presented in this thesis:

Publications:

Segieth, J., Getting, S.J., Biggs, C.S. and Whitton, P.S. (1995). Nitric oxide regulates excitatory amino acids release in a biphasic manner in freely moving rats. Neurosci.Lett., **170**,101-104.

Getting, S., Segieth, J., Ahmad, S., Biggs, C.S. and Whitton P.S.(1996). Biphasic modulation of GABA release by nitric oxide in the hippocampus of freely moving rats in vivo. Brain Res., **717**,196-199.

Segieth, J., Pearce, B.R., Fowler L.J. and Whitton P.S. Nitric oxide modulates N-methyl-D-aspartate (NMDA)-evoked dopamine release in the rat ventral hippocampus as measured by microdialysis in freely moving rats. In preparation.

Meeting abstracts:

Segieth, J., Getting, S., Biggs, C.S. and Whitton P.S. (1995). Effect of nitric oxide on basal and NMDA-evoked release of glutamate and aspartate release in vivo. Brit. J. Pharmacol., **115**, 114P.

Getting, S., Segieth, J., Ahmad, S. and Whitton P.S.(1995). Nitric oxide modulates basal and NMDA-evoked release of GABA in ventral hippocampus in vivo. Brit. J. Pharmacol., **115**, 25P.

Segieth, J., Pallota, M., Pearce, B.R. and Whitton, P.S. (1996). Regulation of hippocampal dopamine release by nitric oxide in the rat hippocampus. Brit. J. Pharmacol., **119**, 109P.

Segieth, J., Pearce, B.R. and Whitton, P.S. (1996). Nitric oxide modulates 5-HT release in rat ventral hippocampus. Brit. J. Pharmacol., **199**, 104P.

Segieth, J., Pearce, B.R., Fowler, L.J. and Whitton, P.S. (1997). Effects of nitric oxide synthase inhibitors on rat brain amino acid levels.An Soc.Neurosci., **319**,106.

Table of Contents   **Page**

LIST OF FIGURES   **Page**

List of Tables **Page**

ABBREVIATIONS

ACh	Acetylcholine
aCSF	Artificial cerebrospinal fluid
AMPA	γ-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ANOVA	Analysis of variance
ASL	Argininosucuccinate lyase
ASP	Aspartate
ASS	Argininosuccinate
L-ARG	Arginine
D-AP5	D (-)-2-amino-5-phosphonopentanoic acid
BSA	Bovine serum albumin
CaM	Ca^{2+}/calmodulin
L-CIT	Citrulline
cGMP	Cyclic guanosine 3', 5' monophosphate
CNS	Central nervous system
DA	Dopamine
DM	Disaggregation medium
DSMO	Dimethyl sulphoxide
DOPAC	3,5-dihydroxyphenylacetic acid
EAAT	excitatory amino acid transporter
EGTA	Ethylene glycol-bis (β-Aminoethyl ether)-N, N, N', N'-tetraacetic Acid
EDRF	Endothelium-dependent relaxing factor
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GABA	γ-aminobutyric acid
GM	Growth medium
L-GLU	Glutamate
L-GLN	Glutamine
Hb	haemoglobin
5-HIAA	5-hydroxyindoleacetic acid
HPLC	High performance liquid chromatography
HPLC-ED	High performance liquid chromatography with electrochemical

	detection
HPLC-FD	High performance liquid chromatography with fluorimetric detection
5-HT	5-hydroxytryptamine; serotonin
HVA	Homovanillic acid
LPS	Lipopolysaccharide
LTP	Long term potentiation
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
MK-801	(+)-5-methyl-10,11, dihydro-5H (a,d), cycloheptan, 5, 10-imine maleate
NA	Noradrenaline
NANC	Non-cholinergic non-adrenergic
NMDA	N-methyl-D-aspartate
L-NAME	N^G-nitro-L-arginine-methyl-esther
L-NMA	N^G-methyl-L-arginine
L-NMMA	N^ω-monomethyl-L-arginine
7-NI	7-nitroindazole
7-NINA	7-nitroindazole monosodium salt
OD	Outer diameter
ODS	Octadecylsilicate
OPA	O-phthaldialdehyde
PCP	Phencyclidine
sGC	Soluble guanylate cyclase
SNAP	S-nitroso-N-acetyl-penicillamine
SNP	Sodium nitroprusside
SNC	S-nitrosocysteine
L-SER	Serine
SWG	Standard wire gauge

TAU	Taurine
THB	Tetrahydrobiopterine
TPH	Tryptophan hydroxylase
PC	Personal computer

Chapter 1

General Introduction

1. Historical background to the discovery of nitric oxide (NO).

The first suggestion that nitrogen oxides were synthesised in mammals dates back to 1916 when it was shown that there was more nitrite in the urine of rats, pigs and humans than was present in their diet. However, these studies were not confirmed or extended until 1981 when it was demonstrated that nitrogen oxides were quantitively significant products of mammalian metabolism and conditions causing inflammation also led to an increase in their production (Green et al., 1981a).

In 1980, Furchgott and Zawadzki showed that vascular relaxation induced by acetylcholine (ACh) was dependent on the presence of the endothelium and provided evidence that this effect was mediated by a labile humoral factor that was later named endothelium derived relaxing factor (EDRF). EDRF-dependent vascular relaxation was found to occur in response to a variety of substances, including thrombin, bradykinin, substance P and the Ca^{2+} ionophore A23187. These findings prompted investigations into the humoral nature of EDRF using a variety of preparations. The most frequently used system consisted of a 'sandwich' arrangement of two rabbit aortic strips in which one strip possessing an intact endothelium (EDRF donor) was placed next to a strip without endothelium (detector) (Furchgott, 1984). Another procedure involved perfusion of the lumen of an intact rabbit isolated aorta, the effluent of which was used to superfuse endothelium-denuded vascular rings (Griffith et al., 1984; Rubanyi et al., 1986). It was established that EDRF was a short-lived substance with a half-life of few seconds in physiological salt solutions (Griffith et al., 1984; Cocks et al., 1985). Release of this substance occurred both under basal and stimulated conditions (Griffith et al., 1984) and was blocked by haemoglobin (Hb). In addition, vascular relaxation was inhibited by methylene blue a known inhibitor of cytosolic guanylate cyclase (sGC), indicating that the effects of EDRF were mediated by stimulation of this enzyme (Martin

et al., 1985).

These studies, performed in the vascular tissues, indicated resemblance in the pharmacological behaviour between EDRF and NO and led to suggestions that these substances may be identical (Furchgott et al., 1987; Ignarro et al., 1988). The first evidence that EDRF may be NO came from experiments in which EDRF was detected by chemical means used to identify NO. It was known that NO could be measured directly as a chemiluminescent product of its reaction with ozone (Downes et al., 1976). Using this method it was demonstrated that concentrations of bradykinin which induced the release of EDRF from porcine aortic cells in culture also caused the release of NO (Palmer et al., 1987: Radomski et al., 1987a). The amount of NO released by these cells was sufficient to account for the relaxation of vascular strips, inhibition of platelet aggregation and adhesion induced by EDRF (Palmer et al., 1987; Radomski et al., 1987a). Thus, the most important observation made during these studies was the correlation between the amount of NO measured by bioassay and that detected by chemiluminescence. In addition, a detailed comparison of the biological actions of NO and EDRF on vascular strips and platelets were found to be indistinguishable (Moncada et al., 1988; Palmer et al., 1987). These findings strongly supported the suggestion that EDRF is NO.

It is now known that NO is a small, relatively unstable, potentially toxic, diatomic free radical. The small size, lipophilic nature and instability of this molecule make it well suited for its role in local trans-cellular communication. Because of its small size and diffusibility, the actions of NO are determined by its chemical reactivity rather than special membrane transporters or receptors. In terms of its biological activity, the most important reactions of NO are with iron groups. The binding of NO to heme is an essential requirement for the activation of sGC and is the first step in the signal

transduction mechanism of this molecule. NO diffuses out of its cell of origin and into target cells where it activates sGC and results in the accumulation of cGMP. Thus, the cellular effects of NO are mediated by intracellular cGMP (Ignarro et al., 1987).

The fact that a simple gas made up of the two most common gases in the atmosphere plays an important role in mammalian systems led to the proposal that its emergence in the animal kingdom might be of an early evolutionary origin (Moncada et al., 1991). To date, NO has been found to be synthesised by animals as diverse as barnacles, fruit flies, horseshoe crabs, chickens, trout and humans (Radomski et al., 1991). The diversity of organisms, some which are very old in the evolutionary terms, suggests that NO may be among the prototypical signal molecules in the animal kingdom (Radomski et al., 1991).

1.1. Synthesis of NO

NO is synthesised from arginine (L-ARG) by nitric oxide synthases (NOS) with a stoichiometric production of citrulline (L-CIT) as a by-product (Mayer et al., 1989; Mulsh et al., 1989; Palmer and Moncada, 1989). The conversion of L-ARG to L-CIT and NO is a five- electron oxidation process of one of the guanidino nitrogen atoms of L-ARG (Palmer et al., 1988a,b; Schmidt et al., 1988). The first step, a two-electron oxidation, is a hydroxylation reaction that forms N^G-hydroxy-L-ARG as an intermediate (Leone et al., 1991; Pufahl et al., 1992;). The second step, a three-electron oxidation involves electron removal, oxygen insertion and carbon oxygen bond rupture to form L-CIT and NO (Stuehr et al., 1992). The same electron donor, reduced nicotinamide-adenine dinucleotide phosphate (NADPH), is required for both steps. The oxygens incorporated into NO and L-CIT are derived from different molecules of oxygen, which means that the enzyme binds and utilises two molecules of oxygen during one catalytic

turnover (Leone et al., 1991). This pathway is summarised in Fig.1.1.

$$O=O \qquad H_2O \qquad\qquad\qquad O=O \qquad H_2O$$

L-Arginine ⟶ ⟶ **N^G-Hydroxy-L-arginine** ⟶ ⟶ **L-Citrulline + NO**

$$NADPH \qquad NADP^+ \qquad\qquad 0.5\,NADPH \qquad 0.5\,NADP^+$$
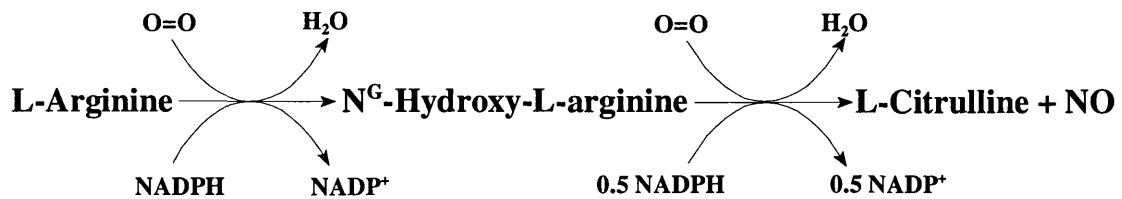
Fig. 1.1: Biosynthesis of Nitric Oxide.

The central route of L-ARG metabolism is the urea cycle. In the brain, the conversion of L-ARG to NO and L-CIT represents the only pathway for L-CIT production because the enzymes, which convert L-ornithine to L-CIT, ornithine transcarbamylase, is absent (Sadasivudu and Rao, 1976). Once produced, L-CIT can be converted back to L-ARG via the intermediate argininosuccinate suggesting that one role of the urea cycle in the brain is to re-synthesise the precursor of NO from its by-product L-CIT (Fig. 1.2).
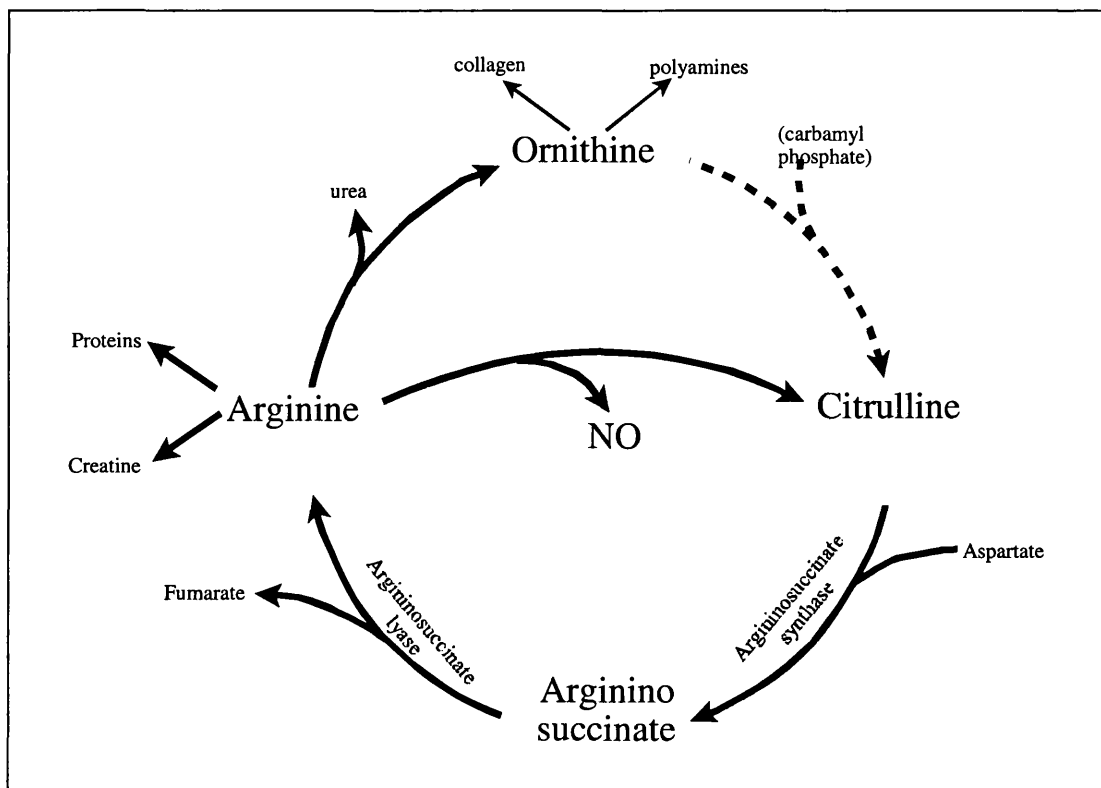
Fig.: 1. 2: L-ARG cycle in the brain (adapted from Garthwaite et al., 1991).

1.2 NOS ISOENZYMES

All known NO synthetases constitute a family of three isoenzymes that represent distinct gene products: the constitutive neuronal (nNOS) and endothelial (eNOS) and the inducible (iNOS) form which was first isolated from murine macrophages (Lamas et al., 1992; Lowenstein et al., 1992; Lyons et al., 1992; Sessa et al., 1992; Forstermann and Kleinert, 1995). All forms of the enzyme have now been cloned from various species and the amino acid sequences are more than 90% identical for nNOS and eNOS and greater than 80% identical for iNOS suggesting that they represent a highly conserved family of isoenzymes (Sessa et al., 1994).

Each NOS isoform has the same layout of catalytic domains: C-terminal

reductase with one binding site each for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH as well as an N-terminal oxygenase section (Bredt et al., 1991; Yamagata et al., 1993). The oxygenase domain contains binding sites for haem, L-ARG and tetra-hydro-pteridine (THB) (Chen et al., 1995; Ghosh et al., 1997). A Ca^{2+}/calmodulin (CaM) binding region separates the two domains (Abu-Sound and Stuehr, 1993).

NOS are strictly dependent on CaM which activates the enzyme by facilitating electron transfer within the reductase. An important difference between the isoenzymes is that while in eNOS and nNOS CaM binding responds to physiological changes in Ca^{2+} concentrations, iNOS binds CaM and is fully active at the lowest concentrations of Ca^{2+} encountered in vivo (Cho et al., 1992). Thus, both eNOS and nNOS are constitutive enzymes which require CaM for activity (Moncada et al., 1991; Pollock et al., 1991) and produce small amounts (pmols) of NO. Conversely, the inducible isoform (iNOS) is Ca^{2+}-independent despite the presence of CaM complex (Cho et al., 1992; Nathan et al., 1992) and produces higher levels (nmols) of NO.

The association of the three NOS isoenzymes with the endothelium, neurones and macrophages is, however, an oversimplification. For example, eNOS may also be found in platelets (Radomski et al., 1991) and certain neuronal populations (Dinerman et al., 1994a; Doyle and Slater, 1997), whereas nNOS has been found in the epithelium of the bronchi and in skeletal muscle (Schmidt et al., 1992; Nakane et al., 1993; Gath et al., 1995) and in some peripheral nerves (Hassall et al., 1992; Saffrey et al., 1992). In addition, eNOS can be induced under certain conditions such as chronic exercise and pregnancy (Weiner et al., 1994) whereas iNOS seems to be present constitutively in the kidney and some foetal tissues (Weiner et al., 1994).

7

nNOS is usually present in the soluble form (Bredt and Snyder, 1991), however, there is evidence that nNOS may also exist in a particulate form, mainly associated with the endoplasmic reticulum (Schmidt et al., 1992; Hecker et al., 1994). Since the particulate enzyme shows the same immunological characteristics as the soluble form, it is thought to represent the same protein (Matsumoto et. al., 1993). Furthermore, the membrane bound and soluble forms of the enzyme are both CaM-dependent and indistinguishable in terms of pH-dependency and inhibitor sensitivity. Although, nNOS is expressed constitutively in cells, its expression is subject to regulation. Its activity can be enhanced by stress, increases in glucose levels and by hormones such as oestrogen (Weiner et al., 1994). This enzyme may be subject to regulation by phophorylation in that nNOS purified from rat cerebellum was found to be a target for CaM protein kinase II, protein kinase A and protein kinase C (Bredt et al., 1991; Okada, 1995). However, contradictory data has been collected regarding the impact of phosphorylation on nNOS activity with investigators reporting both an enhancement and a reduction in enzyme activity (Nakane et al., 1991; Bredt et al., 1992; Dinerman et al., 1994b, Okada, 1995).

Purification of nNOS led to the production of antisera and the immunohistochemical localisation of the enzyme (Bredt et al., 1990a). In addition, molecular cloning enabled NOS mRNA to be localised by in situ hybridisation (Bredt et al., 1991). The highest density of nNOS has been found in the cerebellum where NOS mRNA is concentrated in granule and basket neurones. The Purkinje neurones, on the other hand, were shown to be devoid of nNOS. A high concentration of nNOS was also found in the olfactory bulb, especially in the accessory bulb. In the cerebral cortex and hippocampus, NOS occurs in medium to large aspiny neurones. In addition, the granule neurones of the dendate gyrus express nNOS in abundance but no imunohistochemical

staining was observed in pyramidal neurones of the hippocampal layer. However, studies with the histochemical stain NADPH diaphorase demonstrated the presence of nNOS in this brain structure. All nNOS neurones identified co-localise with the histochemical stain when it is performed under specific paraformaldehyde fixation (Hope et al., 1991). The NADPH diaphorase histochemical stain results from the reduction of tetrazolium dyes by diaphorase enzymes in the presence of NADPH, but not NADP, to a dark blue formazan precipitate (Thomas and Pearse, 1964). Human kidney cells 293 transfected with nNOS cDNA stain for both nNOS and NADPH diaphorase confirming that nNOS is responsible for the diaphorase staining under appropriate fixation conditions. It has been noted that paraformaldehyde fixation inactivates virtually all NADPH-dependent oxidative enzymes with exception of NOS (Matsumoto et al., 1993). In the hippocampus of the mouse, the diaphorase-like activity was found in activated astrocytes of the mouse hippocampus (Wallace et al., 1992), whilst in the human hippocampus nNOS was concentrated in small inter-neurones and pyramidal neurones of the CA1-CA3 regions (Doyle and Slater, 1997). This may indicate some degree of species difference in nNOS localisation. In the striatum, nNOS seems to be distributed in the cell bodies of scattered, medium to large aspiny neurones. nNOS has also been identified in the hypothalamus, mid-brain and at low levels in the medulla (Bredt et al., 1990a). Evidence exists to suggest that nNOS is also expressed in rat cerebellar astrocytes and Bergmann glia, and that it possesses all of the characteristics of the nNOS found in cerebellar granule neurones. This, in turn, suggests that both cell types express the same isoform of the enzyme (Agullo et al., 1995; Arbones et al., 1996; Kugler and Drenckhahn, 1996)

Histological studies have provided evidence that nNOS is also localised within the peripheral nervous system. nNOS was found to be present in the myentric plexus

from the oesophagus to rectum (Dawson et al., 1991;Bredt et al., 1990) and in adrenal glands, epithelial cells of the lung, kidney, pancreatic cells and skeletal muscle (Dun et al.; 1992: Dun et al., 1993; Schmidt et al., 1992; Nakane et al., 1993).

eNOS was first discovered in the vascular endothelium and is structurally similar to nNOS with an overall homology of 60% (Lamas et al., 1992). Unlike the two other isoforms, eNOS is found predominantly in the particulate form where it is attached to the plasma membrane via myristoylation at the N-terminal of glycine (Pollock et al., 1992a). Mutation of the N-terminal myristoylation site converts the eNOS from the particulate to the soluble fraction (Sessa et al., 1993). eNOS is regulated by CaM (Forstermann et al., 1991a; Pollock et al., 1991), thus any events which result in the influx of Ca^{2+} into the cell containing eNOS can cause activation of this enzyme and increased NO levels. For example, ACh, bradykinin, or shear stress will cause an influx of Ca^{2+} into endothelial cells, which results in NO synthesis. eNOS can undergo serine phosphorylation which in turn, leads to the translocation of the enzyme from the particulate to the cytosolic fraction (Michel et al., 1993). Phosphorylation of eNOS has also been described in response to shear stress (Corson et al., 1993); the physiological relevance of this phosphorylation remains to be determined. eNOS is found constitutively expressed in endothelial cells, however, multiple compounds and conditions have been described in recent years that regulate its expression. The activity of eNOS can be increased by shear stress caused by the flow of blood through vessels (Lamontagne et al., 1992) which also up-regulates eNOS expression (Nishida et al., 1992). In addition, oestradiol but not progesterone has been found to increase eNOS activity in guinea pigs at the last stage of pregnancy (Weiner et al., 1994). Also, incubation of human endothelial cells with oestradiol increased eNOS mRNA concentration (Forstermann and Kleinert, 1995).

Using a specific antibody to eNOS, immunohistochemical studies located the

enzyme to various types of arterial and venous endothelial cells in many tissues, including human tissues (Pollock et al., 1993). eNOS has been also been identified in kidney tubular epithelial cells, human placenta (Myatt et al., 1993) and the CA1 and dendate gyrus regions of the hippocampus (Dinerman et al., 1994a; Doyle and Slater, 1997). This, in turn, implies that the production of NO via eNOS may be of importance in neurotransmission.

During the early 1980s several investigators observed that treatment of rats with lipopolysacaccharide (LPS) led to the elevation of nitrate synthesis and correlated with the degree of fever in mammals (Green et al., 1981b; Wagner et al., 1983). Later, it was observed that blood levels and urinary excretion of nitrite and nitrite increased after exposure to LPS in LPS-sensitive mice and that activated mouse peritoneal macrophages exhibited increased nitrite and nitrate production in vitro (Stuehr and Marletta; 1985). In addition, the formation of nitrite and nitrate was dependent upon the presence of L-ARG (Iyengar et al., 1987). The demonstration of the synthesis of NO from L-ARG in mammals (Palmer et al., 1988b) indicated that this molecule was a most likely intermediate in the pathway of nitrite and nitrate synthesis in macrophages (Moncada et al., 1989). Subsequently, it was shown that NO synthesised from L-ARG was indeed the precursor for nitrite and nitrate in these cells (Hibbs et al., 1988; Marletta et al., 1988). In addition, treatment of tumour cells with NO was observed to produce cytostasis in these cells by causing inhibition of mitochondrial respiration, aconitase and DNA synthesis (Hibbs et al., 1988). This action of NO was though to be mediated via inhibition of iron containing enzymes in target cells (Hibbs et al., 1988; Lepoivre et al., 1992). The isoenzyme responsible for NO production in LPS exposed murine macrophages was found to be structurally distinct from the Ca^{2+} -dependent constitutive forms of NOS (Lowenstein et al., 1992; Lyons et al., 1992). This enzyme was not detectable in either

11

macrophage cell lines or peritoneal macrophages that had not been activated by an agent such as LPS (Marletta et al., 1988) and de novo protein synthesis was required for its expression. This isoform was found predominantly in the cytosol (Hevel et al., 1991), although some particulate activity has been reported in activated murine macrophages (Forstermann et al., 1992), but evidence suggests that these are the same proteins (Tracey et al., 1994). iNOS is CaM-independent but possesses the calmodulin-binding site implicating that iNOS binds calmodulin in a Ca^{2+}-independent manner. Since it has been shown that calmodulin binding to the enzyme is very tight, the possibility exists that calmodulin represents the constitutive subunit of iNOS (Cho et al., 1992) allowing the enzyme to be fully active at basal levels of Ca^{2+}.

Immunohistochemical localisation of iNOS in rats treated with LPS demonstrated the enzyme in various cell types including macrophages, some lymphocytes, neutrophils and eosinophils in red pulp of spleen: Kupffer cells, endothelial cells and hepatocytes in liver, alveolar macrophages in lung, mast cells and endothelial cells in colon (Bandaletova et al., 1993). Also, various human cell types can be induced in vitro to express iNOS (Asano et al., 1994; Robbins et al., 1994). Using immunochemistry, Kobzik et al. (1993) demonstrated iNOS-positive alveolar macrophages in inflamed areas of human lung, whilst Tracey et al. (1994) showed strong iNOS immunoreactivity in alveolar macrophages from patients with acute bronchopneumonia. No immunoreactivity was detected in normal human lung tissue. In addition, some molecules, other than cytokines and bacterial LPS, can be effective in inducing iNOS in human monocytes and macrophages. For example, incubation of human monocyte-derived macrophages with HIV Gp120 envelope glycoprotein has been reported to increase NO production (Pietraforte et al., 1994).

1.3 NO and signal transduction.

The first step in the signal transduction mechanism for NO is its interaction with haem iron group in sGC. This is thought be a reversible process (Henry et al., 1991) and an essential requirement if NO is to act as a switch for sGC activation. This enzyme converts guanosine triphosphate to the second messenger molecule cyclic guanosine monophosphate (cGMP). Vascular smooth muscles represent an example of a tissue type where NO elicits its biological action via the intracellular actions of cGMP. It is thought that cGMP activates a specific protein kinase, probably cGMP-dependent protein kinase which phosphorylates and inactivates myosin light chain kinase. The function of myosin light chain kinase is to phosphorylate myosin light chain, thereby resulting in smooth muscle contraction. Inactivation of this enzyme results in the dephosphorylation of myosin light chain and therefore smooth muscle relaxation (Draznin et al., 1989). Because of its physiochemical properties, namely small molecular size and lipophilicity, NO may access sGC with great speed. It has been noted that the addition of NO to vascular smooth muscle leads to an accumulation of cGMP in less than 5s and the onset of relaxation in about 10s (Gruetter et al., 1981; Ignarro et al., 1981).

However, not all effects of NO are a consequence of its stimulation of sGC and accumulation of cGMP. NO is a highly reactive chemical in its own right and so it is likely to have effects unrelated to stimulation of sGC. These include binding to iron-sulphur centres of enzymes, including enzymes involved in the mitochondrial electron transport chain, the citric acid cycle and DNA synthesis (Nathan and Hibbs, 1991; Wink et al., 1991), producing post-translational modifications of proteins via ADP ribosylation (Brune et al., 1994) and via reaction with superoxide to form peroxynitrite (Beckman et al., 1990).

1.4 NOS INHIBITORS

NOS inhibitors represent a class of compounds commonly used for the evaluation of biological responses mediated by NO. These can be divided broadly into the following groups: L-ARG based, haem-interacting amino acids and non-amino acid based inhibitors. The availability of these compounds is advantageous as NOS activity is not easily measured in vivo. This is due to the fact that the half-life of NO is very short (Moncada et al., 1988). The use of NOS inhibitors has provided a wealth of information about the actions of NO in the body. However, it should be noted that none of the commonly used NOS inhibitors are likely to be either completely specific towards a particular NOS isoform or resistant to metabolism to an other biologically active product. For example, N^G-nitro-L-arginine-methyl-esther (L-NAME) has been reported to possess some anti-muscarinic activity (Buxton et al., 1993), while N^G-amino-arginine (L-NNA) can cause seizures unrelated to NOS inhibition (Cobb et al., 1992). In addition, it is difficult to assess accurately the extent of NOS inhibition in a complex system. NOS inhibitors may not be able to access all compartments containing NOS and distinct isoforms of NOS differ in their sensitivity towards different inhibitors. Although the use of NOS inhibitors is not perfect, the advantages of using them greatly outweigh any problems.

L-ARG analogues were the first inhibitors of NOS discovered. The most widely used are L-NAME, N^G-methyl-L-arginine (L-NMA) and L-N^ω-monomethyl-L-arginine (L-NMMA) are   substituted with NH_2 at one of the guanidino (amino or ω-) nitrogens and NNO_2 and NCH_3 at the other (Fukuto et al., 1995). Generally the L-isomers represent the active form of the inhibitor while the D-isomers are biologically inactive but one exception is known. Both isomers of L-NNA have been found to inhibit endothelium-dependent relaxation of rat aortic rings (Wang et al., 1993). The

mechanism of inhibition of the different isoforms of NOS varies between different analogues but always involves occupation of the substrate binding site thus excluding L-ARG and preventing NO production. Many L-ARG analogues are competitive inhibitors such that their effects can be reversed by addition of L-ARG. There are also differences in the sensitivity of the various isoforms of NOS for these inhibitors. For example, L-NAME is a magnitude less potent against iNOS than is L-NNA, an effect that has been attributed to the esterification of the carbon group in L-NAME.

A variety of compounds which are neither L-ARG analogues nor amino acid based have also been developed. Examples include imidazoles (Wolf et al., 1993), 7-nitroindazole (Moore et al., 1993), guanidines (Hasan et al., 1993) and phencyclidine (PCP) (Osava et al., 1993). Amongst the constantly increasing list of new NOS inhibitors, only a very few exhibit any selectivity against particular NOS isoenzymes. Amongst these is 7-nitroindazole (7-NI) which has been reported to exhibit selectivity towards nNOS over iNOS and eNOS (Babbedge et al., 1993). The inhibition produced by this compound appears to involve several steps and involves binding to the haem group of NOS in an L-ARG and or THB reversible fashion depending on the isoform of NOS studied (Wolf et al., 1994) indicating that both the L-ARG and pteridine sites are affected. The important features of 7-NI are its biological actions, particularly its potent nociceptive action which is related to nNOS inhibition (Babbedge et al., 1993). Some characteristic changes in cerebral blood flow, distinct from the effects seen following non-selective NOS inhibition, are seen with 7-NI (Kovah et al., 1993). It also seems to reduce neurogenic oedema formation, presumably by blocking nNOS present in the peripheral nerves (Kajekar et al., 1995).

Aminoguanidine has received much attention as an inhibitor of NOS due to the early recognition of its selectivity towards iNOS (Corbett et al., 1992), its low

acute toxicity and its potential clinical usefulness. There are now numerous reports on the beneficial effects of aminoguanidine in various experimental models of inflammation and shock. In most in vitro systems, aminoguanidine is nearly equipotent with L-NMMA in its ability to inhibit NO production following exposure of macrophages to LPS. On the other hand, aminoguanidine is less potent than L-NMA on eNOS and nNOS in cell homogenates and in vivo (Wu et al., 1995b). The major effects of aminoquanidine range from preventing the interleukin-1- induced decrease in glucose secretion in islets of Langerhans, reducing the glucose- induced vascular dysfunction in rats, restoring contractile responses in vasclur tissue of animals treated with endotoxin in vivo and in vitro to its anti-inflammatory action (Griffith et al., 1993;. Szabo et al., 1994; Wu et al., 1995a). All these biological effects of this inhibitor have been linked to the inhibition of iNOS. A clear evaluation of the in vivo effects of aminoguanidine as an inhibitor of iNOS, however, is hampered by the fact that it has other effects. These include inhibition of histamine metabolism, inhibition of polyamine catabolism and inhibition of catalase (Bieganski et al., 1983; Seiler et al., 1985: Ou et al., 1993).

1.5. NO RELEASING COMPOUNDS

The evaluation of the biological effects of NO may also be performed using various NO-generating compounds. These have been broadly divided into nitrosothiols, iron-nitrosyl complexes, nitrite salts, sydnonimines and polyamine derivatives. In addition, authentic NO gas, in the form of caged NO, has been developed (Williams et al., 1993), which is stable in oxygen containing solutions and release NO upon u.v. radiation. The availability of these compounds is advantageous as they provide

information about the physiological role of NO. However, the effects of these compounds may not always be attributed to NO itself as some of the breakdown products of NO-releasing agents have been found to be biologically active. For example, sodium nitroprusside (SNP) has been observed to block L-GLU-gated channels via production of ferricyanide ions (Manzoni et al., 1992) whilst sydnonimines generate superoxide anions which can react with NO and H^+ to form peroxynitrite anions and hydroxen peroxide respectively (Manzoni et al., 1992). Nitrosothiols occur naturally in human plasma, however, the role of these compounds has yet to be determined. There is the possibility that S-nitrosoalbumin functions as a reservoir for NO by buffering its concentration. The two nitrosothiols which seem to provide the highest concentrations of NO are S-nitroso-cysteine (SNC) and S-nitroso-N-acetylpenicillamine (SNAP). SNC most readily decomposes to NO and native thiol in solution (Kowaluk and Fung, 1990). This compound is more potent than NO itself because its half-life is prolonged by cysteine in a concentration-dependent manner (Feelish et al., 1994). SNC has been suggested to be EDRF (Myers et al., 1990), but to date there is no convincing evidence to indicate that EDRF is a nitrosothiol rather than NO. SNAP, on the other hand, decomposes to penicillamine and NO and the release of NO occurs stoichiometrically. The role of penicillamine is still being questioned but it has been found to influence monoamine release in the brain (Guevara-Guzman et al., 1994). These compounds are, however, widely used in investigations into NO's actions due to their ability to release NO stoichiometrically. In addition, evidence exists to suggest that the effects of SNAP on neurotransmitter release in the brain are comparable to the effects of caged NO (Stout and Woodward, 1994).

1.6 Roles of NO in the body.

1.6.1 Cardiovascular system.

NO synthesised in the vascular endothelium participates in the general homeostatic control of the vasculature. Emerging evidence suggests that NO may act to regulate sympathetic activity (Sakuma et al., 1992) and the release of NO from non-adrenergic non-cholinergic nerves by yet unidentified stimuli may also contribute to vasodilatation (Gaw et al., 1991). However, more commonly, the release of NO occurs in response to pressure or shear force exerted by blood flow on the plasma membrane lining on the blood vessels, thus representing a very basic system of regulation of blood flow and pressure (Rubanyi et al., 1986).

Studies performed with competitive inhibitors of constitutive CaM-dependent NOS (Rees et al., 1989; Moncada et al., 1991) showed an increase in blood pressure in laboratory animals and therefore indicated that tonic NO synthesis is essential for maintenance of normal vasodilator tone. More recently, Huang et al. (1995) showed that disruption of the gene encoding for eNOS in mutant mice resulted in an increase in blood pressure and therefore confirmed that, under physiological conditions, NO synthesis via eNOS is required for the maintenance of vasodilator tone. When this normal level of NO is not produced, the vascular muscles do not relax to an appropriate degree and the resulting vasoconstriction is responsible for increase blood pressure and contributes to hypertension (Sase et al., 1997).

NO produced in endothelial cells relaxes the underlying vascular smooth muscle, opening up the blood vessels and thus flushing away microaggregates; it will also be released towards the lumen of the blood vessels to prevent platelet adhesion to the endothelium and inhibit platelet aggregation. These events are mediated via cGMP

18

(Radomski and Moncada, 1993). Platelets themselves also contain eNOS (Radomski and Moncada, 1993) and appears to be activated in a contraregulatory fashion, so those factors that increase aggregation and adhesion also activate platelet eNOS, thus modifying the increase (Radomski et al., 1990). NO may also be involved in the regulation of leucocyte/vessel wall interaction in vitro (Bath et al., 1991) and it inhibits leucocyte activation in vivo (Kubes et al., 1991). Moreover, NO has been demonstrated to modulate smooth muscle cell proliferation by increasing levels of cGMP (Garg and Hassid, 1989).

1.6.2 HOST DEFENCE

The biochemical basis for NO's action as a cytotoxic agent is its reaction with non haem iron centres in key enzymes of cellular respiration and the synthesis of DNA in target cells (Hibbs et al., 1988; Nathan and Hibbs, 1991). NO produced in stimulated macrophages diffuses into surrounding tissues where it acts with the iron-sulphur centres of several important macromolecules. These include aconitase, an enzyme involved in tricarboxylic acid cycle, and succinate-dependent cellular oxygen consumption at the level of complexes 1 and 2 of the mitochondrial electron transport chain (Hibbs et al., 1988; Sung and Dietert, 1994). NO's high affinity for iron may result in the removal of iron from iron-sulphur centres and the formation of dinitrosyl iron species within the proteins involved. Inhibition of aconitase blocks the metabolism of acetyl coezyme A to carbon dioxide, an important step in the generation of NADH and therefore oxidative phosphorylation (Drapier and Hibbs, 1988). Inhibition of complex 1 blocks the first step of an electron transport process necessary to transfer the energy of NADH into ATP. Inhibition of complex 2 also disrupts energy transfer from a number of processes, including fatty acid oxidation and citric acid cycle, that deliver energy in the form of

reduced flavin. NO has also been shown to inhibit ribonucleotide reductase, the enzyme

that converts ribonucleotides to the deoxyribonucleotides necessary for DNA synthesis

(Lepoivre et al., 1990; Know et al., 1991). NO may cause damage to the ribonucleotide

reductase by targeting one of the following three groups: thiol groups, non-haem iron or

tyrosine groups and therefore depleting the cell of the precursors for DNA synthesis

(Stampler, 1994; Gross and Wolin, 1995). Inhibition of DNA synthesis may represent an

important way in which macrophages inhibit the growth of rapidly dividing tumour cells.

Thus, the resulting inhibition of enzyme activity is thought to participate in cytotoxic

effects of macrophage NO on tumour cells (Marletta et al., 1988; Hibbs et al., 1988;

Stuehr et al., 1989; Moncada et al., 1991). Finally, following induction of iNOS, the

prolonged period of NO release means that NO may itself be cytotoxic. NO can be

cytotoxic not only for invading microorganisms but also for the cells which produce it

and for neighbouring cells (Lepoivre et al., 1989; O'Connor and Moncada, 1991; Palmer

et al., 1992). It appears that NO reacts with superoxide ion to form peroxinitrite which,

in turn, yields highly damaging hydroxyl radicals and nitrogen dioxide (Beckman et al.,

1990). It has been suggested that NO acting via these kind of interactions plays a role in

some forms of tissue damage such as vascular injury produced by tissue deposition of

immune complexes (Mulligan et al., 1991).

NO synthesis is also part of the inflammatory response against invading

pathogens. Thus, NO seems to be involved in a general defence system triggered by

stimuli such as endotoxins, cytokines and live and dead bacteria which are known to

induce non-specific immunity. Evidence suggests that NO is involved in acute and

chronic inflammation. NOS inhibitors were found to reduce the degree of lesion in

models of both acute and chronic inflammation (Ialenti et al., 1992; 1993a, b). In

addition, immune complex-induced vascular injury in rat lungs and dermal vasculature

was attenuated by NOS inhibition (Mulligan et al., 1991). The iNOS protein was detected in brain macrophage/microglia-like cells after viral infection and during experimental autoimmune encephalomyelitis (Van Dam et al., 1995) and elevated mRNA was found in demyelinating regions in multiple sclerosis (Bo et al., 1994). Although these and other observations support a role for NO in the pathogenesis of infectious and inflammatory brain diseases, pharmacological studies on the effects of NOS inhibitors have provided conflicting results (Zielasek et al., 1995). Thus, the origin of NO in the inflammatory process is unclear but it is possible that both the constitutive and inducible forms in blood vessels, neutrophils and macrophages are involved.

It is clear that NO can kill intracellular parasites (Hibbs et al., 1988). Early studies demonstrated that leishmania protozoa growing in macrophages were killed following induction of iNOS and that charide, a constituent of bacterial cell walls that is released in septic shock, can induce synthesis of iNOS in vascular as well as smooth muscle cells (Busse et al., 1990; Beasley et al., 1991; Gross et al., 1994). This induction also occurs in human sepsis and leads to widespread relaxation of vascular smooth muscle and thus hypotension. It has been shown that overproduction of NO by the induced enzyme is responsible for the hypotension seen in septic shock (Kilbourn et al., 1992). These studies have now been extended and confirmed. An enhanced formation of NO due to the activation of eNOS (acute phase of shock) and particularly following the induction of iNOS in the vascular wall (late phase of shock) contributes to hypotension in animals and man with septic shock. Moreover, endotoxin does not cause hypotension in mice in which the gene for iNOS has been inactivated (iNOS knock-out mice) (MacMicking et al., 1995). Thus, the original hypothesis that an enhanced formation of endogenous NO contributes to the hypotension associated with septic shock is now supported by studies using both pharmacological and molecular approaches (reviewed

by Thiemermann, 1997).

1.6.3 Peripheral nervous system.

An NO-dependent mechanism is known to be involved in sensory transmission as NOS inhibitors were found to exhibit anti-nociceptive activity in the mouse (Moore et at., 1991). Evidence has accumulated to suggest that NO may be a transmitter of at least some of the NANC nerves. L-ARG analogues were shown to inhibit NANC-mediated relaxation in the rodent anococcygeus muscle (Gillespie et al., 1989, Gibson et al., 1990) whilst enhancing the contractile response to noradrenergic stimulation (Li and Rand, 1989). NO was demonstrated to be responsible for the NANC-mediated relaxation of gastric fundus (Li and Rand, 1990) and inhibition of the lower oesophageal sphincter (Tottrup et al., 1991). Moreover, NO has also been demonstrated to act as a modulator at some of the NANC nerves. For example, L-ARG has been demonstrated to reverse the constipation induced by morphine in mice, but not that by atropine (Calignano et al., 1991). This selective modulation of opioid-induced constipation is thought to involve L-ARG increasing the amount of NO released by NANC nerves in the gut. The NO pathway has also been shown to have an important role in relaxation of human and rabbit corpus cavernosum (Holmquist et al., 1991). It is likely that NO is the transmitter released during penile erection (Holmquist et al., 1991; Burnett et al., 1992). A possible modulatory role for NO has been proposed in other systems. NO contributes to the NANC-induced vasodilatation and relaxation of guinea pig and human tracheal muscle (Belvisi et al., 1992). In addition, inhibition of NO synthesis in conscious rats induces urinary bladder hyperactivity and decreases bladder capacity (Persson et al., 1992).

1.7 NITRIC OXIDE IN THE CNS.

During the 1970s, well before acidic amino acids were accepted as the major excitatory neurotransmitters in the central nervous system (CNS), it was observed that application of glutamate (L-GLU) by microinjection in vivo or to brain slices in vitro resulted in an increase in cGMP levels (Ferrendelli et al., 1974). Later, it was demonstrated that this response also occurred in vivo (Biggio et al., 1976). At the same time, other investigators found that sodium azide and another nitrogen-containing molecule, hydroxylamine, were also able to cause an increase in cGMP levels (Kimura et al., 1975). In 1977, Deguchi demonstrated that the soluble fraction of rat forebrain contained low molecular weight substance which activated sGC. Similar findings were reported in the rat cerebellum (Miki et al., 1977; Katsuki et al., 1977). It was therefore proposed that NO, an active species formed from nitro and nitroso-compounds, was responsible for the activation of sGC. In 1988, Garthwaite et al. reported that stimulation of rat cerebellar cells with the L-GLU receptor agonist N-methyl-D-aspartate (NMDA) induced an elevation of cGMP levels which was associated with the release of EDRF-like material. In addition, the cells that released this EDRF-like material were not the target cells in which cGMP levels were elevated. Further investigation of NO in the brain revealed that NO was inhibited by Hb, unstable, able to relax smooth muscle and its release following L-GLU receptor stimulation was Ca^{2+}-dependent.

In the CNS, NO has been shown to be intimately linked with the action of excitatory amino acids such as L-GLU. Therefore, as a preface to discussing the functions of NO in the CNS it is necessary to review briefly the action of this amino acid in the brain.

1.7.1 L-GLU AS A NEUROTRANSMITTER.

L-GLU is widely accepted as the predominant excitatory amino acid neurotransmitter in the mammalian brain and spinal cord. The first evidence implying a function for L-GLU in the brain came from a study by Hayashi (1954) who observed that application of this amino acid to the motor cortex led to convulsions. Later, Curtis et al. (1959) demonstrated the direct postsynaptic excitatory effects of L-GLU suggesting the possibility that this amino acid may act as a neurotransmitter in the brain. These initial discoveries led to studies with various L-GLU analogues which elicited the general structural requirements for receptor activation (Curtis and Watkins, 1960, 1963). Consistent with a role as a neurotransmitter, L-GLU shows an uneven distribution in the brain with different neurones exhibiting different sensitivities (McLennon et al., 1968; Duggan et al., 1974). L-GLU is mainly localised in presynaptic terminals where it is stored in vesicles. The release of L-GLU has been shown to be increased on stimulation of afferent pathways in vivo (Jasper and Koyama, 1969) and to be dependent on extracellular Ca^{2+}. Moreover, high affinity transporter systems first identified by Logan and Snyder (1971) have now been purified and cloned (Arriza et al., 1993; Shafgat et al., 1993). To date four different transporters have been identified. Two of the first transporters to be cloned, excitatory amino acid transporter 1 (EAAT1) (Storck et al., 1992) and excitatory amino acid transporter 2 (EAAT2) (Pines et al., 1992) are located predominantly in glia whilst excitatory amino acid transporter 3 (EAAT3) is expressed in neurones (Arriza et al., 1994) and excitatory amino acid transporter 4 (EAAT4) in cerebellum (Fairman et al., 1995). It has been suggested that these distinct transporters play different roles in maintaining L-GLU function in the brain (Rothstein et al., 1996). Using molecular biological methods, where the expression of these transporters was prevented, it was shown that deletion of the glial transporters resulted in an increase in

extracellular L-GLU concentration and cell death, whilst EAAC1 function did not alter the extracellular L-GLU levels but caused epileptic-like seizures. These results indicate that glial transporters may function mainly to keep the L-GLU concentration low in the extracellular space whereas neuronal transporters play a role in synaptic transmission (Rothstein et al., 1996).

L-GLU is known to interact with various receptor subtypes (Watkins and Evans, 1981; Lodge and Collingridge, 1991). These can be broadly divided into ionotropic and metabotropic L-GLU receptors. The ionotropic L-GLU receptors were the first to be characterised and based on electrophysiological studies, three distinct receptors have been characterised. These are ligand-gated ion channels named NMDA, α-amino-3-hydroxy-5-methyl-isoxazole-4-priopionate (AMPA) and kainate receptors (Monaghan et al., 1989; Hollmann and Heinemann, 1994). Since no L-GLU receptor agonist or antagonist clearly distinguishes kainate and AMPA receptors these are often collectively referred to as non-NMDA receptors. However, recent cloning studies have clearly demonstrated that AMPA and kainate receptors are distinct receptor complexes although they can be activated by the same agonists (Hollmann and Heinemann, 1994). The NMDA and AMPA receptors show a parallel distribution in the brain and are mostly found in the hippocampus and the cerebral cortex (Hollman and Heinemann, 1994). Kainate receptors are also abundantly expressed in various brain regions (Hollman and Heinemann, 1994b). Despite the widespread distribution, the characterisation of functional kainate receptors has only been achieved in dorsal root ganglion neurones (Huettner, 1990) and in cultured hippocampal neurones (Lerma et al., 1993).

The metabotropic L-GLU receptors belong to the family of G-protein coupled receptors (Masu et al., 1991; Houamed et al., 1991). This family consists of eight members and these are subdivided into three groups based on the second messenger

system to which they are coupled and amino acid homology in their sequences (Abe et al., 1992; Minakami et al., 1993; Nakajima et al., 1993; Okamoto et al., 1994; Saugstad et al., 1994). Group 1 receptors (mGLU$_1$ and mGLU$_5$) stimulate phospholipase C (PLC) as revealed by an increase in phosphoinositide turnover and Ca^{2+} release from internal stores. The G-proteins involved in the activation of PLC by group 1 mGLU receptors have not been clearly identified but evidence suggests that the Gq family of G-proteins of the PLC-activating, pertussis toxin (PTX)-insensitive are involved (Abe et al., 1992). Group 2 (mGLU$_2$ and mGLU$_3$) and group 3 (mGLU$_4$, mGLU$_6$, mGLU$_7$ and mGLU$_8$) act by inhibition of adenylate cyclase (Tanabe et al., 1993). It is apparent that the G-protein involved in this coupling is of the Gi family (Pin and Duvoisin, 1995). This is based on experiments where addition of PTX terminated the transduction mechanism in all cases. These receptors are widely distributed in the brain. For example, particularly high levels of expression of mGLU$_1$ are found in the hippocampus, the cerebellum and olfactory bulb whilst the expression of mGLU$_6$ is restricted to the retina (Hollman and Heinemann, 1994).

1.7.2 The NMDA receptor

NMDA receptors mediate neuronal signalling, affect gene expression, and are involved in neuronal plasticity, axonal outgrowth or synaptogenesis during brain development. Excessive stimulation of NMDA receptors can lead to neuronal death and may be a common final pathway for the pathogenesis of many neurological diseases. The involvement of the NMDA receptors in such a variety of physiological and pathophysiological events in the brain led to extensive studies of this receptor. To date a wealth of data has accumulated from many different sources to establish that NMDA receptors have a very different molecular biology, pharmacology and excitation

behaviour to those not activated by NMDA.

