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The Effects of Passive Avoidance Learning on the Release of Amino Acids and other Putative Transmitters in the Forebrain of the Day-old Chick

By

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A thesis submitted in partial satisfaction of the degree of Doctor of Philosophy

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Abbreviations

Anatomical

ARCH:	archistriatum
HAD:	hyperstriatum accessorium dorsale
HVc:	higher vocal centre
IMHV:	intermediate medial hyperstriatum ventrale
LNH:	lateral neostriatum and hyperstriatum
LPO:	lobus parolfactorius
MAN:	magnocellular nucleus of the anterior neostriatum
MNH:	medial neostriatum and hyperstriatum ventrale
PA:	palaeostriatum augmentatum
PP:	palaeostriatum primitivum
RA:	robust nucleus of the archistriatum

General

α :	selectivity factor (HPLC)
ACh:	acetylcholine
ACN:	acetonitrile
AHP:	after hyperpolarisation
ANOVA:	analysis of variance
4-AP:	4-aminopyridine
ArA:	arachidonic acid
ATP:	adenosine triphosphate
Ba ²⁺ :	barium ion
B _{max} :	maximal binding of ligand
Ca ²⁺ :	calcium ion
cAMP:	cyclic adenosine monophosphate
cDNA:	complementary deoxyribonucleotide
<i>c-fos</i> :	cellular fos mRNA
cGMP:	cyclic guanosine monophosphate
<i>c-jun</i> :	cellular jun mRNA
CaM kinase:	calcium/calmodulin kinase
CF:	climbing fibre
Cl ⁻ :	chloride ion

cNMDA:	CPP preferring NMDA receptors
CNS:	central nervous system
DAG:	diacylglycerol
2-DG:	2-deoxyglucose
2-DGal:	2-deoxygalactose
dpm:	disintegrations per minute
EAA:	excitatory amino acid
EAAC1:	excitatory amino acid channel
EEG:	electroencephalogram
EPSC:	excitatory post synaptic current
EPSP:	excitatory post synaptic potential
GAP-43:	growth associated protein
GAT1-4:	γ -aminobutyric acid transporter
GLT-1:	glutamate transporter
GluR1-7:	AMPA/KA glutamate receptor subunits
GLYT:	glycine transporter
HCO ₃ ⁻ :	hydrogen carbonate
HPLC:	high performance liquid chromatography
i.c.:	intracerebral

IEG:	immediate early gene
IP ₃ :	inositol triphosphate
IPSC:	inhibitory post synaptic current
IPSP:	inhibitory post synaptic potential
ITM:	intermediate term memory
K':	capacity factor
K ⁺ :	potassium ion
KA1 & KA2:	kainate receptor subunits
KCl:	potassium chloride
K _D :	equilibrium dissociation constant
LTD:	long term depression
LTM:	long term memory
LTP:	long term potentiation
MeA:	methylantranilate
Mg ²⁺ :	magnesium ion
mRNA:	messenger ribonucleic acid
mGluR:	metabotropic glutamate receptor
Na ⁺ :	sodium ion
NCAM:	neural cell adhesion molecule
NDGA:	nordihydroguaiaretic acid

nNMDA:	NMDA preferring NMDA receptor
NO:	nitric oxide
NOS:	nitric oxide synthase
NR1-NR2a-d:	NMDA receptor subunits
NSF:	N-ethylmaleimide sensitive factor
OH:	hydroxide ion
OPA:	o-phthalaldehyde
PC:	purkinje cell
PF:	parallel fibre
PITC:	phenylisothiocyanate
PKA:	protein kinase A
PKC:	protein kinase C
PLA ₂ :	phospholipase A2
PLC:	phospholipase C
PSP:	post-synaptic potentials
PTP:	post-tetanic potentiation
SAA:	sulphur containing amino acid
SCN:	thiocyanate ion
SNAP:	N-ethylmaleimide sensitive factor attachment protein

SNARE:	N-ethylmaleimide sensitive factor attachment protein
	receptor element
SSV:	electron-lucid synaptic vesicles
STM:	short term memory
TEA:	triethylamine
VGCC:	voltage gated Ca ²⁺ channel

Receptor agonists and antagonists

trans ACPD	trans: 1-aminocyclopentyl-1,3-dicarboxylic acid
AMPA:	α -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
2-CADO:	2-chloroadenosine
CGS 21680:	2-p-(2-carboxyethyl)-phenethylamino-5' ^N -ethylcarboxamido adenosine hydrochloride
CHA:	cyclohexyladenosine
7-CIK:	7-chlorokynurenate
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
CPA:	cyclopentyladenosine

CPP:	D-3-(2-carboxypiperazine-4-yl)1-propyl-1-phosphonic acid
CPT:	cyclopentyltheophylline
CSA:	cysteine sulphonic acid
D-AP5:	D-2-amino-5-phosphopentanoic acid
DCS:	D-cycloserine
DHP:	dihydropyridine
DNQX:	6,7-dinitroquinoxaline-2,3-dione
DPCPX:	8-cyclopentyl-1,3-dipropylxanthine
GABA:	γ -aminobutyric acid
GABA-T:	γ -aminobutyric acid transaminase
GAD:	glutamate decarboxylase
γ -LGLA:	γ -L-glutamyl-L-aspartate
HCA:	homocysteate
KA:	kainate
MCPG:	(RS)- α -methyl-4-carboxyphenylglycine
MK-801:	(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine maleate
NECA:	5'-N-ethylcarboxamidoadenosine
NMDA:	N-methyl-D-aspartate

PTX:	pertussis toxin
QA:	quisqualate
R-PIA:	N6-phenylisopropyladenosine
tADA:	trans-azetidine-2,4-dicarboxylic acid

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Abstract

The time course of release of amino acid transmitters was examined in slices of IMHV and LPO from day-old chicks that had completed a one-trial passive avoidance learning task. Amino acids released into an incubation medium were measured using PITC-derivatisation followed by an adaptation of a published method for HPLC analysis. One hour after training chicks to avoid a bead covered with the aversant methylanthranilate (MeA), there was an increase in the Ca^{2+} -dependent release of glutamate, aspartate and GABA from slices of left IMHV compared to chicks trained to peck a similar bead covered in water. Thirty minutes after training glutamate only was increased in MeA-chicks, but from both IMHVs. Glutamate was also increased at 3 and 6.5 hours in the right IMHV as was GABA (6.5 hours) and aspartate (3 hours). Both left and right LPOs of MeA-trained chicks showed increased GABA and glutamate release at 3, 6.5 and 24 hours. The left only showed enhanced release of both at 30 minutes. The release of the neuromodulator adenosine was found to be increased in the IMHV of MeA-trained chicks simultaneous to the increases in glutamate. The adenosine agonist CHA inhibited glutamate release: its actions blocked by the antagonist CPT. An agonist for the excitatory adenosine receptor increased glutamate, but decreased GABA, release according to concentration applied. Pre-training injections of adenosine agonists produced amnesia 30 minutes after training: these effects were lateralised such that injections into the left hemisphere only produced amnesia, those into the right did not. These results demonstrate for the first time increases in amino acid transmitters associated with passive avoidance training, and suggest a role for adenosine modulation of these transmitters.

Chapter 1: Amino Acids as Neurotransmitters

Amino acid neurotransmitters

Amino acids account for the majority of fast synaptic transmission in the vertebrate CNS. On the basis of neurophysiological studies, amino acids have been separated into two general classes: excitatory amino acids, including glutamate and perhaps aspartate and the sulphur containing amino acids, which depolarise neurons; and inhibitory amino acids (γ -aminobutyric acid [GABA], glycine) which act to hyperpolarise mammalian neurons.

Glutamate is the major excitatory neurotransmitter in the mammalian CNS, with estimates of over 50% of all CNS synapses using glutamate as a transmitter i.e. they are glutamatergic (Nicholls, 1994). A variety of well-defined pathways in the mammalian brain have been identified as using glutamate as a neurotransmitter: the lateral olfactory tract (Yamamoto and Matsui, 1976), the parallel fibres of the cerebellum (Sandoval and Cotman, 1978), the perforant path, the mossy and commissural fibres as well as the CA3 pyramidal cell axons in the hippocampus (e.g. Nadler et al., 1976), some corticostriatal fibres (Reubi and Cuenod, 1979) and the cochlear nerve (Canzek and Reubi, 1980).