1.7.2.1 Special features of NMDA receptors

The agonist NMDA, a synthetic analogue of aspartic acid that does not occur naturally in the brain (Watkins, 1962), selectively activates one type of L-GLU receptor channel, hence the name NMDA receptors. Responses to NMDA show a strong block by (D (-)-2-amino –5-phosphonopentanoic acid) D-AP5, a specific competitive antagonist of the NMDA receptor complex and by a non-competitive (channel blocking type) antagonists such as PCP and (+)-5-methyl-10, 11, dihydro-5H (a, d) cyclopentan, 5,1-imine maleate (MK801). In addition, Zn^{2+} produces selective and non-competitive antagonism of NMDA receptors in cultured neurones (Mayer et al., 1987; Sucher et al., 1996). It is thought that the inhibitory actions of Zn^{2+} are due to reduced channel open time. Moreover, responses at NMDA receptors have the unusual properties of a strong voltage-dependence and high Ca^{2+} permeability. Under physiological conditions, the NMDA receptor is blocked by Mg^{2+} ions (Mayer et al., 1984). Mg^{2+} ions sit in the channel of the NMDA receptor and the block is released upon independent depolarisation of the membrane, perhaps by non-NMDA receptors. Thus, the NMDA receptor requires presynaptic activity (L-GLU release) and postsynaptic activity (depolarisation of the postsynaptic membrane). The NMDA receptor is therefore a novel combination of a ligand-activated and a voltage-operated channel requiring both L-GLU and depolarisation for activity. Apart from L-GLU, the NMDA receptor complex requires glycine as a co-agonist for activation (Johnson and Asher, 1987). The binding of glycine potentiates the binding of L-GLU to the NMDA receptor. Kleckner and Dingledine (1988) postulated that the NMDA receptor is characterised by an absolute requirement for glycine. It is now accepted that L-GLU and glycine interact with each other in an allosteric manner, generally two molecules of glycine and two molecules of

L-GLU are needed for NMDA receptor activation (Thedinga et al., 1989). This phenomenon is very important physiologically as glycine is ubiquitous in the extracellular space while L-GLU is released transiently from the nerve terminal. Thus, the NMDA receptor is the only ligand-gated ion channel that requires the binding of two different agonists for activity. Another well established example of an allosteric regulation of NMDA receptors involves the modulation by polyamines. These effects are complex, in part because polyamines appear to act by multiple mechanisms. Electrophysiological studies coupled with the molecular biology technique using recombinant receptors demonstrated that polyamines do not directly activate NMDA receptors but act to potentiate or inhibit L-GLU-mediated responses (Hollman and Heinemann, 1994). Two potentiating effects of polyamines have been described so far: glycine-independent and glycine-dependent stimulation. The first effect occurs when spermine increases L-GLU-induced currents in the presence of saturating concentrations of glycine whilst the latter results in increased affinity of the receptor for glycine. The inhibitory effects of polyamines can also be subdivided into voltage-dependent channel inhibition and a decrease in the affinity of the receptor for L-GLU. The modulatory actions of polyamines are governed by the receptor subtype composition. In addition, NMDA receptor complex possesses a redox modulatory site which consists of thiol groups that form disulphide bonds within the channel itself (Aizenman et al., 1989). The redox site is also critical for normal functioning of the receptor as it acts as a gain control for current flux through NMDA receptor-operated channels and can affect the degree of neurotoxicity produced by excessive NMDA receptor activation. Reducing and oxidising agents, such as dithiothreitol and ascorbic acid, potentiate and inhibit the NMDA receptor complex respectively (Aizenman et al., 1989). Recently, NO generating compounds have been shown to affect the redox modulatory site on NMDA receptor

complex (Lei et al., 1992; Lipton et al., 1993; 1994).

1.7.2.2 Molecular Biology of NMDA receptors.

The current model of the composition of NMDA receptors suggests that these receptors are assembled from two distantly related subunits, the NMDAR1 (NR1) subunit (Moriyoshi et al., 1991) and an NR2 subunit, of which four types (NR2Ato 2D) have been characterised (Ikeda et al., 1992). Many lines of evidence suggest that natively expressed receptors are likely to comprise NR1 and at least one of the NR2 class. Although the primary structure of NMDA receptors are known, the membrane topology of the subunits or the stoichiometry of the subunits in native or recombinant channels have not yet been clarified. The mature subunits of NMDA receptors, and of the other ionotropic L-GLU receptors contain four hydrophobic sequence regions. On this basis the subunits are expected to cross the lipid bilayer four times to assume a membrane topology as established for the nicotinic ACh receptors, whose subunits have extracellulary located amino and carboxy termini.

The NR1 subunit is a principal constituent of the NMDA receptor, being expressed in nearly all central neurones (Moriyoshi et al., 1991). The most important property of this subunit is its ability to self-assemble into functional receptors, sharing many of the properties of native NMDA receptors. These include the requirements for glycine binding for channel activation, voltage-dependent Mg^{2+} block and high permeability to Ca^{2+} (Moriyoshi et al., 1991). The second subunit of the NMDA receptor is named NR2. So far four types of NR2 have been identified (NR2A-NR2D) (Monyer et al, 1992) and are highly related to each other but bear no homology to NR1 subunit. The evidence that NR2 subunits are part of the NMDA receptor complex come from co-expression studies where the presence of both subunits results in potentiation of agonist-evoked currents (Monyer et al., 1992). This, in turn,

suggests that NR1 and NR2 assemble to form a functional NMDA channel as NR2 does not produce a functional channel on its own. The fact that four different NR2 subunits have been cloned suggests that at least four different subtype of NMDA receptor can exist. Evidence suggests that natively expressed NMDA receptors are likely to comprise an NR1 subunit and at least one of the NR2 subunits (Hollman and Heinemann, 1994). Based on studies using recombinantly expressed NR1-NR2 subunit receptor combinations, it was found that each combination of the receptor possess distinctive pharmacological and biophysical properties. These include sensitivity to modulation by glycine (Wafford et al., 1993), affinities for agonists and antagonists (Yamazaki et al., 1992), polyamines (Williams et al., 1994), thestrengthof Mg^{2+} block (Monyer et al., 1992) and sensitivity to reducing agents (Sullivan et al., 1994). The way the subunits assemble themselves into one of the types of the NMDA receptor complex may play crucial role in the effects produced following the NMDA receptor activation. For example, the NR1 subunit lacks the redox modulation site while in combination with NR2 this site is readily expressed suggesting the absolute requirement of the NR2 subunit for the expression of the redox site (Sullivan et al., 1994). Contrary to this suggestion, the NR1 subunit has been found to possess two cysteine residues necessary for formation of redox modulation (Sullivan et al., 1994). This, in turn, implied that the conformation of NR1 subunit, when assembled with the NR2 subunit, might differ from the conformation of NR1 when a homomeric receptor complex is formed. It is possible that the presence of the NR1 subunit leads to the conformation of the NR2 subunit in heteromeric complexes or that the subunits influence each others conformation.

The NMDA receptor can be localised in the brain by receptor radiography using either the NMDA-displacable component of high affinity $[^3H]$-L-GLU binding, or by

30

using high affinity NMDA receptor antagonists while expression can be monitored by in situ hybridisation. The receptor is widely distributed in the CNS, particularly in the cerebral cortex and basal ganglia. Highest levels are found in the CA1 region of the hippocampus where the Schaffer collateral pathway terminates (Hollmann and Heinemann, 1994).

1.7.3 NO IN NEUROTRANSMISSION

Neuronal production of NO is triggered when L-GLU released from one neurone diffuses to a neighbouring neurone and interacts with NMDA receptors on the postsynaptic membrane allowing Ca^{2+} influx and the subsequent binding of this ion to calmodulin. The CaM complex activates nNOS leading to the conversion of L-ARG to NO and its by-product L-CIT. NO then activates sGC within the postsynaptic neurone by binding to the iron of the enzyme's haem prosthetic group. NO may also diffuse back to activate sGC in the presynaptic neurone and astrocytic processes which surround most synapses. As NO diffuses freely in all directions, its true sphere of influence has not yet been determined.

Evidence suggests that NMDA receptor activation appears to be an important trigger for cGMP synthesis in the cerebellum, however, this does not mean that L-GLU stimulated NO formation is under the sole control of the NMDA receptors. In the adult cerebellum, AMPA and kainate are able to elicit an accumulation of cGMP through the formation of NO as is 1-aminocyclopentane-1.3-dicarboxylic acid, an agonist for metabotropic L-GLU receptors (Southam et al., 1991). It is thought that depolarisation evoked by ionotropic L-GLU receptor activation leads to Ca^{2+} entry from the extracelullar space and the subsequent activation of NOS whilst activation of metabotropic receptors results in NOS activation due to the liberation of Ca^{2+} from

intracellular stores.

Astrocyte process

cGMP

GC

GC cGMP

Presynaptic Terminal

Glu

Ca^{2+}

Na^+

NMDA AMPA

⊕

Dendrite

NO

NOS

O_2

Arginine

Citrulline NADP NADPH
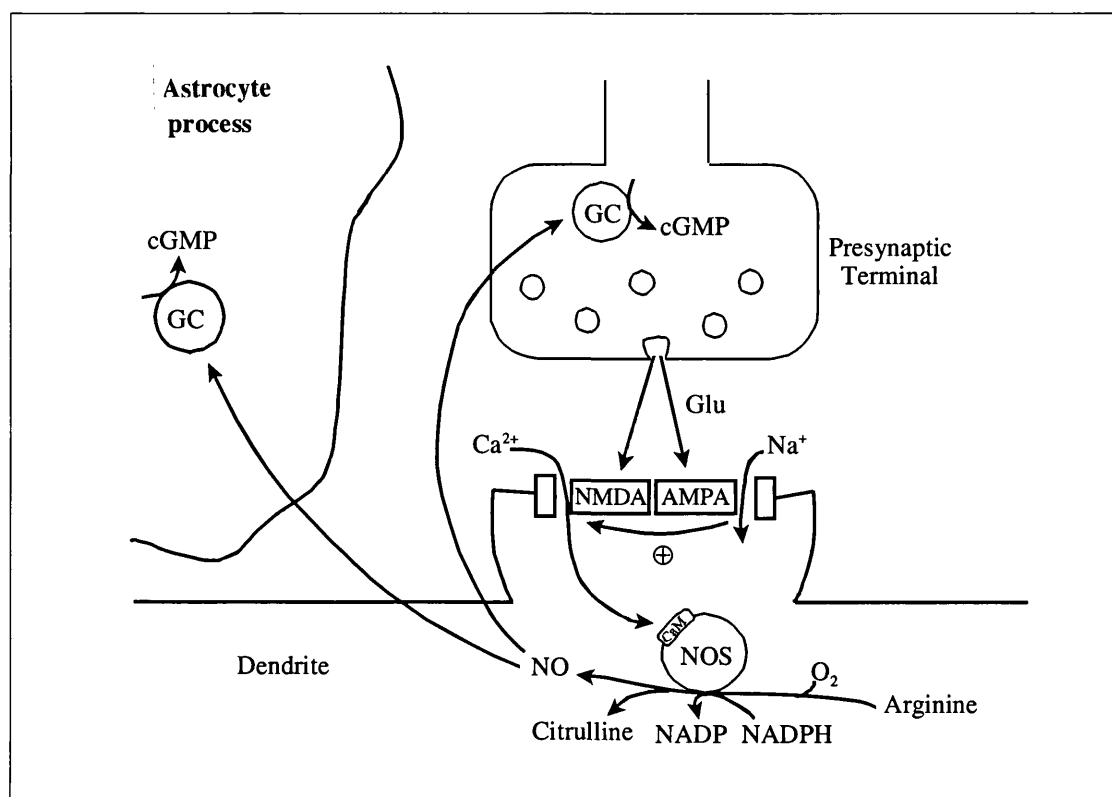
Figure 1.3: Representative model for the operation of the NO system in the CNS (adapted from Garthwaite et al., 1991).

The precise physiological role of neuronal NO has yet to be elucidated although it is thought that NO may serve to relay information about postsynaptic NMDA receptor activation to neighbouring neurones and/or glia (Fig.1.3) (Southam and Garthwaite, 1993). Thus, NO can be generated presynaptically following action potential-dependent Ca^{2+}influx or postsynaptically as a result of a rise in cytosolic Ca^{2+} concentration that is a consequence of NMDA `or other receptor activation.

Evidence also suggests that NO produced in response to NMDA receptor activation can also act on glia. Studies performed in the lesioned cerebellum (Garthwaite and Garthwaite, 1987) and on purified cerebellar cells (Bunn et al., 1986) indicate that the increase in cGMP levels associated with NMDA receptor activation

occurs predominantly in glial cells (Garthwaite et al., 1988). In accordance with these results, immunocytochemical studies revealed that the elevations in cGMP that follow NMDA receptor activation in slices of immature rat cerebellum are located predominantly in astrocytes and Bergmann glia (De Vente et al., 1990), further suggesting the NO-mediated line of communication between neurones and glial cells. It is now evident that astrocytes display both constitutive and inducible NOS activity under various conditions and that activated microglia express inducible NOS (Murphy et al., 1993). Astrocytes from rat cerebellum in primary tissue culture have been observed to express constitutively a Ca^{2+}-dependent NOS activity with the characteristics of the nNOS isoform (Arbones et al., 1996). The NOS activity of astrocytes was shown to possess the same dependency on L-ARG and Ca^{2+}, as well as comparable inhibitor sensitivity to granule cell NOS, indicating that the same isoform is expressed in both cell types. Using chemiluminescence detection of NO or activation of sGC, several agonists have been found to activate the Ca^{2+}-dependent isoform of NOS. Noradrenaline (NA) and quisqualate were found to be most effective. The NA effect depends on extracellular Ca^{2+} and remains under conditions that abolish Ca^{2+} mobilisation. This is supported by the effectiveness of Ca^{2+} ionophore suggesting that intracellular Ca^{2+} is insufficient to activate this isoform of the enzyme. The effect of quisqualate has been found to be mimicked by ibotenate but not by AMPA suggesting the involvement of metabotropic L-GLU receptors. Collectively, these findings indicate NO production from non-neuronal CNS sources and therefore potential "nitrinergic" roles for glial cells.

Neurone-glia signalling by NO may also extend outside the CNS. In some dorsal root ganglia, NOS is mainly contained in neurones, a proportion of which are particularly enriched in the enzyme. Exposure of these ganglia to NO donors leads to

an accumulation of cGMP in the surrounding cells but not in the neurones (Morris et al., 1992).

It appears that glutamatergic activation of NMDA receptors and the subsequent production of NO is associated with a number of physiological and pathophysiological events. These include a long lasting enhancement of synaptic transmission known as the phenomenon of long term potentiation (LTP), (Bohme et al., 1991), convulsions (Mollace et al., 1991) and neurotoxicity (Dawson et al., 1991; Gross et al., 1991).

Current thinking now holds that changes in the synaptic function of the brain underlie the formation and storage of cognitive memories. The hippocampus, because it has been identified as a brain area important in the formation of memory, has been a focus of research in this field. Transient activation of the NMDA receptor has been shown to be prerequisite for the first stage of LTP induction (Bliss and Collingridge, 1993) whilst the expression and maintenance of this phenomenon is believed to involved presynaptic changes (Bliss and Collingridge, 1993). Thus, LTP is induced postsynaptically but may be expressed, at least in part, presynaptically. This, in turn, indicated the need for a retrograde messenger which would process the information to the presynaptic terminal when the postsynaptic requirements have been met. The discovery of NO led to speculation that this molecule may play a role in the LTP. Indeed, a wealth of evidence now exists to suggest an important role for NO in LTP as inhibitors of NOS have been found to block various phases of LTP (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991). Moreover, it has been shown that in hippocampal slices bathed with the NMDA receptor antagonist D-AP5, NO itself induces LTP when paired with tetanic stimulation but not when applied in the absence of this presynaptic activity (Zhuo et al., 1993). This observed activity-

dependence of the NO-induced potentiation can explain how NO may mediate the input-specific nature of LTP; only those synapses that are active during LTP induction become potentiated (Barrioneuvo and Brown, 1983). In contrast, some investigators have suggested that NOS inhibition can facilitate induction of LTP (Kato et al., 1993), whilst Izumi et al. (1993) have shown the development of LTP can be differentially regulated according to the level of NOS activity in hippocampal slices. These authors suggested that the level of NOS inhibition might determine whether induction or inhibition of LTP occurs. For instance, a low concentration of NOS inhibitor L-NMMA was shown to induce NMDA-dependent LTP, whilst at a high concentration this inhibitor blocked NMDA-induced LTP. The ability of NO to induce as well as inhibit LTP indicates the possibility of a dual action of this molecule in the brain.

Considerable evidence exists to suggest that excessive stimulation of NMDA receptors by L-GLU may cause cell death under conditions such as cerebral ischaemia. L-GLU-mediated neurotoxicity has been associated with neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases (Choi, 1988; Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994). The role of L-GLU in ischaemic cell death as well as excitotoxicity has been extensively studied. It is believed that most of the deleterious processes that take place following ischaemia are triggered by the massive rise in intracellular Ca^{2+} levels. Ca^{2+} can enter neurones either through the cationic channel associated with the NMDA receptor or through voltage-operated Ca^{2+} channels. Excessive NO synthesis may be one of the biochemical events triggered by the overstimulation of NMDA receptors during ischaemia. It has been demonstrated that addition of NO-generating compounds such as SNP induces the death of cultured cortical neurones in vitro and that this effect is antagonised by Hb (Dawson et al., 1991). In addition, local injection

of SNP was found to induce a very extensive degeneration of pyramidal cells and dentate granule cells in the hippocampus (Loiacono and Beart, 1992). Collectively, these data show the possibility that excessive NO synthesis might be deleterious to the CNS. However, early studies which investigated the inhibition of NO synthesis on ischaemic brain damage in a rat model of focal cerebral ischaemia proved to be inconclusive. L-ARG analogues such as L-NNA and L-NAME administered following permanent occlusion of the left middle cerebral artery were shown to reduce (Buisson et al., 1992a,b; Nagafuji et al., 1992; Carreau et al., 1994), enhance (Yamamoto et al., 1992) or not affect (Dawson et al., 1992; Buchan et al., 1994) the extent of damage. High doses of L-NAME were shown to worsen the effects of cerebral ischaemia whilst repeated administrations of lower doses often resulted in neuroprotection (reviewed by Iadecola, 1997). Thus, the proposal that NO mediates toxicity in the brain remains controversial, in part because of the use of non-selective agents that block NO formation in neuronal, glial and vascular compartments. It has been observed that in mutant mice deficient in nNOS, infarct volumes were decreased significantly 24 and 72h after middle cerebral artery occlusion and that the neurological deficits were less than those in normal mice (Huang et al., 1994). However, infarct size was shown to be larger in the mutant after NOS inhibition with L-NNA administration. These findings suggest that neuronal NO production appears to exacerbate acute ischaemic injury whereas vascular NO protects after middle artery occlusion. Consequently, more consistent results have been obtained with selective NOS inhibitors. 7-NI reduced cerebral ischaemic damage, an effect associated with inhibition of nNOS but not eNOS (Huang et al., 1994). There is also strong evidence that at high concentrations NO is highly neurotoxic whilst at lower concentrations it might play a neuroprotective role within the brain since it reduces NMDA- or L-GLU-

36

evoked Ca^{2+} entry into striatal cells (Manzoni et al., 1992;).

NO has been shown to mediate an increase in the release of L-GLU in a number of in vitro preparations (Lonard et al., 1992; Meffert et al., 1994; Montague et al., 1994). Various NO donors increased the release of this neurotransmitter from hippocampal slices and application of the NOS inhibitors, L-NMMA and 7-NI, led to inhibition of NMDA receptor-evoked release of L-GLU from cerebellar cortex synaptosomes (Montague et al., 1994; Jones et al., 1995). Likewise, Guevara-Guzman et al. (1994) and Bogdanov et al. (1997) demonstrated a similar relationship between NO and L-GLU release in the striatum in vivo. These observations suggest that NO might primarily exert stimulatory actions within the CNS and, since enhanced L-GLU levels have long been associated with the generation of seizure activity (Hayashi et al., 1954), it led to speculation that this molecule exhibits pro-convulsant effects in the brain. However, the association between NO and seizures in vivo does not appear to be simple. Several reports have indicated that inhibitors of NOS can potentiate the behavioural effects of convulsant drugs which suggests an anti-convulsant role for NO (Penix et al., 1994; Przegalinski et al., 1994; Kelly et al., 1995) whilst other studies suggest that NO has pro-convulsant properties (Mollace et al., 1991; Baggeta et al., 1992; De Sarro et al., 1993). To account for these discrepancies, it has been proposed that the influence of NO may vary with the model of seizure employed (Kirby et al., 1996). It has been demonstrated that both 7-NI and L-NAME greatly exacerbated seizures following the systemic administration of kainate whilst delaying picrotoxin-induced seizures in mice (Kirby et al., 1996). Moreover, these investigators observed that the effects vary both with the dose of the convulsant, the dose of NOS inhibitor, route of administration of NOS inhibitor as well as the species used in the study. Collectively, these findings indicate that specific experimental variables determine

whether NO exerts pro or anti-convulsant actions in the brain.

9. AIMS OF THIS STUDY.

The preceding section of the introduction, containing evidence regarding the involvement of NO in synaptic transmission in the brain, together with a brief review of the known pharmacological tools used to investigate the role of this messenger in biological systems. Whilst in vitro models such as brain slices, synaptosomal preparations and tissue cultures have been extensively utilised in the past to study the action of NO in the CNS, it is impossible to recreate the environment of nervous tissue in vitro. In addition, most of the NOS inhibitors used are non-selective, thus limiting their usefulness in determining the effect of NO in vivo.

Given the overall confusion surrounding the effects of NO on synaptic transmission in the brain, the following study was undertaken in order to clarify the following points:

1) to investigate the role of NO in the regulation of monoamine and amino acid release in the hippocampus in vivo.

2) to examine the relationship between NMDA receptor activation, neurotransmitter and NO release in the hippocampus.

Chapter 2

Materials and Methods

2.1 Introduction to microdialysis

2.1.1 In vivo microdialysis study.

In vivo microdialysis technique is now an established and reliable technique for the measurement of extracellular levels of neurotransmitters. The brain is particularly responsive to study using this technique (Benveniste, 1989) and is now the method of choice for in vivo measurements of the extracellular space. Low molecular weight compounds diffuse down their concentration gradients from the brain extracellular fluid into an artificial cerebrospinal fluid (aCSF) that flows at a constant rate through the dialysis fibre which is implanted into a selected brain region, the samples collected can then be analysed using highly sensitive assay procedures such as high performance liquid chromatography (HPLC). This method allows manipulation of the extracellular environment by drugs that may enter the CNS by any route (either infused by the dialysis probe or applied systematically). The advantages of this technique are summarised in table 2.1 whilst details of the microdialysis procedure are provided in section 2.3 of this chapter. As indicated in the general introduction, the hippocampus is considered to play crucial role in synaptic transmission and this is associated with the production of NO. In addition, this structure is richly innervated by monoaminergic and amino acid neurotransmitter pathways allowing the collection and analysis of a large number of neurotransmitters and metabolites using the microdialysis technique. Since many of these substances share roles in controlling neuronal excitability via interaction with receptors, uptake systems and transporters, thus being able to alter the time and type of response (stimulatory/excitatory), it seemed logical to examine the effects of NO in this structure. Methodologically the hippocampus represents a good region for microdialysis study. Fig 2.2 illustrates the relative ease with which microdialysis

probes can be implanted into this structure. All microdialysis experiments presented in the next chapters used conscious animals, thereby overcoming the problems associated with anaesthesia-induced neurochemical changes, such as the compromising of neuronal function and possible interactions with test drugs which affect neurotransmitter efflux (Marsden, 1985).

2.2 Animals and Husbandry

Male albino Wistar rats (280g-320g; Bantin and Kingman., Ltd., Hull, U.K.) were used in all *in vivo* experiments. Prior to experiments, animals were group housed under conditions of constant humidity and temperature (22 ± 1^0 C). Animals were conditioned to a light-dark cycle with light from 07:00 to 19:00 every day. Access to food (standard rodent diet) and water was ad libitum.

Table 2.1: Major advantages and disadvantages of in vivo monitoring techniques (Modified from Benveniste, 1989).

	Advantages	Disadvantages
In situ Ion-selective Microelectrode	a: Examination of all brain regions b: Time resolution < 1s c: Tip of electrode < 4μm	a: Only detects ions
In situ Carbon fibre microelectrode	a: Examination of all brain regions b: Use in conscious animals c: Time resolution < 1min d: Top of electrode < 300μm	a: Only detects oxidisable compounds b: Selectivity poor without previous HPLC analysis c: Drainage d: Some electrodes have short working life *in vivo*
Ex situ Cortical cup	a: No tissue penetration b: Use of conscious animals	a: Time resolution > 10min b: Only cortex can be analysed c: Drainage d: Deproteination of compounds e: Enzymatic degradation of compounds
Ex Situ Push-pull cannula	a: Examination of all brain regions b: Use in conscious animals c: BBB* intact following implantation	a: Time resolution > 10min b: Drainage c: Deproteination before HPLC d: Enzymatic degradation of compounds e: Tissue trauma following implantation f: Cannula size > 1mm
Ex Situ Microdialysis Probe	a: Examination of all brain regions b: Use in conscious animals c: BBB* intact following implantation d: Probe diameter < 600μm e: Minute tissue trauma within the first 48h f: No need for deproteinisation g: No enzymatic degradation	a: Time resolution > 5min b: Drainage

*Blood brain barrier.

2.3 Principles of microdialysis

2.3.1 Probe preparation

The dialysis probes were constructed (see Fig. 2.1 for schematic diagram) as follows. A steel cannula (24swg stainless steel, 1.5cm length, 0.52mm O.D.; Tomlinson Tube and Instrument Ltd., Warwick.) formed the body of the probe through which two lengths of fine fused silica tubing (Scientific Glass Engineering, Milton Keynes) were inserted. One length of the silica tubing (1cm= probe inlet) emerged from the opposite end of the cannula whilst the other was fed approximately half the distance through the cannula (0.5cm=probe outlet). A drop of epoxy resin (Ciba-Geigy Plastics, Ltd.) adhesive was used to secure the two lengths of silica in position at the appropriate end of the cannula. Once the adhesive had dried, two pieces of non-sterile, fine-bore polyethylene tubing (pp 10 tubing, 0.28mm I.D., 0.61mm O.D.; Portex Ltd., U.K.), both 5cm in length were fitted and formed the inlet and outlet of the probe. The junction between the fused silica and the polyethylene tubing was coated with epoxy resin and allowed to dry. This was followed by trimming any excess silica from the inlet cannula to 4mm. To complete the probe, a hollow fibre dialysis membrane (10 KD molecular weight cut-off point; Cuprophan capillary membrane, type Fl 8 200, 0.2mm O.D., Gambro, GFE9, Hechingen, Germany) was fed over the trimmed silica tube such that when cut, the dialysis fibre extended 0.5mm beyond the end of this tube. The cut end of the dialysis fibre was carefully sealed with a small drop of epoxy resin and the opposite end of the fibre secured to the inner circumference of the 24swg cannula in the same manner. Each stage of the construction was performed under a binocular microscope. After the epoxy resin had set, probes were gently perfused with (aCSF) (composition: KCl 2.5mM, NaCl 125mM, $MgCl_2$ 1.18mM and $CaCl_2$ 1.26 mM, pH 7.0.) to test their

43

functional integrity. At this point any probe which showed high resistance to the flow

of aCSF or failed to allow passage of fluid was discarded . For all experiments, very

low internal volume tubing (1.2 µl/100mm; FEP tubing, Carnegie Medicine, Sweden)

was fitted to the probe output tube. This facilitated the rapid transit of perfusate via

the dialysis membrane to a collecting vial allowing a more accurate temporal

correlation between collection of substances at the dialysis membrane/ brain tissue or

dialysis membrane/bathing fluid interfaces and their appearance in the collecting vial.

This was particularly important in the case of monoamine neurotransmitters since

these are subject to oxidation.

2.3.2 In vitro recoveries of the microdialysis probes

The term recovery is defined as the ratio between the concentration of the

particular substance in the outflow solution and the concentration of the same

substance in the solution outside the probe. Absolute recovery is the amount of the

substance harvested in the outflow per unit time. In order to obtain an estimation of

the extracellular concentration of monoamines and amino acids from the dialysis

experiments, the recovery of these species from the bathing medium by probes

perfused at an appropriate flow rate were calculated.

INLET   OUTLET

POLYETHYLENE
TUBING

EPOXY RESIN

FUSED SILICA

STAINLESS STEEL
SHAFT

DIALYSIS
MEMBRANE

EPOXY RESIN
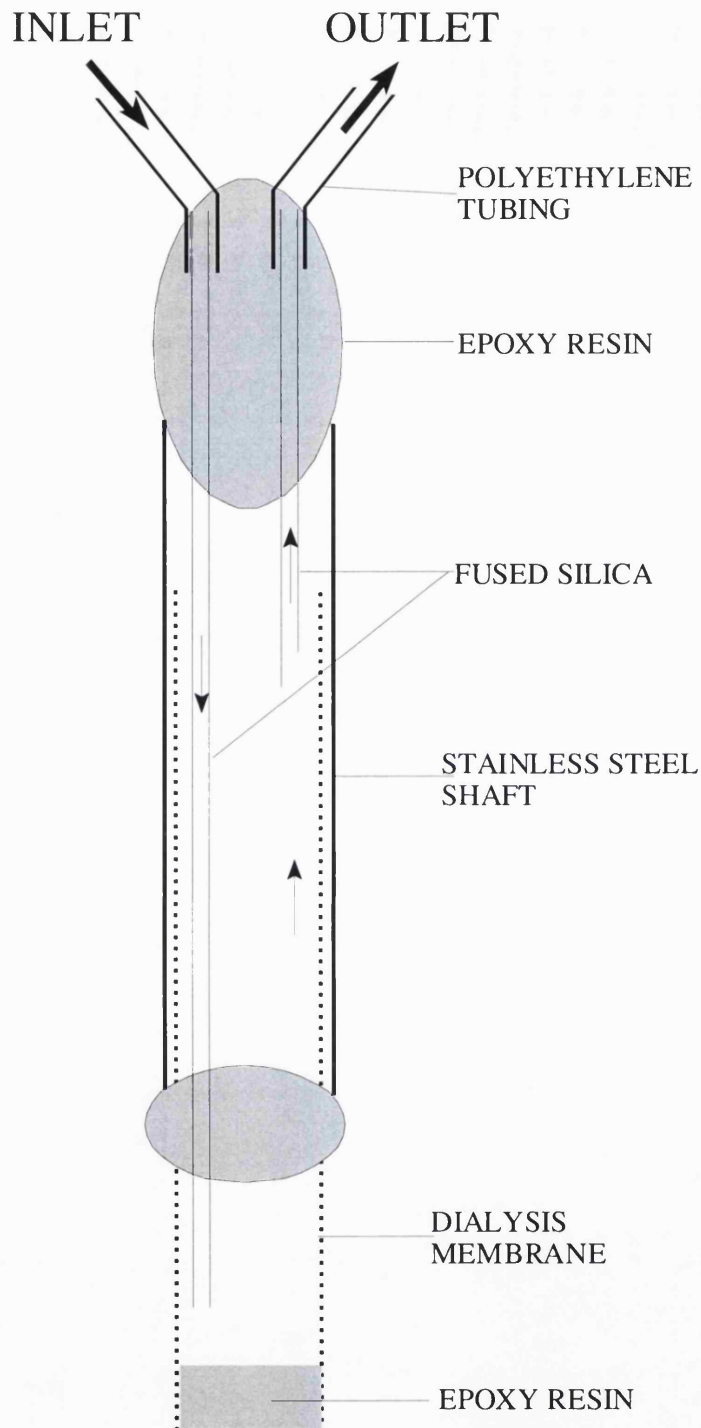
Fig. 2.1: Schematic representation of a concentric dialysis probe. Arrows depict direction of flow.

The probe was continuously perfused at a constant flow rate of 0.5μl/min via a model 22 infusion pump (Harvard Apparatus, U.S.A.) with aCSF while being suspended in bathing medium containing a solution of dopamine (DA), 5-hydroxytryptamine (5-HT), 3,4-dihydroxyphenyl acetic acid (DOPAC), 5-hydroxyindole acetic acid (5-HIAA) and homovanillic acid (HVA). The concentration of monoamines and their metabolites was 1μM with L-cysteine (20mg/100ml) added to limit oxidation (Chai and Meltzer, 1992). In all experiments, aCSF was delivered to the probe input tubing via an appropriate length of pp10 tubing fitted to a gas tight 500μl microsyringe (Hamilton). Collected fractions were immediately analysed for monoamine and metabolite content using HPLC with electrochemical detection (HPLC-ED). All monoamines and metabolites were obtained from Sigma, U.K.

The estimations of amino acid recoveries were carried out in a similar manner as described for the monoamines. The bathing medium contained aspartate (L-ASP), L-GLU, γ-amino-butyric-acid (GABA) and serine (L-SER), at concentrations of 1μM in distilled double-deionised water without addition of L-cysteine. The perfusion fluid was identical to the medium outside the probe except for the substance of interest. The probe was infused for at least 30min before samples were collected for analysis using HPLC with fluorimetric detection. All amino acids were obtained from Sigma, U.K.

From these experiments, the % recovery of analytes from bathing was calculated as follows: (Reproduced from Benveniste, 1989).

The % of recovery from the bathing medium for both amino acids and monoamines was calculated using the following formula.

a.) $\text{Recovery}_{vitro} = C_o / C_i$

Where C_o is the concentration of the substance in the outflow and C_i is the substance

concentration in the medium. Bearing in mind that the conditions in vitro can only approximate to those in vivo, estimates of actual brain extracellular levels of monoamines and amino acids were made in subsequent microdialysis experiments on the basis of the relationship;

b). $C_i = C_{out} / Recovery_{vitro}$

Where C_i is the extracelullar concentration and C_{out} is the extracellular concentration in vivo outflow solution

ANALYTES	% RECOVERY (range; n=6)
DA	26-27
DOPAC	25-27
5HT	18-20
5HIAA	19-21
HVA	18-20
ASP	37-41
GLU	49-53
GABA	48-52
SER	50-53

TABLE 2.2. % Recovery of dialysate monoamines and amino acids from mixed standard solutions.

The material that dialysis probes are made of not only allows the passage of water and small solutes but it may also interact with some of these solutes. Therefore, the presence of surface charges may explain, for instance, the lower recoveries of acidic amino acids as compared to neutral ones. It is important to stress here that probes with unusually low recoveries were not used in microdialysis experiments.

2.3.3 Stereotaxic surgery and probe implantation

All animals used in these experiments were subjected to the same surgical technique for implantation of microdialysis probes. Rats were anaesthetised with chloral hydrate (Sigma, UK) dissolved in 0.9% saline (400mg/kg injected i.p.) then placed in a stereotaxic frame (David Kopf, U.S.A.) with the incisor bar set according to the size of the animal. For rats weighing between 220g-350g, the setting was adjusted to between -3.3mm to 2.5mm. Once the incisor bar was correctly adjusted, the head of the animal was placed parallel to the frame base plate. The surface of the skull was cut using a scalpel and two skin flaps reflected laterally to expose the skull surface, excess blood was removed using cotton wool. Membranous material was removed from the top of the skull and bregma located visually. This was followed by locating the ventral hippocampus using stereotaxic co-ordinates (Paxinos and Watson, 1982). Once located, a pencil was used to mark the insertion point for the probe and a dental drill fitted with a tungsten carbide burr tip (2mm) was positioned directly above it. The co-ordinates used were as followed: A-5.0mm, L-5.0mm from bregma, 7.5 mm below dura. A hole was drilled at the insertion point of the skull and the dura matter exposed. Two additional holes were drilled in the skull and 2mm stainless steel grub screws (size 10B, Clerkenwell Screw Company, Holborn, London) fastened into each using a jeweller's screwdriver. One of the screws was placed posterior and the other anterior to bregma. All the probes were implanted unilaterally into the brain without prior insertion of guide cannulae. The probe was mounted into a probe holder and secured by means of a bolt to the frame. Insertion into the hippocampus was performed using a magnifying lens. Once the tip had made contact with the dura, it was lowered gently to the required depth by means of a graduated thumbwheel on the instrument manipulator. During this process the probe was gently perfused with aCSF

in order to limit compression of the dialysis membrane by the underlying tissue. This was particularly important as it prevented the collapse of the membrane in situ. The probe was secured by putting dental acrylic (Heathco, Bolton, Lancs.) around the probe and allowing it to dry, thus anchoring the whole assembly. After the acrylic was completely solid, the probe holder was removed and more acrylic spread around the skull to ensure durability. Once the acrylic was dry, rats were removed from the stereotaxic frame, wrapped in paper towels to limit post-surgical hypothermia and placed in cages.

All the cages were purpose built from perspex and animals were allowed constant access to food and water. Implanted animals were allowed a postoperative recovery period for approximately 18h prior to experimentation. This recovery period was necessary since evidence suggests that implantation results in tissue damage, principally oedema (Edvinsson et al., 1971). Therefore, the implanted microdialysis probes often recover excessive quantities of analytes that do not reflect true estimations of extracellular basal levels. This is supported by findings (Westerink and De Vries, 1988) where dialysate neurotransmitters recovered following acute probe implantation were demonstrated to be derived from damaged nerve terminals rather than steady-state release from neuronal stores.

2.3.4 General procedures: microdialysis

This section details the experimental protocol common to all microdialysis studies performed during this study. The day following implantation, rats were prepared for dialysis. All experiments were performed using freely moving rats. A gas-tight microsyringe was filled with aCSF and the needle connected to the pp10 tubing fitted. The tubing was then flushed with fluid and connected via a short length

of wider bore tube to the probe inlet and the syringe plunger gently depressed to assess the efficiency of the system. Once the probe was found to be working, its outlet was connected to a length of FEP tubing (20 $\pm$ 3cm) and the system flushed with aCSF. A gas tight 500μl syringe was then connected to the tubing and gently placed on a Harvard Apparatus syringe infusion pump type 22. The aCSF was perfused via the probe at a rate of 0.5μl/min and in all cases a 60min fraction was collected and discarded prior to the collection of further dialysates. This allowed the elimination of abnormally high levels of analytes that would have accumulated in the vicinity of the dialysis membrane during the post-operative period. Apart from the initial 60min pre-dialysis sample collection, all other samples were collected at 30min intervals. In all experiments, four consecutive samples were collected to establish basal release of monoamines and amino acids. This was followed by infusion of various drugs via the dialysis probes and experiments continued for up to 420 min.

All reagents used for the aCSF came from Sigma U.K. In the experiments where monoamines were measured, the aCSF contained 1.0μM citalopram in order to elevate basal 5-HT to detectable levels.

2.3.5 Anatomical verification of probe placement

After completion of each of the microdialysis experiment, the location of the probe in the brain was verified. An overdose of sagital (expiral, 250 mg/kg i.p.) was used to sacrifice rats. Their brains were removed and quickly frozen in isopentane at -40^0C. Coronal brain sections ($10\mu m$) were cut on a cryostat and mounted on gelatin-coated slides. Inspection of probe placement was made by visualisation of the probe tract. A typical probe placement is shown in Fig 2.2 and the tract of the probe stem can clearly be seen entering the hippocampus.

Fig 2.2: Photomicrograph illustrating a typical microdialysis probe placement.

The arrow shows the entry track of the probe into the hippocampus, with the

membraneous area (area for diffusion extending 4mm below thisMagnification x15)

2.4 High performance liquid chromatography

All collected dialysates obtained from in vitro recovery and in vivo experiments were analysed for amino acid and monoamine content. Samples were not subjected to pre-analysis treatments such as acidification or deproteination. As a general procedure, dialysates to be analysed for monoamine content were injected into the HPLC-ED system on the day of collection or were stored at -80° C for a period not exceeding one week. Samples to be analysed for amino acid content were injected into the HPLC-FD system or stored at -80° C stored for a period not exceeding one month.

2.4.1 Chromatographic conditions and system configuration

The HPLC system used for the detection and quantification of monoamines consisted of the following components: Gilson solvent delivery pump (model 303), a C18 reverse phase column (3μm particle size ODS; 4.6mm I.D.X 100mm;Rainin Dynamax Instrument Co. INC., U.S.A.) protected by a microsorb guard column (4.6mm I.D.x 15mm; C18 5μm: Rainin Dynamax Instrument Co. Inc., U.S.A) and ESA electochemical detector model 5100A with porous carbon electrode analytical cell 5011(Severn Analytical, Bedfordshire,U.K.). Both column and analytical cells were protected by a ESA model 5020 guard cell.

Data were collected via a Dell Corporation PC system 310 (Dell Corporation, U.S.A.) interfaced with the electrochemical detector via a data collection unit (Drew Ltd., U.K.) and via an in line BBC Goerz Metrawatt SE 120 chart recorder. This latter equipment allowed a further 10-fold amplification of the detector output. Fig. 2.3 illustrates a representative trace for separation of dialysate monoamines.

The separation technique used was based on that of Hutson et al. (1989) with

modifications to allow concurrent detection of DA and its metabolites. All separations were isocratic and the mobile phase had the following composition: Sodium acetate (90mM), citric acid (35mM), EDTA (0.43mM) and 0.06mM sodium octylsulphonic acid (as an ion pairing reagent) with 11% methanol, pH 4.2. Helium- degassed mobile phase was pumped at a flow rate of 1.0ml/min.

2.4.2 Detection

A guard cell was set at +500mV and placed in front of the column and the analytical cell. This arrangement was necessary in order to reduce the background interference, occurring due to pre-oxidation of the mobile phase ahead of the injector. The analytical cell detected electroactive species. The ECD settings were as follows: Electrode 1: -175mV and the second electrode was set at +400mV, which appears to be an optimal sensitivity for the oxidation of 5-HT, 5-HIAA and HVA.
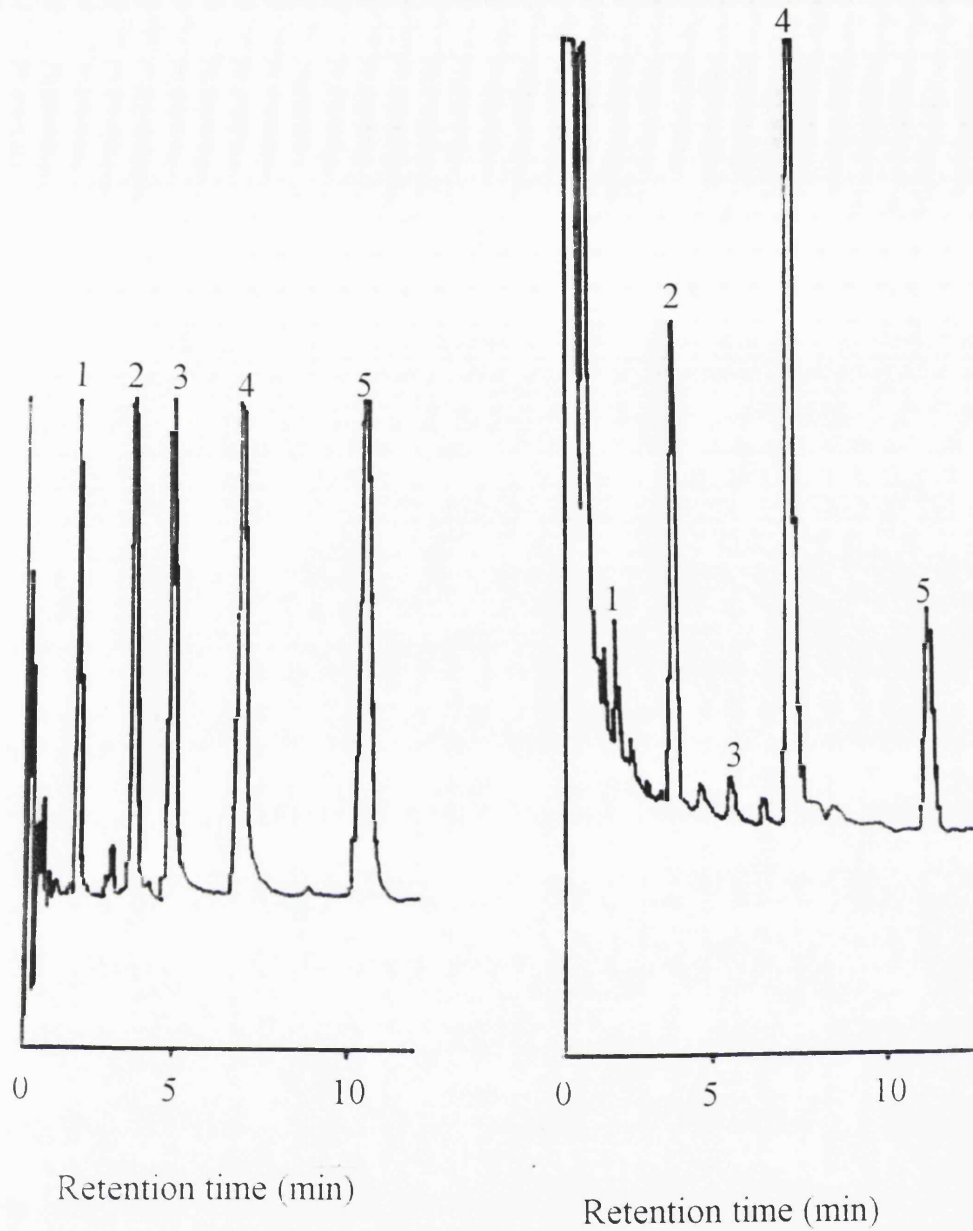
Fig 2.3: Typical chromatograms obtained following separation of monoamines using HPLC with ED. (A) shows a representative standard mixture and (B) is typical representation of basal monoamine output obtained in the ventral hippocampus. Horizontal axis represents elution times in min. Peaks denoted are: (1) DA, (2) DOPAC, (3) 5-HT, (4) 5-HIAA, (5) HVA.

2.4.4 Maintenance and calibration of the HPLC-ED

After completion of each experiment the flow rate was decreased to 0.1 ml/min and the electrochemical cells switched off. The mobile phase was changed approximately every three weeks and the equilibration time of a new mobile phase took approximately one day. The HPLC-ED was regularly calibrated with standard solutions of monoamines and metabolites to ensure that peak identification following dialysate analysis could be made. Stock solutions (0.5mM) containing a mixture of DA (as the hydrochloride), DOPAC, 5-HT, 5-HIIA and HVA were prepared in the mobile phase with addition of few drops 1mM HCl and stored as 1ml aliquots at - 80^0C. On the day of the experiment aliquots were removed from the freezer and diluted in double–distilled water to give a final concentration of 50pmols/ml and stored in the fridge for use. Dilutions of the standard were injected into the HPLC-ED system via an injection valve (Rheodyne,U.S.A.) with a 10 µl loop (see Fig. 2.4. for monoamine standard curves). The loop was always overfilled (approximately 15µl of standard mixture or dialysate to ensure reproducibility).

Monoamine peaks were quantified by measuring peak area (software-generated). A low gain setting on the detector (to ensure larger peaks were accurately measured) was employed. Identification of peaks was achieved by comparison of retention times with authentic monoamine standards (see Fig. 2.4) for typical calibration plots for monoamine standard.
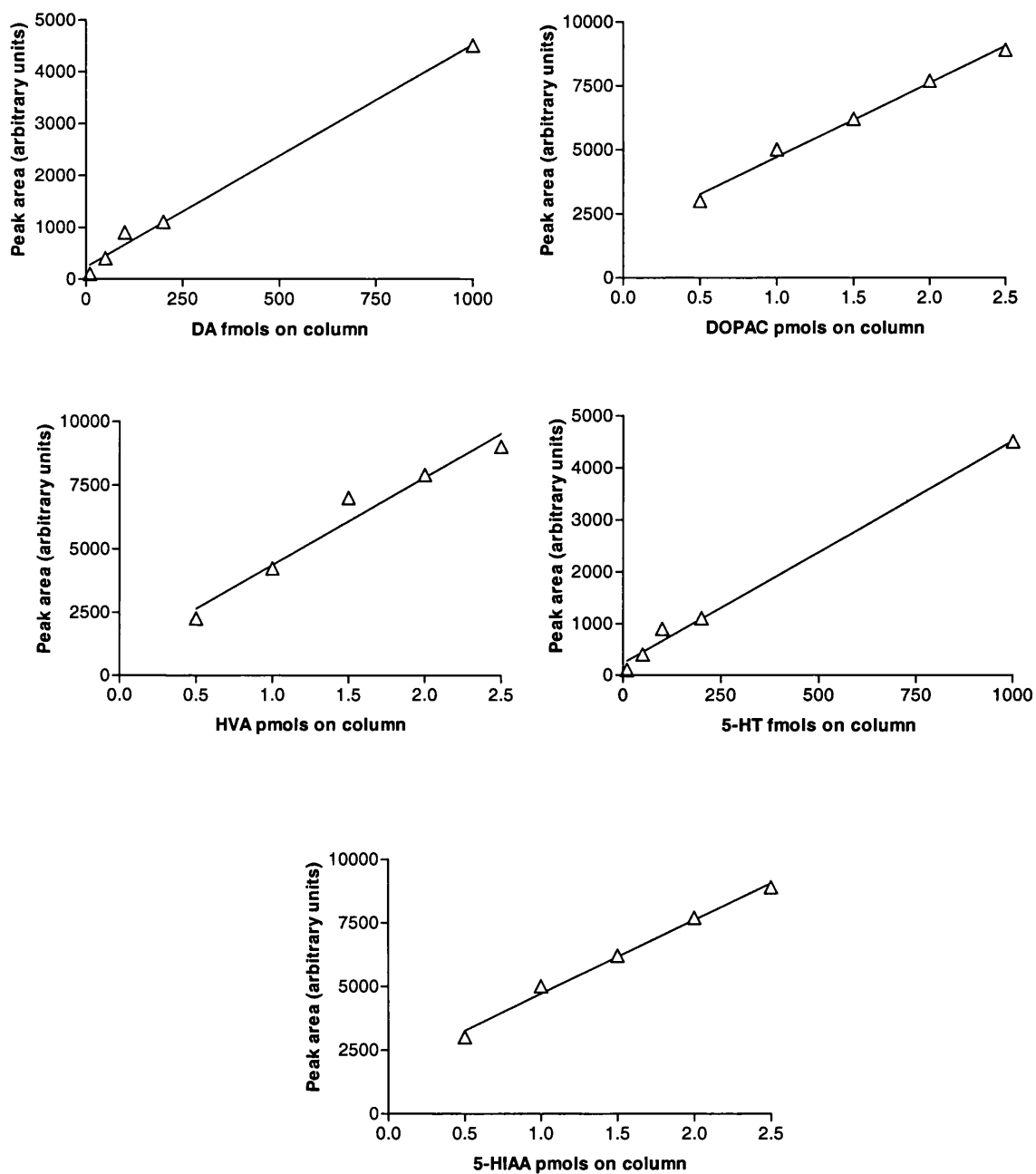
Figure 2.4: Typical calibration plots for monoamines obtained using HPLC with electrochemical detection. Refer to text for details of chromatographic technique.