GABA is the main inhibitory transmitter in the higher brain areas, being present primarily in interneurons. It is thought that 25-45% of all nerve terminals are GABAergic judged by the occurrence of a specific re-uptake

pathway (Nicholls, 1994). Interneuronal locations for GABA have been described in the visual cortex (Ribak, 1978), in the basket cells lying close to the pyramidal and granule cell bodies and other interneurons in the hippocampus (Storm-Mathiesen, 1977), and in both the hypothalamus and olfactory bulb (Fagg and Foster, 1983). In addition, GABAergic pathways are found in the basal ganglia and cerebellum (Fagg and Foster, 1983).

Glycine is the main inhibitory transmitter in the brain stem and spinal cord of mammals, where it is released by segmental interneurons (including the Renshaw cell-motor neuron synapse) and propriospinal fibres (Fagg and Foster, 1983). Glycinergic transmission is important for the control of most motor and sensory functions. In addition, it was suggested that glycine may also mediate transmission in the cortex, cerebellum, striatum and in the hypothalamus (Fagg and Foster, 1983), but these data should be treated with caution due to the glycine modulatory site on the NMDA receptor (see later).

Another potential excitatory amino acid is aspartate, although its physiological role in the intact nervous system is in doubt. Evidence for a transmitter role for aspartate includes that it is a potent agonist at NMDA receptors (though not at non-NMDA receptors) (Verdoorn and Dingeldine, 1988). In a study by Dickie et al. (1992) NMDA-induced aspartate release was found to be tetrodotoxin-sensitive, suggesting a neuronal site of release from cerebellar slices. Endogenous and labelled aspartate has been found to be released following electrical stimulation (e.g. Corradetti et al., 1983), with this release being reduced when Ca^{2+} is at low levels. Also, aspartate has been demonstrated as being present in synaptic vesicles (Villanueva et al., 1990). However, the presence of aspartate was postulated to be due to contamination due to a lack of both an ATP-dependent uptake and of a temperature sensitive efflux (Villanueva et al., 1990). Interpretation of the release studies also suffer from the fact that release is induced by pre-synaptic release of an endogenous

excitatory neurotransmitter, which will increase post-synaptic Na^+ and will therefore increase the efflux of post-synaptic cytoplasmic aspartate (Orrego and Villanueva, 1993). In addition, other data suggest that aspartate is not released from synaptosomes and that there is no evidence for vesicular uptake (Klanchnik et al., 1992).

All the studies suggest, that at best, the role of aspartate as a neurotransmitter in the brain is uncertain. Perhaps, as has been suggested by Klanchnik et al. (1992), non-vesicular release of aspartate may occur as part of an exchange process when glutamate is taken up, which may account for the stimulation-induced increase in the levels of aspartate, but not of glutamate.

A number of sulphur-containing amino acids have also been suggested as potential neurotransmitters. Homocysteate (HCA) has been localised in the glia of the cerebellar cortex of rats (Grandes et al., 1991), perhaps suggesting a non-neurotransmitter role for the substance, whilst the biosynthetic and metabolic enzymes for cysteine sulphinic acid (CSA) have been identified in nerve endings (Klanchnik et al., 1992). The different patterns of release found in that study (Klanchnik, et al., 1992), CSA levels being increased during electrical stimulation and the levels of HCA increased post stimulation, also suggest a differential distribution of the two amino acids. The release of HCA may be mediated by a glial receptor or, as discussed in the case of aspartate, by an exchange mechanism. The uptake of HCA and other sulphur-containing amino acids (SAAs) is thought to share the plasma membrane transport system with L-glutamate and L-aspartate (Grieve et al., 1992). Also, SAAs are active at metabotropic glutamate receptors where they are more potent than L-glutamate (Porter and Roberts, 1993).

Pathways of amino acid synthesis and degradation

Aspartate and glutamate are synthesised from glucose and other precursors in the Krebs cycle. This cycle takes place in mitochondria where the appropriate enzymes are present. Transaminase action on oxaloacetate or α -ketoglutarate produces glutamate and aspartate respectively. In addition, glutamate may be synthesised by reduction of 2-oxoglutarate by glutamate dehydrogenase and deamination of glutamine by glutaminase (Nicholls, 1994). Glutamine is synthesised by glutamine synthetase from glutamate. This would allow a recycling of released glutamate following uptake into either the glia or presynaptic nerve terminals.

GABA is formed by the α -decarboxylation of glutamate as catalysed by glutamate decarboxylase (GAD). The cofactor for this reaction is confined to the terminal cytoplasm, which accounts for the high concentrations of GABA in these terminals as opposed to the dendrites and cell bodies (Nicholls, 1994). GABA is degraded by GABA- α -oxoglutarate transaminase (GABA-T), which yields succinic semialdehyde (which is returned to the Krebs cycle as succinic acid via oxidation by succinate semialdehyde dehydrogenase) and glutamate. GABA-T is contained in the mitochondria of all neuronal regions, but appears to be concentrated in the terminals of GABAergic neurons and glia and dendrites of neurons that are in the vicinity of GABA synapses (Roberts and Sherman, 1993). GABA-T and GAD are equi-active (in that both possess the same percentage of maximal potential activity) at pH 7.5. Acidification of the intracellular space produces an increase in GAD activity and thus an increase in the amount of GABA, whilst alkalinisation increases GABA-T activity and a decrease in GABA available for release (Roberts and Sherman, 1993). Indeed, the increased release of GABA that was found during inhibition of GABA-T activity indicates that the coupling between uptake and GABA-T, not uptake

alone, is important for removing extrasynaptic GABA (Bakkellund et al., 1993). Glycine synthesis requires no specialised metabolic pathways, and is synthesised from serine.

Amino Acid Release

“Regulated exocytosis is the process whereby vesicles fuse with the plasma membrane, following depolarisation of the neuron and a subsequent influx of calcium through voltage sensitive calcium channels, and release their contents into the synaptic cleft.” (Nicholls, 1994). Two types of synaptic signalling can be separated: point-to-point and rapid (milliseconds) transmission of chemicals that are ionotropic (their activity involves the gating of an ion channel); and longer term, tonic release of the transmitter over a period of seconds to minutes which tends to have a metabotropic action involving second messenger systems (Sihra and Nicholls, 1993).

Synaptic vesicles

Amino acids such as GABA and glutamate are stored in small electron-lucid synaptic vesicles (SSVs), as determined by immunocytochemistry (Ottersen, 1989), and have been shown to be transported into these SSVs *in vitro* (Fykse and Fonnum, 1988). The release of transmitters via SSVs takes place exclusively at the active zone, a specialised area of the presynaptic membrane which is in precise register with the receptor-enriched area of the postsynaptic membrane. Distinct pools of SSVs are involved in transmitter release (Pieribone et al., 1995). A proximal pool, without the protein synapsin, appears to be docked to the cytoplasmic side of the active zone, whilst a distal pool, containing the important neuronal protein synapsin, appears to be a reserve pool.

Calcium and transmitter release

Exocytosis of SSVs is dependent upon the Ca^{2+} influx resulting from

the depolarisation of the neuron. Ultrastructural evidence from freeze-fracture studies indicates that Ca^{2+} channels are clustered at the active zones (Robitaille et al., 1990). It has been calculated that the sub-synaptic Ca^{2+} concentrations achieved on initial channel activation may be as high as $100\mu\text{M}$ (Smith and Augustine, 1988). This high concentration in the vicinity of the calcium channel means that the affinity of the release may be low with respect to Ca^{2+} . This would be appropriate for a phasic release event, since following activation and release, the Ca^{2+} channel will close, and owing to the low affinity binding, the Ca^{2+} will rapidly dissociate from the site ensuring rapid termination of release and also a rapid mechanism to reset itself for the next action potential (Nicholls, 1994).

Ca^{2+} channels may be categorised into several types: the N-type (high threshold, rapidly inactivating, ω -conotoxin sensitive); L-type (high threshold, inactivating, dihydropyridine (DHP) sensitive); T-type (low threshold, rapidly inactivating); and P-type (high threshold, inactivating, funnel web spider toxin sensitive) (Sihra and Nichols, 1993). Ca^{2+} influx into brain synaptosomes is biphasic with a transient, fast component and a slow, sustained component (Sihra and Nichols, 1993). The latter, non-inactivating phase of Ca^{2+} entry is the mechanism by which amino acids such as GABA and glutamate are released (Verhage et al., 1991). Indeed, a non-inactivating Ca^{2+} -channel is thought to be responsible for glutamate release from mossy fibre terminals (Nicholls, 1993). The channels responsible for the slow component have not been found to be DHP- or ω -conotoxin-sensitive and therefore appear to be neither N or L-type channels, and it is not a T-channel as it requires depolarisation to beyond -30mV for activation; although it may be that there is a subtype of L-type channel which binds DHP without a complete block of the Ca^{2+} channel (Sihra and Nichols, 1993). A fourth channel, the P-channel, has been described that is inhibited by Aga-GI, a toxin that inhibits the sustained phase of KCl-evoked Ca entry and totally blocks the evoked release of glutamate (Pocock and Nicholls, 1992).