2.4.4   Analysis of amino acids using HPLC-FD

Separation of amino acid-containing samples was carried out by a gradient, reverse phase HPLC system using the method of Lindroth and Mopper (1979).

2.4.5 System configuration

The HPLC system used for amino acid separation and quantification comprised of: two delivery pumps (Gilson, Model 303), a detector (Gilson, Model 121), a Dynamix C18 reverse- phase column (15 cm in length; 0.4 cm internal diameter; particle size 5μm; Rainin Dynamax Instrument Corporation Inc., U.S.A.) with column heater (30 ^{0}C: Anachem), a 5μm C18 guard column (Rainin Dynamax) and a refrigerated autosampler (model 231) with Rheodyne injection valve. Data were collected from the fluorimetric detector via a Gilson data collection module (model 621) which acted as an interface to a Dell PC system. The PC system was equipped with Gilson chromatography software with in built gradient manager.

2.4.6   Preparation of o-pthaldialdehyde/ thiol derivatisation reagent.

Prior to analysis, amino acids were conjugated to a fluorescent moiety by a non-enzymatic reaction for subsequent detection. The amino acids were pre-column derivatised with o-phthaldialdehyde (OPT) in the presence of 2-mercaptoethanol under alkaline conditions (sodium tetraborate buffer, pH 9.5) to produce an isoindole derivative sufficiently stable to undergo chromatographic separation (Fig. 2.5). The OPA (27mg, (Sigma, U.K.) was dissolved in 500 μl of absolute ethanol and 5ml of a 0.1M solution of sodium tetraborate buffer. After the solution was thoroughly mixed, 50 μl of mercaptoethanol for thiol reduction was added and the mixture stored in a

container in the dark for up to two weeks. The OPA was always prepared 24h prior to

being used and was changed on a weekly basis.
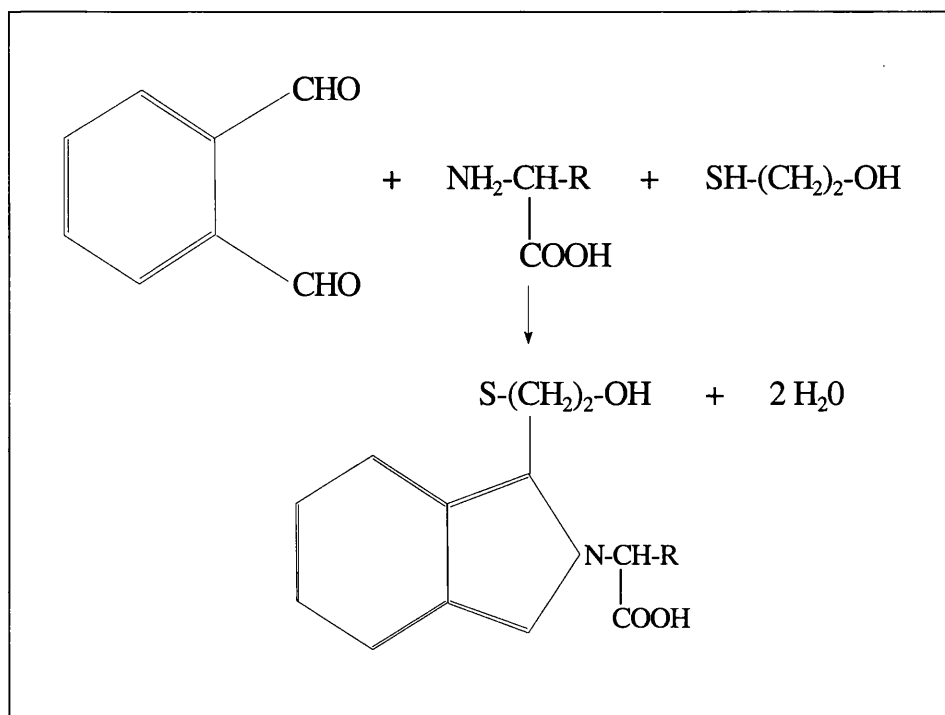
Fig 2.5: Formation of fluorescent amino acid derivatives by reaction of primary

amine with OPA and 2-mercaptoethanol.

2.4.8   Chromatographic conditions

All separations were carried out under gradient conditions such that the

mobile phase became progressively non-polar with elution time. This allowed for the

separation of acidic (ie, ASP) through to relatively non-polar amino acid derivatives

(ie, GABA) from a mixture over a period of 40 min. Two mobile phase components (A)

mobile phase and (B) methanol were mixed prior to delivery to the column inlet. For

gradient elution the mobile phase consisted of the following two components: (A)

50mM sodium dihydrogen orthophosphate, adjusted to pH 5.5 (with 10mM NaOH)

to which 20% methanol was added and (B)100% methanol. The proportions of (A) and (B) in the mixture were determined by the relative outputs of the two mobile phase delivery modules which were controlled by the gradient manager. The column was held at a constant temperature of 30° C by the column heater. The solvent delivered to the top of the column was free of particles as it was previously filtered (HPLC grade filter) and degassed with helium. Samples for analysis (15ml) were placed into vials seated in an autosampler rack. Each sample was mixed with 15ml of OPA/thiol reagent and incubated for 2min at 4°C before application of the mixture (20ml) to the column via the Rheodyne. At the moment of injection of the derivatised sample, the system received a signal, triggering the gradient manager and data collection. OPA/thiol-amino acid conjugates were detected fluorimetrically (excitation: 360nm, emission: 455nm). The entire process was repeated until all the samples were analysed. Chromatograms and peak area data were represented as software general printouts. Representative chromatograms illustrating the separation of OPA thiol derivatised amino acids from hippocampal dialysates are presented in Fig. 2.6.
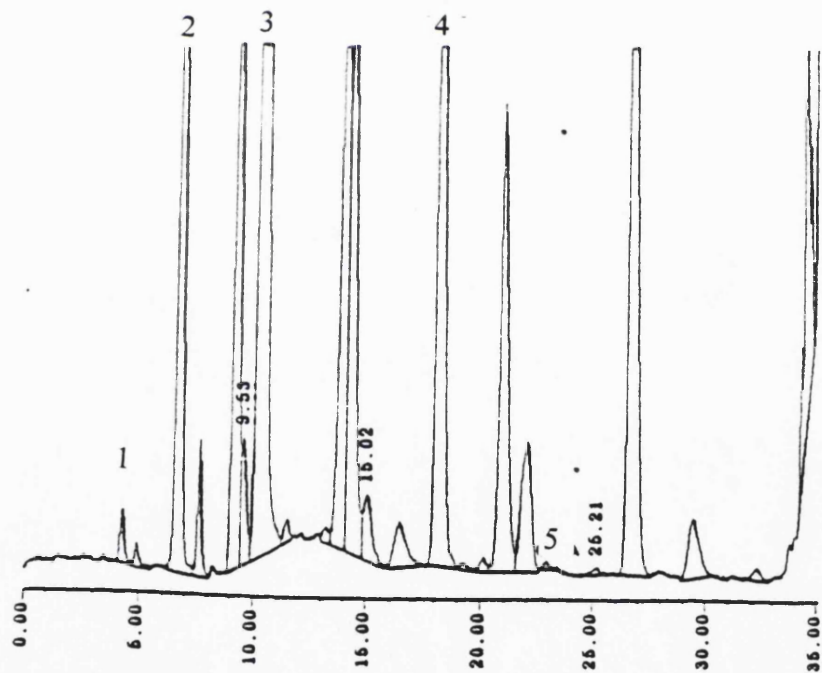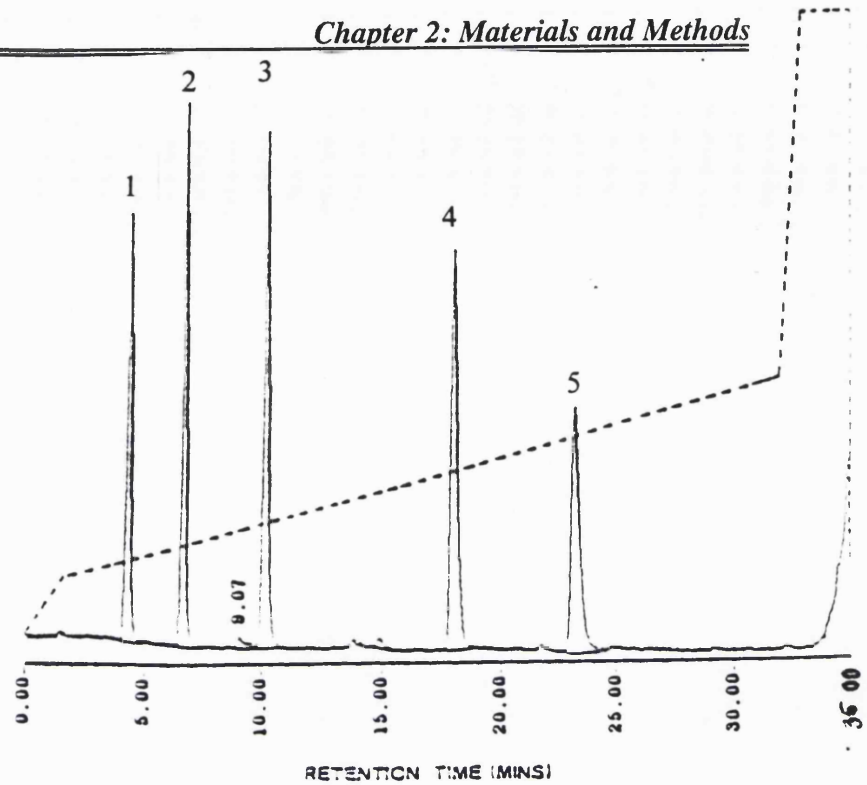
Fig 2.6 Typical chromatograms obtained following separation of amino acids using HPLC with FD. (A) shows a representative standard mixture and (B) is typical representation of basal amino acid output obtained in the ventral hippocampus. Horizontal axis represents elution times in min. Black line shows gradient buffer conditions. Peaks denoted are: (1) L-ASP, (2) L-GLU, (3) L-GLN, (4) TAU, (5) GABA.
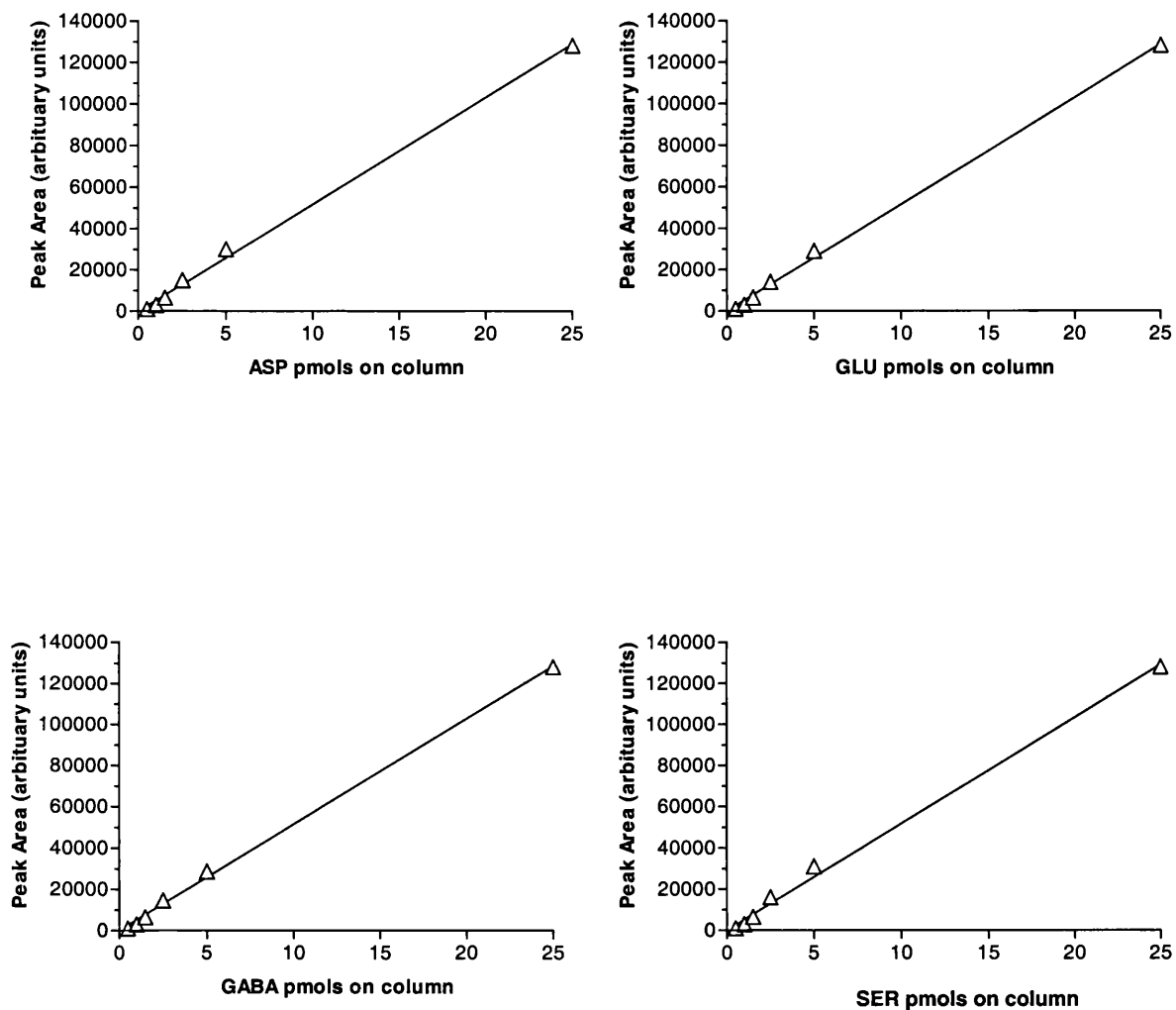
Fig. 2.7: Typical calibration plots for amino acid standard mixture obtained using

HPLC with fluorimetric detection. Refer to text for details of chromatographic

technique.

2.4.8   System calibration.

If not in use, the HPLC was left with 100% methanol being pumped through the system. This prevented the crystallisation of buffer A in pump (A) as well as getting rid of any contamination on the column. On the day of an experiment the HPLC was equilibrated by allowing a progressive change of the gradient from 100% methanol to 100% mobile phase   Standard curves for amino acid quantification in samples were routinely obtained. Stock solutions (2.5mM standard mixture) of ASP, GLU, GLN, TAU, SER and GABA were prepared in mobile phase A, diluted to 2.5µM on the required days and run at regular intervals amongst dialysate samples. The resultant chromatograms were used to establish a quantity/peak area relationship for each species. In the case of GABA, the lowest two-calibrant peak areas were obtained from the chart recorder to allow the very low levels of this amino acid found in some dialysates (< 1pmol) to be estimated with some accuracy. Fig (2.7) shows a separation typical for amino acid standard mixture using HPLC-FD . Examples of amino acid standard curves are provided (Fig. 2.7)

2.5   Statistical analysis

All data included in this thesis were calculated as the percentage of amino acid or monoamines levels. Statistical comparisons were made between drug and aCSF perfused control for each of the collection periods. In addition, in some cases, statistical comparisons were made between two drug treatments. A one-way ANOVA test followed by Dunnett's test was use throughout the study. A P value of<0.05 was considered statistically significant.

Chapter 3

Effects of nitric oxide on monoamine and amino acid release in the rat ventral hippocampus as measured by microdialysis in freely moving rats.

3.1 Introduction

As reviewed in the general introduction, research has indicated a possible role for NO in synaptic transmission in the brain where it has been associated with the activation of L-GLU receptors (Garthwaite et al., 1988; Moncada et al., 1991 Schuman and Madison, 1994). Although all types of L-GLU receptors have been implicated, the role of the NMDA receptor has received most attention. It is thought that L-GLU released from presynaptic terminals acts on postsynaptic NMDA receptors leading to the opening of Ca^{2+} channels and the activation of NOS which, in turn, produces NO from L-ARG (Bredt and Snyder, 1990b). The NO formed may then act intra and extracellularly or diffuse extracellulary to adjacent neurones or glial cells where its effects are mediated by the activation of sGC and the resultant elevation of cGMP levels. It would appear that the postsynaptic formation of NO following the activation of NMDA receptors is widespread, given that NMDA evokes cGMP accumulation in several brain regions in vivo (Southam and Garthwaite, 1993) and may be involved in many aspects of synaptic transmission.

NMDA receptors can modulate the release of many neurotransmitters. It has been shown that activation of NMDA receptors leads to a stimulation of the release of L-GLU and monoamines in many brain regions including the nucleus accumbens (Imperato et al., 1990), prefrontal cortex (Karremann et al., 1990), striatum (Carter et al., 1988;Young and Bradford, 1991) and hippocampus (Whitton et al., 1994a,b). Infusion of NMDA into the striatum, for example, has been shown to promote DA and L-GLU release (Carter et al., 1988; Whitton et al., 1994a), whilst systemic treatment with the non-competitive NMDA receptor antagonist MK-801 leads to reduced extracellular levels of these neurotransmitters (Whitton et al., 1992a,b). In contrast, similar experiments performed by Whitton et al. (1994a) in the hippocampus have

shown that L-GLU release is elevated whilst DA release is inhibited in response to NMDA infusion. This suggests that L-GLU has the potential to either facilitate or inhibit DA release depending upon the brain area under investigation. The association between NMDA receptor activation, NO production and the modulation of neurotransmitter release has been investigated both in vivo and in vitro. However, there is some confusion in the literature regarding the manner in which NO modulates neurotransmitter release in the brain.

Infusion of the competitive NOS inhibitors L-NAME and L-NMMA into the striatum was shown to produce a concentration-dependent inhibition of NMDA-evoked L-GLU release (Bogdanov et al., 1997) whilst enhancing DA release (Shibata et al., 1996). In addition, NO donors were found to increase L-GLU and decrease DA release in this structure in vivo (Guevara-Guzman et al., 1994). These findings were not, however, supported by other studies which suggested that NO donors increase spontaneous DA release from striatal slices whilst decreasing L-GLU release in vitro (Zhu and Luo, 1992; Lonart et al., 1993; Black et al., 1994; Steward et al., 1996). Furthermore, Hanabaur et al. (1992) demonstrated that the non-selective NOS inhibitor L-NNA attenuated NMDA-induced DA release in striatal slices, an effect which was the opposite to that observed by Shibata et al. (1996). Evidence supporting the idea that NO regulates neurotransmitter release in the hippocampus has also yielded contradictory data. The role of NO in this brain structure has mainly been investigated using different NO releasing agents in brain slice preparations. Hydroxylamine had been shown to stimulate $[^3H]$-NA and $[^{14}C]$-ACh release from rat hippocampal slices whereas high concentrations of SNP stimulated the release of $[^3H]$ NA (Lonart et al., 1992: Jones et al., 1995). In contrast, Stout and Woodward (1994) observed that SNP inhibited NMDA-evoked NA release from rat hippocampal slices.

Likewise, depolarisation-induced L-GLU release from hippocampal slices was inhibited by SNP but enhanced by SNAP (Woodward and Stout, 1994). Moreover, Mefferet et al. (1994) observed that various NO donors enhanced L-GLU release from hippocampal slices. A recent report by Nei et al. (1996) demonstrated that the NOS inhibitor L-NNA blocked NMDA-evoked L-GLU release from the dentate gyrus whilst a reduction in NMDA-stimulated release of endogenous L-ASP was observed by Dickie et al. (1992). These studies strengthen the case for the involvement of NO in NMDA-induced neurotransmitter release in the hippocampus.

The reasons for the apparent contradictory data are not clear. As more information is collected on the biochemistry and physiology of NO, some of the opposing effects of this molecule have been clarified. It is thought that certain discrepancies may be related to the different capacities and mechanisms whereby NO is produced from different donors (Woodward and Stout, 1994; Jones et al., 1995). For example, SNAP and SNP are broken down by simple dissociation to produce NO whereas SIN-1 undergoes a more complex pH-dependent conversion to SIN-1A which then decomposes in association with the production of a supreroxide ion, to NO and SIN-1C. Generated superoxide can react with NO and H^+ to form peroxynitrite and hydrogen peroxide which have deleterious effects on biological membranes. Moreover, the varying effects seen with the exogenous donors may involve the NO produced extracellularly altering the redox modulatory site(s) on the NMDA receptor channel complex (Aizenman et al., 1989; Lei et al., 1992; Lipton et al., 1993; 1994). NO donors may generate one of three redox related states of NO. These are NO^+ (nitrosonium ion), NO and NO^- (nitroxyl ion). Lipton et al. (1994) proposed that depending on which of these species are generated, NO might exert stimulatory or inhibitory effects on NMDA-evoked neurotransmitter release. For example, NO

donors possessing NO^+ characteristics (e.g. SNP and nitroglycerine) can form S-nitroso-compounds with sulphydryl groups in the NMDA receptor channel therefore causing down-regulation of its activity. NO is also capable of accepting an electron to form NO^- which also down-regulates responses at the NMDA receptor complex. However, it is important to stress here that these studies were performed in vitro and some of these reactions may not occur in vivo. Moreover, this work, mostly performed on the recombinant NMDA receptor redox site is determined by the availability of NR2 subtypes. Thus, NMDA receptors of different neurones may have redox properties which are dependent on the composition of NR2 subtypes.

It has also been suggested that the stimulatory and inhibitory effects observed in vivo may be due to the experimental conditions used such as species differences, animal model and the choice of NOS inhibitors. For example, it has been suggested that NO plays a role as an anti-convulsant substance only in case of seizures induced by picrotoxin whilst being pro-convulsant in seizures induced with kainate (Kirby et al., 1996). It appears that genetic factors are also important determinants of convulsive responses in L-NAME treated rats; L-NAME was shown to potentiate kainate-induced convulsions in Spraque-Dawley rats but not in Wistar rats (Kirby et al., 1996). Regarding the use of NOS inhibitors, nearly all conclusions are based on studies with L-ARG analogues which lack specificity and will inhibit not only neuronal but also endothelial NOS with equal potencies. Based on the suggestion that neuronal NO production promotes seizures and vascular NO synthesis suppresses seizures (Penix et al., 1994) it is reasonable to speculate that the source of NO may be a critical factor in determining the final response.

In this section, an attempt has been made to clarify the role of NO in synaptic transmission in the hippocampus. This study was performed in vivo using freely

moving rats as it was reasoned that this model offers a realistic assessment of the

function of NO in the modulation of neurotransmitter release in this brain structure.

The following questions were addressed:

i)   Does NO regulate basal and NMDA-evoked neurotransmitter release in the
       ventral hippocampus in vivo.

ii)   Does this regulation depend on the concentration of NO.

iii)   Is the source of NO production important in determining the resultant response.

3.2 RESULTS

3.2.1. Basal levels of amino acids and monoamines measured in ventral hippocampal dialysates.

Basal extracellular amino acid and monoamine levels were derived from all of the experiments performed in this study and were calculated from the first four samples taken from each dialysis experiment. Basal levels of L-GLU, L-ASP, GABA and L-SER were found to be 36.20 ± 5, 25.5 ± 3, 2.65 ± 3 and 33 ± 0.9 (pmols/15μl dialysate/30min sample, n=65) whilst those of DA, DOPAC, 5-HT, 5-HIAA and HVA were 47.7 ± 2.1, 530 ± 10.6, 97 ± 3.0, 966 ± 25.7 and 880 ± 34.9 (fmols/10μl dialysate/30min sample, n=70) respectively.

3.2.2. Effects of NMDA on amino acid and monoamine release in ventral hippocampus.

A 30min infusion of NMDA at three different concentrations elicited a concentration-dependent increase in the efflux of L-GLU, L-ASP and GABA. NMDA at 1mM increased L-GLU levels by up to $555 \pm 55\%$ over control (Fig. 3.1 A, B and C), an effect that was rapid in onset and persisted for 30min after the drug infusion ceased before declining to basal levels. L-ASP and GABA levels were also increased following NMDA infusion but to a much lesser extent with the 1mM concentration of NMDA increasing L-ASP levels by $300 \pm 55\%$ and GABA by approximately $110 \pm 25\%$ over basal values (Fig. 3.1 A, B and C). To ensure that these effects were specific for amino acids considered to be neurotransmitters, we also examined the effect of NMDA on L-SER. Fig. 3.1 D shows that application of NMDA did not significantly alter the levels of this amino acid in the extracellular space.
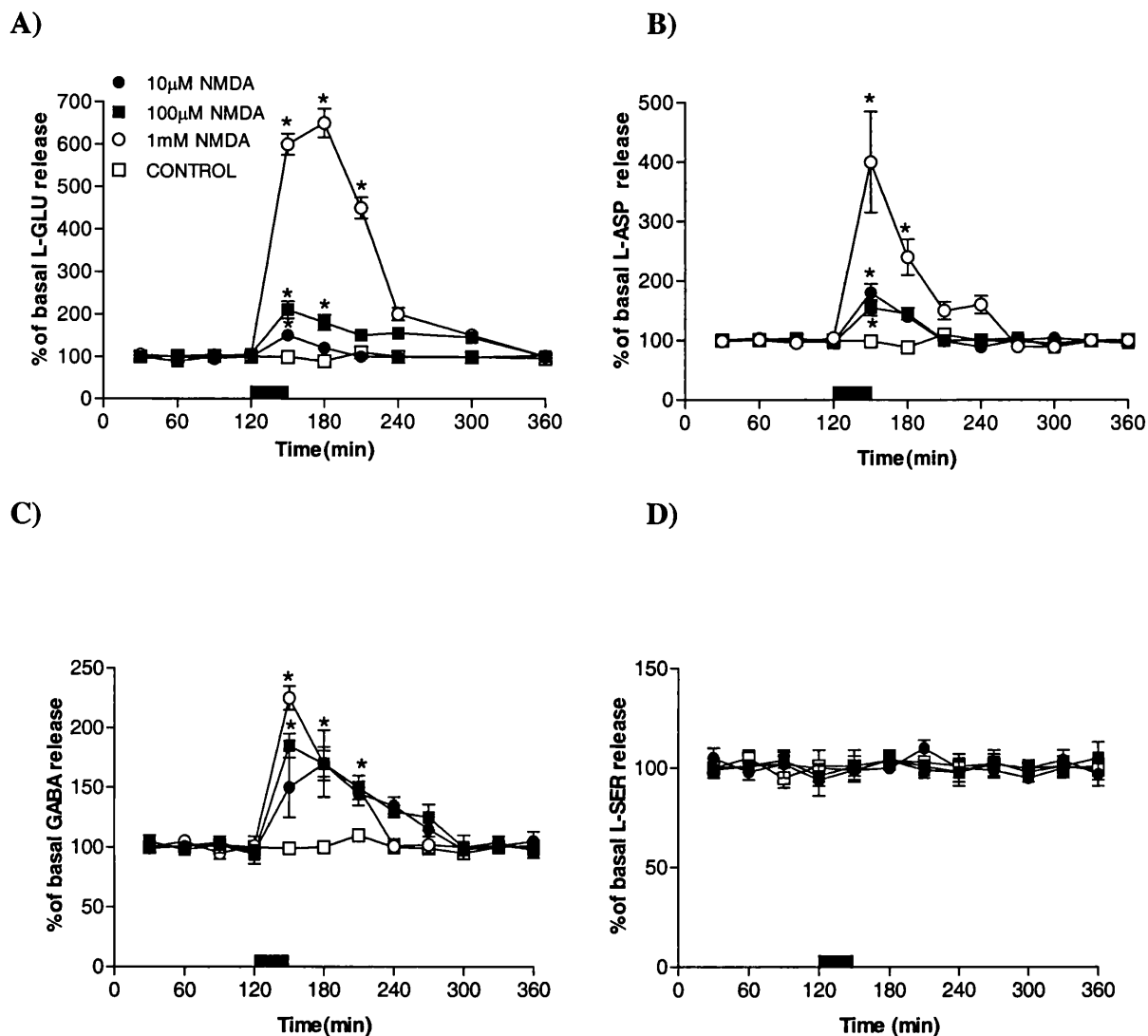
Fig. 3.1: Effect of NMDA infusion into the ventral hippocampus via the microdialysis probe on L-GLU (A), L-ASP (B), GABA (C) and L-SER (D) release. Bars denote the duration of NMDA infusion. All values are means ± s.e.m. (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control, ($p < 0.05$).
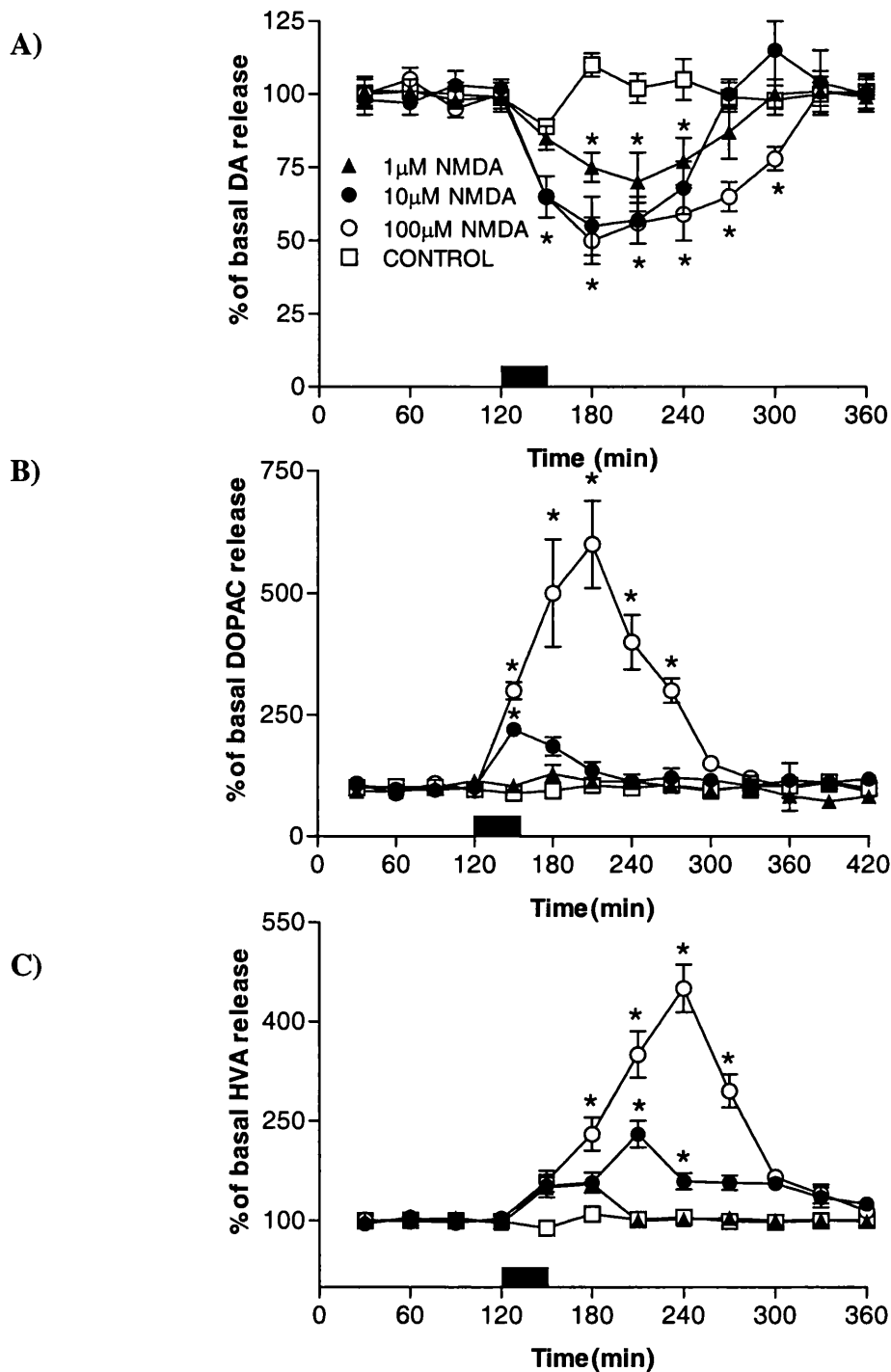
Fig. 3.2: Effect of NMDA infusion into the ventral hippocampus via the microdialysis probe on DA (A), DOPAC (B) and HVA (C) release. Bars denote the duration of NMDA infusion. All values are means ± s.e.m. (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control, ($p<0.05$).
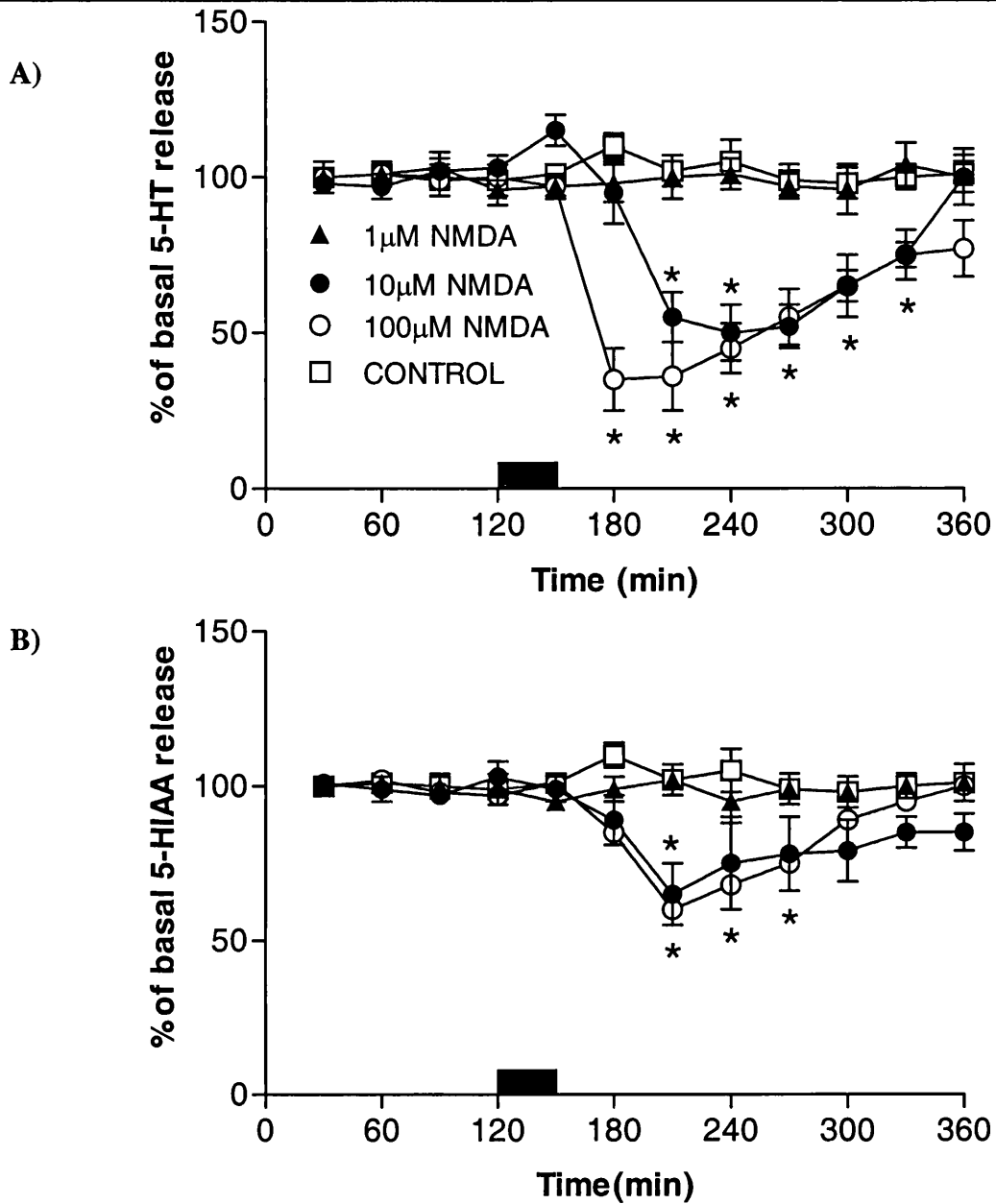
Fig. 3.3: Effect of NMDA infusion into the ventral hippocampus via the microdialysis probe on 5-HT (A) and 5-HIAA (B) release. Bars denote the duration of NMDA infusion. All values are means ± s.e.m. (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control, $p < 0.05$.

In contrast to its effect on amino acids, NMDA produced a concentration-dependent decrease in the efflux of both DA and 5-HT with a maximum reduction of approximately $50 \pm 10\%$ occurring at 100μM NMDA (Fig 3.2 A and 3.3 A). These effects were long lasting as it took approximately 3h for the levels of these monoamines to return to basal. The efflux of DA and 5-HT metabolites were also altered following the infusion of NMDA. DOPAC and HVA levels increased by up $400 \pm 38\%$ and $430 \pm 75\%$ respectively at 100μM NMDA (Fig 3.2 A and B) whilst 5-HIAA levels declined by approximately $50 \pm 10\%$ (Fig. 3.3 B).

To confirm that the effects of NMDA infusion were the result of NMDA receptor activation, experiments were performed in the presence of the competitive antagonist D-AP5. The NMDA-evoked increase in L-GLU release was reduced by approximately $50 \pm 10\%$ in the presence of D-AP5 whilst the increase in L-ASP efflux was totally abolished. Likewise, the inhibition of DA and 5-HT release produced by NMDA infusion were reversed by the administration of D-AP5. The NMDA-evoked increases in DOPAC and HVA levels and the decrease in 5-HIAA efflux were blocked by the infusion of D-AP5 (Fig. 3.4 A, and B, Fig. 3.5 A, B and C, Fig. 3.6 A and B).

Infusion of D-AP5 alone decreased basal levels of L-GLU by $50 \pm 10\%$ below basal values. No significant changes were observed in L-ASP and DA levels when compared to controls. In contrast, a transient increase in the levels of 5-HT was observed 30 min after the infusion of D-AP5 ceased. Of the three monoamine metabolites measured only HVA levels were affected by infusion of D-AP5, showing a transient but significant decrease to $50 \pm 15\%$ below control levels (Fig. 3.4 A and B, Fig. 3.5 A, B and C, Fig. 3.6 A and B).
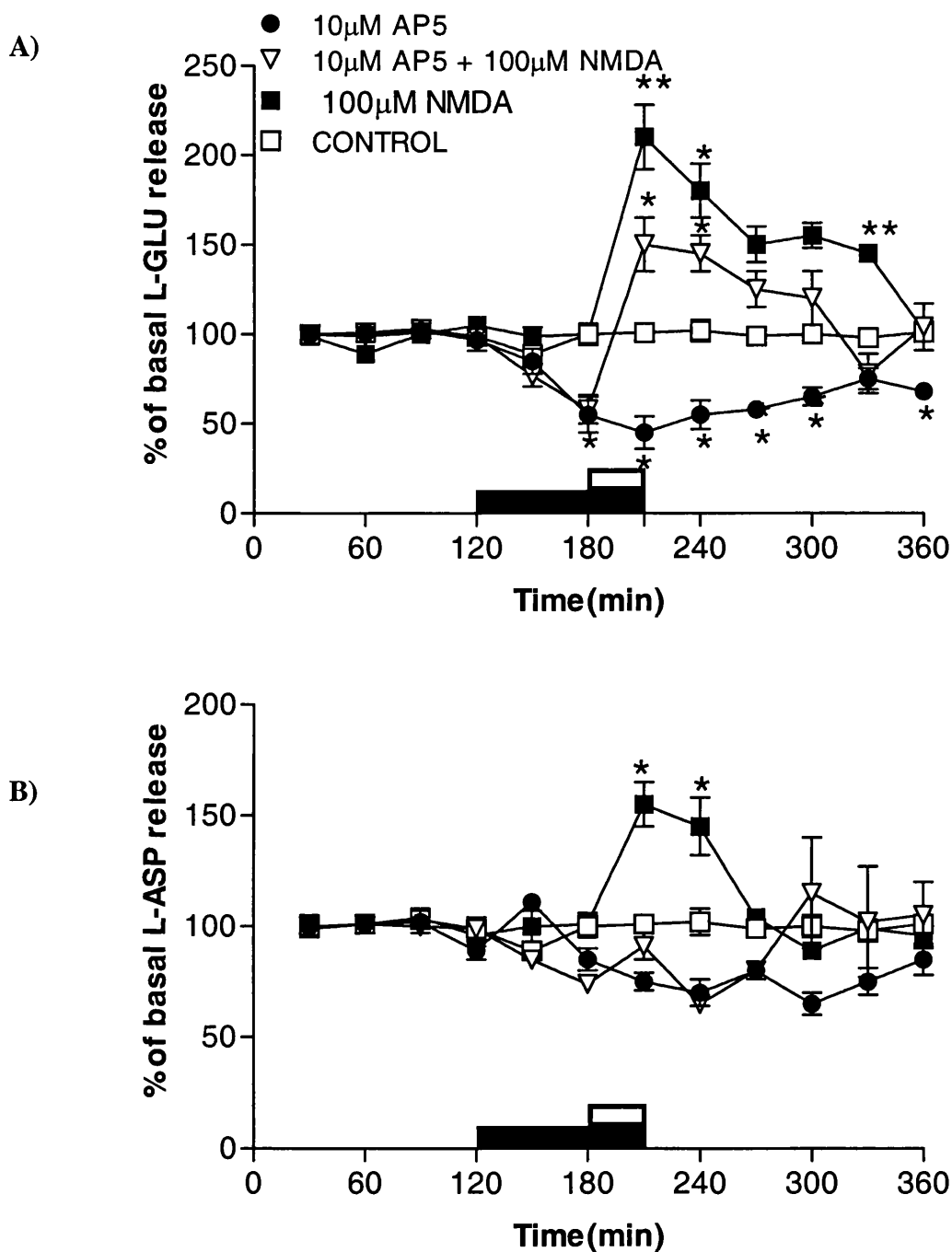
Fig. 3.4: Effect of D-AP5 infusion administered for 60 min prior to co-infusion with

NMDA on hippocampal dialysate levels of L-GLU (A) and L-ASP (B). The solid bars

represent the period of D-AP5 addition while the open bars indicate the combination

of D-AP5 with NMDA. All values are means ± s.e.m. (shown when larger than the

symbol) from 4 experiments. * indicates data points significantly different from

control ($p < 0.05$) and ** significant difference between NMDA and the combination

of NMDA with D-AP5 treatment ($p < 0.05$).
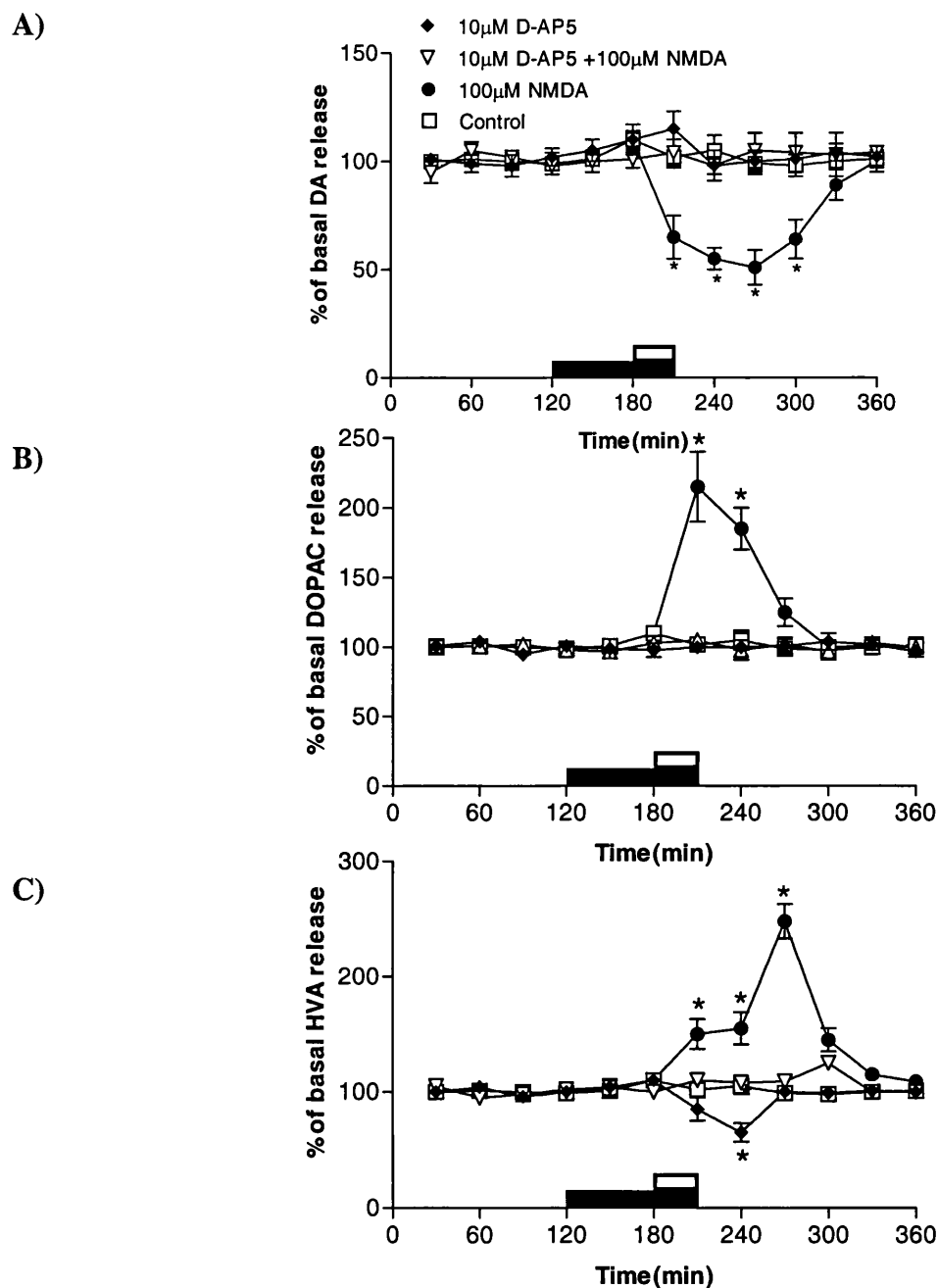
Fig. 3.5: Effect of D-AP5 infusion administered for 60 min prior to co-infusion with NMDA on hippocampal dialysate levels of DA (A) and its metabolites, DOPAC (B) and HVA (C). The solid bars represent the period from D-AP5 addition while the open bars indicate the combination of D-AP5 with NMDA. All values are means ± s.e.m. (shown when larger than the symbol) from 4 experiments. * indicates data points significantly different from control ($p < 0.05$).
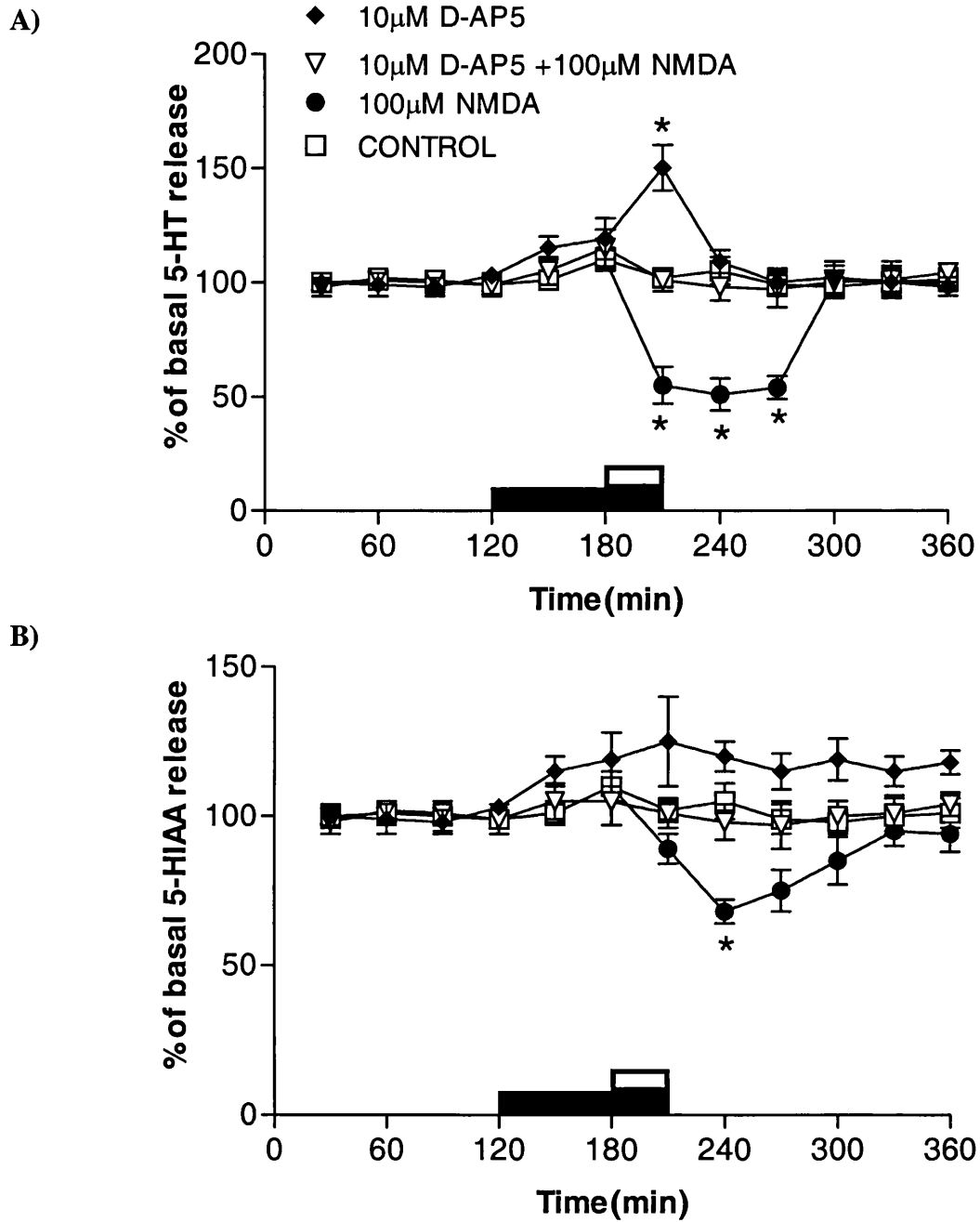
Fig. 3.6: Effect of D-AP5 infusion administered for 60 min prior to co-infusion with NMDA on hippocampal dialysate levels of_5-HT (A) and its major metabolite 5-HIAA (B).The solid bars represent the period from D-AP5 addition while the open bars indicate the combination of D-AP5 with NMDA. All values are means ± s.e.m. (shown when larger than the symbol) from 4 experiments. * indicates data points significantly different from control ($p < 0.05$).