The *in vitro* preparation

Brain slices

It is pertinent, at this point, to appreciate the limitations of neurochemical preparations, such as the brain slices used in the work presented in this thesis, for monitoring amino acid release. If a direct postsynaptic response is not measured electrophysiologically, it is not possible to determine the release from a single terminal in response to an action potential. Instead, the preparation must be depolarised by one of the methods discussed below and the total release detected over a time interval. As the depolarisation is generally prolonged, compared to normal physiological processes, there may be a depletion in available transmitter to be released, and a requirement for replenishment from a reserve pool of vesicles. This would produce a biphasic release of the transmitter that must be distinguished from that due to inactivation of presynaptic Ca^{2+} -channels.

Brain slices have been widely used to study the biochemistry of the CNS. Incubated slices have been shown to retain many vital functions: they respond to electrical stimulation and to chemical stimulation (Jones & McIlwain, 1971); they respire at a high rate for several hours even in grossly non-physiological media and at low temperatures (Orrego, 1979). They have adequate levels of ATP for amino acid transport and maintain their high potassium and low sodium ion concentrations (Orrego, 1979). Important advantages of brain slices over *in vivo* methods of studying CNS are the absence of a blood-brain barrier, the lack of extracerebral metabolism and the fine control of the extracellular environment. The latter is particularly pertinent in the present study, allowing the possibility of uncovering the form of transmitter release, e.g. calcium dependency etc.

Three methods of ionic depolarisation are recognised. These are KCl elevation, Na⁺-channel activation and K⁺-channel inhibition.

Depolarisation by elevated KCl

KCl depolarises the plasma membrane by "clamping" the potassium-equilibrium potential to a depolarised level, and decreasing the potassium concentration gradient which causes a rapid influx of calcium, producing a rapid, transient rise in internal calcium that correlates with the release of different transmitters (Adam-Vizi, 1992). The method does have some severe limitations however. It has been shown that a high [K⁺] can also cause the release of glial amino acids and the release of inulin and sucrose from brain slices (Orrego, 1979). Also, as KCl produces just a single depolarisation it will allow transient channels to fire only once before inactivating, even though the membrane remains depolarised. It is therefore difficult to observe processes which rely upon the modulation of such channels, since their effect on the clamped membrane will not be apparent.

4-Amino pyridine

The aminopyridines induce transmitter release by what is termed chemical potentiation. Freeze-fracture studies on frog motor neurons showed an increase in the number of exocytotic sites on the presynaptic membrane at the moment of impulse-evoked transmitter releases as a consequence of 4-aminopyridine (4-AP) treatment (Heuser et al., 1979). It is thought that 4-AP works by lengthening the action potential by blocking potassium channels (Nicholls, 1994). It is possible to induce the firing of spontaneous repetitive action potentials by inhibiting the potassium A-channel (the "fast" potassium channel). This channel normally operates to stabilise the plasma membrane potential by opening rapidly following subthreshold depolarisations and by increasing the duration of the after hyperpolarisation(AHP). 4-AP inhibits this channel so that the membrane is destabilised, and the terminals fire

spontaneous, tetrodotoxin-sensitive action potentials (indicating repetitive firing of sodium channels). In comparison to an elevated potassium level, 4-aminopyridine may expose the membrane to conditions more closely mimicking repetitive stimulation *in vivo*:

- a. Repetitive activation of transient calcium channels seems to occur;
- b. The time course of membrane potential changes is more physiological;
- c. The brief depolarising spikes avoid the calcium-independent efflux of cytoplasmic amino acid pools as seen during potassium or veratridine depolarisation.

Veratridine

Veratridine depolarises membranes that possess voltage-sensitive sodium channels (therefore not glial cells). This is probably via the inhibition of the inactivation mechanism of these channels, thus allowing a large amount of sodium to enter the axon down its concentration gradient, either spontaneously or following stimulation (Adam-Vizi, 1992). This depolarisation in turn leads to the opening of voltage-sensitive calcium channels and, therefore, to calcium influx. Together there is an enhanced release of transmitters, due to the elevated intracellular calcium, and also of soluble substances that are actively transported by concentration of sodium. The types of release may be distinguished according to their calcium dependency. However this agent has the disadvantage of promoting a massive release of cytoplasmic amino acids by reversal of the sodium-coupled plasma membrane carrier (Nicholls, 1989).