3.2.3. Effect of infusing an NO donor on amino acid and monoamine release

To assess the role of exogenous NO on neurotransmitter release in the rat ventral hippocampus, the NO releasing agent SNAP was infused via the microdialysis probe. A 30min SNAP infusion exerted an inverse concentration-dependent effect on amino acid and monoamine release. High concentrations of the donor increased amino acid but decreased monoamine release, whilst low concentrations of SNAP had the opposite effect (Fig. 3.7 A, B, and C). The increase in amino acid release was rapid in onset with 2mM SNAP elevating L-GLU, L-ASP and GABA by approximately 550 ± 25%, 400 ± 55% and 700 ± 106% above baseline, respectively. At this concentration the effect of SNAP on L-GLU and GABA efflux persisted for 30min after the infusion of SNAP ceased before returning to basal levels. In contrast, the increase in L-ASP levels was slower in onset and after a maximum was reached, followed by immediate decline and return to the basal levels. SNAP at 500µM decreased the efflux of these amino acids (Fig. 3.7 A, B, C and D), however, this effect was delayed, manifesting itself only after the drug infusion was stopped. A return to basal levels was observed within the time course of the experiment. The levels of all these amino acids decreased by approximately 50 ± 15%. To ensure that these effects were specific for amino acids considered to be neurotransmitters, the effect of the NO donor was also examined on L-SER. No statistically significant changes were observed in the release of this amino acid following the 30min infusion of SNAP at all concentrations used (Fig. 3.7 D).

5mM SNAP produced a 50 ± 15 % decline in monoamine levels, showing no return to the basal levels despite of the termination of the drug infusion. At 1mM SNAP increased the release of 5-HT and DA by approximately 50 ± 10% above control values (Fig. 3.8 A, Fig. 3.9 A). In both cases, the increase was
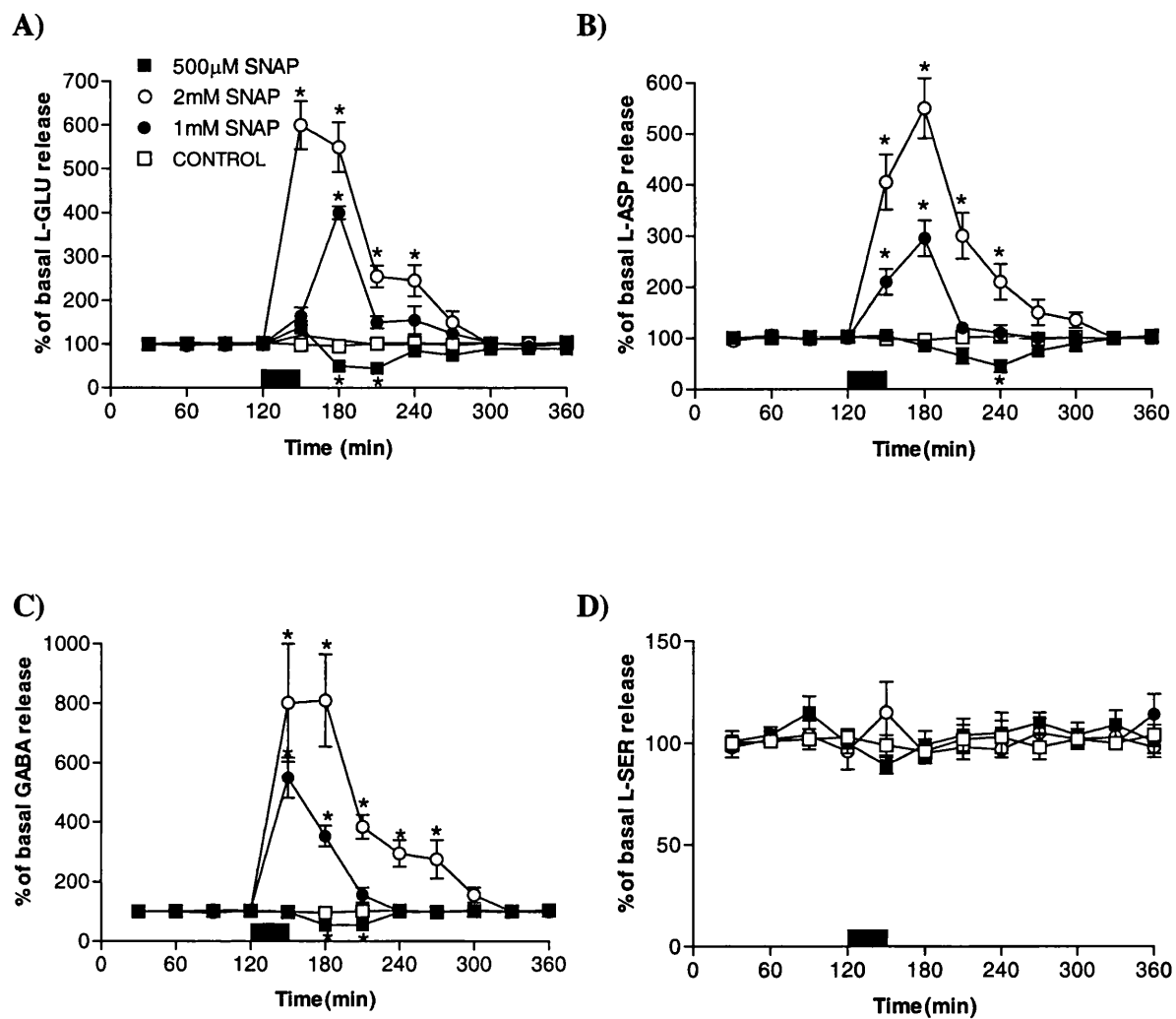
Fig. 3.7: Effect of SNAP infusion into the ventral hippocampus on L-GLU (A), L-ASP (B), GABA (C) and L-SER (D) efflux. Bars denote the period of SNAP infusion. All values are means ± s.e.m. (shown when larger than the symbol) from 4 experiments. * indicates data points significantly different from control, p< 0.05.

Fig. 3.8: Effect of SNAP infusion on hippocampal DA (A), DOPAC (B) and HVA (C) release. Bars denote the period of SNAP infusion. All values are means ± s.e.m., (shown when larger than the symbol) from 4 experiments. * indicates data points significantly different from controls ($p<0.05$).
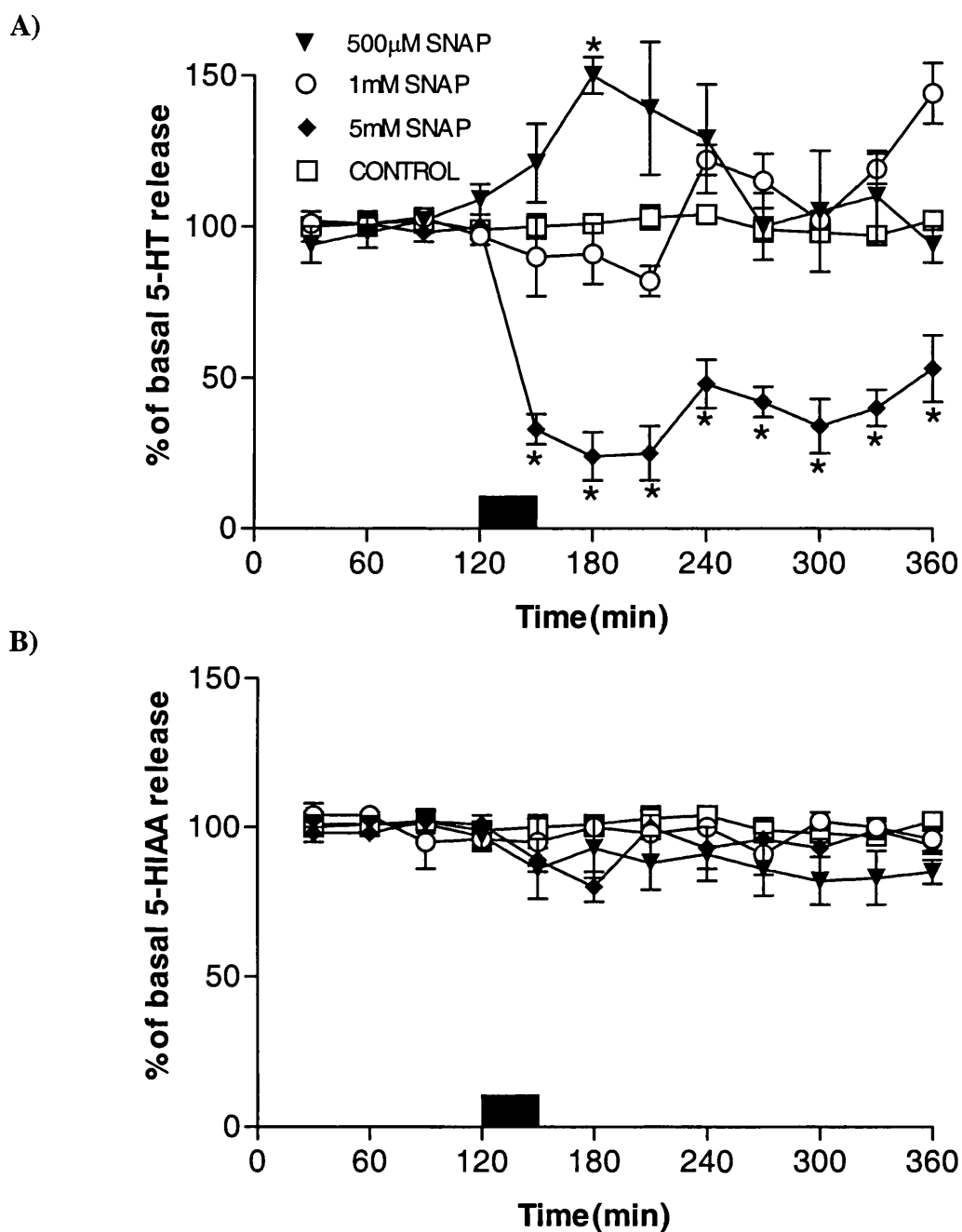
Fig. 3.9: Effect of SNAP infusion on hippocampal 5-HT (A) and its metabolite 5-HIAA

(B) release. Bars denote the period of SNAP infusion. All values are means ± s.e.m.

(shown when larger than the symbol) from 4 experiments. * indicates data points
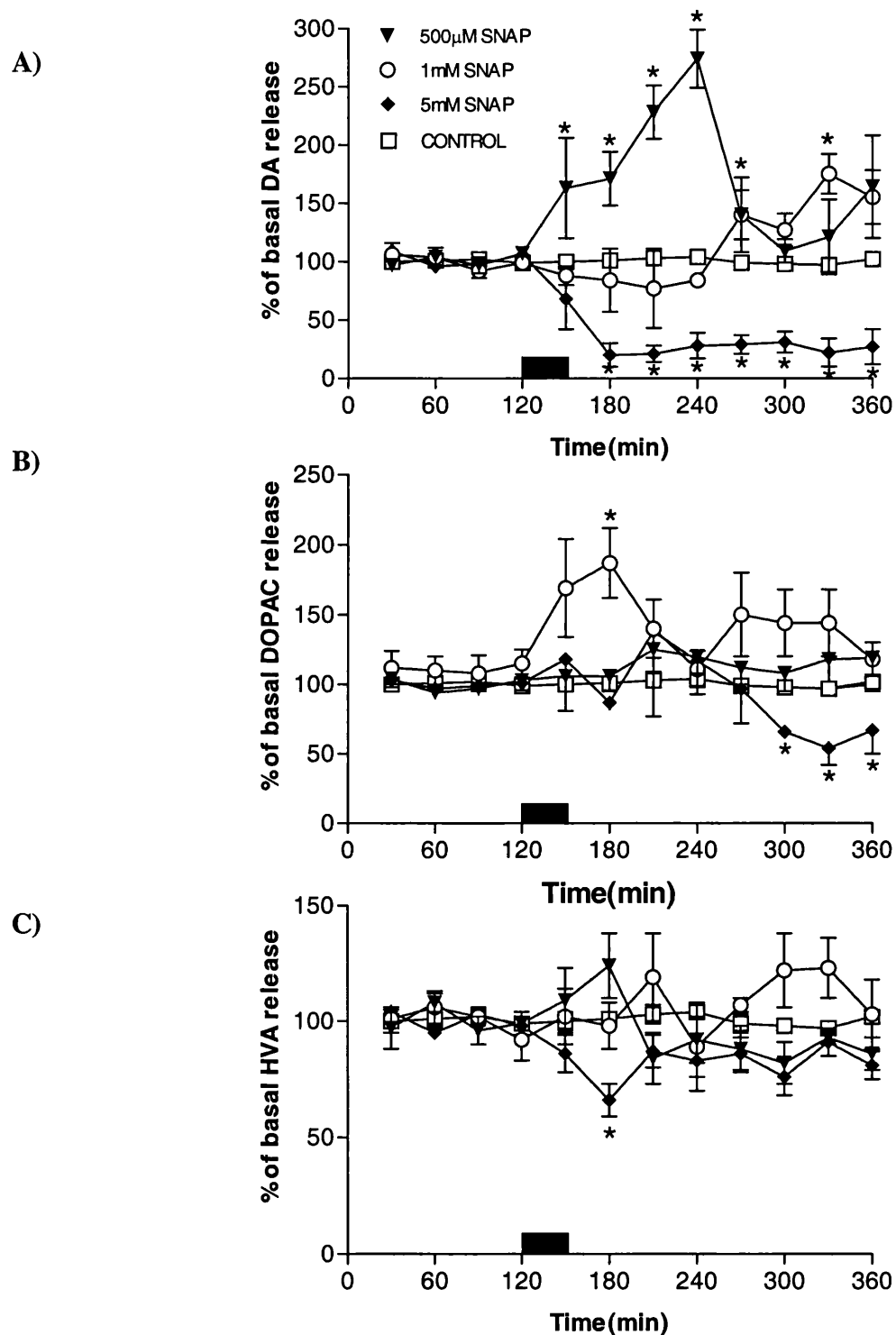
significantly different from controls ($p < 0.05$).

delayed and found to be statistically significant at the end of the experiment. Although the low concentration (500µM) of SNAP produced an augmentation of DA and 5-HT levels, the magnitude of the responses differed. DA levels increased by approximately 150 ± 15% above control while 5-HT levels reached a maximum at 50 ± 10% above baseline. Unlike the rapid onset of the effects seen with application of the high concentration (5mM) of SNAP, these responses were slow, reaching a maximum at 90min and 60min, respectively, after the infusion of the drug was ceased. Infusion of 1mM SNAP transiently elevated DOPAC levels by approximately 70 ± 15% whilst 5mM decreased HVA levels by up to 45 ± 5% below basal levels (Fig. 3.6 B and C). No significant changes in the levels of 5-HIAA were observed (Fig. 3.9 B). The effects of the NO donor SNAP at 5mM for monoamines and 2mM for amino acids were inhibited by Hb, a well know scavenger of NO (Fig. 3.10 A, B, C and D, Fig. 3.11 A, B and C, Fig 3.12 A and B.).

Since penicillamine is a carrier molecule for SNAP the effect of this on neurotransmitter release was also investigated. Previous evidence suggested that whilst penicillamine did not alter amino acid release (Guevara-Guzman et al., 1994), some investigators reported that monoamine release was altered by this molecule (Jones et al., 1993). In this study, 5mM penicillamine did not produce statistically significant changes in monoamine levels (Fig. 3.13 and Fig. 3.14 A and B).

When D-AP5 (10µM) was infused for 60 min prior to co-infusion with SNAP (5mM), the SNAP- evoked inhibition of DA release was not altered whilst that of 5-HT was diminished (Fig. 3.15 A and Fig. 3.16 A). Interestingly, whilst the presence of D-AP5 did not alter the effect of 500µM SNAP on 5-HT release, DA levels declined which is the opposite to that seen with SNAP alone at this concentration (Fig. 3.15 A and Fig. 3.16A). No statistically significant changes were observed in the levels of
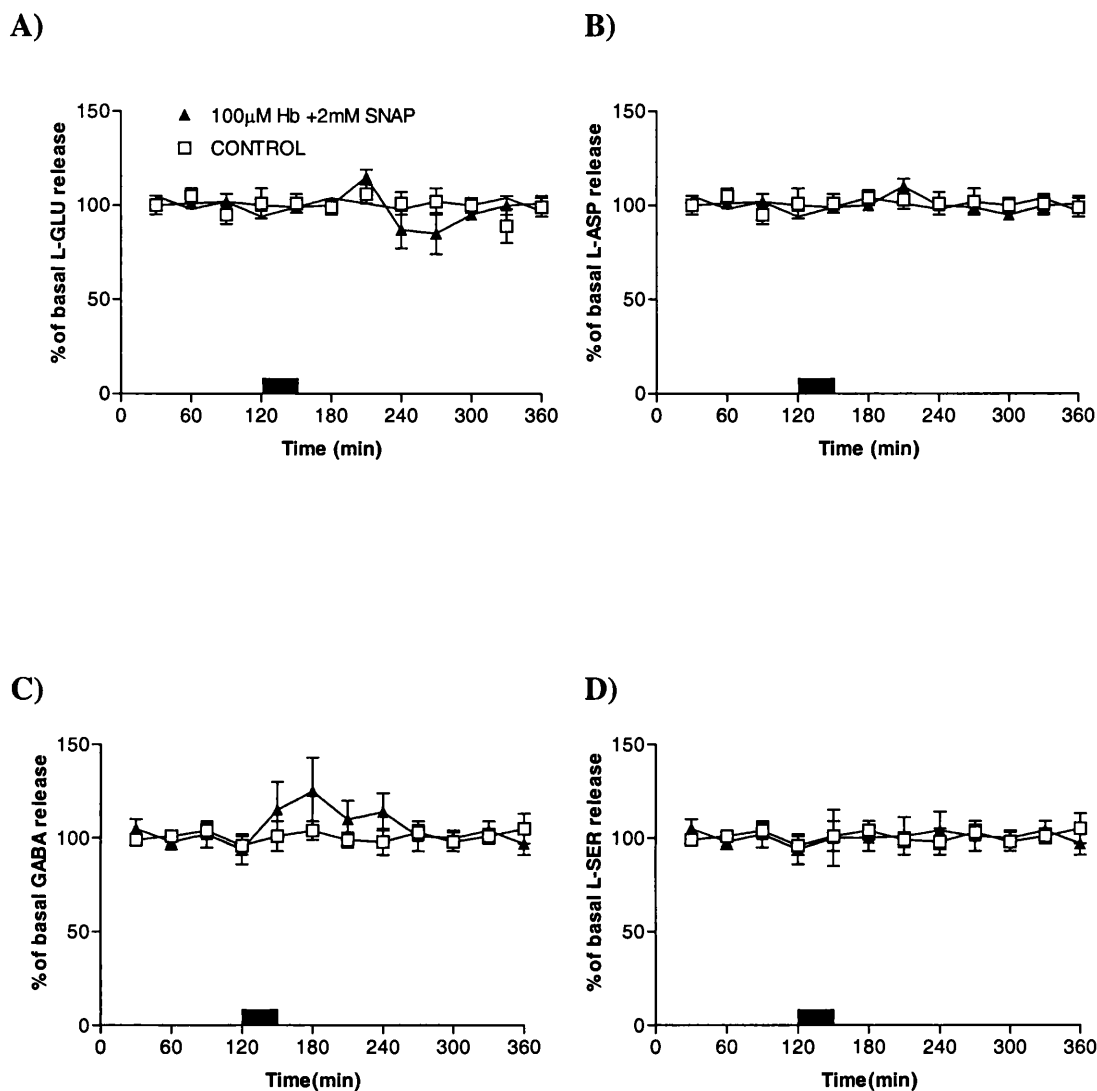
Fig. 3.10 Effect of SNAP infusion together with Hb on hippocampal L-GLU (A), L-ASP (B), GABA (C) and L-SER release. Bars denote the period of drugs infusion. All values are means ± s.e.m. (shown when larger than the symbol) from 4 experiments.

A)

B)

C)
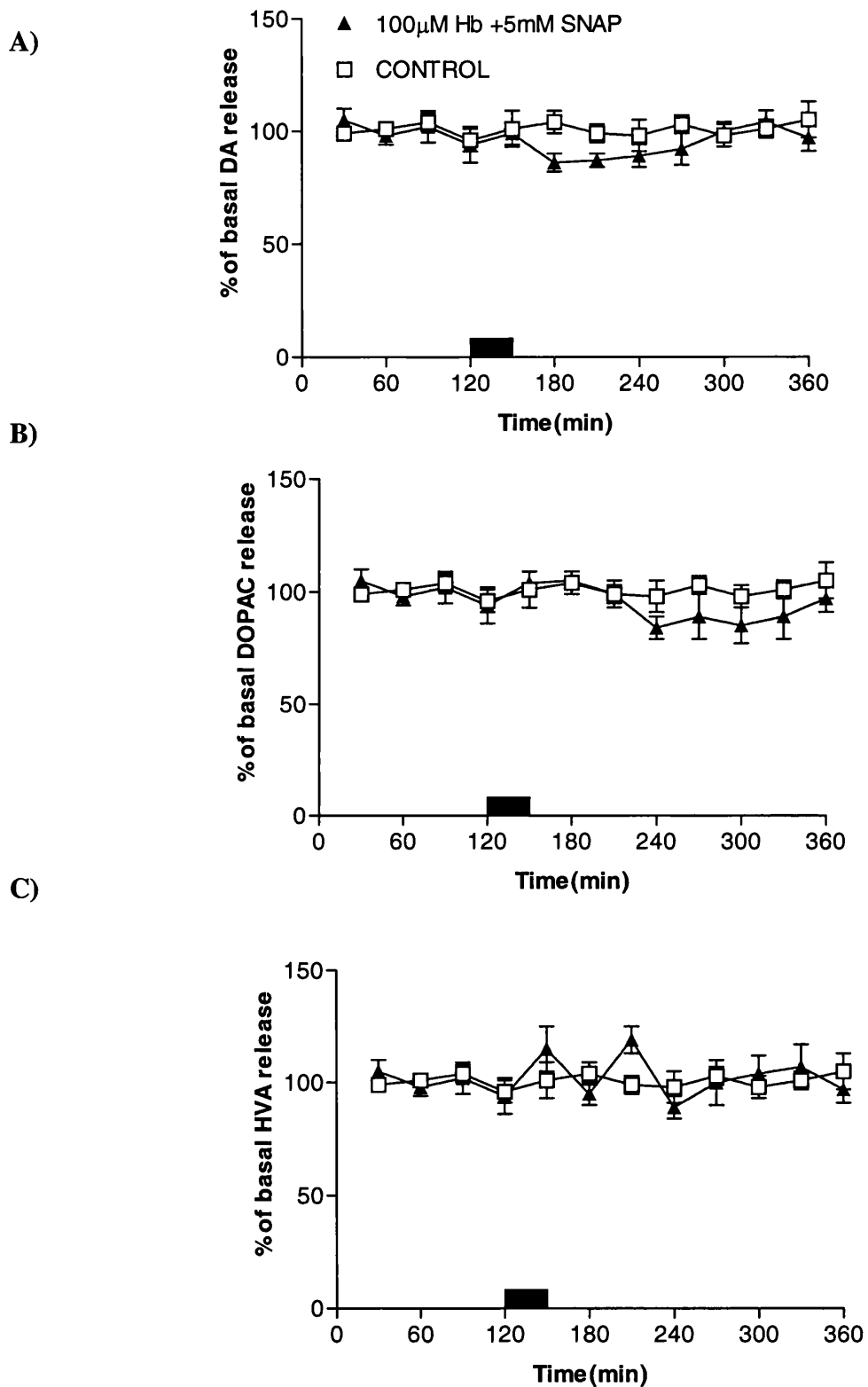
Fig. 3.11: Effect of infusion of SNAP together with Hb into the ventral hippocampus on

DA (A), DOPAC (B) and HVA (C) release. Bars denote the duration of drug infusion.

All values are means ±s.e.m. (shown when larger than the symbol) from 6 experiments.

84

A)
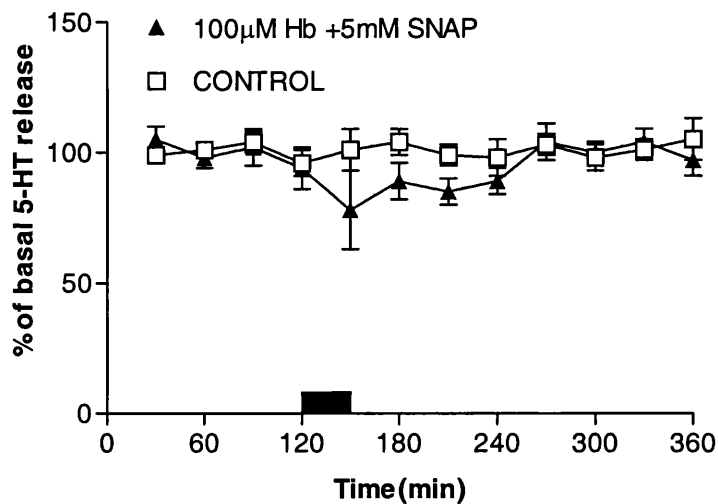
B)
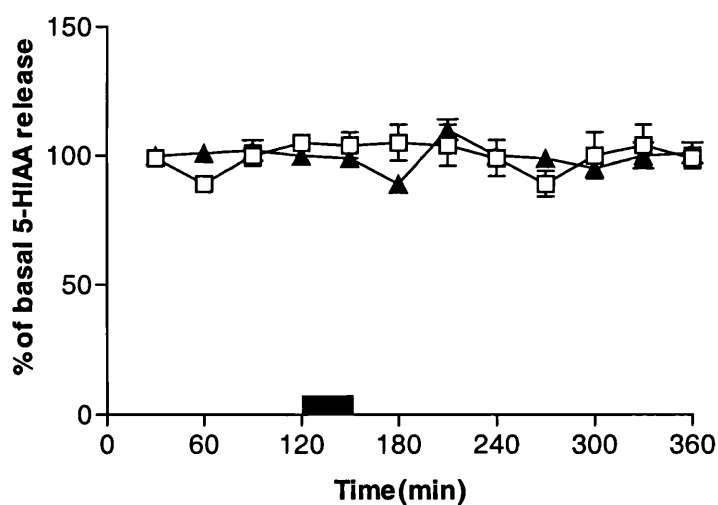
Fig. 3.12: Effect of infusion of SNAP together with Hb into the ventral hippocampus on

5-HT (A), 5-HIAA (B) release. Bars donate the duration of drug infusion. All values are

means ±s.e.m. (shown when larger than the symbol) from 6 experiments.

A)
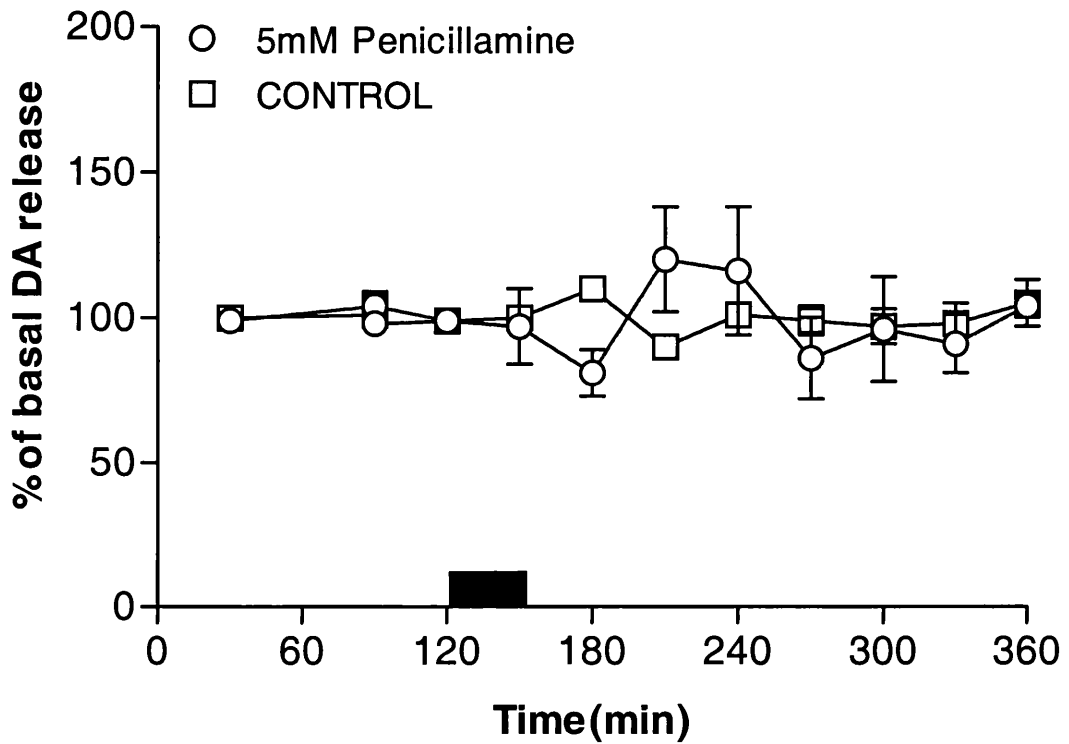
Fig. 3.13: Effect of penicillamine infusion on hippocampal DA release. Bar denotes the

period of penicillamine infusion. All values are means ± s.e.m. (shown when larger than

the symbol) from 4 experiments.

A)
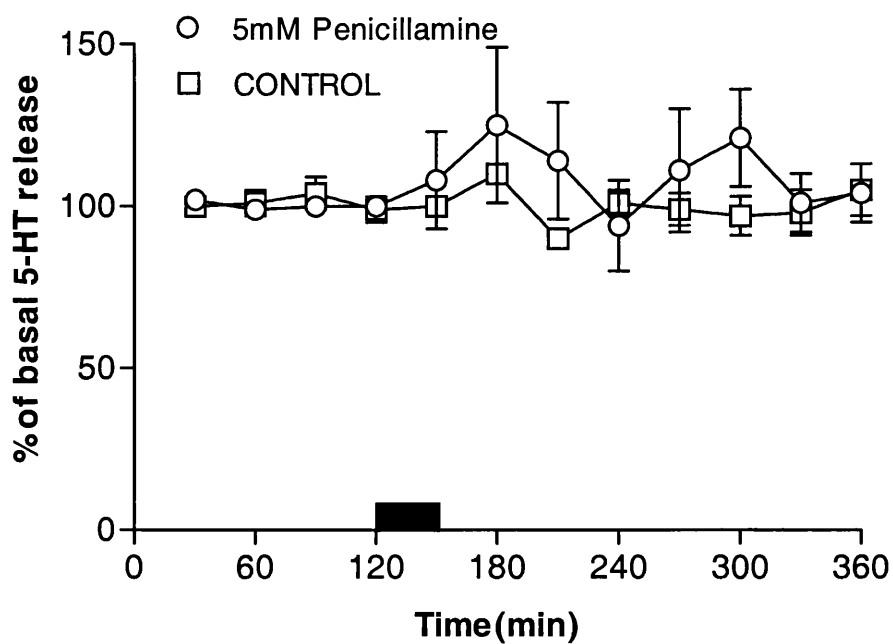
B)
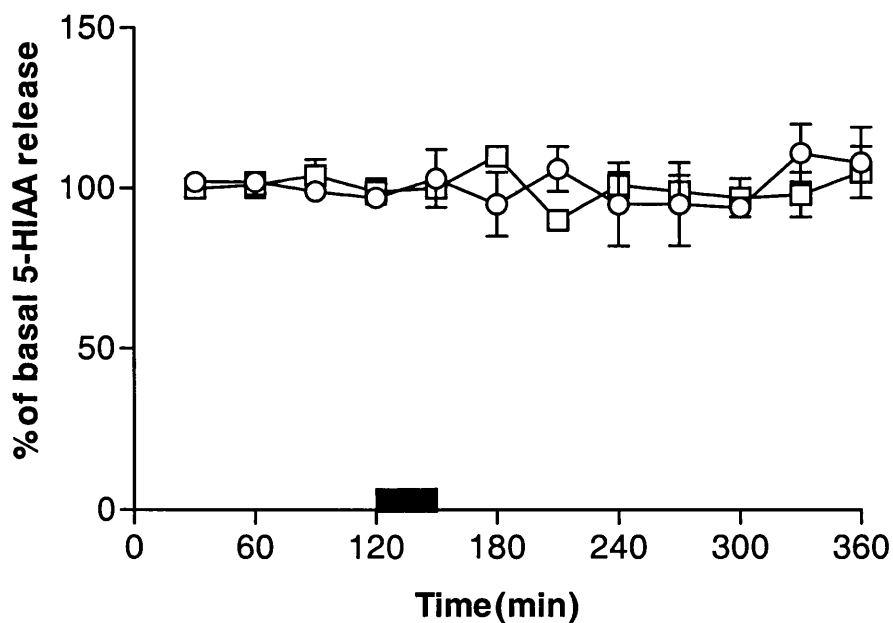
Fig. 3.14: Effect of penicillamine infusion on hippocampal 5-HT (A) and its metabolite

5-HIAA (B) efflux. Bars denote the period from penicillamine infusion. All values are

means ± s.e.m. (shown when larger than the symbol) from 4 experiments.
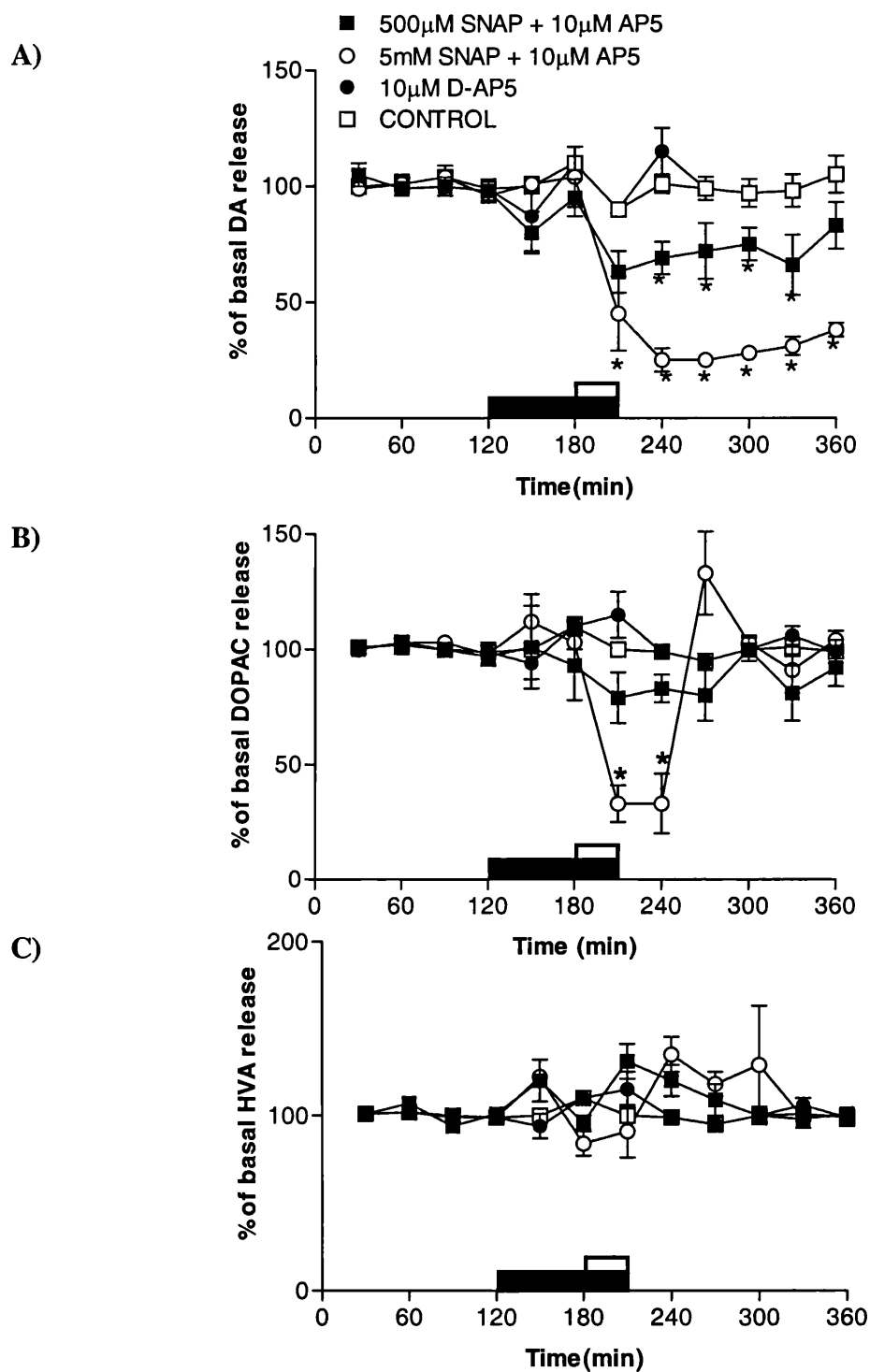
Fig. 3.15: Effect of D-AP5 infusion prior to addition of SNAP on hippocampal DA (A), DOPAC (B) and HVA (C) efflux. The solid bars represent the period of D-AP5 infusion whilst the open bars denote the combined drug treatment. All values are means ± s.e.m., (shown when larger than the symbol) from 4 experiments. * indicates data points significantly different from controls (p<0.05).

A)

B)
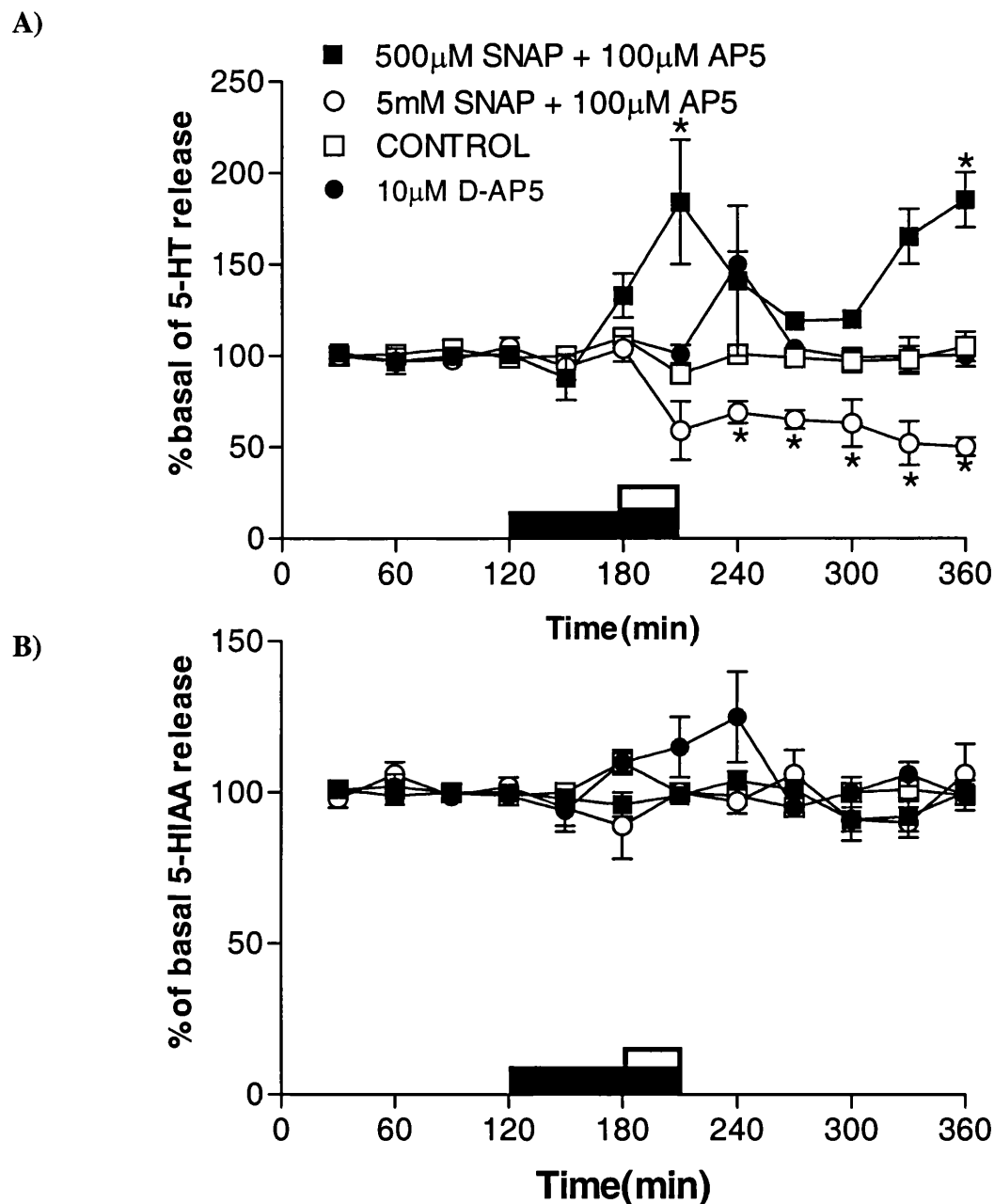
Figure 3.16: Effect of D-AP5 infusion prior to addition of SNAP on hippocampal 5-HT (A) and 5-HIAA (B) efflux. The solid bars denote the period of D-AP5 infusion whilst the open bars represent the addition of SNAP. All values are means ± s.e.m. (shown when larger than the symbol) from 4 experiments. * indicates data points significantly different from controls ($p < 0.05$).

HVA and 5-HIAA (Fig. 3.15 C, Fig 3.16 B). The extracellular levels of DOPAC decreased by up to 60 ± 15% on addition of 5mM SNAP (Fig. 3.15 B).

3.2.4. Effects of NOS inhibition on basal and NMDA evoked release of amino acids and monoamines.

Continuous infusion of L-NAME elicited differential effects on the release of amino acids when compared to that of monoamines. L-GLU levels were increased by approximately 20 ± 10% at 1mM L-NAME, a level which was generally maintained for the duration of the experiment. The increase in L-ASP efflux was immediate reaching a maximum of approximately 50 ± 10% before declining (Fig. 3.17 A and B). In contrast, 1mM L-NAME elicited a decrease in GABA levels by up to 50 ± 10%, an effect which was not maintained (Fig. 3.17 C). Monoamine levels were elevated by up to 160 ± 10% for DA and 100 ± 15% for 5-HT following infusion of the inhibitor (Fig. 3.18 A, Fig. 3.19 A). The increase in DA levels was gradual whilst that of 5-HT was rapid in onset and followed by a plateau period. Despite continuous infusion of L-NAME, the levels of the monoamines returned to baseline before the administration of the inhibitor ceased. The levels of monoamine metabolites were unaltered (Fig. 3.18 B and C, Fig. 3.19 B). Continuous infusion of 7-nitoro-indazole-monosodium salt (7-NINA) (1mM), on the other hand, produced an overall decrease in neurotransmitter release (Fig. 3.20 A B and C, Fig. 3.22 A). L-GLU and L-ASP declined by approximately 70 ± 15%, while 5-HT and GABA decreased by 50 ± 10% below baseline (Fig. 3.20 B and C, Fig. 3.22 A.). The observed decrement in neurotransmitter release persisted for the duration of the experiment.
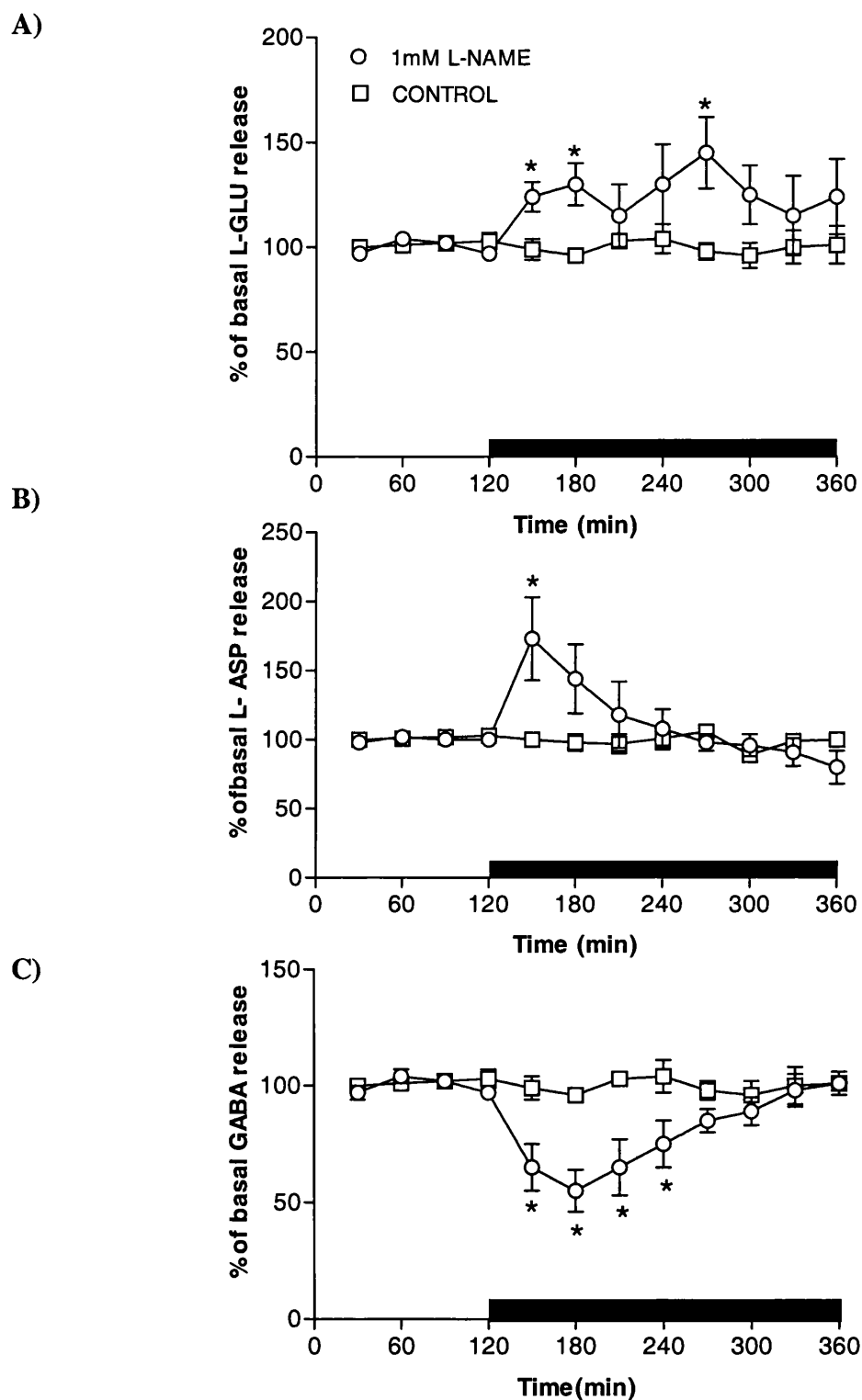
Fig. 3.17: Effect of L-NAME infusion into the ventral hippocampus on L-GLU (A), L-ASP (B) and GABA (C) release. Bars represent the period of L-NAME infusion All values are means ± s.e.m. (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from controls (p<0.05).

Fig. 3.18: Effect of L-NAME infusion into the ventral hippocampus on DA (A), DOPAC (B) and HVA (C) levels. Bars represent the period of L-NAME infusion. All values are means ± s.e.m. (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from controls ($p < 0.05$).
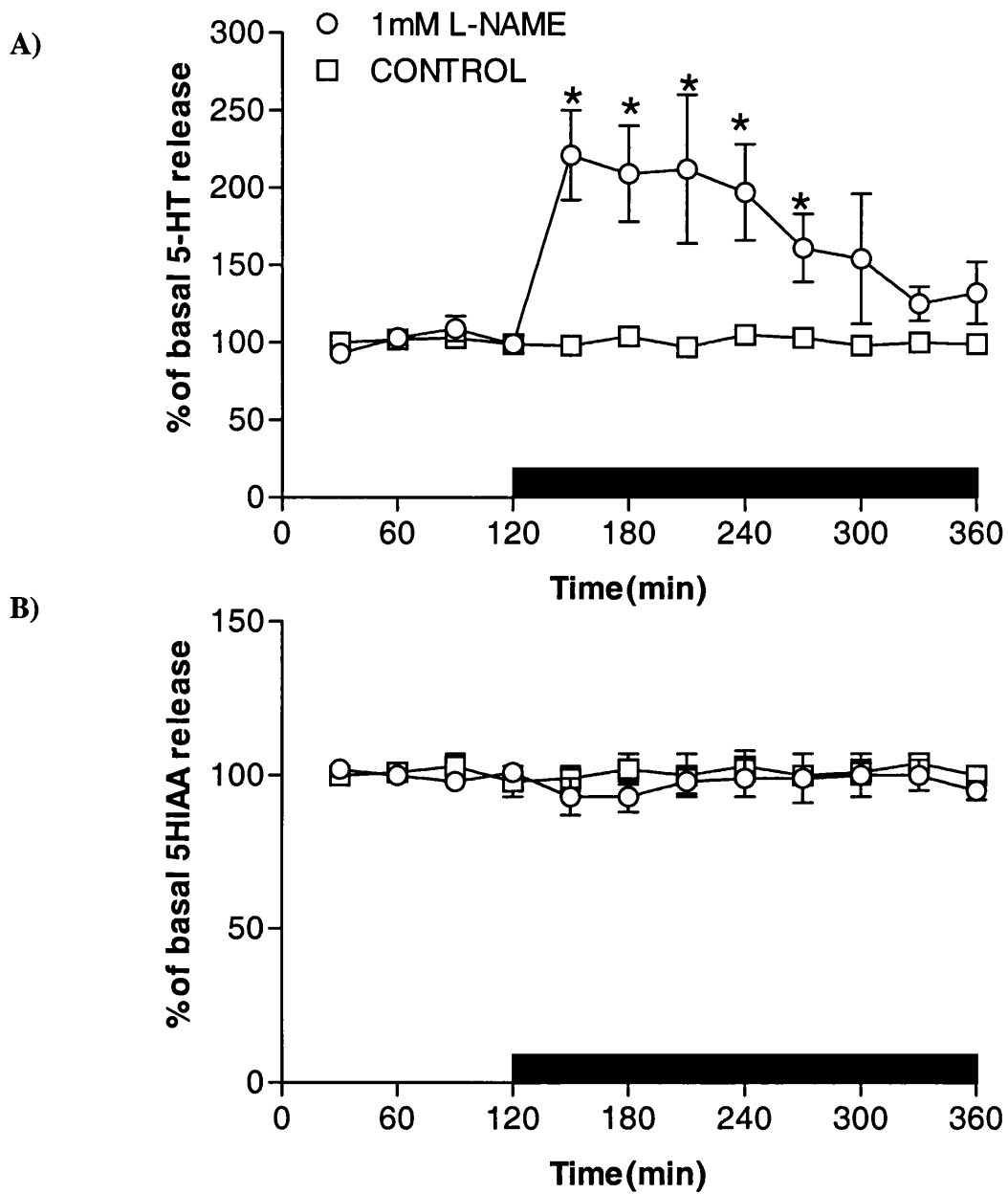
Fig. 3.19: Effect of L-NAME infusion on 5-HT (A) and 5-HIAA (B) levels. Bars represent the period of L-NAME infusion. All values are means ± s.e.m. (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from controls ($p < 0.05$).

Fig. 3.20: Effect of 7-NINA infusion on hippocampal L-GLU (A), L-ASP (B) and GABA (C) efflux. The bars denote the period of 7-NINA infusion. All values are means ± s.e.m., (shown when larger than the symbol) from 7 experiments. * indicates data points significantly different from controls (p<0.05).

Fig. 3.21: Effect of 7-NINA infusion on hippocampal DA (A), DOPAC (B) and HVA (C) efflux. The bars denote the period of 7-NINA infusion. All values are means ± s.e.m, (shown when larger than the symbol) from 7 experiments. * indicates data points significantly different from controls (p<0.05).
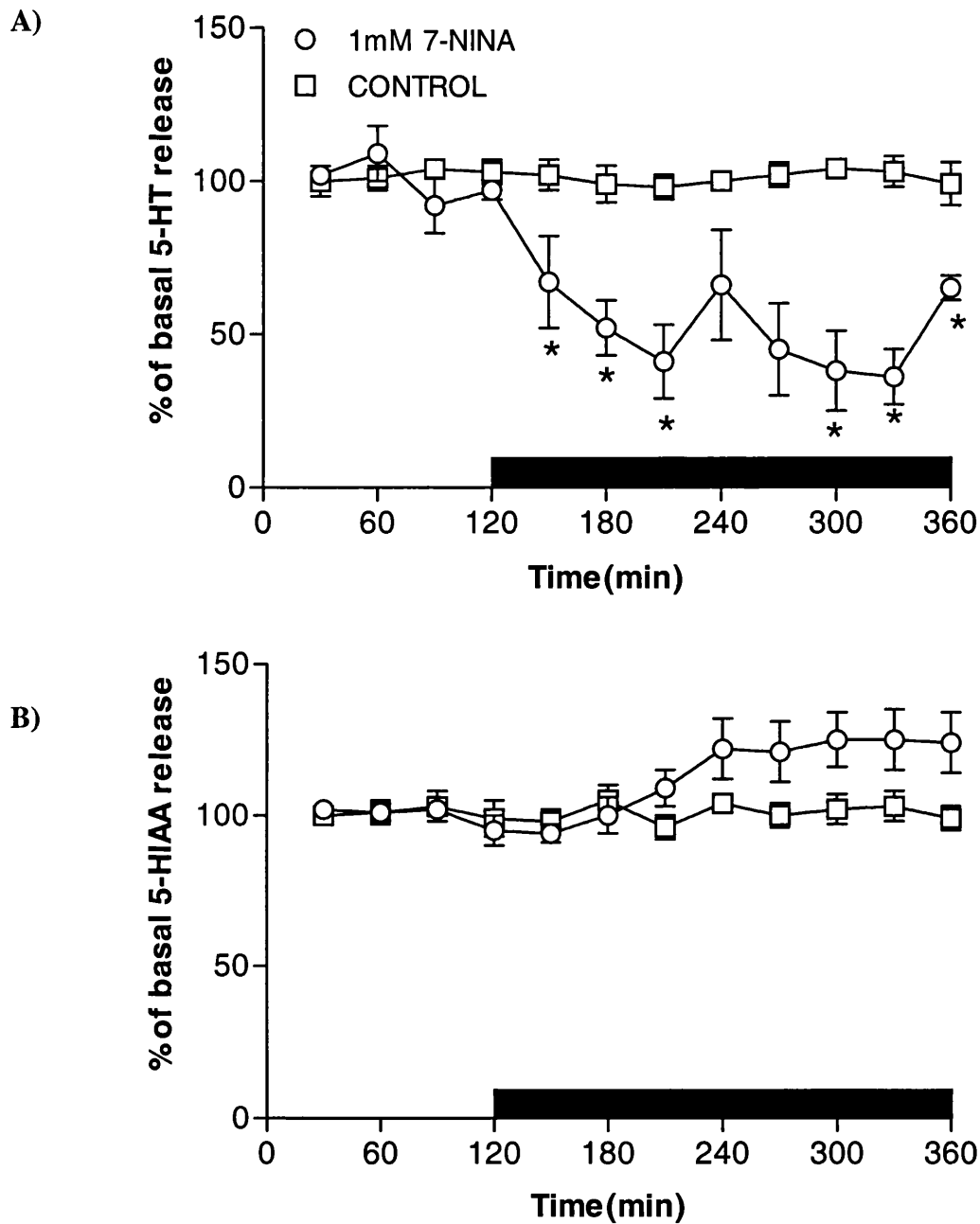
Fig. 3.22: Effect of 7-NINA infusion on hippocampal 5-HT (A) and 5-HIAA (B) efflux. The bars denote the period of 7-NINA infusion. All values are means ± s.e.m., (shown when larger than the symbol) from 7 experiments. * indicates data points significantly different from controls ($p < 0.05$).

Conversely, extracellular DA and DOPAC levels were elevated following the administration of 7-NINA at 1mM (Fig. 3.21 A and B). The augmentation of DA, DOPAC and HVA levels was slow in onset and persisted during the time course of the experiment (Fig. 3.21 A, B and C.). No statistically significant differences, in comparison to controls, were observed in 5-HIAA levels (Fig. 3.22 B).

To ensure that the inhibition of neurotransmitter release observed following the infusion of 7-NINA was due to the action of this compound on NOS, the effect of 7-NINA in the presence of SNAP was examined. Fig. 3.23 A and B shows that when 7-NINA (1mM) was infused into the hippocampus for a period of 60 min a significant decrease in basal amino acids levels was observed. L-GLU and L-ASP levels declined by approximately $50 \pm 15\%$ and this decrement was sustained in that the levels of these amino acids did not return to basal levels within the time course of the experiment. When 7-NINA (1mM) was infused together with SNAP at (5mM) the inhibition of L-GLU and L-ASP release produced by the inhibitor was reversed and the release of amino acids was increased by up to $500 \pm 55\%$ and $250 \pm 25\%$ respectively (Fig. 3.23 A and B). Infusion of 7-NINA together with 500μM SNAP on the other hand, showed a small but significant increase in L-GLU $50 \pm 10\%$ and no significant changes in L-ASP levels (Fig. 3.23 A and B.).

When 1mM L-NAME was infused for 60 min prior to co-infusion with NMDA (100μM), the NMDA-evoked release of L-GLU was less than that seen following NMDA infusion alone (Fig. 3.24 A) whilst L-ASP levels declined by approximately $50 \pm 10\%$ below control values (Fig. 3.24 B). The decrease in L-ASP levels became significant after the infusion of drugs was stopped and the levels of the amino acid returned to basal values within the time course of the experiment.

Fig. 3.23: Effect of 7-NINA infusion prior to co-infusion with SNAP on hippocampal L-GLU (A) and L-ASP (B) release. The solid bars denote a period of 7-NINA infusion while the open bars indicate the combination of 7-NINA with SNAP. All values are means ± s.e.m., (shown when larger than the symbol) from 5 experiments. * indicates data points significantly different from controls (p<0.05).

**Fig. 3.24:** Effect of L-NAME infusion prior to co-infusion of NMDA on hippocampal

L-GLU (A), L-ASP (B) and GABA (C) release. The solid bars represent the period of

L-NAME infusion and the open bars the addition of L-NAME together with NMDA.

All values are means ± s.e.m., (shown when larger than the symbol) from 5

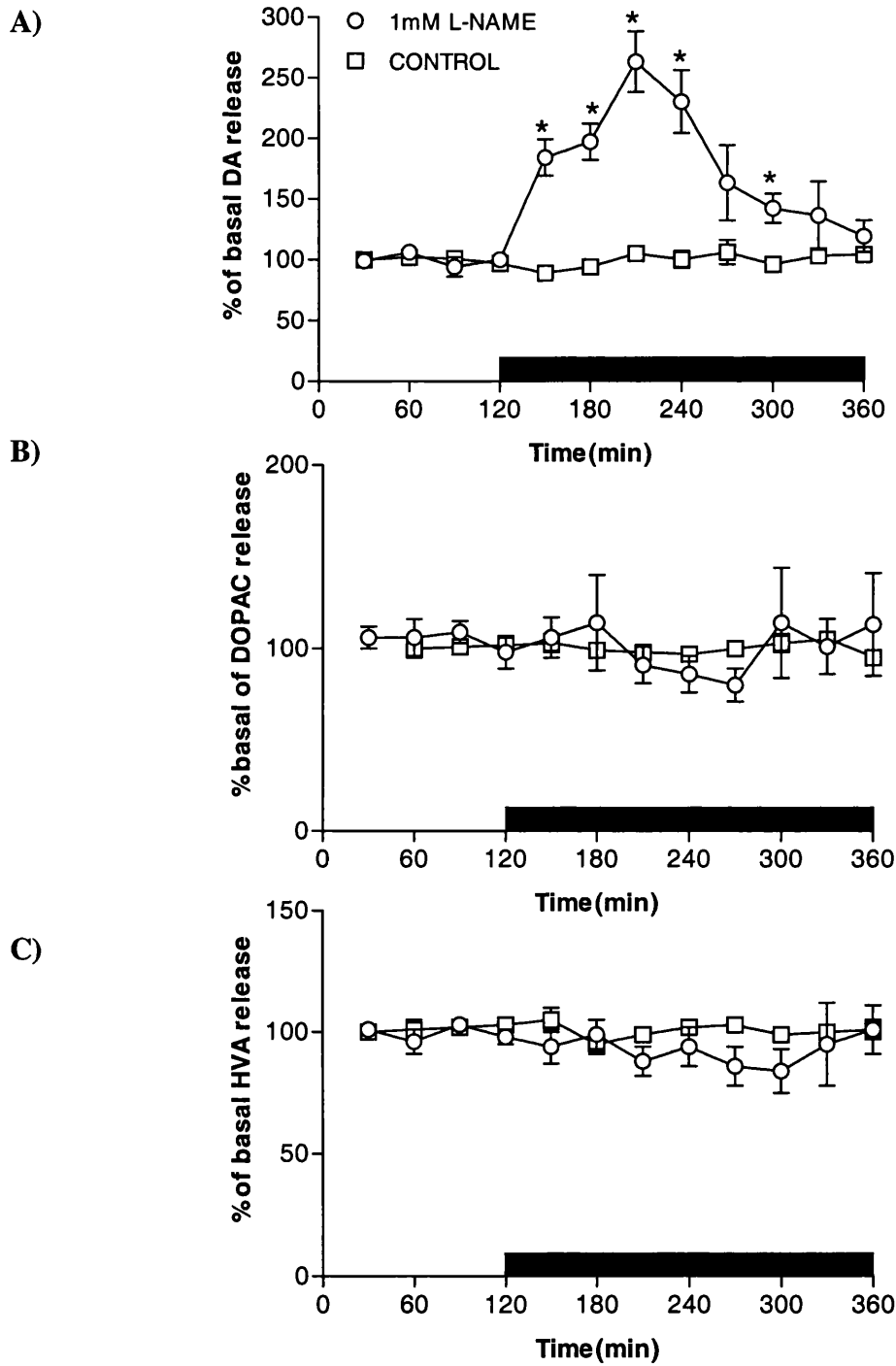experiments. * indicates data points significantly different from controls (p<0.05).

A)

■ 100µM NMDA
O 1mM D-NAME +100µM NMDA
□ CONTROL

B)

C)

D)
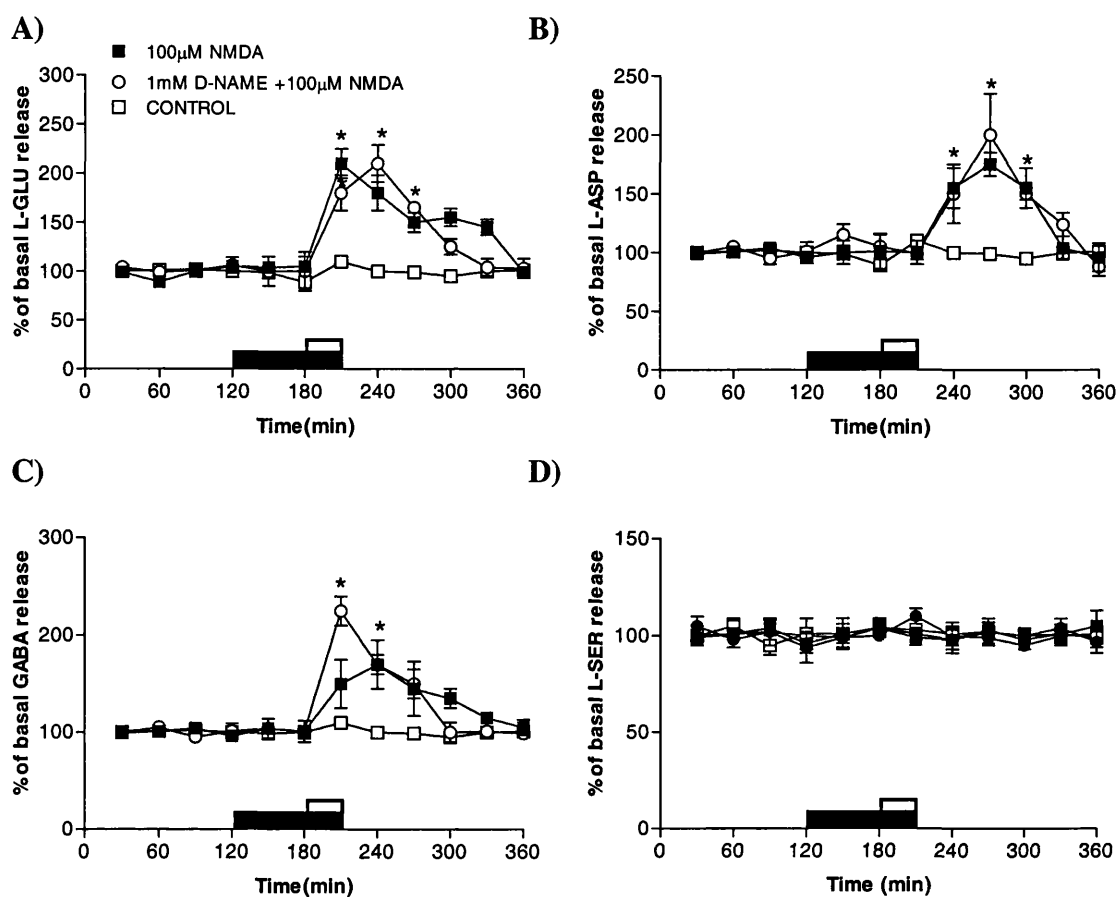
Fig. 3.25: Effect of D-NAME infusion prior to co-infusion with NMDA into the ventral hippocampus on L-GLU (A), L-ASP (B), GABA (C) and L-SER (D). The solid bars represent the period of D-NAME infusion, while the open bars the addition of D-NAME together with NMDA. All values are means ± s.e.m., (shown when larger than the symbol) from 5 experiments. * data points significantly different from controls (p< 0.05).

The initial decrease in GABA release was reversed on addition of NMDA (Fig. 3.24 C). Infusion of 1mM L-NAME prior to co-infusion with NMDA, on the other hand, reversed the inhibition of monoamine release produced following the infusion of NMDA alone. Interestingly, the effect of L-NAME was greatly potentiated by the addition of NMDA with DA and 5-HT levels rising by up to $450 \pm 25\%$ and $200 \pm 15\%$ above control values respectively (Fig. 3.26 A Fig. 3.27 A). These effects were transient as it was noted that the levels of these monoamines returned to the baseline shortly after the drug infusion was stopped. Both of these transmitters reached basal levels within the time course of the experiment but the effect was more immediate for 5-HT compared to DA. The effects of NMDA infusion alone on monoamine metabolites were abolished by L-NAME (Fig. 3.26 B and C, Fig. 3.27 B). All the effects of L-NAME were stereospecific, since D-NAME did not alter the NMDA-evoked neurotransmitter release (Fig. 3.25 A, B, C and D, Fig. 3.28 A, B and C, Fig. 3.29 A and B.).

When 7-NINA (1mM) was infused for 60min prior to co-infusion with NMDA (100μM), the NMDA-evoked increase in L-GLU and L-ASP was abolished (Fig. 3.30 A and B). The augmentation of the extracellular DA levels produced by 7-NINA infusion alone ceased on addition of NMDA and DA levels returned to baseline within the time course of the experiment (Fig. 3.31 A) whilst 5-HT levels were increased by $50 \pm 10\%$ above basal values (Fig. 3.32 A). This effect was transient in nature and followed by an immediate decline to the basal levels. The effects of NMDA on all monoamine metabolites were abolished by 7-NINA (Fig. 3.31 B and C; Fig. 3.32 B).
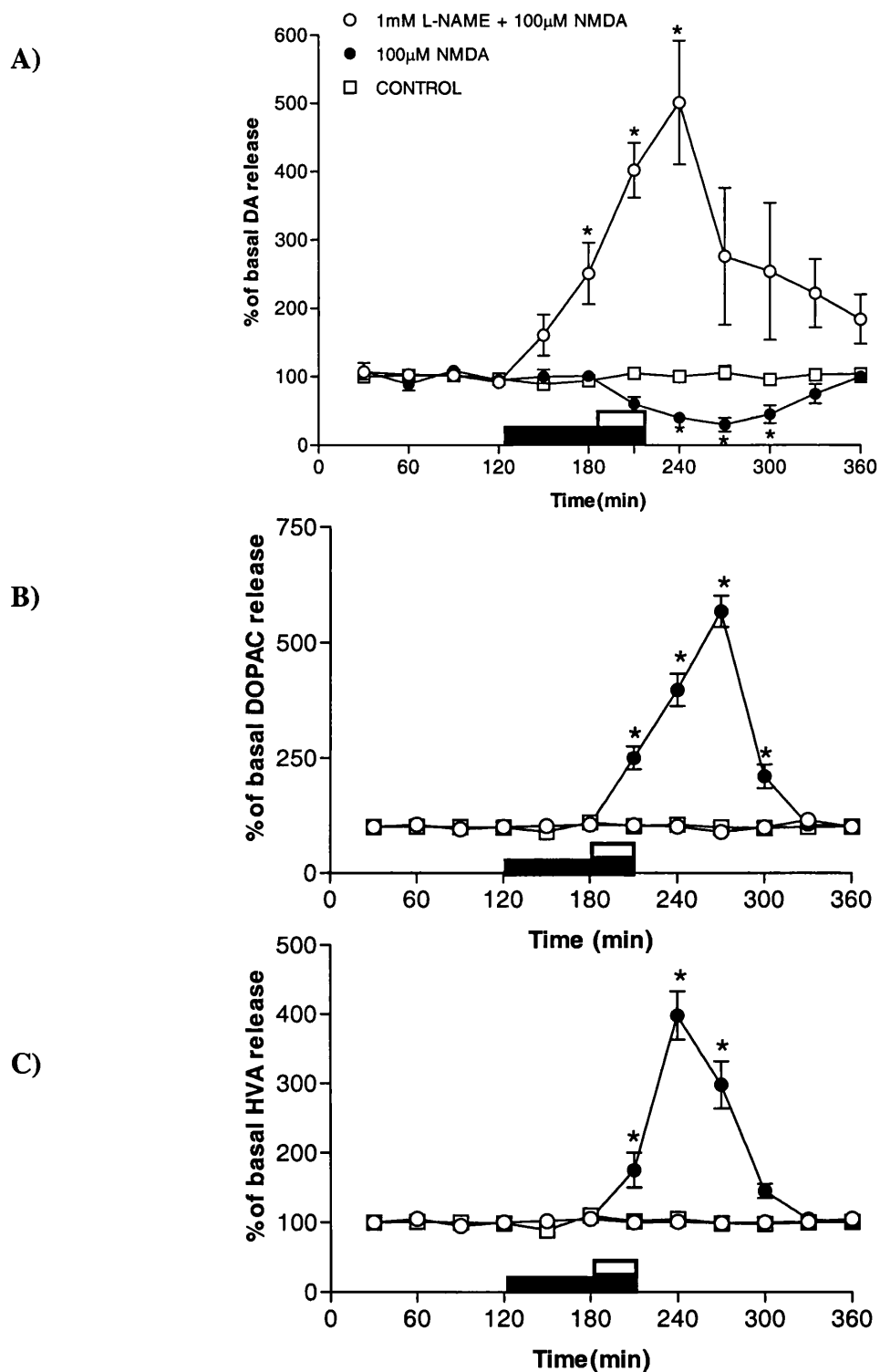
Fig. 3.26: Effect of L-NAME infusion prior to co-infusion with NMDA on hippocampal

DA (A), DOPAC (B) and HVA (C) levels. The solid bars represent the period of L-

NAME infusion while the open bars the addition of L-NAME together with NMDA. All

values are means ± s.e.m., (shown when larger than the symbol) from 5 experiments. *

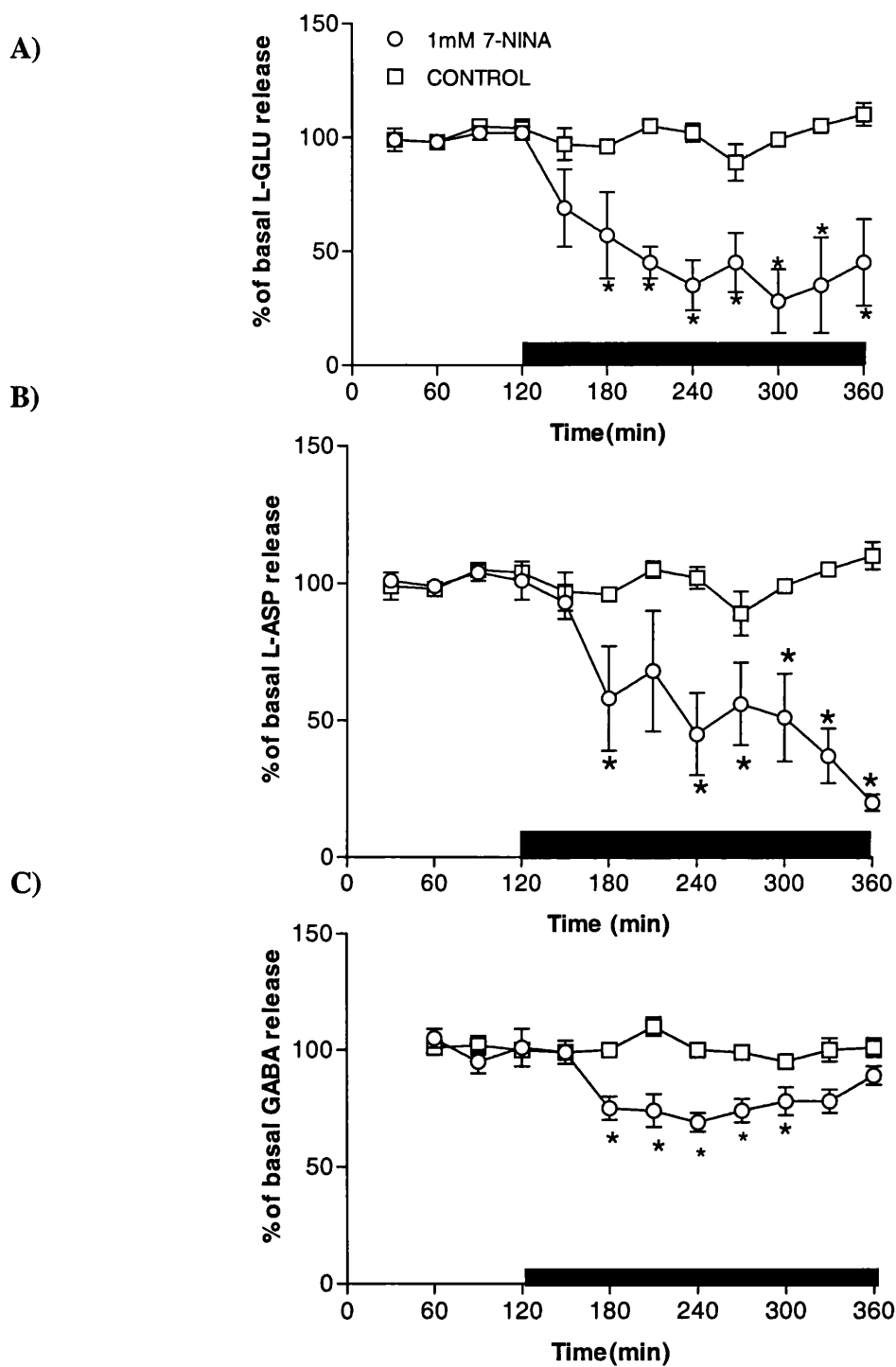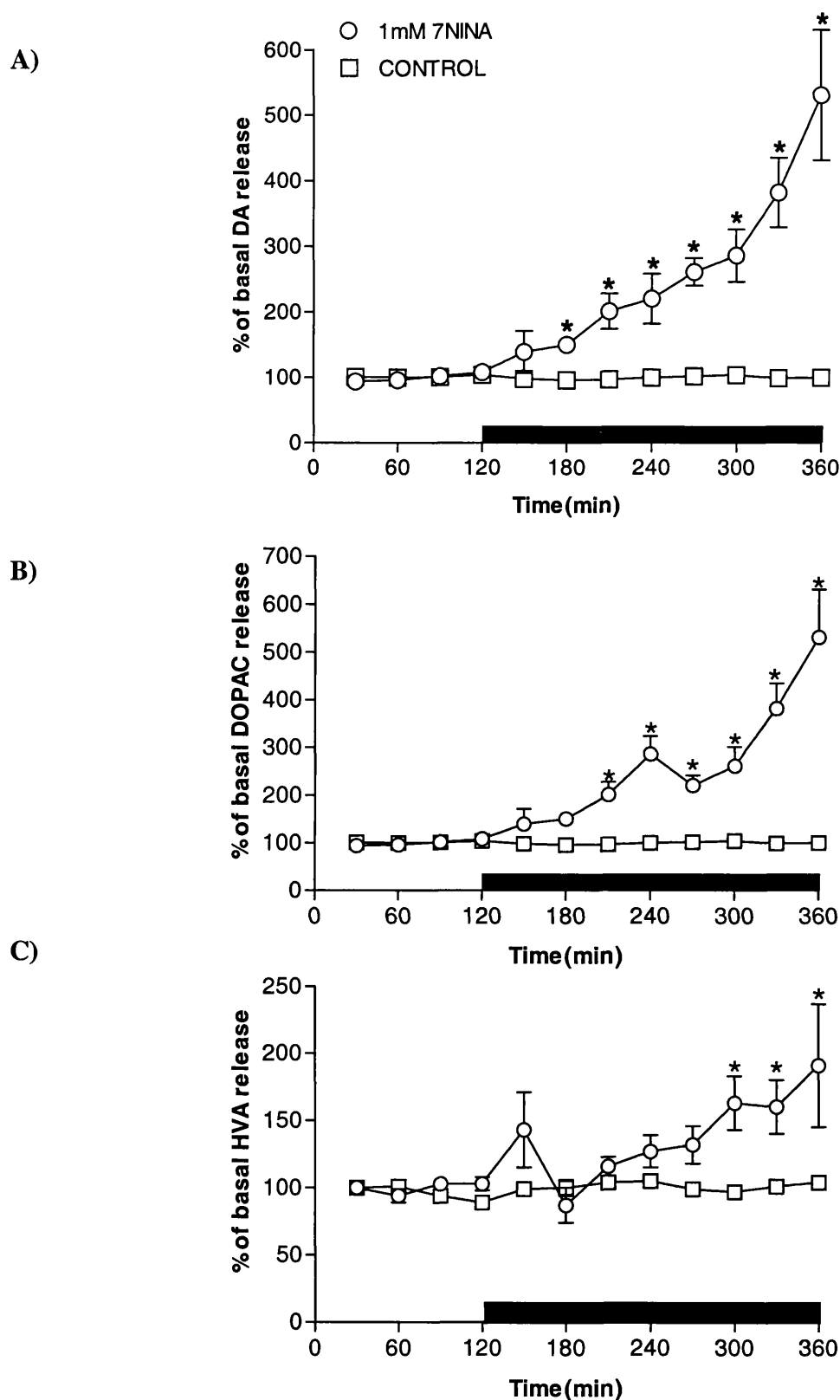indicates data points significantly different from controls (p<0.05).

A)

B)
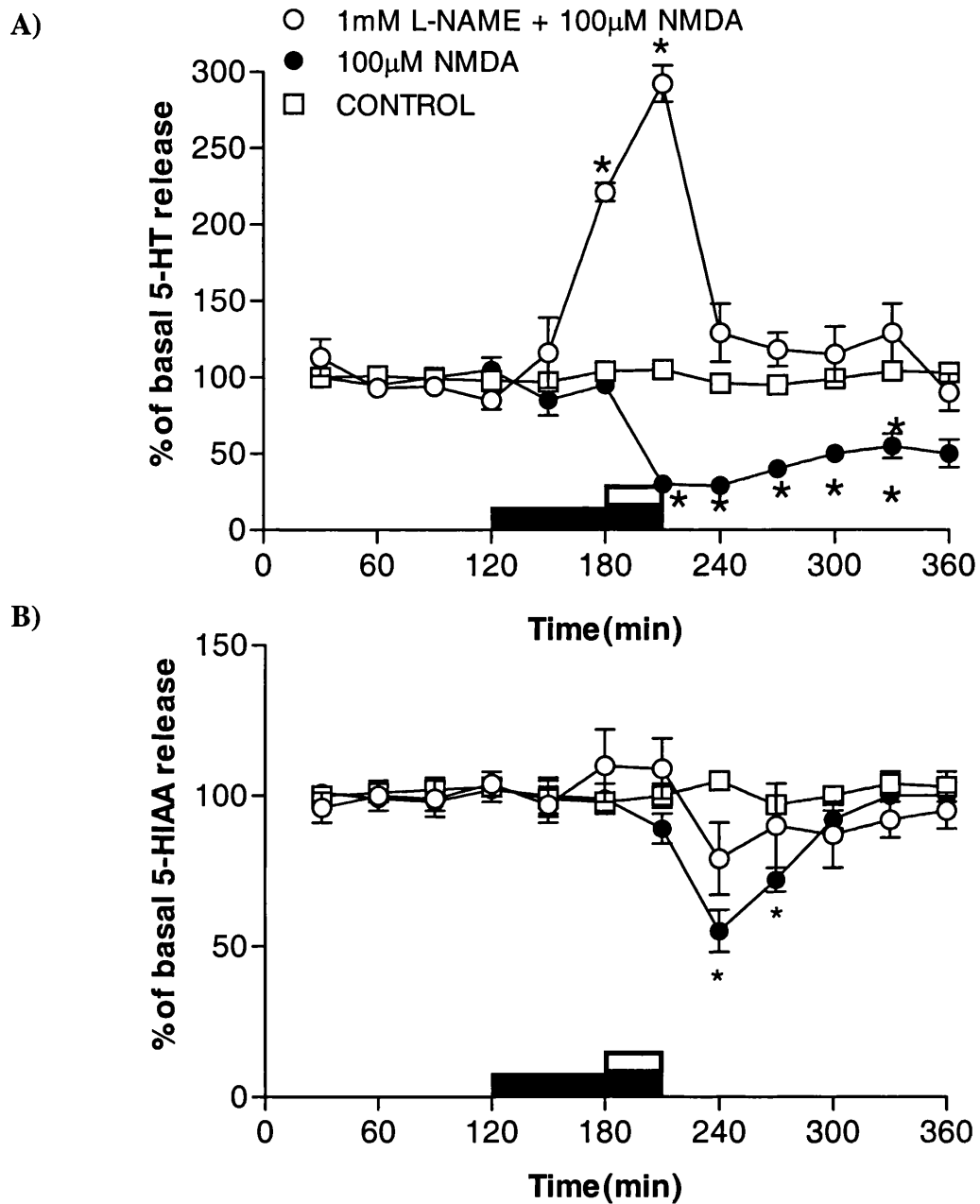
Fig. 3.27: Effect of L-NAME infusion prior to co-infusion with NMDA on hippocampal

5-HT (A) and 5-HIAA (B) levels. The solid bars represent the period of L-NAME

infusion while the open bars the addition of L-NAME together with NMDA. All values

are means ± s.e.m., (shown larger than the symbol) from 5 experiments. * indicates data

points significantly different from controls (p<0.05).
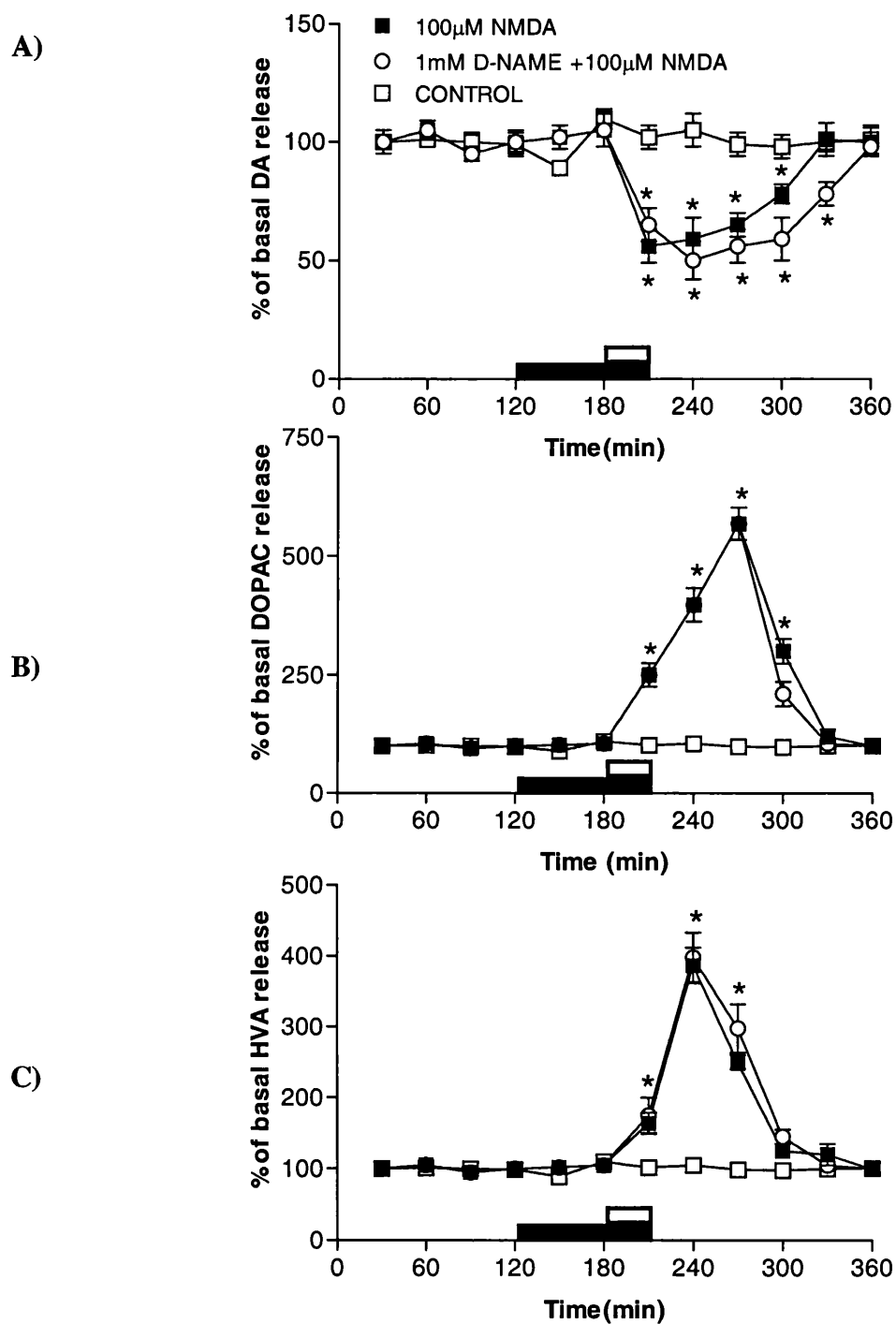
**Fig. 3.28:** Effect of 60 min D-NAME (1mM) infusion prior to co-infusion with NMDA into the ventral hippocampus on DA (A), DOPAC (B) and HVA (C) release. The solid bars represent the period of D-NAME infusion, while the open bars the addition of D-NAME together with NMDA. All values are means ± s.e.m. (shown when larger than the symbol) from 5 experiments. * indicates data points significantly different from controls (p<0.05).
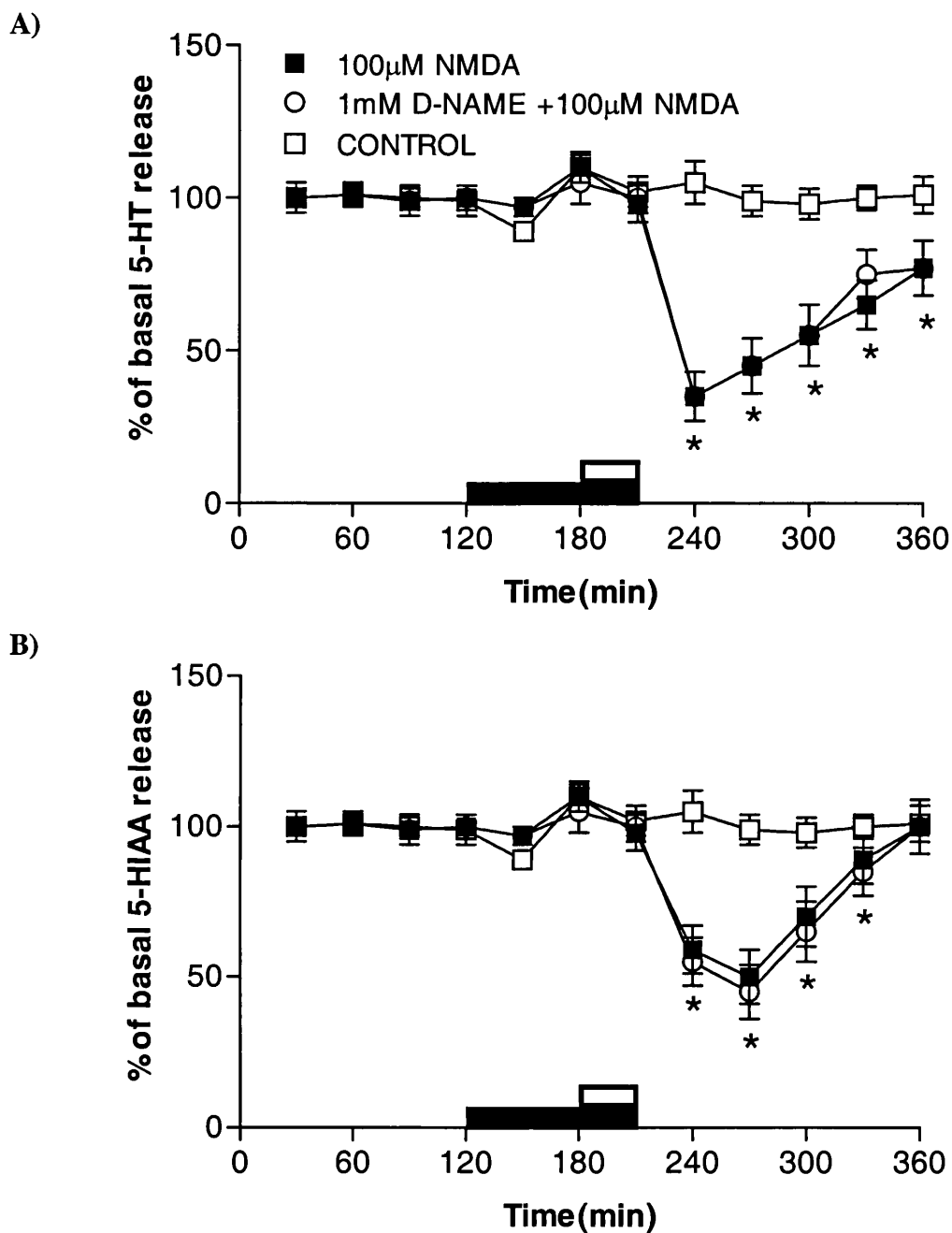
Fig. 3.29: Effect of 60 min D-NAME infusion prior to co-infusion with NMDA on 5-HT

(A) and 5-HIAA (B) release. The solid bars represent the period of D-NAME infusion

and the open bars the addition of D-NAME together with NMDA. All values are means