Amino acid release from tissue

With the previous sections in mind, it has been found that endogenous amino acids have been recorded as being released in a calcium-dependent manner from brain slice and synaptosomal preparations by several different depolarising methods including electrical-field stimulation, high potassium concentration and veratridine (Fagg & Foster, 1983). Examples of amino acid synaptic release after specific stimulation of nerve pathways have also been carried out *in vitro*. Exogenously added D-aspartate or L-glutamate is released after stimulation of the Schaffer collaterals and of the commissural pathway (Skrede and Malthe-Sorensen, 1981), in hippocampal slice preparations, or from the lateral septum in slice preparations (Fagg and Foster, 1983). Endogenous aspartate and glutamate are also released by stimulation of the lateral olfactory tract, both directly and by the activation of pyramidal cells in the olfactory cortical slice (Fagg & Foster, 1983).

Despite the evidence described above demonstrating the release of amino acids from CNS preparations, there is still a lack of consensus as to whether they are released exocytotically or directly from the cytoplasm, or whether by both mechanisms.

Calcium-dependent release of amino acids

The release of amino acids by KCl depolarisation is biphasic (Nicholls, 1994). Detection by fluorometric assay has shown that there is a release component of glutamate that is complete within 2-5 seconds from the initial stimulation, of approximately 20% of total release (Nicholls et al., 1987). After this, prolonged depolarisation results in continued Ca^{2+} -dependent release of glutamate until no more can be evoked after 3-5 minutes. In addition it has been shown that Ca^{2+} entry is also biphasic, although it has been noted that, as it is the slow, non-inactivating component of Ca^{2+} entry that is linked to glutamate release, the biphasic entry of Ca^{2+} does not explain the kinetics of

the release (Nicholls, 1994). From this, it is suggested that the biphasic effect may be related to the dual localisation of synaptic vesicles, those that are proximal to the presynaptic membrane and those that are distal (Pieribone et al., 1995). In most preparations there appears to be a time, 3 to 5 minutes, after which there is little or no release (Nicholls, 1989). This time point may well represent the time at which the recycling of synaptic vesicles, known to lag behind the exocytotic release of the vesicle package, is such that there is a depletion of total vesicle number (Ryan and Smith, 1995). The concentration of the elevated KCl has a profound effect on the release of amino acids with a steep increase found from 10mM K⁺ to a plateau at 30mM K⁺ (Verhage et al., 1989).

Whether the release of amino acids is vesicular or from a cytoplasmic pool can be determined by showing that the pool which is released in a Ca²⁺-dependent manner originates from a noncytoplasmic compartment and is released without equilibrating with the cytoplasm. The exchange of labelled amino acid into the vesicle is slower than across the plasma membrane, therefore, if the Ca²⁺-dependent release is of low specific-activity amino acid, then it can be assumed that exocytotic vesicular release is the mechanism. Sihra and Nicholls (1987) exposed synaptosomes to labelled GABA and evoked release with KCl. The released GABA had a lower level of label as compared to the cytoplasm, thus the data was consistent with the hypothesis that the Ca²⁺-dependent release was from the cytoplasmic compartment of the synaptosome. The Ca²⁺-dependent pool of glutamate released from guinea pig cerebral cortical synaptosomes following 60 seconds of depolarisation was only 50% equilibrated after 15 minutes compared to the cytoplasmic glutamate which equilibrated within 3 minutes (Wilkinson and Nicholls, 1988).

Calcium-independent release of amino acids

There is substantial evidence that amino acids are released in a calcium-independent manner from physiological preparations (reviewed by

Adam-Vizi, 1992). They appear to be released in this way due to their transporters and uptake carriers coupling the influx of the amino acid with Na^+ ions. Thus, following prolonged stimulation by one of the release mechanisms described (KCl, veratridine etc.) there will be a reduction in the Na^+ -electrochemical potential causing an efflux of the transmitter. Indeed, the K^+ -evoked Ca^{2+} -independent release of GABA was found to be abolished in Na^+ -free medium suggesting a role for the GABA transporter (Haycock et al., 1978). In addition, aspartate, which is thought not to be present in the lumen of synaptic vesicles (see above), accompanies the release of glutamate following depolarisation with KCl (McMahon et al., 1990). The exocytosis of the amino acid is energy dependent, and any factor that lowers the ATP level decreases the KCl-evoked release of transmitter until the plasma membrane carrier reverses (Nicholls, 1994). The reversal may lead to an accumulation of the amino acid in the extracellular space, and in the case of glutamate, to excitotoxic effects.