± s.e.m., (shown when larger than the symbol) from 5 experiments. * indicates data

points significantly different from controls (p<0.05).
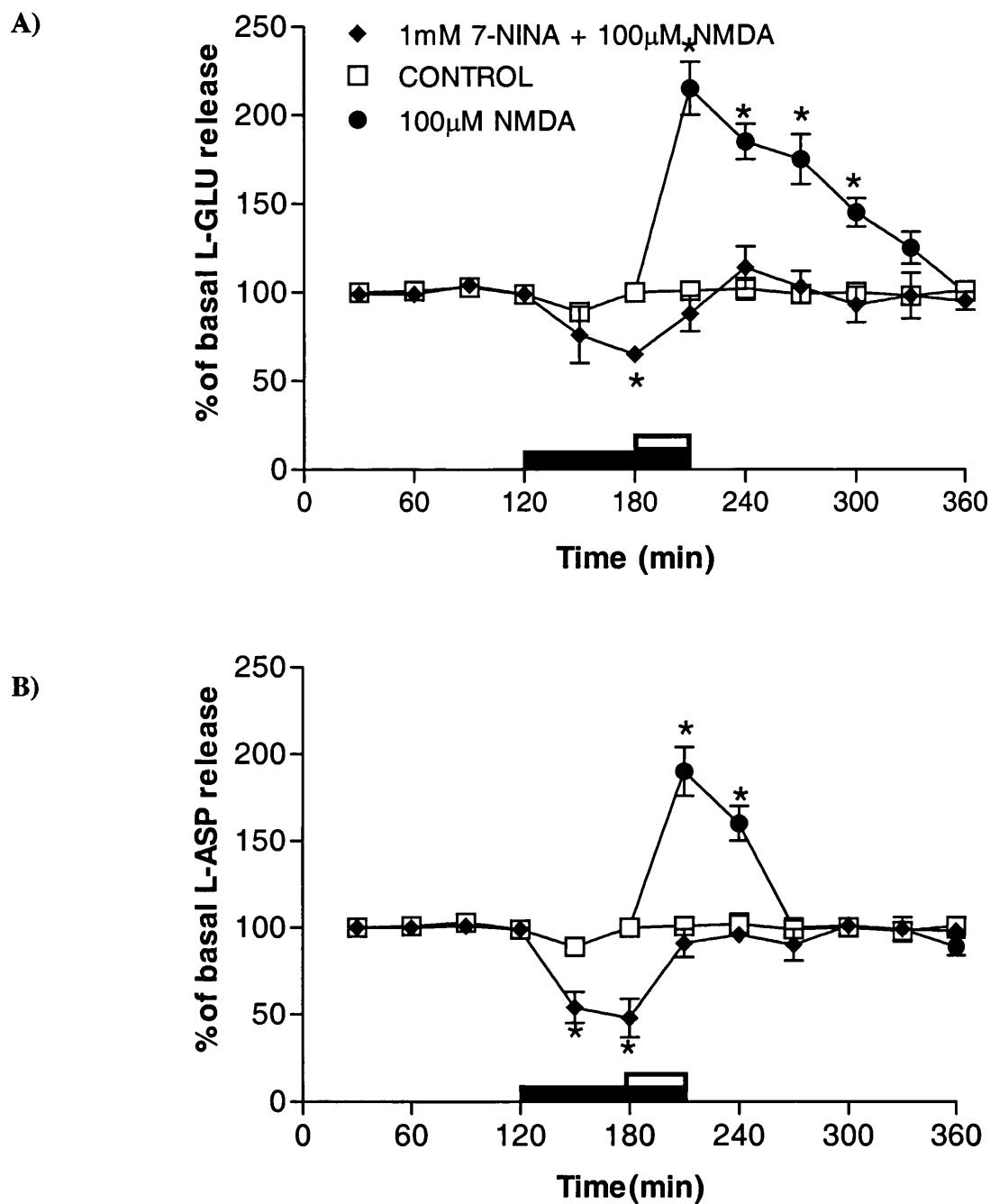
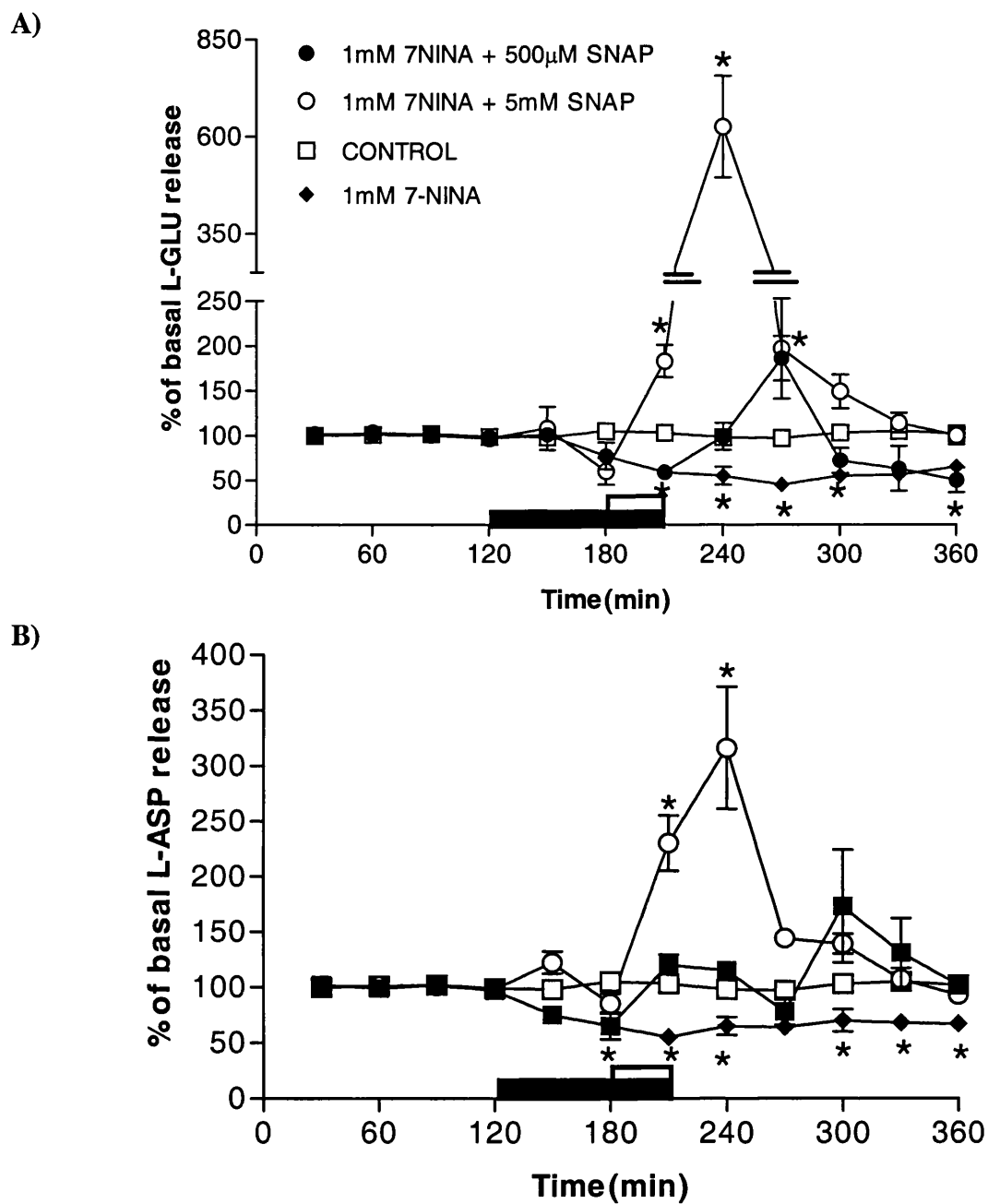**Fig. 3.30:** Effect of 7-NINA infusion to co-infusion with NMDA on hippocampal L-GLU (A) and L-ASP (B) release. The solid bars represent the period of 7-NINA infusion and the open bars the addition of NMDA and 7-NINA together. All values are means ± s.e.m., (shown larger than the symbol) from 4 experiments. * indicates data points significantly different from controls ($p < 0.05$).
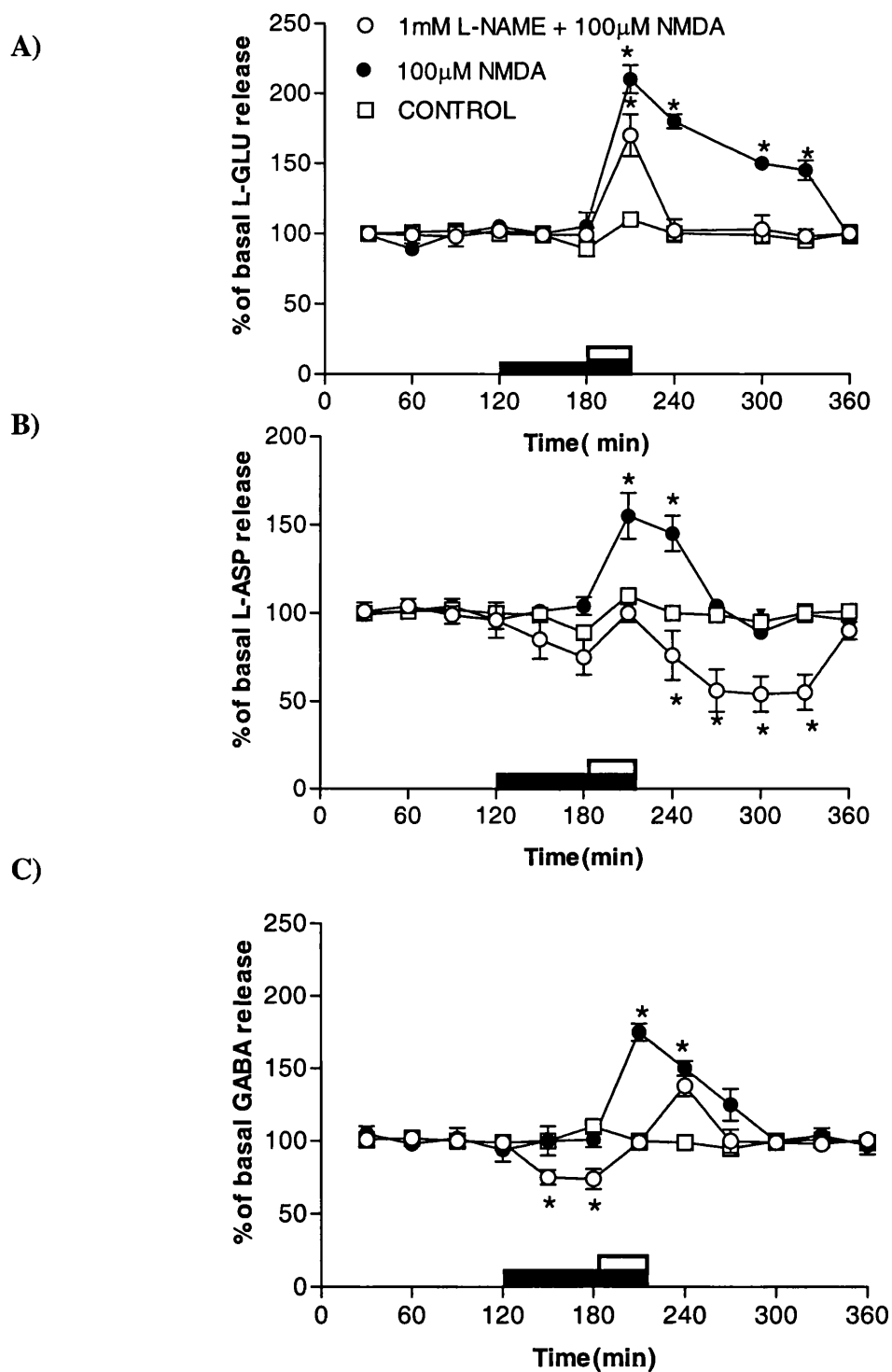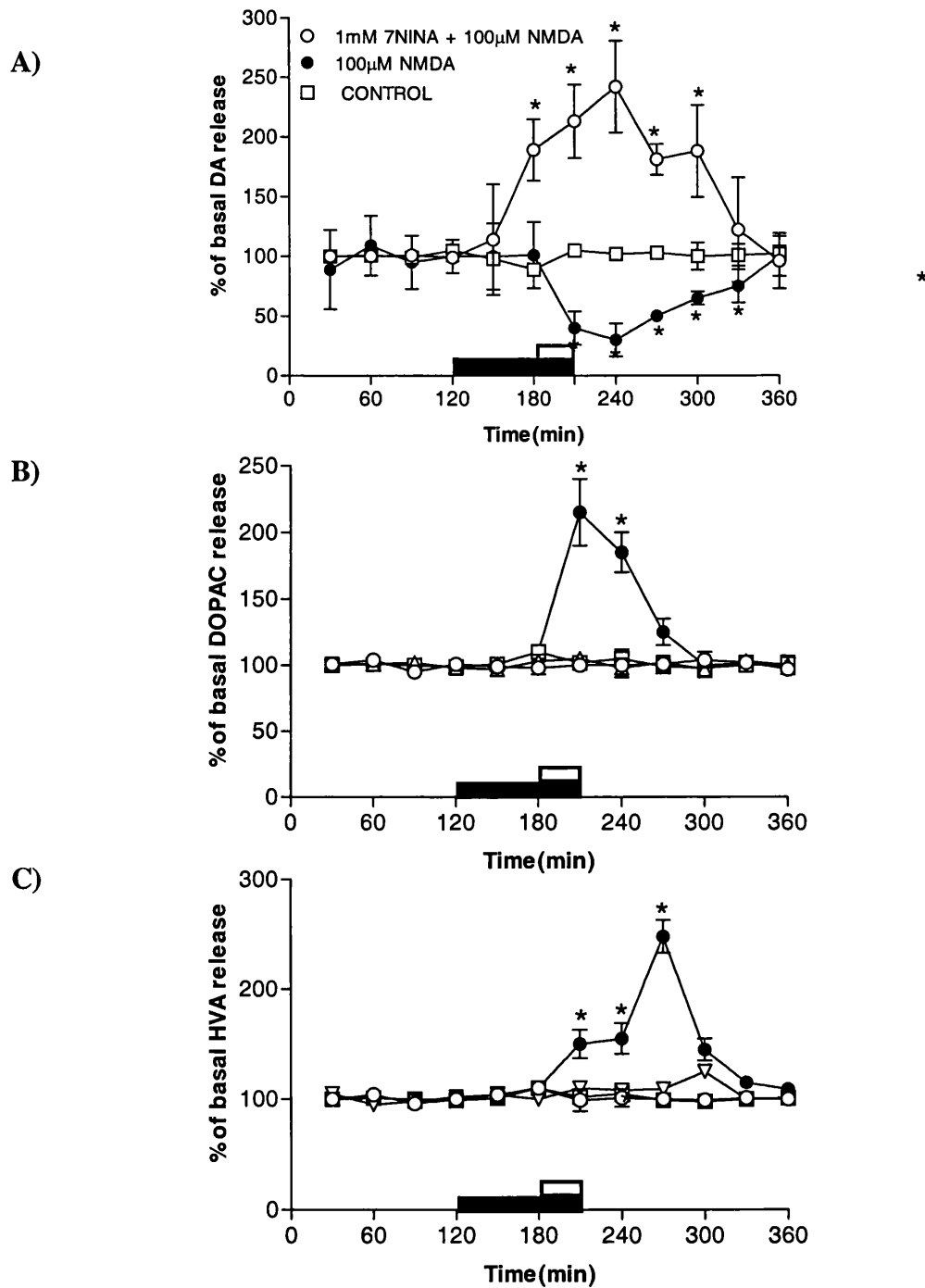
Fig. 3.31: Effect of 7-NINA infusion prior to co-infusion with NMDA on hippocampal DA (A), DOPAC (B) and HVA (C) levels. The solid bars represent the period of 7-NINA infusion while the open bars the infusion of NMDA and 7-NINA together. All values are means ± s.e.m., (shown when larger than the symbol) from 5 experiments. * indicates data points significantly different from controls ($p<0.05$).

A)

B)
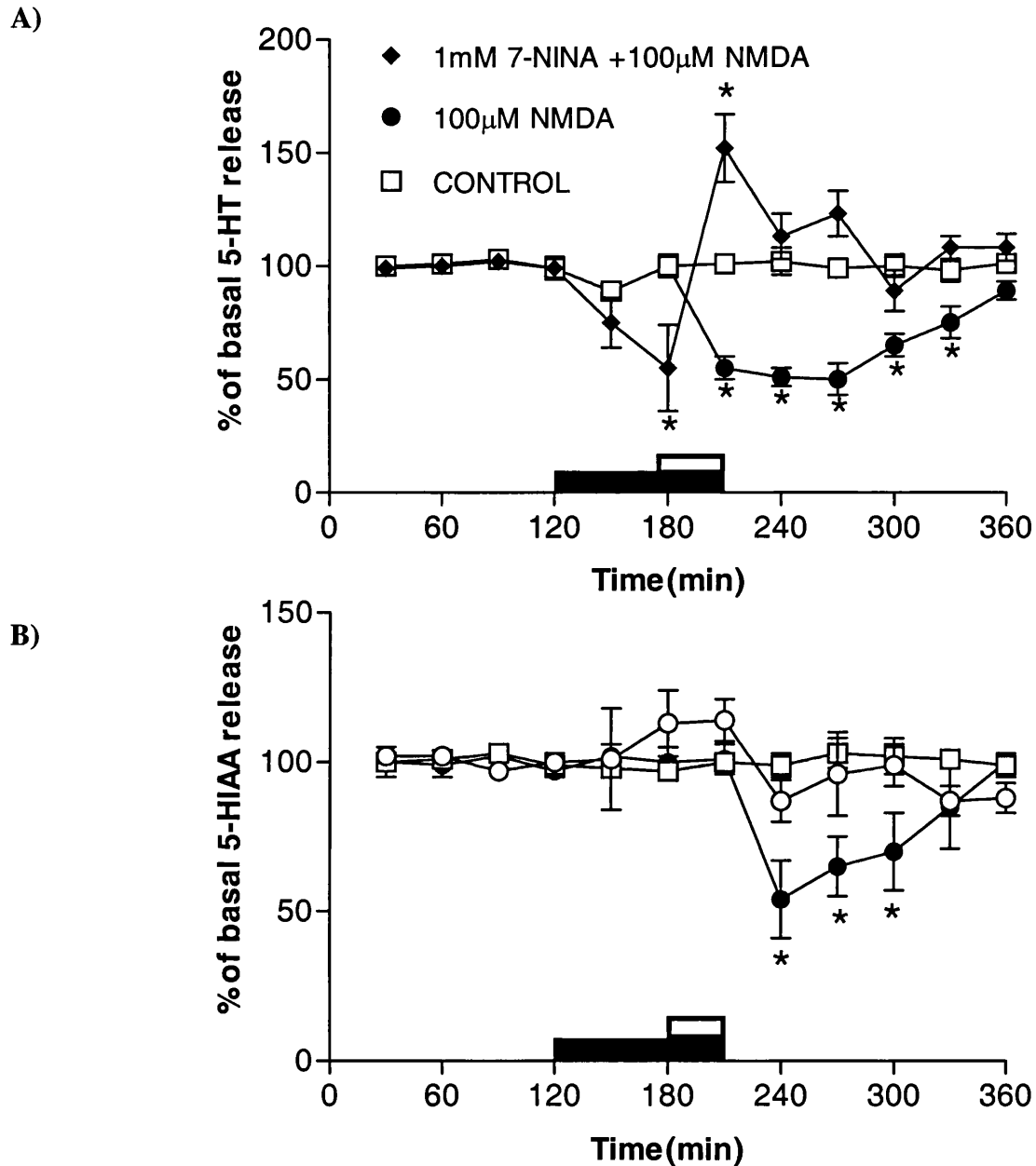
Fig. 3.32: Effect of 7-NINA infusion to co-infusion with NMDA on hipocampal 5-HT (A) and 5-HIAA (B). The solid bars represent the period of 7-NINA infusion and the open bars the infusion of NMDA and 7-NINA together. All values are means ± s.e.m., (shown when larger than the symbol) from 5 experiments. * indicates data points significantly different from controls (p<0.05).

3.3 Discussion

3.3.1.Overview

As stated in the introduction to this chapter, the exact role played by NO in the control of communication between hippocampal neurones remains to be fully elucidated. Indeed, some of the published data are contradictory suggesting both stimulatory and inhibitory actions of NO on neurotransmitter release (Nei et al., 1996; Stout and Woodward, 1994). In an attempt to resolve some of these discrepancies this study was undertaken to investigate the role of NO in the regulation of basal and NMDA–evoked neurotransmitter release in freely moving animals.

The data presented in this chapter indicate that NMDA receptor activation leads to neurotransmitter release in the rat ventral hippocampus. It also suggests that L-GLU may play a major role in regulating both dopaminergic and serotoninergic efflux, possibly via NO production. However, inhibition of NOS has also been found to modulate amino acid and monoamine release suggesting the existence of tonic NO-mediated regulation of neurotransmitter release. The observed inverse concentration-dependence between the prevailing NO levels and the resultant responses may suggest a complex interaction between NO, NMDA receptor activation and neurotransmitter release. These observations serve to illustrate the possibility that NO exerts a dual action (inhibitory/stimulatory) on neurotransmitter release in this brain region.

3.3.2 The relationship between NMDA receptor activation and neurotransmitter release in rat ventral hippocampus in vivo.

The observed elevation of L-GLU, L-ASP and GABA levels following the infusion of NMDA was in contrast to its effect on monoamine release where both DA and 5-HT levels declined. These effects were shown to be the result of NMDA

receptor activation because they were inhibited by D-AP5, a selective NMDA receptor antagonist. A decrease in L-GLU and a transient elevation of 5-HT levels were observed following the infusion of D-AP5 alone. This is consistent with the findings of a previous study (Whitton et al., 1994 a, b) and implies that these receptors are involved in the tonic regulation of both L-GLU and 5-HT release. NMDA was also observed to alter hippocampal DA and 5-HT metabolism. DOPAC concentrations were sharply increased following NMDA infusion reflecting increased metabolism of DA within hippocampal nerve terminals. The changes in the extracellular levels of DOPAC were also abolished by D-AP5. HVA concentrations were also increased by NMDA infusion, possibly because changes in HVA are secondary to alterations in DOPAC concentration (Rao et al., 1990) and inhibited by D-AP5. In addition, D-AP5 alone caused a small but significant decrease in HVA levels suggesting presence of tonic glutamatergic tone regulating DA release and/or metabolism in the hippocampus. Comparatively little change in extracellular 5-HIAA levels was seen after NMDA or D-AP5 infusion.

Overall, these data are in agreement with previous studies (Crowder et al., 1987b; Connick and Stone, 1988; Whitton et al., 1994 a, b; Jones et al., 1995;). However, a recent report by Liu et al. (1995), whilst indicating an increase in amino acid release following the application of NMDA and therefore confirming the current results, also contradicted the observation of the effect of D-AP5 infusion alone on L-GLU release. In the present study it is reported that infusion of D-AP5 leads to a decrease in L-GLU release. This may be attributed to some methodological differences (e.g. probe size and flow rate) but more importantly these authors studied the release of amino acids from dorsal hippocampus. The reason for these discrepancies is yet unknown but may suggest distinct interactions between L-GLU and inhibitory neuronal systems (e.g.

GABA neurones) in these two regions of the hippocampus. It is important however, to stress here that collectively these data indicate that there are ongoing processes in the hippocampus that control the basal outflow of L-GLU. For example, L-GLU may be tonically activating GABA interneurones and the increase in the release of GABA from interneurones or axon collaterals could in turn inhibit release of L-GLU from adjacent terminals.

In support of this hypothesis, the current data shows that application of NMDA increases the release of GABA whilst NMDA receptor antagonists have been reported to decrease its release (Young and Bradford, 1991: Giovannini et al., 1994).

Regionally specific effects of NMDA on amino acid and monoamine release have been reported (Whitton et al., 1994 a, b)) and the data presented here would suggest that the activation of NMDA receptors in the hippocampus produces a different neurochemical profile than in other brain regions. Previous investigations have revealed that both amino acid and monoamine levels rise in the striatum and frontal cortex following the infusion of NMDA (Bogdanov et al., 1997, Young and Bradford, 1991; Sved et al., 1993; Whitton et al., 1994 a, b,) whilst the current data show an increase in amino acid and decline in monoamine levels in the hippocampus. Of particular interest is a clear trend towards an increase in amino acid release in all brain regions studied to date whilst monoamine efflux seems to be brain structure dependent. There are several possible explanations for these observations. First, a number of reports suggest the presence of a positive feedback system controlling L-GLU release in the brain (Choi et al., 1988; Adamson et al., 1990; Young and Bratford, 1991; Herrero et al., 1992; 1994). For example, kainate and NMDA (Monagham et al., 1989) as well as the metabotropic L-GLU receptor agonist trans-

aminocylopentanedicarboxylic acid (Irving et al., 1990) have been shown to increase the extracellular

levels of L-GLU in vivo and synaptic release of L-GLU in vitro (Young et al., 1988;

Herrero et al., 1992; 1994). A recent report by Breukel et al. (1998) suggested the

presence of presynaptic NMDA receptors in hippocampal synaptosomes. These

investigators demonstrated that the NMDA-induced potentiation of amino acid

superfusate levels was blocked by MK-801 and Mg^{2+}, was slow in onset and returned

to baseline on removal of the agonist. In addition, these receptors were found to be

modulated by Ca^{2+}-independent release of amino acids from a cytoplasmic pool.

Based on this experimental evidence it was postulated that the presynaptic NMDA

autoreceptor at glutamatergic nerve terminals in the hippocampus might provide a

positive feedback on the release of amino acids probably by reversing Na^+-dependent

L-GLU transporters. In general terms, the findings presented in this study support this

theory as the levels of L-GLU and L-ASP are elevated following the application of

NMDA. However, the current data also shows that NMDA receptor activation results

in the increase in the extracellular GABA release. Breukel et al. (1998) showed

different effects on GABA release depending upon the assay applied. Thus, under

physiological conditions, i.e. in the presence of Mg^{2+}, where high K^+ was used to

relieve the Mg^{2+} block, no evidence for NMDA autoreceptor control of GABA release

was seen. These results therefore suggest that NMDA autoreceptors modulate L-GLU

and L-ASP release whilst indicating a different mechanism for control of GABA

release.

Presynaptic L-GLU receptors have been reported to control not only amino

acid but also monoamine release in the brain. Application of NMDA, AMPA and

kainate have been shown to control the release of monoamines in the rat striatum by

providing a positive feedback system thus increasing the extracellular levels of these

neurotransmitters (Patel et al., 1997: Ohta et al., 1994a). In this study the infusion of

NMDA led to a reduction in monoamine levels. This may suggest that these

presynaptic receptors by exhibiting a facilitatory effect over amino acid release inhibit

monoamine efflux. This interpretation seems probable when considered in association

with my results using D-AP5. The effects of D-AP5 infusion suggest the existence of

a tonic release of L-GLU in the hippocampus and its control of amine efflux. Several

lines of evidence have indicated an association between the glutamatergic,

dopaminergic and serotoninergic systems. For example, Hata et al. (1990) have shown

that NMDA receptor antagonists increase DA turnover and that NMDA decreases DA

release in the pre-frontal cortex. Similarly, Bequet et al. (1990b) have shown that the

infusion of NMDA into the caudate of anaesthetised cats leads to a decrease in

extracellular 5-HT levels. The non-competitive NMDA receptor antagonist MK-801

has been found to induce behavioural states which are characteristic of increased

activity in dopaminergic and serotoninergic transmission (Tricklebank et al., 1989;

Loscher and Honack, 1991; Whitton et al., 1994 b). These effects have generally been

attributed to the possibility that these drugs lead to an increase in extracellular

monoamine levels which results in marked locomotor activity and stereotypic

behaviour (Carsson et al., 1990; Loscher et al., 1993). However, Whitton et al.

(1992a,b) found MK-801 to decrease striatal DA levels whilst increasing 5-HT efflux

in freely moving rats. It was also noted that the animals exhibited circling behaviour

under these conditions. It has been postulated that this kind of behaviour reflects

unilateral damage in one hemisphere of the striatum, possibly by the dialysis probe

itself, resulting in motor asymmetry and stereotypic behaviour after infusion of MK-

801. This may suggest that antagonism of NMDA receptors and the reported

behavioural activation in rodents is due to the involvement of other brain structures as

PCP has been found to increase DA release in the nucleus accumbens as well as evoking circling behaviour (McCullough et al., 1992). In contrast to the striatum, it was found that MK-801 produces an increase in both DA and 5-HT levels in the hippocampus (Whitton et al., 1992 b). This maybe of functional significance since the hippocampus is associated with seizure activity and monoamines have been shown to be of importance in epilepsy (Simialowski et al., 1990; Lu et al., 1998; Watanabe et al., 1998). It may be, therefore, that the NMDA-induced decrease in extracellular DA may contribute to the pro-convulsant effect following NMDA receptor stimulation. Although there is strong evidence supporting the regulation of monoamine release by L-GLU in the brain, the nature of NMDA receptor-mediated regulation of monoamine release is not clear. DA release following direct infusion of NMDA into the striatum may occur via three different mechanisms. A direct effect of NMDA on DA nerve terminals, an indirect action on presynaptic receptors following activation of inter-neurones and NMDA also leads to increase DA release as a result of increased firing of the striatal-nigral pathway. One key question regarding the control of DA release in the brain is whether the effect of L-GLU exerts a phasic, tonic or dual regulation of DA release. The results presented here indicate the presence of an ongoing glutamatergic tone and that NMDA-evoked L-GLU release appears to control monoamine efflux. Therefore, the following mechanism for control of neurotransmitter release is proposed. Under normal conditions, small amounts of DA are released tonically by L-GLU from glutamatergic fibres (Grace et al., 1991). Following NMDA receptor activation, L-GLU levels are elevated and this, in turn, leads to the loss of normal control of DA levels. This pattern of events is observed in epilepsy where the loss of epileptiform activity following NMDA receptor activation is manifested by a reduction in DA release (Ferraro et al., 1991). In addition,

biochemical (Chesselet, 1984), electrophysiological (Prisco et al., 1994) and behavioural studies (Copeland et al., 1980) have provided evidence for a functional relationship between 5-HT and DA neuronal systems. Recently, a microdialysis study performed by Matsumoto et al. (1996) suggested regulation by DA receptors of 5-HT release in the hippocampus. This study showed that exogenous and endogenous DA could facilitate the release of 5-HT via the activation of D_2 receptors. It would be tempting to speculate that the decline in DA levels observed in my study leads to a decrease in 5-HT efflux. It would follow that NMDA–evoked L-GLU release leads to the inhibition of DA release which, in turn, inhibits 5-HT efflux. This kind of interaction between L-GLU, DA and 5-HT systems has been reported in the caudate nucleus (Hertting et al., 1988). However, the possibility of L-GLU regulation of 5-HT release without the involvement of dopaminergic system cannot be totally excluded here given the evidence of direct modulatory action of this amino acid on 5-HT efflux in the brain (Whitton et al., 1994 b).

The possibility that local inter-neurones, predominantly GABAergic, are involved in the control of amino acid and monoamine release cannot be ruled out (Bonanno et al., 1991). Some investigators reported that depolarisation-evoked L-GLU release was modulated by GABA acting on presynaptic terminals (Collins et al., 1982, Bonanno et al., 1992) and, conversely, GABA release was found to be modulated by L-GLU acting on presynaptic GABA terminals (Becquet et al., 1990a; Bonanno et al., 1991). These observations may reflect a possible reciprocal relationship between the release of L-GLU and GABA. Indeed, results presented here show that the application of NMDA leads to an increase in GABA efflux in the hippocampus suggesting that L-GLU release may enhance GABA efflux. Since the increase in GABA release can be blocked by NMDA receptors antagonists (Young

and Bradford, 1991: Giovannini et al., 1994), this kind of modulation may represent an important mechanism in the control of inhibitory/excitatory transmission in the hippocampus (Jolkkonen et al., 1996). DA levels have been shown to decrease in the frontal cortex following the administration of an NMDA receptor agonist whilst increased DA metabolism was observed after an NMDA antagonist was applied (Nishijima et al., 1994; Jedema et al., 1996). These authors proposed that the release of DA is likely to be mediated through GABAergic inter-neurones. It is possible that GABAergic neurones control the release of monoamines in the hippocampus. It has previously been reported that applications of GABA receptor antagonists facilitate the release of DA in the brain (Santiago et al., 1993). Furthermore, the data presented here indicate that administration of NMDA leads to an increase in GABA release. These combined findings may suggest that GABAergic inter-neurones inhibit the release of DA in the rat ventral hippocampus following application of antagonists at the NMDA receptor complex. In addition, GABAergic inter-neurones may stimulate the release of DA in this brain region following the addition of an NMDA receptor agonist. Local inhibitory GABAergic circuits in the brain have also been suggested to control the release of 5-HT (Becquet et al., 1990a). It is possible that the NMDA-evoked inhibition of 5-HT release observed in the present studies occurs via the activation of GABAergic inter-neurones. As mentioned above, inhibition of 5-HT release following the application of NMDA may be a consequence of a decline in DA efflux. It is entirely possible that the regulation of this amine by GABA may occur directly or through transmitters such as DA (Becquet et al., 1991).

3.3.3 Effects of the NO releasing agent SNAP on neurotransmitter release in vivo.

Since the current data indicate that the basal release of amino acids and monoamines maybe under the tonic control of L-GLU in the hippocampus, the role of NO on neurotransmitter release was also investigated. The first objective of this study was to determine whether the basal release of amino acids and monoamines is mediated by NO.

The effects of NO on neurotransmitter release in the hippocampus in vivo have been poorly studied. This study reports findings that suggest a dual (stimulatory/inhibitory) action of NO through which this messenger may exert its modulatory effects on neurotransmitter release in this brain structure. Of particular interest is the observation that infusion of the NO donor SNAP results in changes in basal amino acid and monoamine release which have an inverse relationship to the concentration of drug applied. Infusion of high concentrations of the donor caused an increase in amino acid release while lower concentrations of SNAP elicited a decrease. On the other hand, the effect of SNAP on monoamine release showed an increase in the extracellular DA and 5-HT levels at lower concentrations and an increase at high concentrations of the donor. It was also noted that DOPAC levels were increased following infusion of SNAP at 1mM although HVA levels were unchanged. The effect of SNAP on the extracellular levels of DOPAC is particularly puzzling when compared its effect on DA at 1mM concentration. SNAP (1mM) did not alter the levels of DA initially but produced a transient increase 180min after the infusion of the donor was stopped. However, the levels of DOPAC increased immediately then returned to basal levels 60min after the infusion of the donor was stopped. It would appear that SNAP, at least at this concentration, causes an increase

in DA metabolism. It is not clear however, why SNAP at 1mM should increase DOPAC levels but have no effect on HVA. Previous reports suggest that the extracellular pool of DOPAC as estimated by microdialysis, may be derived largely from newly synthesised DA and not from the deamination of re-uptaken DA (Soares-da Silva and Garett, 1990). A substantial portion (40%) of hippocampal DA is thought to be derived from noradrenergic terminals (Scatton et al., 1980). It follows, therefore, that the transient increase in DA levels observed with 1mM SNAP could have been the result of enhanced monoamine synthesis in hippocampal dopaminergic and/or noradrenergic terminals. In addition, Guevara-Guzman et al. (1994) reported that the effects of SNAP on monoamine release might be attributed to its carrier molecule penicillamine. These investigators reported that infusion of penicillamine (1mM and 1M) into the striatum of urethane-anaesthetised animals increased the extracellular concentrations of 5-HT and DA. However, no statistically significant changes in monoamine levels at 5mM of penicillamine were observed in the present study. The discrepancy maybe due to the fact that the present study was performed in a freely moving animals whilst Guevara-Guzman et al. (1994) used anaesthetised animals. Since anaesthesia itself can alter neurotransmitter release this may have contributed to the results of the above mentioned study (Marsden, 1985).

The functional implications of the effects of SNAP on amino acid and monoamine release are, at present, unclear and difficult to explain. Previous investigations have reported contradictory observations concerning the effects of exogenous NO on neurotransmitter release in the brain, moreover, no corroborative data are available regarding the effects of SNAP on monoamine metabolism. It is entirely possible that these discrepancies can be attributed to differences between the experimental protocols used by different investigators, distinct effects of NO donors

and regional brain differences (Kirby et al., 1996). For example, in vitro studies using tissue slices have shown NO to increase DA efflux in striatum (Steward et al., 1996), whilst microdialysis studies suggest the opposite to be true (Shibata et al., 1996). SNP has been found to inhibit NMDA receptor-induced L-GLU and DA release in striatum, whilst SNAP enhanced NMDA-induced L-GLU release from hippocampal synaptosomes (Woodward and Stout, 1994). Furthermore, whilst NA release from hippocampal synaptosomes was inhibited following the addition of SNP, the release from cerebellar cortex was stimulated (Montague et al., 1994). Thus neurotransmitter release may be differentially affected not only by different NO donors but also by the same donor. The reasons for this are presently unknown. It has been suggested that in pancreatic acinar and colonic epithelial NO exerts a fine-tuning of store operated Ca^{2+} channel activity via the generation of cGMP (Xu et al., 1994). This effect was stimulated at low cGMP levels but inhibited at higher concentrations (Xu et al., 1994). Moreover, these responses could be sustained by changes in endogenous NO (Pandol et al., 1990a,b). It is plausible to speculate here that the effects of NO on neurotransmitter release may be dependent upon the level of cGMP being generated by NO donors and correlated with changes in the concentration of cytosolic Ca^{2+} concentrations. Evidently, the mechanism of action of NO on neurotransmitter release is very complex and the physiological significance of this phenomenon will be dealt with later on in this chapter.

3.3.4 Effects of 7-NINA and L-NAME on neurotransmitter release in vivo.

Amongst the many questions arising from the findings previously discussed, the most important are those which relate to the existence of an NO-mediated regulatory tone and the involvement of the NMDA receptor complex in determining

neurotransmitter release. Since the conversion of L-ARG to NO and its by-product L-CIT is known to be catalysed by one of three isoforms of NOS, the inhibition of this enzyme in the presence and absence of NMDA represent a logical approach for tackling these questions. In this study, two NOS inhibitors, L-NAME a non-selective NOS inhibitor (Rees et al., 1990) and 7-NINA a selective nNOS inhibitor (Babbege et al., 1993, Moore et al., 1993b) were used. These two inhibitors were used for the following reasons: To date, no studies have reported the effects of a selective nNOS inhibitor on neurotransmitter release in the hippocampus in vivo. Furthermore, hippocampal neurones have been shown to express both forms of constitutive NOS (eNOS and nNOS) (Endoh et al., 1994, Hara et al., 1996: Doyle and Slater, 1997), suggesting a functional importance for both isoforms in the modulation of neurotransmission in this brain structure. Thus, the source of NO production, neuronal or endothelial, may determine whether neurotransmitter release is attenuated or stimulated. The ability of NO to diffuse from its site of origin and activate distant target cells may suggest that the location of NOS with respect to its target be of major significance. Several lines of evidence indicate that the NMDA receptor complex may be one possible target for NO (Lei et al., 1992; Lipton et al., 1993, 1994; Fagni and Bockaert, 1996). Evidence exists to suggest that NMDA receptors are present on cells producing NO in the brain (Dohrn et al., 1994). Studies performed by Gracy et al. (1997) have shown that NOS is localised throughout the cytoplasm of somata and dendrites of aspiny neurones in the nucleus accumbens some of which also possess NMDA receptors. In addition, immunochemical studies detected the presence NMDA receptors in glial processes, dendrites and axons apposed to NOS- containing neurones (Gracy et al., 1997). These authors suggest that NO may exert its effects directly on the dendrites of aspiny neurones or via a more indirect route involving the

regulation of substrate availability for NOS synthesis in dendrite and axon terminals. These results show the relationship between NOS and the NMDA receptor localisation in the nucleus accumbens. Thus, it is possible that NO modulates neurotransmitter release following the activation of postsynaptic NMDA receptors by L-GLU or more directly by regulating substrate availability for NOS synthesis. This dual action of NO may explain the effectiveness of 7-NINA (Smith et al., 1996a) and NMDA receptor antagonists (Loscher, 1998) in the treatment of convulsions and, in addition, accounting for the convulsant effects of L-ARG analogues (Mulsh et al., 1994). In general terms, these findings appear to be consistent with my observations. The NOS inhibitors L-NAME and 7-NINA exert different effects on amino acid and monoamine release. Continuous infusion of L-NAME increased the basal release of L-GLU, L-ASP, DA and 5-HT but decreased GABA release. Contrary to this, 7-NINA decreased the extracellular levels of all neurotransmitters studied with exception of the extracellular DA which was elevated. The addition of NMDA in the presence of these inhibitors also yielded contrasting results. When L-NAME was infused prior to co-infusion with NMDA, the increase in L-GLU release was less than that seen with NMDA alone. The slight inhibition of the NMDA-evoked increase in L-GLU release was not statistically significant when compared to the effect of NMDA infusion alone. This is of particular interest when compared to the results with the other amino acids measured. As mentioned above, L-NAME infusion produced a decrease in L-ASP and GABA efflux. Addition of NMDA further decreased L-ASP levels whilst reversing the inhibition of GABA release. Why inhibition of NOS should significantly affect NMDA-evoked GABA release but not L-GLU release is unclear. The inhibition of DA and 5-HT levels produced by NMDA was reversed by addition of the L-NAME. When 7-NINA was infused for 60min prior to co-infusion

with NMDA, the inhibition of L-GLU and L-ASP release was reversed. The inhibition of 5-HT release produced by 7-NINA was, however, reversed and in fact potentiated by the addition of NMDA. The augmentation of DA levels produced by 7-NINA was abolished on addition of NMDA with DA levels returning to basal values within the time course of the experiment. Collectively, the present data suggests the involvement of NO in modulating basal and NMDA-evoked neurotransmitter release in the hippocampus in vivo.

It appears that activation of NMDA receptors is associated with pathophysiological events such as convulsions (Mollace et al., 1991) and neurotoxicity (Dawson et al., 1991). Given the conflicting data regarding NO's action in the control of these events, it has been suggested that NO production via the activation of distinct isoforms of NOS may be a crucial factor in determining the final response (Huang et al., 1994; Schulz et al., 1995; Smith et al., 1996a,b). It has been suggested that systemic administration of non-selective NOS inhibitors may result in the promotion of seizures via inhibition of vascular NO whilst application of selective nNOS inhibitors lead to the suppression of this phenomenon (Penix et al., 1994). Consistent with this hypothesis, in vivo studies in which nNOS production was selectively inhibited within the brain have revealed anticonvulsant effects (De Sarro et al., 1991; Mulsh et al., 1994; Smith et al., 1996a) whilst non-selective blockade of NOS showed the reverse (Penix et al., 1994). Furthermore, 7-NINA, by selectively inhibiting nNOS, has been found to protect against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced neurotoxicity (MacKenzie et al., 1997). On the other hand, the blockade of NOS by L-NAME has been reported to exacerbate toxicity and seizures in animals (Yamamoto et al., 1992b) whilst having no effect on MPTP-induced toxicity in the marmoset (Mackenzie et al., 1997). These studies

provide evidence that the effects of NO are determined, to some extent, by its source. In general terms this is in agreement with the present findings since a decline and an increase in L-GLU release was observed following infusion of 7-NINA and L-NAME, respectively. However, it should be remembered that the evidence concerning pro-convulsant and anti-convulsant effects observed by other investigators were the result of the systemic administration of 7-NI. There is now evidence that nNOS is not necessarily confined to the brain but may be present in other organs of the body e.g. epithelial cells of the lung and kidney (Dun et al., 1992; 1993). This in turn may indicate that following systemic drug administration 7-NI may inhibit nNOS in the brain as well as in other organs in the body. Whether the inhibition of nNOS by 7-NI in the periphery contributes to its effect in the CNS is presently unknown and remains to be elucidated.

It is now known that nNOS and eNOS are widely distributed throughout the body (Dinnerman et al., 1994a). A recent report by Doyle and Slater (1997) showed the presence of eNOS in CA1 pyramidal neurones and nNOS in CA1 –CA3 pyramidal neurones and granule neurones of the dentate gyrus in the human hippocampus. It is entirely possible that the selective targeting of one or the other isoenzyme results in different effects. Furthermore, the possibility that eNOS with nNOS may coexist in the same neurone (Doyle and Slater, 1997) could indicate a complex relationship between NO produced by different forms of NOS and neurotransmission in the brain. The differing effects of the NOS inhibitors reported here and in the literature cannot be explained entirely by their selectivity for particular NOS isoforms. There are still many unresolved questions concerning the selectivity of these drugs towards all three NOS isoforms. For example, L-NAME is a non-selective NOS inhibitor and will inhibit eNOS, nNOS and also iNOS in vivo but its potency

against the inducible isoenzyme appears to be less than that for both constitutive forms (Southam and Szabo, 1996). 7-NINA is considered to be a nNOS selective inhibitor but it has also been suggested to inhibit iNOS in vivo (Wolff and Gribin, 1994). The potential for 7-NINA to inhibit both iNOS and nNOS might be of importance in my study. Implantation of the probes may lead to tissue damage that could, in turn, result in the expression of iNOS. A recent report by Golomb et al. (1996) suggested that LPS-stimulated iNOS in the hippocampus might exist in the same neurones that express nNOS and eNOS. The possibility exists, therefore, that during the microdialysis experiments all three isoforms of NOS are present and that the induction of iNOS within neurones contributes to the different effects on neurotransmitter release observed with L-NAME and 7-NINA.

The findings of Kelly et al. (1995) and Kovah et al. (1993) may indicate that 7-NINA is not entirely devoid of direct actions on the vasculature. This in turn may indicate that cerebrovascular sequelae of 7-NI treatment in conscious rat are very similar to those observed with L-NAME and may suggest that NO synthesised and released from neuronal, as opposed to endothelial sources, in the brain has the potential to regulate cerebral blood flow. However, Faraci et al. (1995) found that 7-NI did not alter the diameter of cerebral arterioles suggesting that the basal tone of cerebral vessels is endothelium derived. The source of NO may, therefore, be responsible for the differing effects of L-NAME and 7-NINA on neurotransmitter release in the hippocampus in vivo.

The distinct effects of L-NAME and 7-NINA on neurotransmitter release could be due to their differing mechanisms of action of these inhibitors. L-NAME being an L-ARG analogue exerts its inhibition by displacing this amino acid from its binding site while 7-NINA acts by binding to the haem group of NOS in such a way

that oxygen cannot bind. By binding to the haem site, the binding of 7-NINA interferes not only with the L-ARG binding site on the enzyme but also with THB. This might explain the apparent competitive inhibitory nature of this compound (Wolff and Gribin, 1994). One important observation that has emerged from this study is that, with the exception of DA, 7-NINA decreased the release of all the neurotransmitters studied. The levels of other amino acids including L-SER, TAU, glycine, glutamine (data not shown) were also decreased. Since the extracellular L-SER levels were unchanged by infusion of NMDA, SNAP and L-NAME, the fact that 7-NINA produced a decline in the release of this amino acid may indicate that some of the effects of this inhibitor are unrelated to NOS inhibition. If 7-NINA has the capacity to block a variety of iron-dependent systems as do some other NOS inhibitors in vitro (Peterson et al., 1992), the decline in all neurotransmitters measured may be due to the inhibition of enzymes such as catalase. Catalase is an iron-containing enzyme that could potentially interact with the iron binding groups of 7-NINA. Since the normal function of this enzyme is to remove hydrogen peroxide, its inhibition of iron groups could be toxic to the cells. However, to my knowledge no reports exist to suggest that such an effect occurs in vivo.

In the evaluation of the action of these drugs on neurotransmitter release it is important to consider their time course of action. Both L-NAME and 7-NINA have previously been reported to produce time-dependent NOS inhibition in the brain (Traystman et al., 1995). It was concluded that sufficient time has to be allowed for full inhibition to be produced by L-NAME as the action of this drug relies on diffusion into the cytosol. These studies are in agreement with present findings in that the effects of L-NAME on neurotransmitter release were found to be time-dependent. For example, the rise in extracellular DA levels was slow in onset, reaching the

maximum 90min after the infusion of the inhibitor began, a result which is in agreement with that of Traystman et al. (1995). However, despite continuous infusion of L-NAME, the elevation in neurotransmitter release caused by this compound was not sustained during the time course of the experiment. After the maximum was reached, the levels of neurotransmitters returned to the baseline. This effect might be explained by the mechanism of action of this inhibitor. L-NAME is a competitive inhibitor of NOS in that it displaces L-ARG from its binding site. This competitive inhibition of NOS can be reversed by addition of exogenous L-ARG (Strasser, 1994) which may suggest that the prevailing L-ARG concentration may determine the time course of NOS inhibition by L-NAME. It is tempting to speculate here that continuous infusion of L-NAME leads to the displacement of L-ARG from its binding site and into the extracellular space. The increased extracellular concentration of L-ARG may then lead to a stimulation of the uptake system (Cynober et al., 1995) and thus increasing the intracellular levels of this amino acid. This may result in competition between L-ARG and L-NAME for the binding site on NOS and a consequent loss of NOS inhibition.

7-NINA, on the other hand, produced a different pattern of events. The continuous infusion of this inhibitor resulted in a long lasting inhibition of neurotransmitter release. This effect is surprising as MacKenzie et al. (1994) reported that the action of this compound is shorter than that of L-ARG based inhibitors such as L-NNA. However, a long lasting effect of 7-NINA on nNOS has been observed in the hippocampus (Kalisch et al., 1996). A high dose of this drug produced a 60% inhibition of nNOS in this brain structure which was followed by a slow recovery over a 4h period before normal NOS activity was restored. Kalisch et al. (1996) also observed that the maximal NOS inhibition was dose-dependent but that the recovery

period was the same for all doses applied. The data presented here are in agreement with these investigators and show that the inhibition produced by 7-NINA is long lasting.

Monoamine metabolites were also differentially affected by the application of L-NAME and 7-NINA. No changes in extracellular DOPAC and HVA levels were found following L-NAME infusion. This is consistent with the findings of Yamada et al. (1995) who observed a lack of significant changes in these metabolites in the brain, with exception of the striatum, following i.p. administration of L-NAME. Contrary to the effects of L-NAME, 7-NINA greatly increased DOPAC and HVA levels suggesting that long-term inhibition of NOS may cause an increase in DA turnover in the hippocampus. The effect of NMDA on monoamine release was blocked by the administration of both inhibitors.

The present data on DA efflux appears to be consistent with the observation that NO inhibits DA release in the hippocampus and with the finding that MK-801 and PCP, which are considered to inhibit NO production by blocking NMDA receptors in striatum, cause an increase in DA metabolism (Loscher et al., 1991). On the other hand, no statistically significant alterations in 5-HT metabolism were observed with neither L-NAME nor 7-NINA. This contrasts sharply with the findings of Yamada et al. (1995) which demonstrated that application of L-NAME results in a decrease in the metabolism of this amine in the striatum. Given the lack of currently available data concerning the effects of NO production on 5-HT release, it is impossible to determine the reasons for these discrepancies. It is, however, of interest to note here that these two studies were performed in different brain regions and may suggest brain-selective effects of NOS inhibition on 5-HT metabolism.

Why one inhibitor of NOS should affect DA metabolism but not the other is presently unclear. However, it is of interest to note that the changes in both DA and 5-HT metabolism caused by administration of L-NAME were observed in different brain regions and occurred in different directions. Although a plausible explanation for this is not obvious, it is possible that NO modulates the release of both excitatory and inhibitory neurotransmitters which could then elicit either an increase or a decrease in the metabolism of these neurotransmitters. Alternatively, the lack of changes in 5-HIAA levels observed in my study may be due to the inclusion of citalopram in the dialysates although subsequent investigations in the absence of the re-uptake inhibitor failed to demonstrate any changes in 5-HIAA accumulation (data not shown). Since the extracellular ratio of 5-HIAA to 5-HT was found to be very high (approximately 98 to 1 in hippocampal dialysates), it is possible that a relatively modest increase in 5-HT output would not significantly contribute to the total 5-HIAA pool.

3.3.5 NO and neurotransmitter release in the hippocampus.

It is believed that many of the physiological functions of NO in the CNS are mediated by stimulation of sGC and subsequent cGMP production (Southam and Garthwaite, 1991). NO has been observed to increase L-GLU release whilst inhibiting DA release via the production of cGMP (Meffert et al., 1994, Hirsh et al., 1993). However, NO has been shown to exert other effects on cellular function including protein ribosylation, ADP ribosylation (Griffith et al., 1995), nitrosylation of thiol groups (Stamler et al., 1994), nitrosylation of cysteine residues of the DA transporter (Lei et al., 1992) and the regulation of NMDA receptor function by nitrosylation of the putative redox modulatory site of the receptor (Lipton et al., 1993). In addition,

NO has been found to alter both NA and L-GLU uptake systems (Lonard and Johnson, 1995a,b). The results show an inverse concentration-dependence between the prevailing NO concentration and neurotransmitter release that may suggest a dual (inhibitory/stimulatory) role for this messenger in the hippocampus. Infusion of SNAP at a high concentration enhanced L-GLU release but decreased the release of this amino acid at a lower concentration. A recent report by Sistiaga et al. (1997) showed that SNAP decreased the release of L-GLU from cerebral synaptosomes whilst enhancing cGMP levels. These investigators postulated that the increase in the cGMP levels in nerve terminals induced the depression of L-GLU release, probably via an activation of cGMP-dependent protein kinase G. Interestingly, these investigators found no evidence that the NO/cGMP pathway was capable of enhancing the release of this amino acid, an effect reported by others (Vallebuona and Raiteri, 1994). However, they observed a reversal of inhibitory effect of cGMP on L-GLU release following the application of a high concentration of a membrane permeable analogue of cGMP. This, in turn, suggested a dual role for the NO/cGMP pathway in the control of L-GLU release. It is also possible that an increase in L-GLU release observed with high SNAP concentrations might, for example, be the result of the inhibition of L-GLU transporter. This concept is supported by the study performed by Pogun et al.(1994b) which showed that NO enhances striatal extracellular L-GLU via inhibition of its transport in synaptosomes.

Monoamine release was also affected by the prevailing NO concentration but in the opposite direction to that seen with L-GLU. The release of monoamines was found to under glutamatergic control because when D-AP-5 was infused prior to co-infusion with SNAP, the levels of both monoamines decreased which is the opposite to that seen with SNAP infusion alone. This may suggest that the release of

monoamines is, at least in part, dependent on L-GLU activation of NMDA receptors and the subsequent release of NO. These results are in agreement with those of other investigators (Ohmo et al., 1994; Shibata et al., 1995). The effect of 5mM SNAP, on the other hand, was unaffected by co-infusion with D-AP-5 suggesting that at this concentration NO modulates the release of these neurotransmitters via a mechanism independent of NMDA receptor activation. It has previously been observed that NO may inhibit L-GLU transporter system and consequently increase the extracellular L-GLU levels (Pogun et al., 1994b). In addition, NO has been demonstrated to inhibit DA uptake system (Pogun et al., 1994b) and cause a reduction in proton activity in monoamine storage vesicles (Gronberg et al., 1990), a condition that favours the reversal of the transport system (Levi and Raiteri, 1993). It would follow, therefore that a high concentration SNAP inhibits the uptake of L-GLU and increases L-GLU levels whilst causing a reversal of the vesicular DA transporter and a decrease in synaptic levels of the amine . This scheme could account for the opposing effects on L-GLU and monoamines observed in my study. In addition, the decrease in the release of monoamines may be due to NO acting at the level of synaptic vesicles. Studies, performed by Kuhn et al. (1996) suggested that NO or NO-releasing agents might inactivate tryptophan hydroxylase (TPH), the rate limiting enzyme in 5-HT synthesis, via nitrosylation of iron-sulphur complexes present in the enzyme. TPH belongs to the family of monooxygenase enzymes which also includes tyrosine hydroxylase, the rate limiting enzyme in DA synthesis, indicating that this enzyme could also represent a target for NO (Dix et al., 1987). The inhibition of this enzyme would result in the termination of monoamine synthesis and subsequent decline in DA and 5-HT levels in the extracellular space. As mentioned above, NO could also reduce the proton gradient that is the driving force for monoamine storage in vesicles (Gronberg et al.,

1990). This, in turn, could result in the leakage of monoamines into the cytoplasm thereby promoting the reversal of the transport systems for DA and 5-HT.

In the evaluation of the effects of drugs, such as NO donors which are transient in nature, the metabolism and pharmacokinetics of the compound must be taken into consideration. An important component of the clearance of NO is its conversion to redox related forms. This molecule could appear as NO, NO^+ and/or NO^- (Stamler et al., 1992). These different states may vary according to the donor used or redox properties of its environment (Butler et al., 1995). According to observations made by Lipton et al. (1993), NO mediates toxicity by reacting with superoxide to form peroxynitrite. If this hypothesis is correct, the increase in amino acid release produced by NO donors could be explained as follows. SNAP undergoes spontaneous decomposition to NO. The NO group of SNAP (in the form of NO^+) gains an electron from a donor to liberate NO^o. NO^o then reacts with superoxide anion to generate peroxynitrite. Peroxinitrite or one of its breakdown products is neurotoxic (Lipton et al., 1993, Saran et al., 1990). These reactions occur readily under physiological conditions (Ischiropoulos et al., 1992) and could result in an increase in amino acids efflux due to cell damage. Such a mechanism of action has been suggested by Ohkuma et al. (1995) who observed an increase in GABA release following the application of SNAP and SNP and that this was due to the rapid formation of peroxynitrite. Here, it was concluded that if the NO donor was neurotoxic, then the levels of amino acids would not return to basal after the infusion of the drug had ceased. Instead, the extracellular levels of L-GLU would remain elevated due to the damage inflicted on cells in the vicinity of the probe.

The basal efflux of monoamines was decreased by administration of the NO donor. Interestingly, neither DA nor 5-HT levels returned to the baseline following

the infusion of 5mM SNAP. NO can be readily oxidised to nitrite and nitrates. Since

this is a second order reaction (Wink et al., 1993), the lifetime of NO depends on its

initial concentration. In agreement with this, it was observed that at a low

concentration (500μM) of SNAP the efflux of DA and 5-HT was very transiently

affected but at high concentrations the effects produced by SNAP were long lasting

and the levels of monoamines did not return to baseline. This effect may indicate that

at above a critical concentration NO can activate different mechanisms resulting in an

irreversible modification of target molecules such as iron-sulphur-containing proteins

and consequently destroying their catalytic function. It is also possible that at higher

NO concentrations a significant portion of it is stored as nitrosated compounds such

as metal nitrosyl complexes and thionitrates (Vanin, 1991). The storage and slow

release of NO from these complexes could then evoke long lasting effects

In my view, the circulating NO concentration is important in determining of

the final response observed. NO controls the release of neurotransmitters in an inverse

concentration-dependent manner. But it is possible that the inverse concentration-

dependence of SNAP on neurotransmitter release may be the result of its action on

NOS. In recent years evidence has accumulated which suggests that exogenous NO

may lead to an inhibition of NOS activity (Assreuy et al., 1993; Vickroy et al., 1995).

SNP and SNAP have been shown to directly inhibit the brain constitutive NOS. This

direct feedback inhibition was found to be dependent on a high concentration of donor

which is probably due to the slow release of NO by these agents (Radomski et al.,

1992). The mechanism by which NO inhibits NOS activity is at present unknown. It is

possible that NO exerts a direct effect on the haem moiety of the enzyme. Following

this hypothesis, the effect of SNAP on the efflux of amino acids in the presence of the

selective NOS inhibitor 7-NINA was assessed. If NO at high concentrations (5mM,

2mM) inhibited NOS, then the combination of the NOS inhibitor and the NO-releasing agent might be expected to lead to a further decrease in L-GLU release. Contrary to this, the data presented here showed that on addition of SNAP the effect of 7-NINA was reversed, an effect that was evident at both low and high concentrations of SNAP. Overall, my findings do not support the view that exogenous NO leads to the inhibition of NOS activity in vivo.

CONCLUSIONS

1. Activation of NMDA receptor in the release of amino acid neurotransmitters in the hippocampus as measured by in vivo microdialysis in freely moving rats.

2. It appears that NMDA-evoked monoamine release is linked to the release of L-GLU in this brain structure.

3. NO modulates basal and NMDA-evoked neurotransmitter release in the hippocampus in vivo.

4. The source of NO, neuronal or endothelial, may be important in determining the final response as indicated by the different effects of 7-NINA and L-NAME

5. The inhibitory and stimulatory effects of NO may be dependent on the prevailing extracellular NO concentration.

Chapter 4

Effects of nitric oxide on L-ARG and L-CIT release in rat ventral hippocampus in vivo and glial cells in vitro.

4. Part A.

4.1 Introduction

Data presented in the previous chapter suggest that NO can mediate changes in extracellular neurotransmitter and metabolite levels in the brain and that the prevailing extracellular NO concentration may be important in determining whether neurotransmitter release is stimulated or attenuated. However, it was noted that there were differences in the responses exhibited by the two NOS inhibitors used. This might be due to any one of the following reasons: a.) all three isoforms of NOS may contribute to the resultant response, b.) the mechanism of action of the NOS inhibitor may alter the final response and/or c.) the actions of these two compounds may not be exclusive of NOS inhibition.

Comparing these data with results obtained by other investigators reveals that the alterations in neurotransmitter release may vary with the type of NOS inhibitor applied in vivo. L-NAME has been shown to increase DA release following its infusion into the striatum (Shibata et al., 1996) yet another inhibitor from the family of L-ARG analogues, L-NNA, attenuated the release of this neurotransmitter (Hanabaur et al., 1992). Thus, controversy still remains concerning the precise role of NO in regulating neurotransmitter release in the brain.

Some of the difficulties in evaluating the importance of NO in the brain relate, at least in part, to the differences in potencies of NOS inhibitors in vivo and the inability of these inhibitors to access equally all of the NOS containing compartment5. In addition, none of the inhibitors is likely to be completely NOS specific or resistant to metabolism to other biologically active products. For example, L-NMA has been shown to be metabolised by NOS to L-CIT (Feldman et al., 1993), a by-product of NO production which, in turn, is recycled to L-ARG (Hecker et al., 1990). L-ARG

supports NO synthesis and therefore decreases the inhibition of NOS produced by L-NMA. Moreover, another L-ARG analogue, L-NAME, has been demonstrated to possess anti-muscarinic properties (Buxton et al., 1993) and L-NAA has been found to cause seizures in dogs that appear to be unrelated to NOS inhibition (Cobb et al., 1992). These effects reflect the need for a quantitative measurement of NOS activity during any study where the effects of NO are being assessed.

The characteristics of NO, such as its relatively short half-life, make the direct quantitative assessment of this molecule difficult to perform. The measurement of nitrite and nitrate levels, the primary metabolites of NO, is one of the methods used to assess NO production in the brain in vivo. Luo et al. (1993) combined the technique of intracerebral microdialysis with the Griess azo dye detection method to assess the changes in NO metabolites following infusion of NMDA whilst Yamada et al. (1997a) measured the levels of nitrite and nitrate by HPLC in dialysates following i.p. treatment with L-NAME. In both of these studies the application of NMDA led to an increase whilst the inhibition of NOS produced a decrease in the levels of nitrite and nitrate. However, there are disadvantages associated with these methods. The sensitivity of these techniques is limited by the levels of nitrite/nitrate already present in buffers (~0.5μM) and contributed by the tissue samples (e.g. nitrate in tissues is present at > 10μM). It is now thought that the primary reaction of NO in oxygenated water is its conversion to nitrite (Kharritonor et al., 1994). Thus, a simplified variant of this type of assay only measures the nitrite formed. However, several reactions (especially that with oxyhaemoglobin) can result in NO and nitrite being converted to nitrate (Spagnuolo et al., 1987) and therefore this simplified assay has to be used with caution.

NOS activity may also be measured by assaying the conversion of L-ARG to L-CIT. This is usually achieved by using radiolabelled L-ARG that is separated from radioactive L-CIT by cation exchange chromatography (Bredt and Snyder, 1989), the limits of detection being determined by the amount of radiolabelled L-ARG used in the assay. Variations in this parameter make it difficult to compare results from different studies. Recently, two HPLC methods have been developed which provide a non-radioactive and sensitive assay for NOS activity (Carlberg, 1994; Ohta et al., 1994b). Both involve sample derivatisation with OPA reagent but the separation of L-ARG and L-CIT were carried out under different conditions. Carlberg (1994) used HPLC with fluorimetric detection and a mobile phase containing acetonitrile, tetrahydrofuran (THF) and methanol which allowed a good separation of both amino acids with elution times of 5min for L-CIT and 15min for L-ARG reported. However, an OPA artefact peak made the quantitative assessment of L-ARG difficult as it preceded the elution time for this amino acid. This method only allows the assessment of NOS activity by measuring the changes in L-CIT levels, the by-product of NO synthesis. Ohta et al. (1994b), on the other hand, used an electrochemical detector which consisted of a graphite working electrode at +650mV versus a Ag/AgCl reference electrode. The mobile phase consisted of 100mM sodium phosphate buffer (pH 6), EDTA (54 mg/l) and 30% methanol. According to these investigators a good separation of L-ARG and L-CIT was achieved, however no representative traces were included in this publication.

The above discussion illustrates the approaches that can be used for a quantitative assessment of NOS activity during any in vivo study. Each of these methods provides selective information about the effects of NO and the choice of method depends on the aims of the study. In this section, an attempt has been made to

discover if the effects of two NOS inhibitors on neurotransmitter release reported in chapter 3 are due exclusively to NOS inhibition. Following microdialysis experiments, the samples collected were analysed for their L-ARG and L-CIT content by HPLC with fluorimetric detection. It was reasoned that by monitoring the extracellular levels of L-ARG and L-CIT the following questions could be answered.

1) Do 7-NINA and L-NAME produce inhibition of NOS in vivo?

2) Is the inhibition long lasting?

3) Are L-ARG levels affected by infusion of NOS inhibitors?

4.2 Experimental details

All microdialysis experiments were carried out according to the procedure outlined in chapter 2.

4.2.1 HPLC ANALYSIS

4.2.2 Separation of L-ARG and L-CIT

A new method was established for the separation of two basic amino acids L-CIT and L-ARG using HPLC with fluorimetric detection (section 2.4.1; chapter 2). Both amino acids were pre-column derivatised with OPA, detected and quantified as detailed in sections (2.4.2; 2.4.4: 2.4.5) of chapter 2. All separations were carried out under a linear elution buffer gradient. The gradient started at of 25% (B) rose to 75% (B) at 20min and to 100% (B) at 25min and returned to 25% (B) at 28min with a flow rate of 1ml/min and a total run time 30min. The mobile phase (A) consisted of (50mM) NaH_2PO_4, 11.5% methanol and 1% THF, pH 7.0). The sample standard curves are provided in Fig 4.1 and representative chromatograms illustrating the separation of OPA thiol derivatised L-ARG and L-CIT from hippocampal dialysates in Fig. 4.2 A and B.
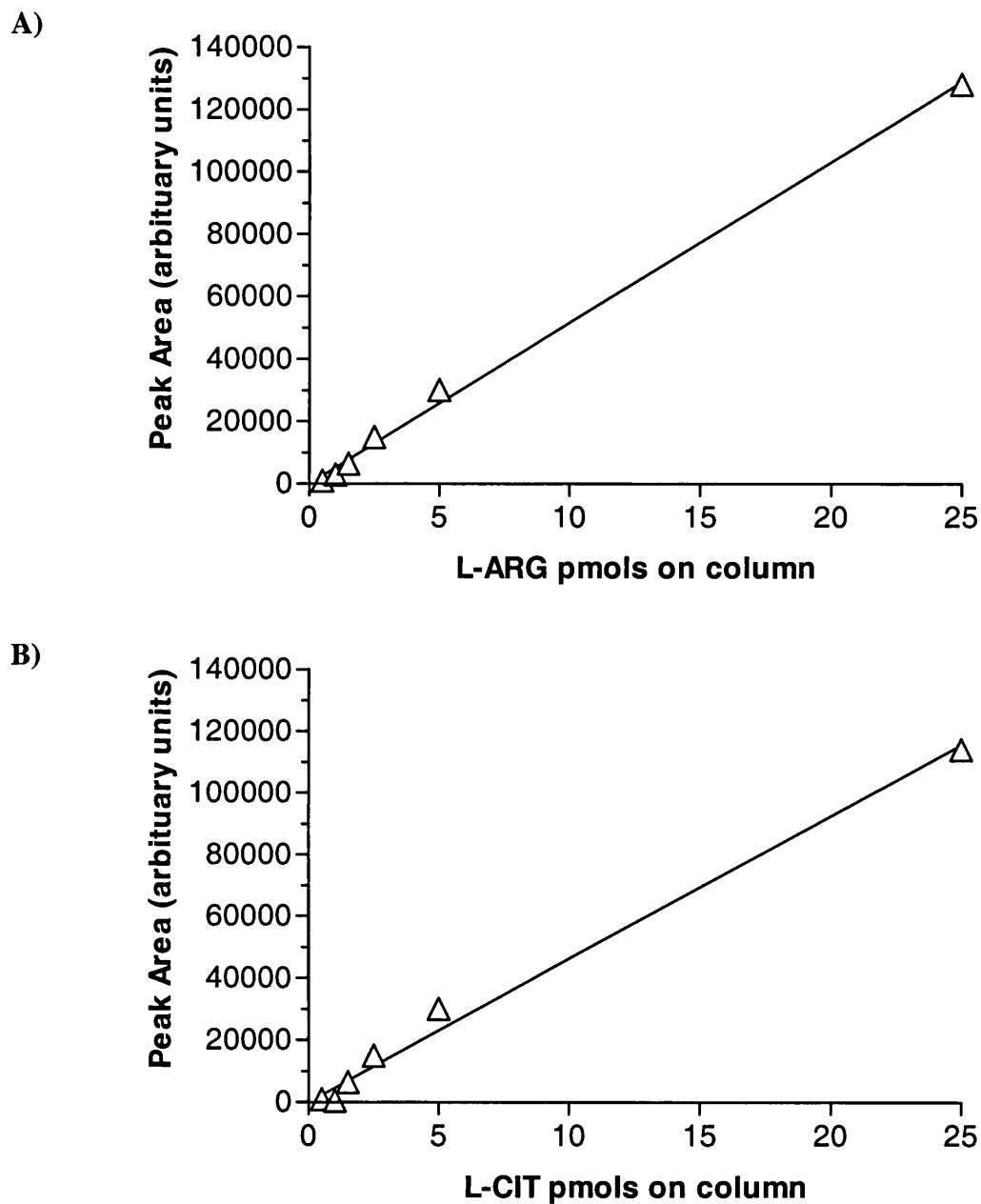
Fig. 4.1: Typical calibration plots for L-ARG (A) and L-CIT (B) obtained using HPLC with fluorimetric detection. Refer to text for details of the chromatographic technique (section; 4.2.2).
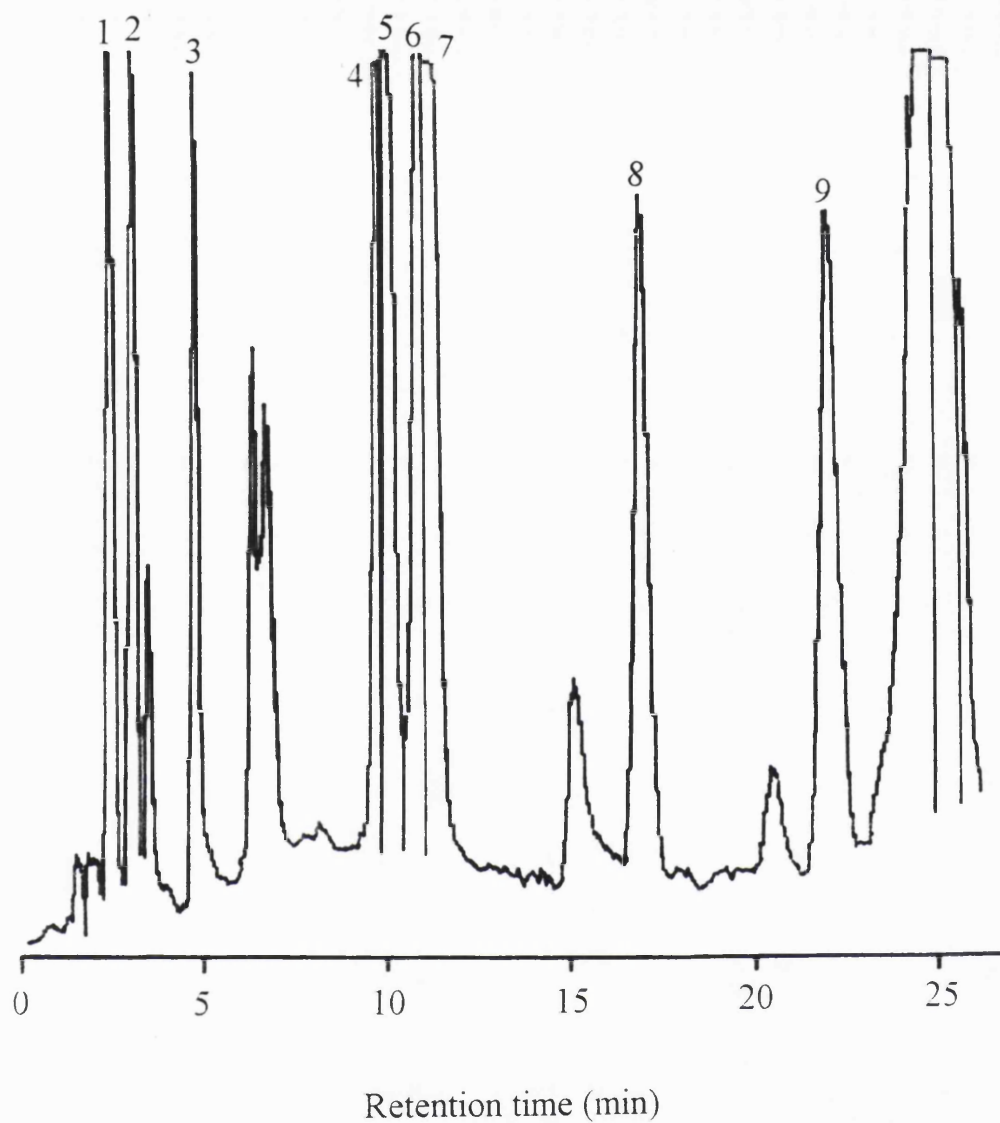
Fig. 4.2 A: Typical chromatogram obtained following separation of amino acid standard mixture using HPLC with FD. Horizontal axis represents elution time in min. Peaks denoted are: (1) ASP, (2) L-GLU, (3) L-GLN, (4) L-ARG, (5) L-CIT, (6) Threonine, (7) glycine (8) TAU, (9) GABA.

Retention time (min)
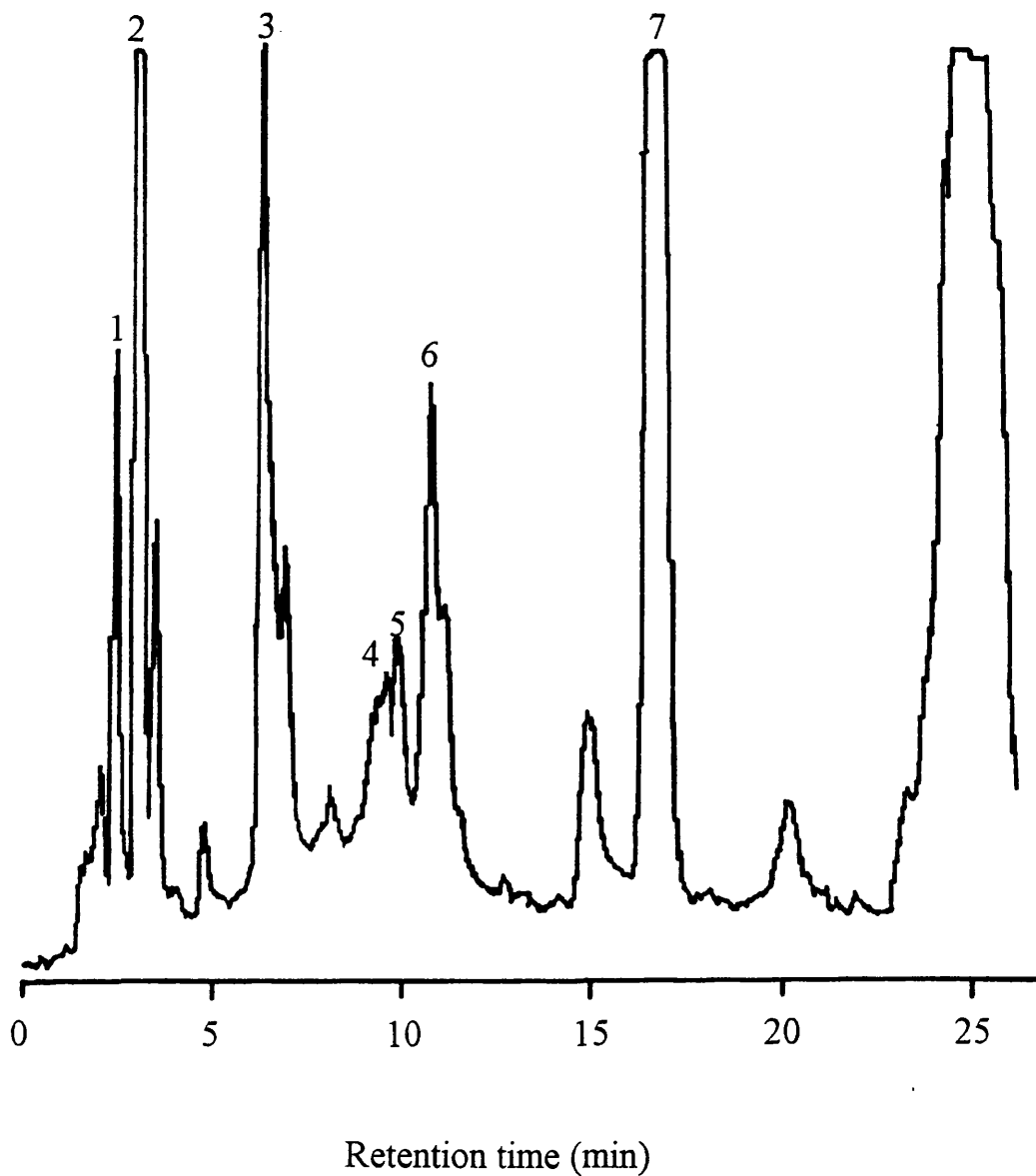
Fig. 4.2B: Typical chromatogram obtained following separation of amino acid from

hippocampal dialysates using HPLC with FD. Horizontal axis represents elution time

in min. Peaks denoted are: (1) ASP, (2) L-GLU, (3) L-GLN, (4) L-ARG, (5) L-CIT,

(6) Threonine/glycine (7) TAU.

4.3 RESULTS

Basal levels of L-ARG and L-CIT in dialysates collected from ventral hippocampus were found to be 14.4 ± 0.8 and 25.5 ± 3 pmols /15μl/ dialysate/ 30 min sample (n=64) respectively. Continuous infusion of L-NAME led to an elevation of L-ARG levels with a maximum of $150 + 15\%$ above baseline being achieved at 1mM (Fig. 4.3 A). The increase was slow in onset but persisted for the time course of the experiment. L-CIT levels declined following the administration of 1mM L-NAME (Fig. 4.3 B). The slow but progressive decline in the level of this amino acid reached a minimum 150min after the drug infusion commenced and returned to the baseline within the time course of the experiment (Fig. 4.3 B).

In contrast to the effects of L-NAME, infusion of 7-NINA at 1mM led to a decrease in both L-ARG and L-CIT levels. This decline was rapid in onset and persisted for the duration of the experiment. The level of both amino acids declined by approximately $50 \pm 15\%$ below control values (Fig. 4.4 A and B).

A)

B)
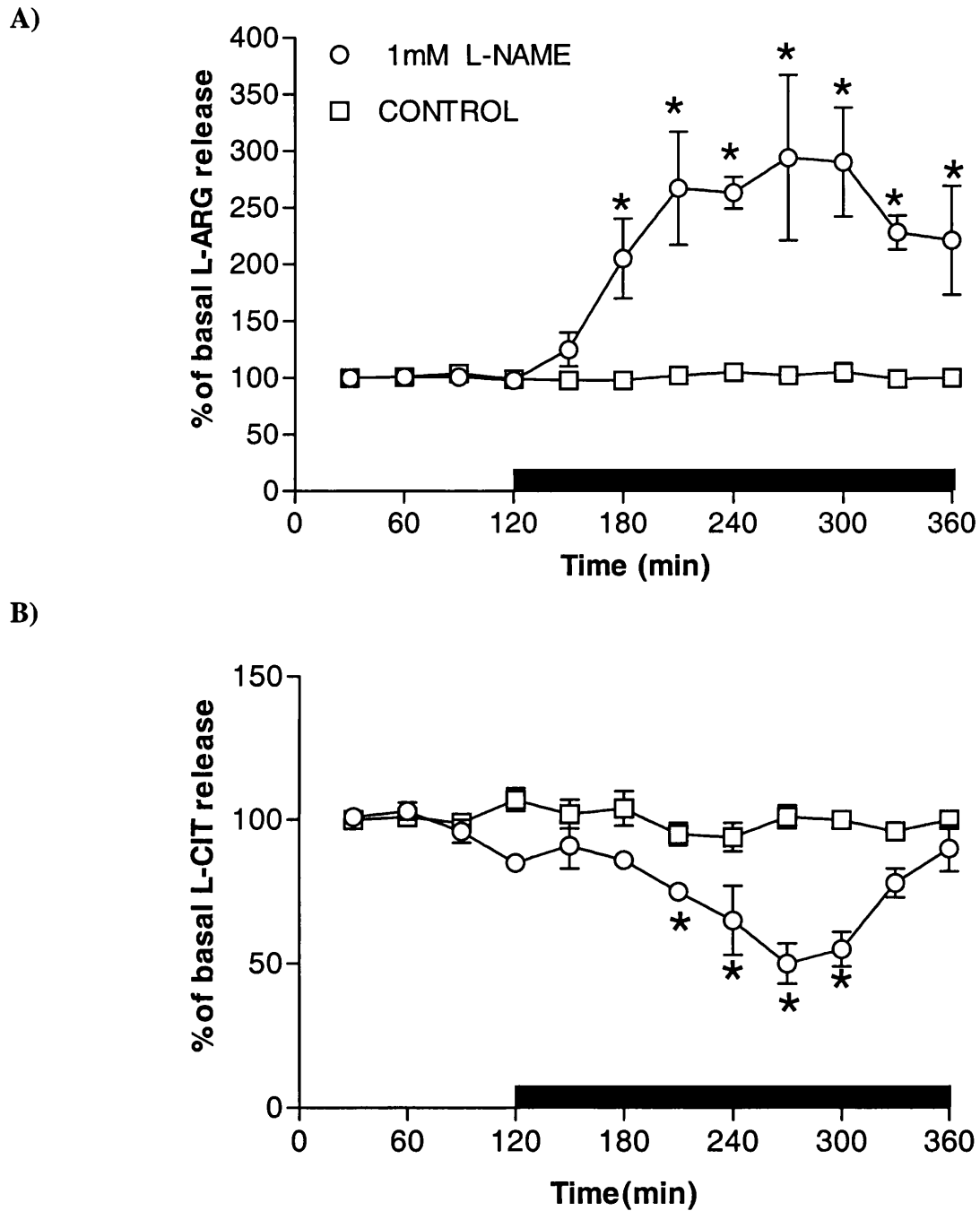
Fig. 4.3: Effect of L-NAME infusion into ventral hippocampus on L-ARG (A) and L-CIT (B) release. The solid bars represent the period of L-NAME infusion. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls ($p < 0.05$).
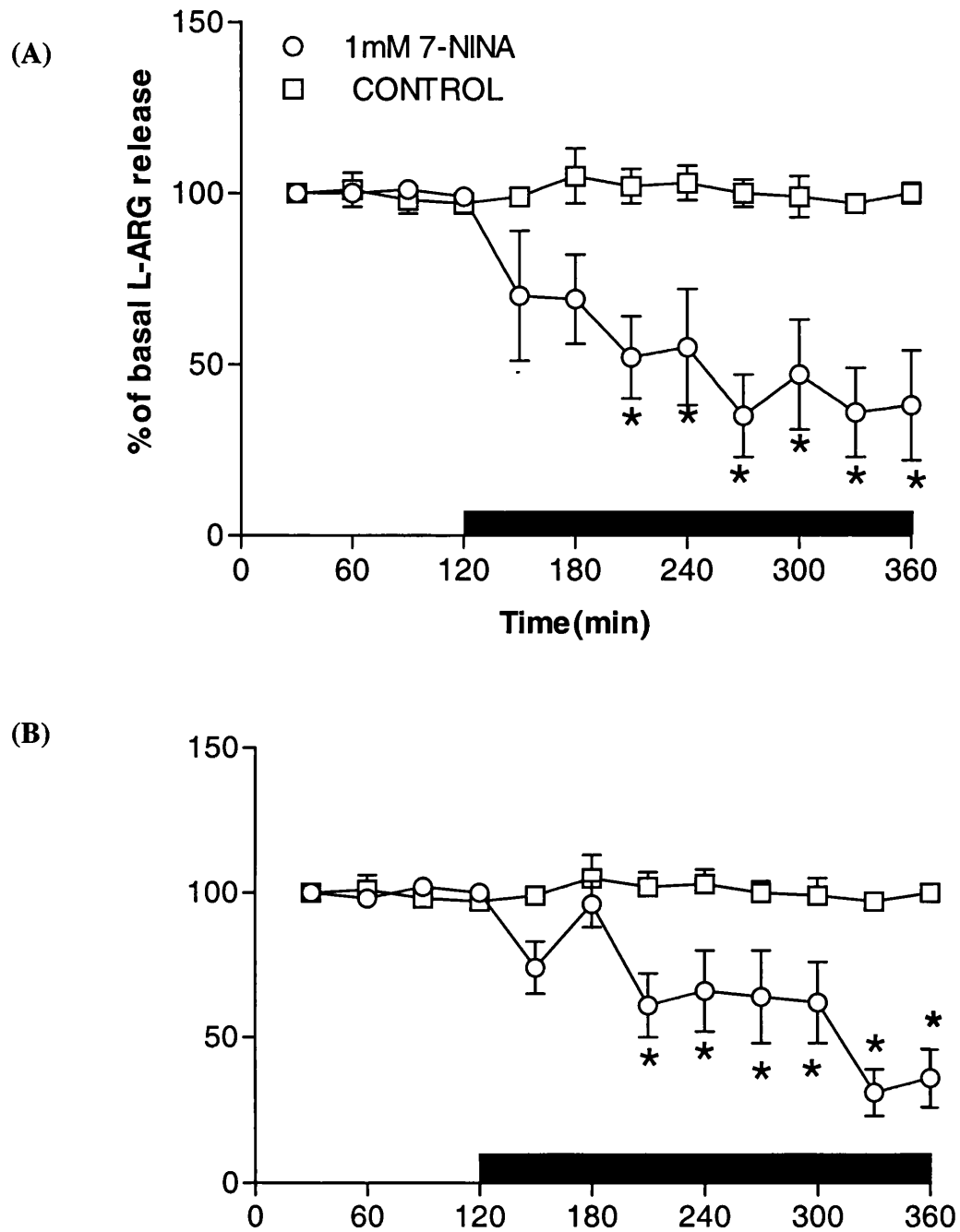
Fig. 4.4: Effect of 7-NINA infusion into ventral hippocampus on L-ARG (A) and L-CIT (B) release. The solid bars represent the period of 7-NINA infusion. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls (p<0.05).

A 30min infusion of SNAP into the ventral hippocampus elicited a concentration-dependent increase in the extracellular L-ARG levels with the donor at 5mM eliciting an increase of up to 200 ± 35% above control values. This effect was slow in onset and showed an initial plateau period followed by a more rapid increase to the maximum 90 min after the infusion ceased. No statistically significant changes occurred at the lower concentration of SNAP (500μM) (Fig 4.5 A). L-CIT levels were also elevated by SNAP at 5mM and a maximum response of 150 + 15% above control values was obtained (Fig 4.5 B). The increase was slow in onset and persisted for 120 min after the infusion of the donor stopped before declining rapidly to the baseline. SNAP at 500μM led to a decline in the levels of this amino acid, reaching a minimum of 67 + 10% below control values for a short period some 120 min after the infusion of the drug ceased (Fig. 4.5 B).

When 7-NINA (1mM) was infused for 60 min prior to co-infusion with SNAP at 500μM, the inhibition of L-ARG and L-CIT levels produced by the NOS inhibitor was reversed. Furthermore, the extracellular levels L-ARG and L-CIT increased above control values by up to 70 ± 10% and 50 ± 15% respectively (Fig 4.6A and B).

(A)
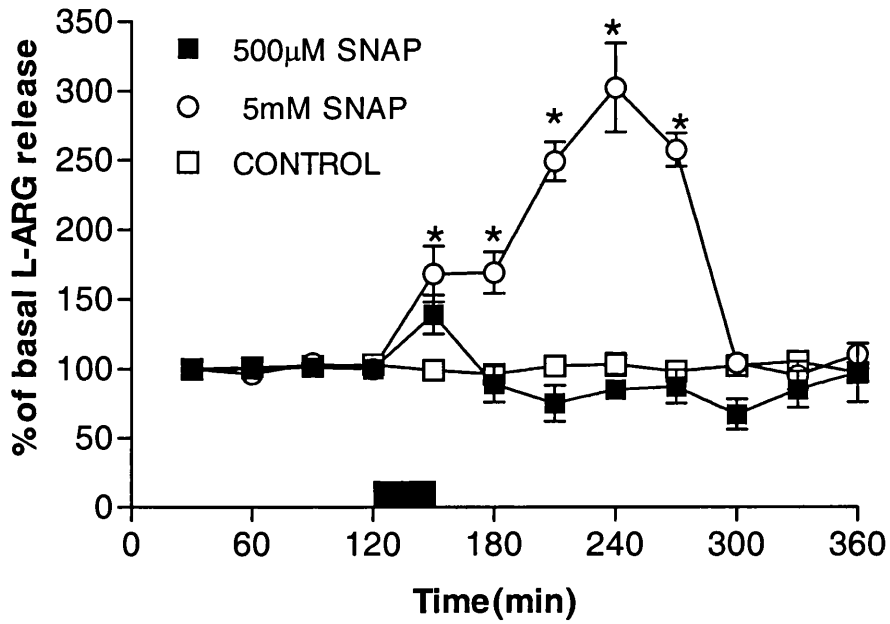
(B)
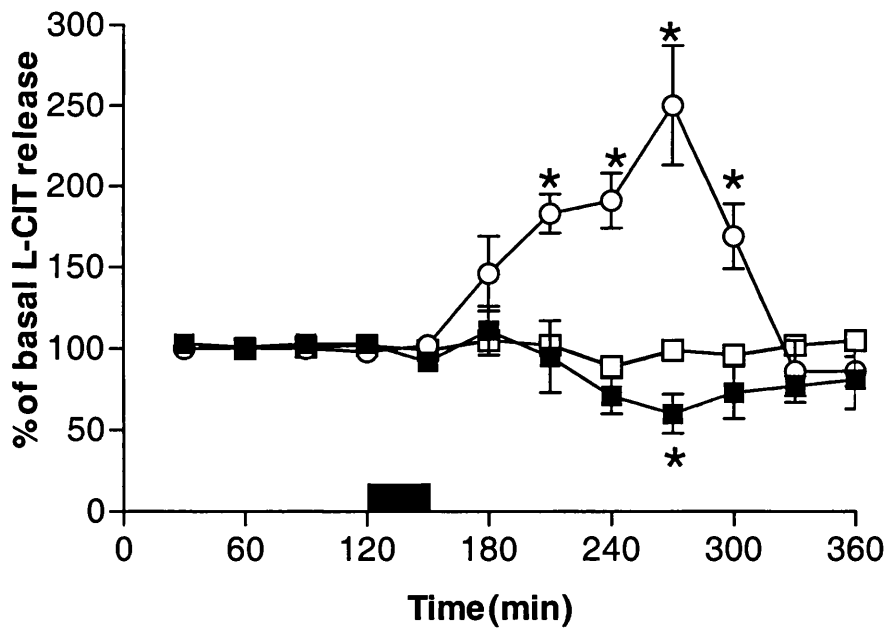
Fig. 4.5: Effect of SNAP infusion into the ventral hippocampus on L-ARG (A) and L-CIT (B) release. Bars denote the period of SNAP infusion. All values are means ±s.e.m (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls ($p < 0.05$).
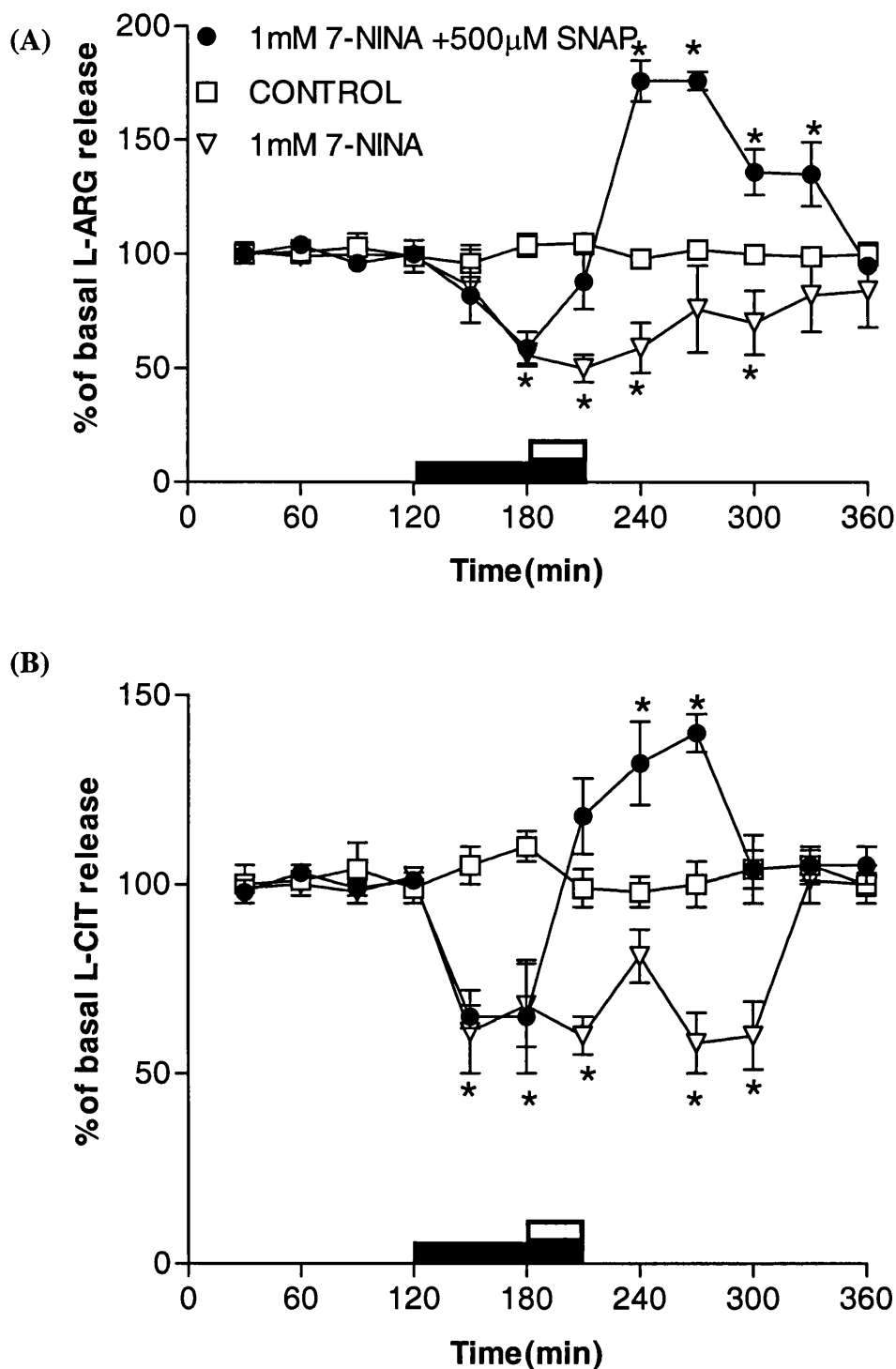
Fig. 4.6: Effect of 7-NINA infusion into ventral hippocampus on L-ARG (A) and L-CIT (B) release in the presence and absence of SNAP. The solid bars represent the period of 7-NINA infusion whilst the open bars denote the combined drug treatment. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls (p<0.05).

4.4 Discussion

The central route for L-ARG metabolism in the brain is the urea cycle. Ornithine transcarbamylase, an enzyme of the urea cycle which catalyses the synthesis of L-CIT from ornithine and carbamoyl phosphate, is absent in the brain (Sadasivudu and Rao, 1976). Therefore, the NOS reaction represents the only pathway for the formation of L-CIT from L-ARG. Production of NO occurs with stoichiometric formation of its by-product L-CIT, thus a decline in L-CIT levels has been used as an index of NOS inhibition (Ohta et al., 1994b; Sessa et al., 1990). Enhanced extracellular L-ARG levels and reduced L-CIT levels during infusion of NOS inhibitors is generally considered to reflect suppression of NOS activity. With regard to the present study, the effect of L-NAME on the levels of these amino acids seems to follow this general trend. This observation is in agreement with previous study performed by Ohta et al. (1994b) who infused L-NAME at 1mM into striatum for a period of 60 min and also observed an increase in L-ARG and decrease in L-CIT levels, the latter being permanent. In contrast, the current data show that L-CIT levels returned to the baseline despite the continuous presence of the inhibitor. It is reasonable to assume that this effect is due to the competitive nature of the inhibitor, such that the prolonged increase in the levels of extracellular L-ARG reversed the L-NAME-evoked inhibition of NOS.

Of particular interest are the data obtained following the infusion of 7-NINA where the extracellular levels of both L-ARG and L-CIT declined. The reduction in L-CIT levels may indicate a suppression of NOS activity but the decrease in extracellular L-ARG is more difficult to explain. The mechanism of action of this inhibitor is different from that of L-ARG analogues, such as L-NAME, in that its inhibition of NOS is, at least in part, independent of L-ARG and cannot be attributed

solely to competition with this amino acid (Wolf et al., 1994). To date, two working hypotheses have been suggested to account for the mechanism of action of 7-NINA on nNOS. It may be that the binding of 7-NINA (which is much larger than oxygen) to the Hb-iron causes it to project into the space normally occupied by L-ARG and THB during the reaction of oxygen with L-ARG and/or N-hydroxy-arginine to form L-CIT and NO. Alternatively, the binding of 7-NINA may produce a competitive interaction pattern causing reversible dissociation of L-ARG and THB from their binding sites on the enzyme (Wolf et al., 1994).

Evidence suggests that the effects of NOS inhibitors on L-ARG and L-CIT efflux may not be exclusive to NOS inhibition. L-ARG analogues have been found to inhibit L-ARG uptake systems (Schmidt et al., 1995). The transport of L-ARG via blood brain barrier is mediated by system y^+ (Stoll et al., 1993) which is also responsible for uptake of this amino acid by neocortical neurones and cerebellar granule cells (Westergaard et al., 1993). L-NMA has been found to be a substrate for this particular uptake system whilst another inhibitor from this family has been shown to inhibit arginase, the enzyme which converts L-ARG to L-ornithine (Robertson et al., 1993). These effects are, however, properties of particular inhibitor rather then general properties of all L-ARG based inhibitors. To my knowledge, no reports exist to suggest that these types of effects occur following the infusion of L-NAME or 7-NINA in vivo.

Evidence suggests that L-ARG release occurs following the stimulation of selective sensory afferents in the thalamus in vivo (Do et al., 1994). These investigators showed that application of L-ARG onto thalamic relay neurones facilitated sensory synaptic transmission possibly via the synthesis of NO. This, in turn, may indicate the possibility of L-ARG being released into the extracellular space

following the application of appropriate stimuli. The question was asked as to whether

NO plays a role as a trigger in the release of L-ARG into the extracellular space. If

this hypothesis is correct, then application of an NO donor should alter the levels of

extracellular L-ARG. Given the possibility of L-CIT recycling to L-ARG, we have

also measured changes this amino acid. The results show that the application of SNAP

led to an increase in both amino acid levels. At 500µM no statistically significant

changes in L-ARG and a decline in L-CIT levels were observed but at 5mM SNAP

elevated the extracellular levels of both L-ARG and L-CIT. The inhibition of L-ARG

and L-CIT levels produced by 7-NINA was not only reversed but release was

increased upon the addition of SNAP (500µM). This effect is puzzling since this

concentration of SNAP alone did not alter the basal release of L-ARG. Collectively,

these results suggest that there is a complex interaction between the prevailing L-

ARG concentration and the production of NO. To date, the mechanism underlying the

tonic production of NO and the importance of the availability of its substrate in the

brain is unclear. It is generally accepted that in the CNS, as well as the periphery, NO

and L-CIT are produced stoichiometrically from L-ARG by NOS (Moncada et al.,

1991; Moncada and Higgs, 1993). Although the role of L-CIT recycling to L-ARG in

the CNS is still being elucidated, its role in the gastrointestinal tract has now been

recognised (Rattan et al., 1996). It has been shown that L-ARG and L-CIT can

overcome the inhibition of NOS which leads to the attenuation of smooth muscle

relaxation in the colon. This study suggests the presence of enzymatic machinery

responsible for the recycling of L-CIT into L-ARG (Yu et al., 1995). Chakder and

Rattan (1997) have proposed that a critical level of L-ARG and the recycling of L-

CIT to L-ARG are important in the maintenance of smooth muscle relaxation in the

periphery. Moreover, production of NO by activated macrophages has been found to

be critically dependent on extracellular L-ARG (Keller et al., 1990; Bogle et al., 1992b). At sites of macrophage activation in vivo, a rapid depletion of extracellular L-ARG has been found to occur due to the metabolism of L-ARG by both arginase and NOS (Granger et al., 1990). It has been suggested that under these conditions the availability of L-ARG may become rate limiting for NO production (B aydoun et al., 1994). With respect to the study described here, one might suggest that during application of 7-NINA the extracellular L-ARG levels declined as a result of an inhibition of NO production and a consequent decline in the extracellular L-CIT levels. Studies in vivo suggest that L-CIT is synthesised and released from the intestine as an end product of glutamine nitrogen metabolism (Windmueller, 1985). In the circulation, once cleared by the kidney, L-CIT will no longer be available for metabolism and released as L-ARG (Dhanakoti et al., 1990). Thus uptake and metabolism of L-CIT may contribute to plasma L-ARG level which is important for sustained NO production in vivo (Aisaka et al., 1989). Baydoun et al. (1994) observed that under basal conditions the metabolism of L-CIT to L-ARG in order to provide substrate for NO production was limited, perhaps emphasising the importance of an adequate supply of extracellular L-ARG to sustain NO synthesis in activated macrophages. The current data suggests that the prevailing L-ARG and L-CIT concentrations may play an important role in the determination of the rate of NO production. This explanation is possible when viewed in the light of the results observed here following the addition of SNAP. These show that infusion of SNAP (5mM) led to an enhancement of L-ARG and L-CIT levels whilst (500μM) SNAP did not alter L-ARG and decreased L-CIT release. Based on this data, it is tempting to speculate that exogenous NO may cause the release of L-ARG into the extracellular space and by doing so regulates the rate of its own production. Moreover, the effects of SNAP (5mM) appear be time-

dependent. Addition of SNAP led to an immediate rise in extracellular L-ARG levels whilst the increase in L-CIT levels appeared to be delayed and was only evident once infusion of the donor was stopped. A recent report by Hu et al. (1995) showed that the NO donors SIN-1 and SNP caused the conversion of $[^3H]$-L-ARG to $[^3H]$-L-CIT in neuroblastoma N1E-115 cells. The stimulatory effect of these two donors on radiolabelled L-CIT formation was inhibited by L-NNA which suggests that the measured signal was a result of enhanced NOS activity. Furthermore, the increase in L-CIT formation was found to be time- and concentration-dependent in that the response was only observed at high donor concentrations and after prolonged (90min) incubation periods. With regard to the current data, these findings suggest that exogenous NO may cause the release of L-ARG into the extracellular space. This, in turn, may lead to an increase in NO production and consequently NOS activity. L-ARG is converted to NO and L-CIT, hence the levels of L-CIT increase. The time needed for L-ARG to produce NO and L-CIT may on the other hand, account for the delay in enhancement of L-CIT levels via the NO pathway.

The current hypothesis that NO may indeed cause the release of L-ARG into the extracellular space is mainly supported by the experiments where the inhibition of L-ARG release produced by 7-NINA was reversed on addition of SNAP. This also suggests that the rate of NO production in the brain may be conditioned by the availability of its substrate. These findings combined with the evidence which demonstrate that L-ARG is primarily stored in glial cells (Aoki et al., 1991) strongly support the notion this amino acid may be released from these cells on demand to sustain NO production in neurones.

4.5 Conclusions-part A

1. L-NAME and 7-NINA exert their effects on neurotransmitter release via inhibition of NOS as reflected by the decline in L-CIT levels.

2. The prevailing L-ARG concentration may determine the rate of NO production in the brain.

3. NO may control the release of L-ARG into the extracellular space in vivo.

PART B

4.6 Introduction

It is known that NO generation occurs as a part of the urea cycle and leads to stoichiometric production of L-CIT (Palmer and Moncada, 1989). In the brain, the NO pathway represents the only possible route for L-CIT production as the enzyme which converts L-ornithine to L-CIT, ornithine transcarbamylase, is missing. It has previously been shown that L-CIT is re-cycled to L-ARG via argininoscuccinate (Sadasivudu and Rao, 1976; Lee et al., 1996; Chakder and Rattan, 1997). This process is dependent on two enzymes, argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). ASS catalyses the synthesis of L-argininosuccinate from L-ASP and L-CIT and ASL cleaves L-argininosuccinate to yield fumarate and L-ARG. In the cerebellum, immunohistochemical studies have demonstrated that the two enzymes ASS and ASL which are involved in the recycling of L-CIT to L-ARG are not always found in the same neurones nor do they always colocalise with the NOS (Nakamura et al., 1990; Arnt-Ramos et al., 1992). Therefore, NO producing neurones may not have the facility to generate sufficient L-ARG to ensure continuous NOS activity and may be reliant on other cells.

Immunocytochemical evidence indicated that L-ARG, the substrate for NO synthesis, is primarily stored in glial cells especially in the cerebellum (Aoki et al., 1991) and thalamus (Kharazia et al., 1997). In addition, studies performed by Yamada et al. (1997b) showed that in fluorocitrate-treated rats, inhibition of NOS by L-NAME resulted in a decrease in the production of nitrite and nitrate Fluorocitrate has been characterised as a suicide substrate for the enzyme aconitase (Clarke et al., 1991) which is selectively taken up by glial cells and leads to alteration of the tricarboxylic acid cycle thus impairing the function of these cells. The effect of this agent persisted

for approximately 2h following the cessation of administration and had little effect on

cerebellar NOS activity in vivo (Yamada et al., 1997 a), thus indicating a role for glial

cells in the maintenance of NO production. Studies performed by Largo et al. (1996)

showed that infusion of fluorocitrate via a microdialysis fibre increased the

extracellular L-ARG levels in the hippocampus in anaesthetised rats whilst Yamada et

al. (1997a) demonstrated that i.p. injection of L-ARG as well as infusion into the

cerebellum through a dialysis probe increases nitrite and nitrate levels in the

dialysates. Collectively, these findings suggest that glial cells play a role in

maintenance of NO production and may argue for shuttling of intermediates between

different cell types.This possibility is further supported by evidence which showed

that stimulation of the white matter of cerebellar slices leads to the release of

endogenous L-ARG (Hansel et al., 1992) and from the thalamus by activation of

sensory afferents (Do et al., 1994). Thus, following application of appropriate stimuli

the observed L-ARG release may therefore represent its transfer from glial cells to

neurones in order to supply NOS with its substrate. A recent report by Grima et al.

(1997) proposed that L-ARG release from glial cells occurs via the activation of

excitatory amino acid receptors. It was demonstrated that the activation of non-

NMDA receptors by their respective agonists, but not NMDA or metabotropic L-GLU

receptors, resulted in the release of L-ARG from glial cells. These investigators

suggested that L-GLU might be a stimulus that causes the release of L-ARG from

glial cells. However, if L-GLU represented the only signal for L-ARG release from

glial cells, the transfer of L-ARG to neurones in order to supply nNOS with its

substrate would have to be restricted to glutamatergic terminals. This seems unlikely

since studies with NOS inhibitors have demonstrated a presence of tonic, NO-

mediated tone in the brain (Silva et al., 1995; Nei et al., 1996). It is plausible to

propose here that other stimuli may also lead to release of L-ARG from glial cells. NO itself is a strong candidate because of its freely diffusible nature and speed with which it may access different compartments.

It has been previously observed (part A of this chapter) that the prevailing NO concentration controls the release of L-ARG into the extracellular space in vivo. Based on the above evidence and the current findings it was reasoned that NO may regulate its own rate of synthesis by releasing L-ARG from glial cells to supply neuronal NOS with substrate. This hypothesis would be difficult to test in an in vivo model because of the structural complexity of this system. Therefore, cerebellar glial cultures were used as a model system as they offer a convenient and simple way to test the hypothesis.

4.7 Methods

4.7.1 Glial cell culture.

Primary cerebellar glial cultures were prepared using neonatal rats aged 5 to 7 days old according to the method of Dutton et al. (1981). Following decapitation, the cerebella were removed from the brain then thoroughly cleaned of meninges and vasculature. The tissue was then placed in a few drops of sterile disaggregation medium (DM) of the following composition: glucose (14mM), bovine serum albumin (BSA) (3mg/ml) and $MgSO_4$ (1.5 mM) in Ca^{2+}-and Mg^{2+}-free Earle's Balanced Salt Solution (Gibco) then coarsely chopped with a sterile scalpel. The tissue suspension was transferred to a trypsinisation flask containing trypsin (250µg/ml) in 10 ml of DM, the flasks were then placed in a shaking water bath at 37 °C for 15min. A 10ml solution containing trypsin inhibitor (192µg/ml), diluted DNase (6.4µg/ml) and $MgSO_4$ (240µM) in DM was added then the mixture was transferred to 50 ml sterile plastic tubes and centrifuged at 100x g for 5s (Denley, BS 400) to sediment the tissue blocks. The supernatant was removed and the tissue resuspended in a few drops of a solution containing trypsin inhibitor (1.2 mg/ml), DNase (40µg/ml), and $MgSO_4$. $7H_2O$ (1.5 mM) in DM.

The tissue was then mechanically dissociated by gentle trituration through a 1.5mm diameter stainless steel cannula attached to a 2ml sterile syringe. The resultant cell suspension was removed and transferred to a sterile 15ml tube then underlaid with 2ml of a solution containing a 4% w/v BSA in DM. The cells were pelleted through the BSA underlay by centrifugation at 100x g for 5min. The supernatant and the BSA underlay, which contained cell debris, were discarded and the cell pellet resuspended in a small volume of growth medium (GM) of the following composition: Minimum

Essential Medium with Earle's Salts (Gibco) supplemented with foetal calf serum (10% v/v), glutamine (2mM), glucose (33mM) and gentamicin (65μg/ml). The cell suspension was diluted appropriately in GM to give a seeding density of 100, 000 cells per dish. Cells were then seeded onto poly-D-lysine (50μg/ml)-coated 35mm diameter dishes (Nunc).

The cells were maintained for 14 days in vitro in a humidified atmosphere containing 5% CO_2 in air at 37° C. All experiments were performed on confluent cultures at approximately 14 days in vitro.

4.7.2 Release experiments

Cultures grown on 35mm dishes were washed three times with buffer of the following composition: NaCl (116mM), $NaHCO_3$ (26mM), NaH_2PO_4 (1mM), $MgSO_4$ (1.5mM), KCl (5mM), $CaCl_2$ (1.3mM) and glucose (20mM) gassed with 5% CO_2 in O_2.. The cultures were then incubated at 37 ^{0}C in 5% CO $_2$/air atmosphere in buffer for 60 min, prior to starting the experiment. Cultures were then incubated in 2ml volumes of buffer for twelve consecutive 2min periods, the buffer being collected after each incubation period and replaced with fresh buffer. The first five samples were collected to establish basal levels of L-ARG and L-CIT. Drug additions were made for 2-6min depending upon the experiment, before returning to drug-free buffer for the reminder of the experiment. All incubations were carried out at room temperature.

4.7.4 Sample analysis.

All samples were analysed by HPLC with fluorimetric detection as outlined in

part A of this chapter. A typical chromatogram illustrating the sample from cultures is

provided in Fig. 4.7.
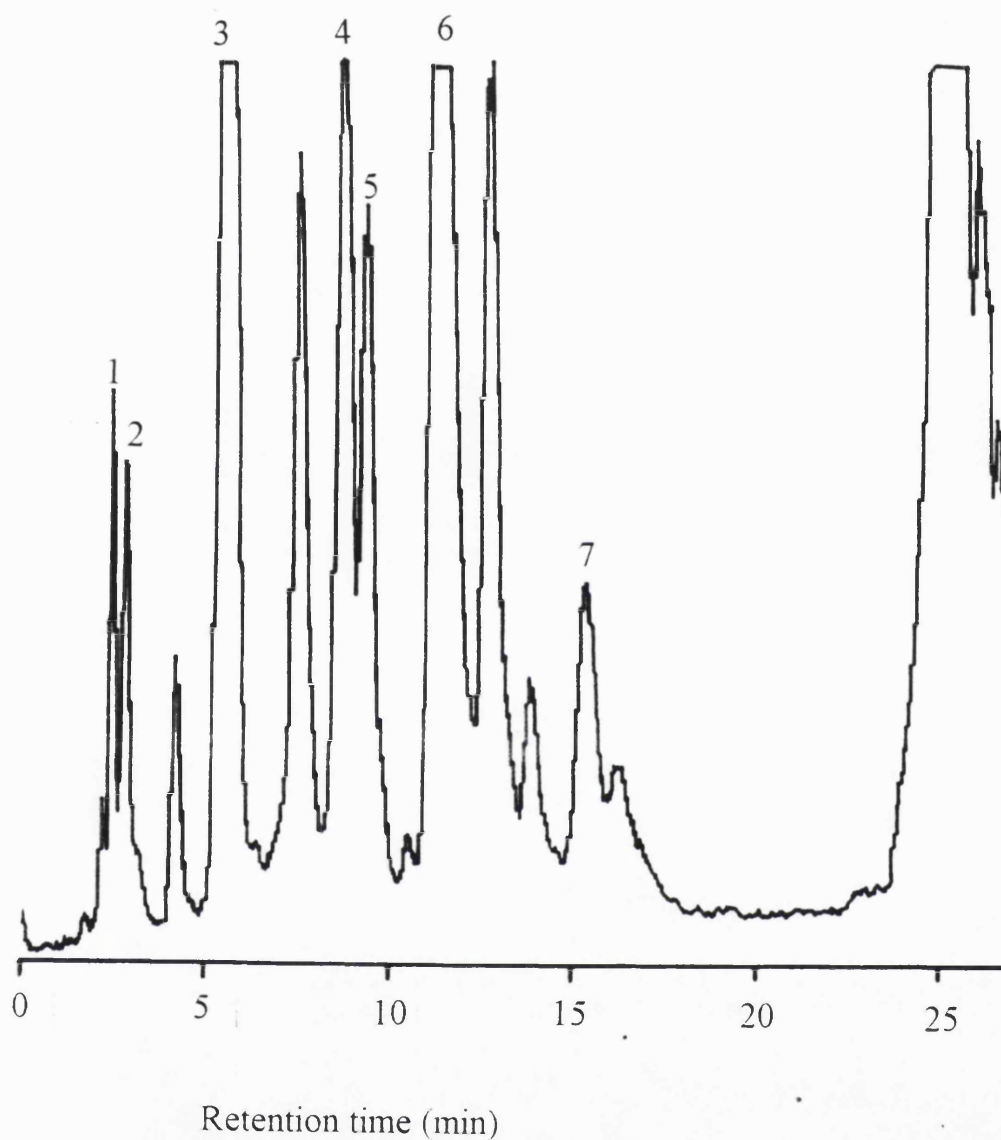
Fig. 4.7: Typical chromatogram obtained following separation of amino acid from glial cells using HPLC with FD. Horizontal axis represents elution times in min. Peaks denoted are: (1) ASP, (2) L-GLU, (3) L-GLN, (4) L-ARG, (5) L-CIT, (6) Threonine/glycine (7) TAU. Separation of the standard amino acid mixture is presented in Fig. 4.2 A.

4.8 Results

The extracellular levels of L-ARG and L-CIT were 20 ± 7 and 56 ± 8 pmols/15µl/2min sample, (n=25) respectively.

A 2min addition of SNAP at 50µM and 500µM to cultured cells produced a concentration-dependent increase in L-ARG efflux with 500µM elevating the amino acid level by 150 + 33% above control values. The increase was rapid in onset and persisted for 2min after the drug infusion ceased before returning to the control levels (Fig. 4.8 A). Likewise, L-CIT levels were also elevated following the addition of SNAP at both concentrations. The amino acid levels increased by up to 100 ±25% above control values but showed no statistically significant differences between concentrations (Fig. 4.8 B). Interestingly, 500µM SNAP caused a rapid increase in L-CIT levels whilst the effect of the lower concentration was delayed, reaching a maximum 2min after the administration of the donor had ceased. This was followed by a decline in L-CIT levels which reached a minimum of 40 ± 15% and 50 ± 10% below control values for 500 and 50µM SNAP, respectively.

To ensure that the effects of the donor on the release of amino acids from glial cultures were due to the action of NO, Hb a known NO scavenger was applied on its own and in the presence of SNAP. A 6 min administration of Hb at 10µM did not significantly alter the levels of L-ARG whilst L-CIT levels declined by 45 ± 15% below control values (Fig. 4.9 A and B). When Hb (10µM) was infused for 4 min prior to co-infusion with SNAP (50µM) the elevations in both amino acids previously seen with this donor were abolished (Fig. 4.9 A and B).
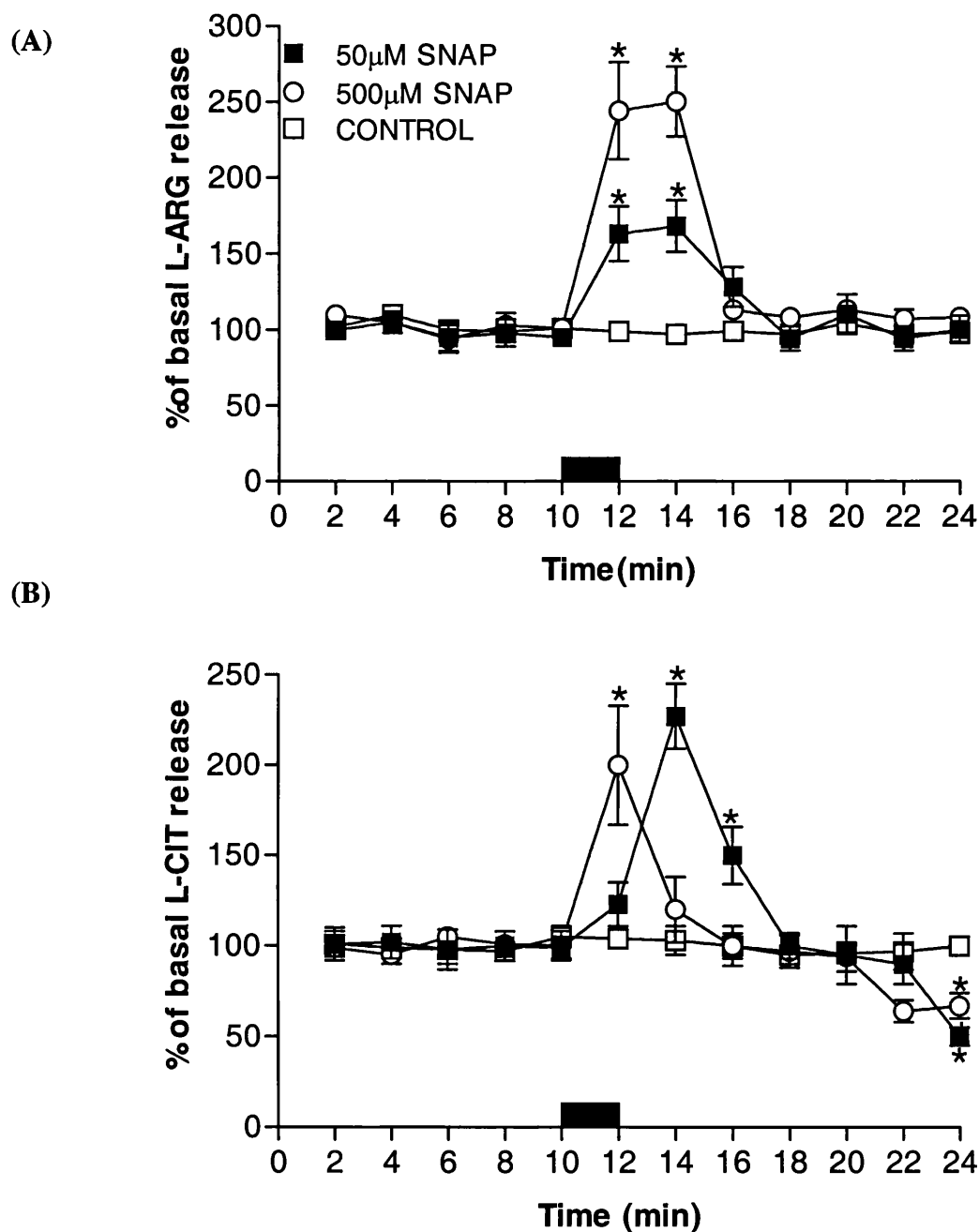
Fig. 4.8: Effect of SNAP administration on L-ARG (A) and L-CIT (B) release from glial cells. The solid bars represent the period of SNAP administration. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls (p<0.05).
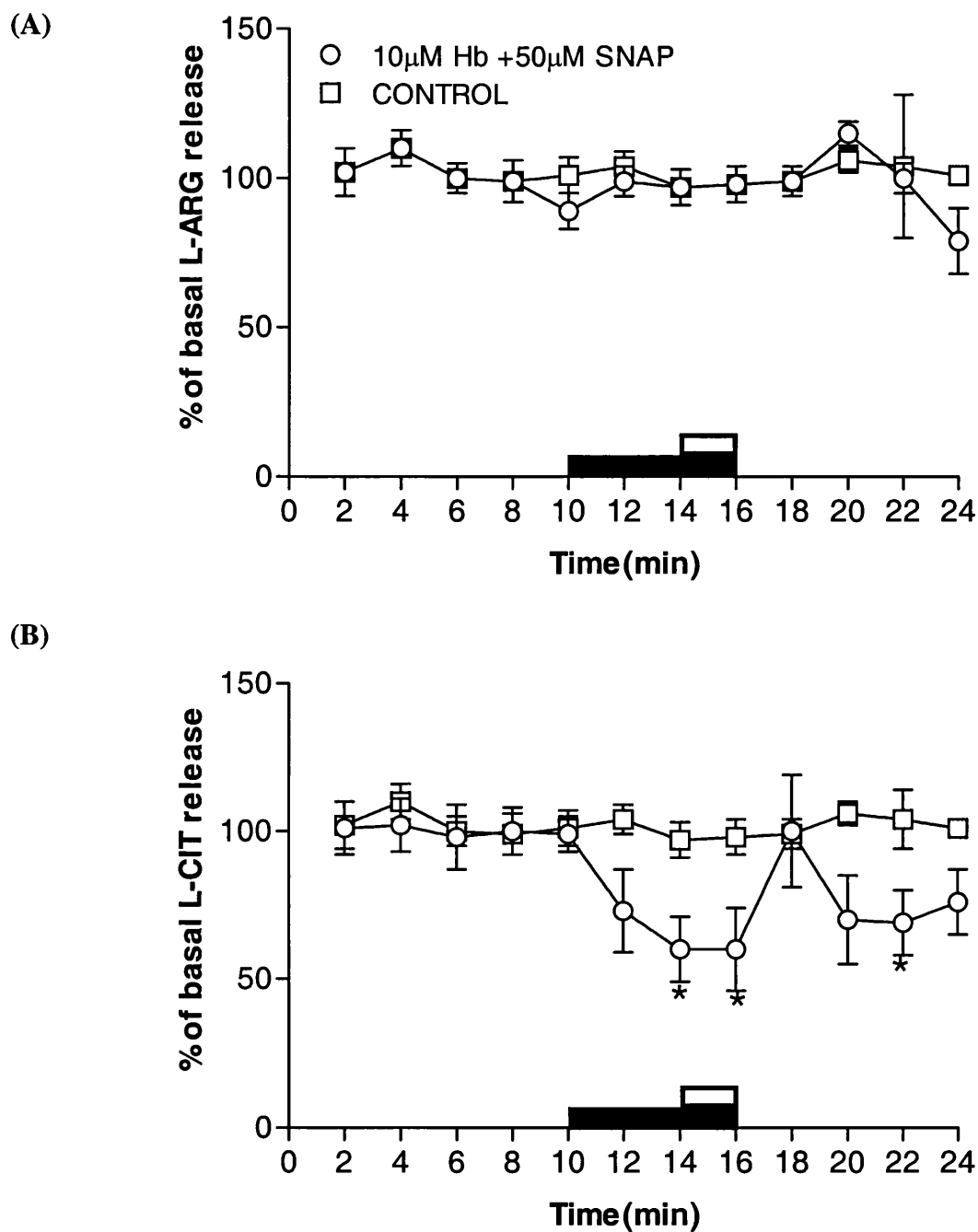
Fig. 4.9: Effect of SNAP administration on L-ARG (A) and L-CIT (B) release from glial cells in the presence of Hb. The solid bars represent the period of SNAP administration and the open bars denote SNAP and Hb together. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls ($p < 0.05$).

A 6min application of L-NAME at 10μM decreased the extracellular L-ARG and L-CIT levels by up to 55 ± 15% and 50 ± 15% below control values, respectively. This effect was rapid in onset and long lasting in that the level of the amino acid did not return to baseline within the time course of the experiment (Fig. 4.10 A and B). When L-NAME (10μM) was infused for 4min prior to co-infusion with SNAP (50μM), the inhibition of L-ARG and L-CIT efflux produced by L-NAME was reversed (Fig. 4.10 A and B). The combined treatment of L-NAME (10μM) and SNAP (50μM) led to an elevation of L-ARG and L-CIT levels by up to 35 ± 5% and 100 ± 15% above control values, respectively.

A 2min administration of 8-bromo-cyclic-guanosine-monophosphate (8 Br-cGMP) at 100μM produced an elevation of L-ARG efflux by up to 50 ± 10% above baseline (Fig. 4.11 A). The increase was rapid in onset and was followed by a long plateau before returning to control levels (Fig. 4.11 A). In contrast, L-CIT levels declined rapidly with the levels of the amino acid decreasing to 50 ± 10% below basal levels. This effect was permanent in that the levels of L-CIT did not return to baseline within the time course of the experiment (Fig. 4.11 B).

When cells were incubated in Ca^{2+}-free medium to which 0.2mM EGTA had been added, the increase in extracellular L-ARG efflux observed with 50μM SNAP was abolished. L-ARG levels declined by approximately 45 ± 10% below baseline within 2min after the challenge with SNAP ceased (Fig. 4.12 A) then returned to baseline within the time course of the experiment. The removal of Ca^{2+} from the incubation medium did not alter the effect of 50μM SNAP on L-CIT release (Fig. 4.12 B).

Fig. 4.10: Effect of L-NAME administration on L-ARG (A) and L-CIT (B) release from glial cells in the presence and absence of SNAP. The solid bars represent the period of L-NAME application whilst the open bars denote the combined drug treatment. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls ($p < 0.05$).

TAU release is often used as an indicator of cell swelling as its release is associated with cell volume regulation (Vitarella et al., 1994). In order to assess whether cell swelling may account for the release of L-ARG seen in this study the effect of L-NAME and SNAP on TAU release was determined. Neither a 2min application of SNAP (50μM) nor administration of L-NAME (10μM) for 6min altered the basal level of extracellular TAU (Fig. 4.13 A and B).
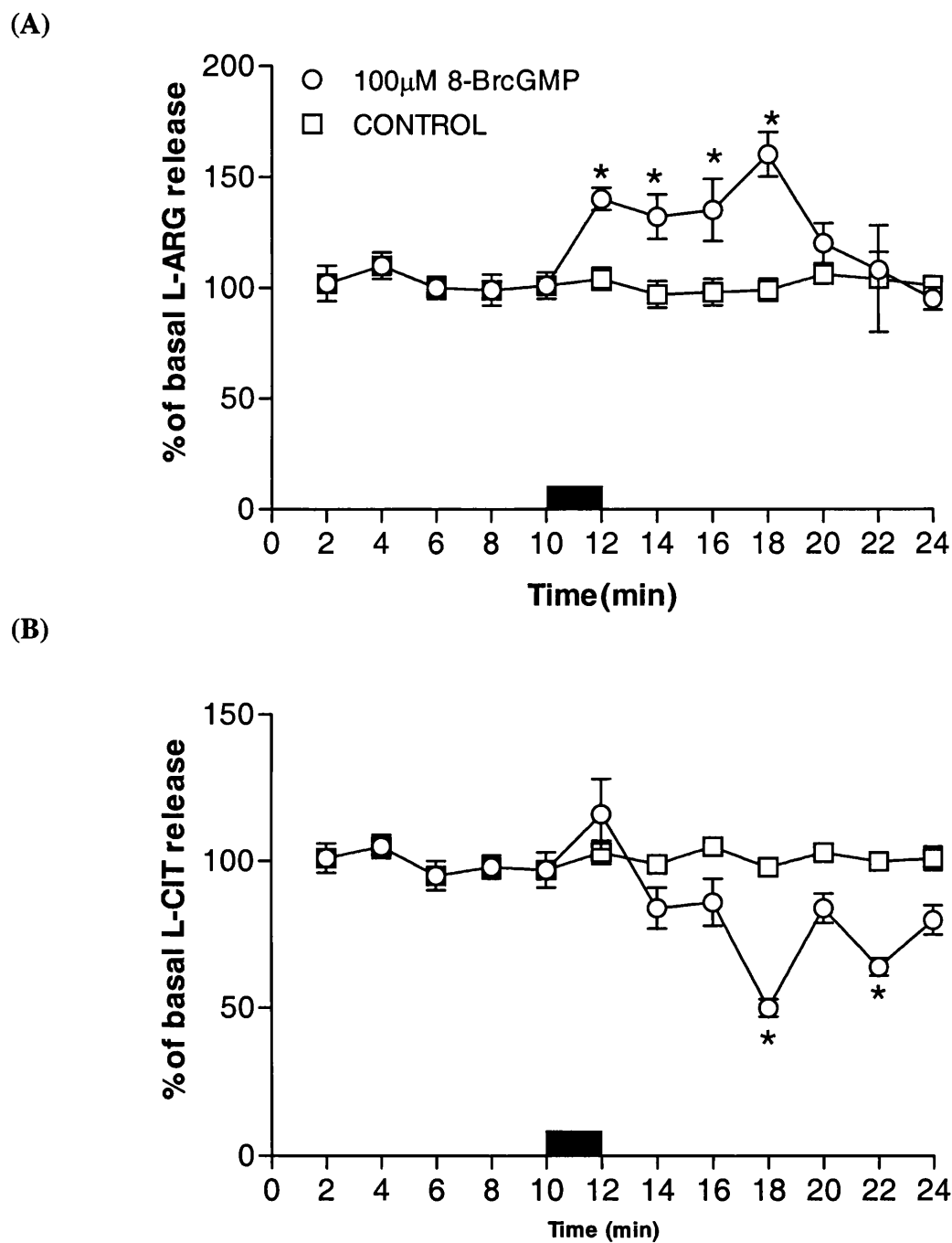
Fig. 4.11: Effect of 8-BrcGMP administration on L-ARG (A) and L-CIT (B) release from glial cells. The solid bars represent the period of 8-BrcGMP application. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls (p<0.05).

(A)

(B)
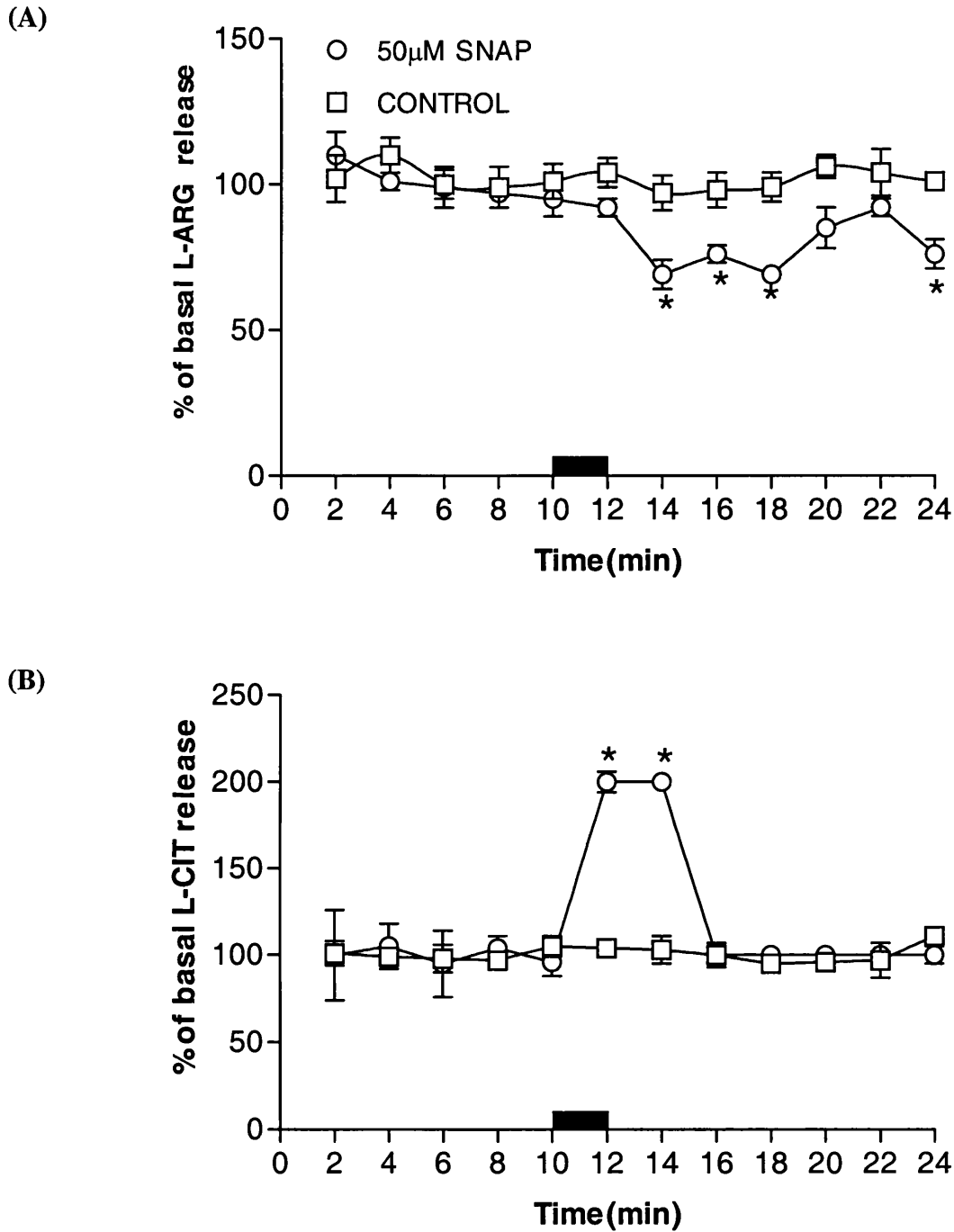
Fig. 4.12: Effects of SNAP administration on (A) L-ARG and (B) L-CIT release from glial cultures in the absence of extracellular Ca^{2+}. Bars represent the period of SNAP application. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments. * indicates data points significantly different from controls ($p < 0.05$).

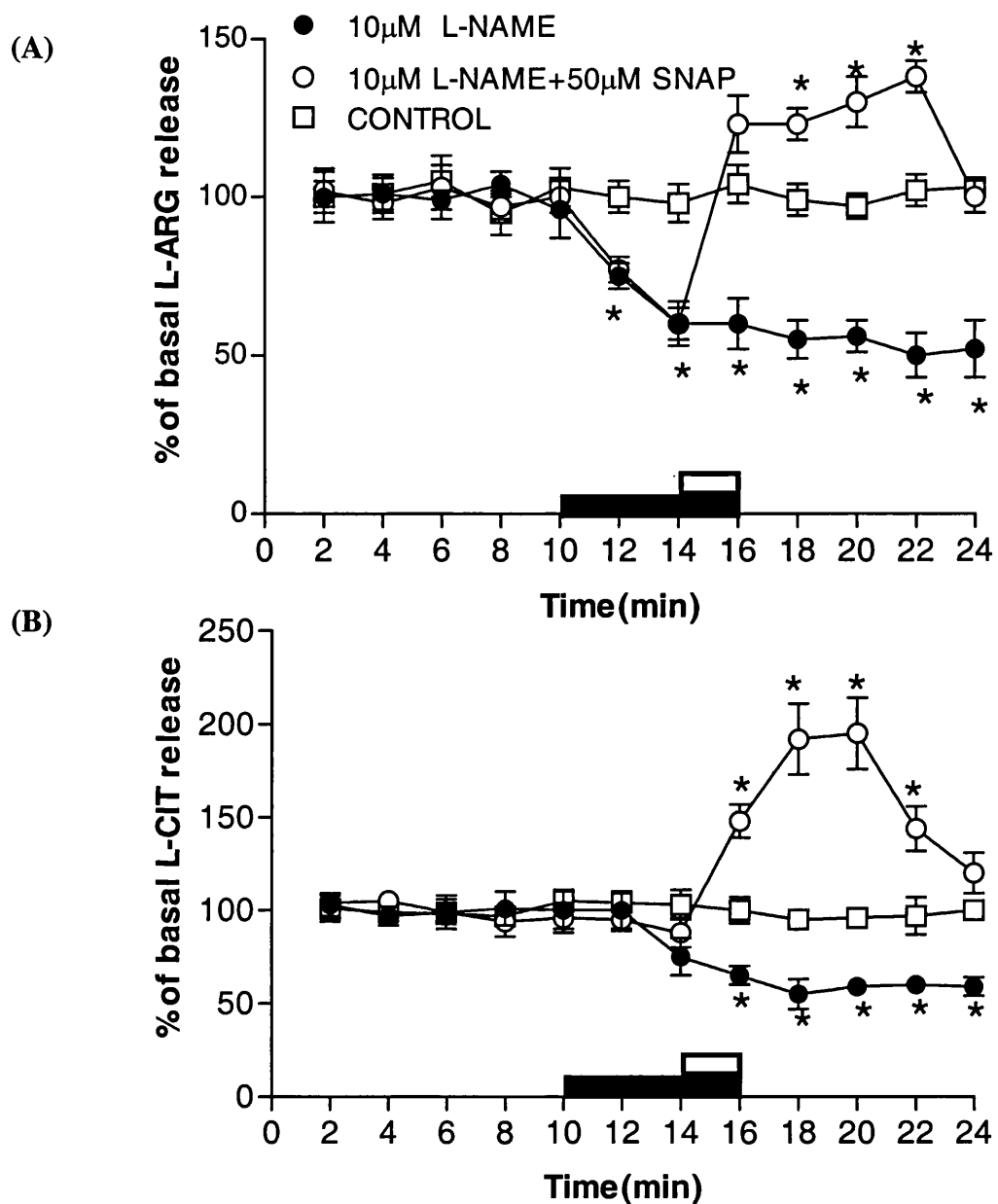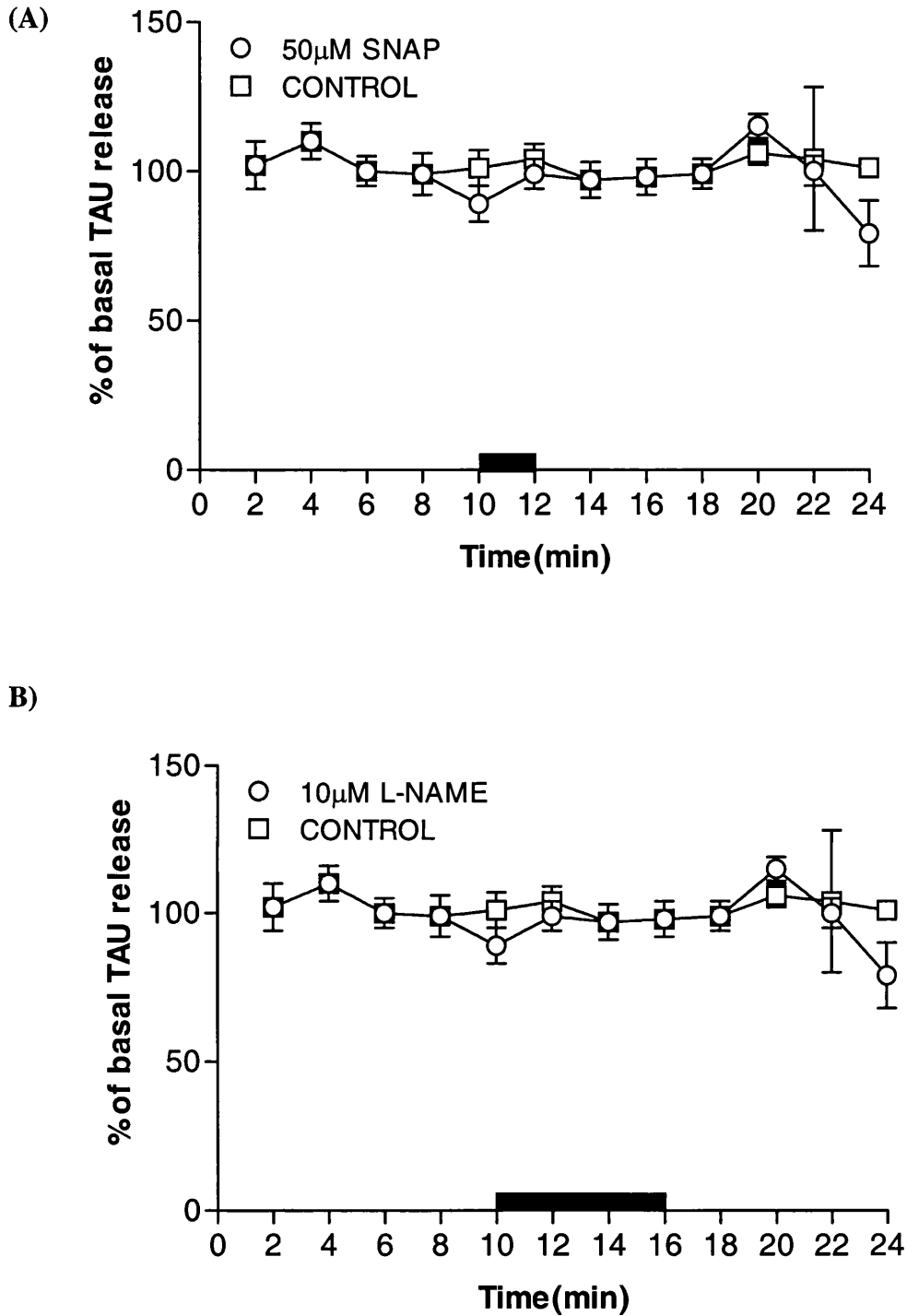**Fig. 4 13:** Effect of (A) SNAP and (B) L-NAME administration on TAU release from glial cultures. Bars represent the period of SNAP and L-NAME infusion. All values are means ±s.e.m. (shown when larger than the symbol) from four experiments.

4.9 Discussion

The results obtained in this study show that L-ARG, the precursor of NO, is released from cerebellar glial cells following the administration of the NO donor SNAP. The release was found to be Ca^{2+} and concentration-dependent and was inhibited by Hb, a known scavenger for NO. These results support the hypothesis that NO may control the release of L-ARG from glial cells.

The mechanisms controlling the release of substances from glial cells are poorly understood. The term release is usually applied to the efflux of compounds by one of the following mechanisms: reversal of an uptake system, Ca^{2+}-dependent vesicular release and release caused by swelling. A major problem in studying the release from glial cells is distinguishing between these alternatives. There is evidence to suggest that bradykinin causes Ca^{2+}-dependent release of L-GLU from cortical astrocytes and that this occurs by a mechanism similar to the neuronal secretory process (Parpura et al., 1995). The current data show that the release of L-ARG from glial cells is Ca^{2+}-dependent. This is in agreement with the results obtained by Grima et al. (1997), where the application of Ca^{2+} ionophore A23187 induced a rise in the extracellular L-ARG levels in astroglial cultures. It is possible that the release of L-ARG from glial cells occur via a 'vesicular like' mechanism, similar to that observed with L-GLU. This possibility is further supported by immunocytochemistry studies which indicate that astrocytes possess a secretory system that is similar to that found in neurones (Jeftinija et al., 1996). Astrocytes have been shown to express synaptobrevin 2, a vesicular protein that together with the plasma membrane protein synaxin and synaptosomal associated protein (SNAP-25) participate in formation of the anchoring core complex required for the initiation of exocytosis. Moreover, while these investigators were unable to demonstrate the presence of SNAP-25 and syntaxin in

cultured astrocytes, pre-treatment with botulinum toxin B (which cleaves SNAP 25) and botulinum toxin C (which cleaves syntaxins) diminished the bradykinin-evoked release of L-GLU from cultured astrocytes. Collectively these findings support the notion that astrocytes may release neurotransmitters using mechanism similar to the neuronal secretory process and the lack of expression of SNAP-25 and synataxin in these cells can be explained by the low amounts of these two proteins in cultured astrocytes. It is tempting to speculate that the release of L-ARG from glial cells in response to NO occurs via a vesicular like mechanism. Based on the freely diffusible nature of NO and its high affinity for metalloproteins, NO itself has been proposed to interact with synaptic protein, which has a domain similar to metal binding domains of metalloproteins (Oyler et al., 1989), SNAP-25 (Lonart and Johnson, 1995a,b). It has been suggested that the release of NA from hippocampal slices takes place via NO interacting with this protein and therefore participating in the process of exocytosis. This docking protein may associate with Ca^{2+}-sensitive channels and together they form "synaptosecretosomes" which can organise the process of neurotransmitter release (O' Connor et al., 1993). It may be that NO by interacting with synaptic proteins causes Ca^{2+}-dependent, 'vesicular like' release of L-ARG into the extracellular space from glial cells.

Alternatively, L-ARG may be released from glial cells by reversal of the membrane transporter system. Such a mechanism has previously been described for L-GLU and GABA (Gallo et al., 1990: Szatkowski et al., 1990). The L-ARG uptake system into glial cells has been shown to be of the y^{+} type (Schmidlin and Wiesinger, 1994). This transporter can function as a two directional system and has been shown to be pH-and Na^{+}-independent (White et al., 1985). The release of L-ARG from glial cells in response to NO through the reversal of a L-ARG transporter is unlikely as L-

ARG is released in Ca^{2+}-dependent manner and is not stimulated by depolarising stimuli (Kavanaugh et al., 1993: Wayte et al., 1996), which have been shown to reverse the L-GLU uptake.

A further release mechanism is tension–controlled release due to glial swelling. This has been reported for various amino acids such as L-GLU, L-ASP and TAU (Kimelberg et al., 1990). It is unlikely that this process is involved in the release of L-ARG shown here. TAU is highly correlated with glial cell swelling (Vitarella et al., 1994) but the current experiments show no increase in the extracellular TAU levels, following the application of SNAP or L-NAME. Taken together, the release of L-ARG might take place via an as yet unknown 'vesicular like' mechanism.

It is now generally accepted that the primary target molecule for NO is sGC (Martin et al., 1985) and that stimulation of sGC leads to increases in cGMP levels (Southam et al., 1991). Cerebellar astrocytes have been proposed to represent a major target for NO released from granule cells (Southam and Garthwaite, 1991) and immunohistochemical findings indicate cGMP formation in astrocytes following application of the NO donor SNP (Garthwaite et al, 1987; Feelish et al., 1987; De Vente et al., 1990). In the study described here, an application of the cGMP analogue, 8-BrcGMP, caused an enhancement in the release of L-ARG from glial cells. Thus it appears that not only NO but also an increase in cGMP may be involved in the release of L-ARG from glial cells.

The present study also demonstrates that L-CIT, the by-product of NO synthesis, is released from cerebellar glial cells in response to exogenous NO. In contrast to L-ARG, SNAP caused a Ca^{2+}-and concentration-independent release of L-CIT. Application of Hb resulted in a decrease in L-CIT release, an effect which was briefly reversed by SNAP. It is difficult to speculate how L-CIT release relates to L-ARG

release from glial cells. Evidently, two distinct mechanisms of release operate here. Whilst L-ARG may be released from glial cells in order to supply neurones with the substrate for NO synthesis, L-CIT may simply be released as an additional co-substrate. The current study also reports that basal extracellular levels of L-CIT were approximately twice as high as that of L-ARG. This is in agreement with earlier studies performed by Sadasivudu and Rao, (1976) which indicated that the L-CIT concentration in the brain is high. These investigators suggested that L-CIT is converted to L-ARG as administration of high levels of the amino acid led to increases in L-ARG levels in all brain regions studied. It is known that the only known metabolic pathway in which L-CIT can serve as a substrate by the synthesis of L-ARG is via argininosuccinate (Sadasivi and Rao, 1976). Thus it may be that the high extracellular levels of L-CIT contribute to the maintenance of L-ARG levels (Aisaka et al., 1989).

Studies indicate that under certain conditions glial cells can express both constitutive and inducible form of NOS (Chao et al., 1992; Schmidt et al., 1992; Zielasek et al., 1992). The presence of the constitutive NOS suggests a tonic production of NO in glial cells. Infusion of L-NAME ($10\mu M$) decreased the release of L-CIT and L-ARG. The decrease in extracellular L-CIT levels is considered to reflect the inhibition of NOS activity whilst the effect on L-ARG further supports the hypothesis that the prevailing concentration of NO may control the release of L-ARG from glial cells. Interestingly, the effect of L-NAME on L-ARG release in vitro was in contrast to its effect in vivo but similar to that of 7-NINA in vivo. The reasons for this discrepancy between the effects of L-NAME in vivo and in vitro are presently unknown.

Studies on the L-ARG transporter have shown that the availability of intracellular L-ARG is rate limiting for NO production (Bogle et al., 1996). Moreover, intracellular L-ARG levels are mainly regulated by uptake (Westergaard et al., 1993; Bogle et al., 1996). Addition of exogenous L-ARG has been demonstrated to potentiate AMPA-induced cGMP accumulation in cerebellar slices (Southam et al., 1991). Thus the availability of L-ARG might be one of the control mechanisms for NO production by constitutive NOS. In contrast, it could be argued that the L-ARG pool in glial cells serves as a reservoir of substrate for iNOS. This notion can be excluded for the following reasons. First, nNOS can be induced in a variety of cells including neurones and glial (Golomb et al., 1996) thus an L-ARG pool should also be present in neurones. This is not, however, the case (Aoki et al., 1993). Second, the enzyme involved in recycling L-CIT to L-ARG, ASS, which converts L-CIT to argininosuccinate, is co-induced with iNOS by LPS whilst ASL, which converts argininosuccinate to L-ARG, is constitutively expressed (Nussler et al., 1994). This, in turn, may suggest that the cells have the capacity to synthesise NO without the need for extracellular L-ARG. Therefore, iNOS production may be independent of extracellular L-ARG, whilst the production of NO by the constitutive NOS expressing cells could be based on L-ARG release from glial cells in response to NO and subsequent uptake by neuronal cells.

It is now generally accepted that glial cells are involved in intercellular communication in the brain (Porter et al., 1996; Tsocopoulos et al., 1996). The study described here provides an example of cross-talk between glia and neurones which may be a key element in NO synthesis in the CNS. The prevailing extracellular NO concentration provides a signal for glial cells to release L-ARG which is then taken up by neurones in order to sustain synthesis of NO.

4.10 Conclusions- Part B.

1) The release of L-ARG from glial cells may occur via vesicular-like mechanism and is dependent upon the prevailing extracellular NO concentration.

2) The release of L-CIT may represent another form of control over NO production

3) The release of L-ARG from glial cells appears to involve an increase in intra – cellular cGMP levels.

Chapter 5

Concluding remarks

5.1 Overview

The main aims of this thesis were

1) to investigate the effects of NO on the regulation of monoamine and amino acid transmitter release in the hippocampus in vivo using microdialysis in freely moving rats.

2) to examine the relationship between NMDA receptor activation, neurotransmitter release and NO in the hippocampus.

3) to explore the role of glial cells in sustaining neuronal NO production.

NO has been shown to exert a dual inhibitory/stimulatory effect on synaptic transmission in the brain. The work presented in chapter 3 has extended these observations to examine the relationship between NO, amino acid and monoamine release in rat ventral hippocampus in vivo. These results showed that different concentrations of NO donor SNAP were capable of stimulating or inhibiting neurotransmitter release in this brain structure in vivo. In addition, it was demonstrated that   NO drugs alter the NMDA-receptor-evoked responses on neurotransmitter release in the hippocampus. The precise role of NO in the regulation of neurotransmitter release remains to be elucidated and given the limitations of the available pharmacological tools, conclusions must be dependent upon those which are. Nonetheless, the responses observed appeared to be determined by the prevailing NO concentration.

There were curious discrepancies between the effects of the two NOS inhibitors used and the functional significance of this has yet to be determined. For example, 7-NINA decreased the extracellular levels of all amino acids and monoamines measured

with exception of DA whilst the opposite was observed with L-NAME. Overall, the results presented in chapter 3 have demonstrated the presence of a tonic NO-mediated regulatory tone in the control of neurotransmitter release. This conclusion has been supported by the observations presented in chapter 4 (part A) showing that the application of these inhibitors led to a decrease in L-CIT levels which was used as an index of NOS activity. Although; the infusion of these inhibitors resulted in a decrease in L-CIT levels, suggesting that the effects of 7-NINA and L-NAME were due to NOS inhibition, criticism may be levelled at such study. The differential effects these two inhibitors on neurotransmitter release may be attributed to their differing mechanisms of action, interference with other enzyme systems or lack of selectivity in the inhibition of all the NOS isoforms. These findings do, however, suggest that the source of NO (neuronal or endothelial) may be crucial in determining the final response.

The results presented in chapter 4 (part A) demonstrated that inhibition of NOS by 7-NINA resulted in a decrease in L-ARG levels and that this decrease was reversed upon addition of exogenous NO. These were interesting observations since they suggested that the prevailing NO concentration might be important in the control of L-ARG release. We were interested to examine this hypothesis in detail and investigate whether L-ARG, which has been found to be stored primarily in glia (Aoki, et al., 1990), was released on demand by NO in order to supply nNOS with its substrate. The work presented in chapter 4 part (B) is the first demonstration that NO may indeed regulate synthesis by releasing L-ARG from glial cells. The release of L-ARG can be enhanced by increases in cGMP levels and was dependent on the influx of extracellular Ca^{2+}.

The role of NO and /or cGMP on intracellular Ca^{2+} in glial cells remains to be fully elucidated, and this reflects the current understanding of Ca^{2+} homeostasis in non-excitable cells in general. It is not known, for example, how cGMP-dependent mechanism regulates intracellular Ca^{2+} concentrations, whether it involves Ca^{2+} entry via various families of Ca^{2+} channels such as voltage-operated Ca^{2+} channels, store-operated Ca^{2+} channels or due to the activation of intracellular Ca^{2+} stores. It has been postulated that NO exerts a fine tuning of Ca^{2+} channels activity via the generation of cGMP (reviewed by Milbourne et al., 1995). It was noted that cGMP levels were elevated when pancreatic cells were treated with different concentration of SNP (Xu et al., 1994). cGMP levels increased concurrently with increases in the concentration of SNP and appeared to have a dual effect on Ca^{2+} entry. Ca^{2+} entry was stimulated when cGMP levels were low and inhibited at higher levels.

FUTURE DIRECTIONS

The hypothesis that NO regulates its own synthesis by releasing L-ARG from glial cells clearly requires more attention. Moreover, the potential role of L-CIT in the regulation of NO synthesis ought to be examined. It would be particularly interesting, for example, to compare and contrast the concentration-dependency between increases in cGMP levels and NO donors causing the release of these amino acids from glial cells. These experiments could be extended to include the effects of NO on intracellular Ca^{2+} concentration and examine the interaction between the levels of cGMP and Ca^{2+} in glial cells.

In conclusion, this thesis presents evidence for a functional relationship between NO and neurotransmitter release in the hippocampus in vivo and that NMDA-receptor induced modifications in amino acid and monoamine

neurotransmitter release might be mediated by NO. Finally, glial cells appear to be of

importance in providing neurones with substrate for NO synthesis and that NO itself

may act as the signal for L-ARG release from these cells

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