The data suggests that the efflux, of Ca^{2+} -independent GABA and glutamate, comes from cytoplasmic sources (Adam-Vizi, 1992). The work by Haycock et al. (1978) demonstrated that the independent release of GABA came from accumulated GABA (assumed to be cytoplasmic in origin), whilst the Ca^{2+} -dependent release was found to be both accumulated and endogenous GABA. GABA release was found to continue in a preparation that had already been depleted of its Ca^{2+} -dependent stores (Szerb, 1979). Also, glutamate is observed to be released from two separate pools: the labelled, accumulated glutamate could be released without external Ca^{2+} whilst the endogenous glutamate required external Ca^{2+} (Nicholls and Sihra, 1986).

The physiological relevance of calcium-independent release is not established. Adam Vizi (1992) suggests that a role may be that, "in the absence of axonal firing, a continuous release of transmitters in a Ca^{2+} -independent way could be important in maintaining the sensitivity and proper trophic

function of the postsynaptic region."

Amino Acid Receptors

Excitatory Amino Acid (EAA) Receptors

Glutamate receptors can be divided into two groups: the ionotropic glutamate receptors which are coupled to ion channels and mediate fast responses (AMPA, NMDA and kainate receptors); and a second group linked via G-proteins to a second messenger system and which are responsible for slower effects (the metabotropic glutamate receptors).

Non-NMDA glutamate receptors

The non-NMDA receptors comprise two distinct groups of receptors; those that are identified as being specifically activated by the agonist AMPA and those that have kainate as the agonist, although there is a certain amount of overlap in their binding. Non-NMDA receptors mediate fast excitatory transmission. L-glutamate is thought to be the main endogenous agonist for the receptor ($EC_{50}=11\mu M$) as L-aspartate does not produce an inward current at a concentration up to 10mM (Curras and Dingeldine, 1992).

The AMPA receptors are the most abundant class of excitatory receptor in the vertebrate brain. The kinetics of activation suggest that they mediate fast excitatory neurotransmission via a fast desensitising component and a steady state component, and are permeable to Na^+ and K^+ but have only a low permeability to Ca^{2+} ions (Seeburg, 1993). The desensitisation process, which determines for how long the receptor is active, is much faster in AMPA receptors than for NMDA receptors (Mayer et al., 1991).

The kainate receptor is present in a few specific areas of the vertebrate CNS and tends to be more apparent later in development (Monaghan and Anderson, 1991). As long as glutamate is present the kainate receptor is not inactivated: kainate is associated with excitotoxicity, and dihydrokainate also

inhibits the re-uptake of glutamate into the presynapse (Nicholls, 1994).

NMDA glutamate receptors

The NMDA receptor is a receptor complex coupled to ion channels which show a voltage-dependent blockade by magnesium (Watkins, 1989). In order for the magnesium block to be lifted, the membrane must first be depolarised, allowing the activation of the NMDA receptor complex and further depolarisation. The NMDA receptor activates slowly, and is active for several hundred milliseconds in the presence of an agonist at the synapse. The endogenous agonist is probably L-glutamate ($EC_{50}=2.2\mu M$) although other amino acids may have some effect: HCA ($EC_{50}=13\mu M$); L-aspartate ($EC_{50}=13\mu M$); although quinolinate has an EC_{50} value of over 7200mM (Curras and Dingeldine, 1992). NMDA receptors mediate a low amplitude, slow activating and slow decaying current (Mayer et al., 1992). In addition to being able to conduct Na^+ and K^+ ions, the NMDA receptor is also permeable to Ca^{2+} ions (Ascher and Johnson, 1989).

The NMDA receptor complex also contains a number of regulatory binding sites in addition to the Mg^{2+} block. These include a site that binds zinc ions, another for polyamines such as spermine, and others for glycine (Fletcher et al., 1989). The activation of the glycine site potentiates the responses to NMDA via allosteric actions with the NMDA receptor site. Both glycine and L-glutamate are thought to have two binding sites on the NMDA receptor (Clements and Westbrook, 1991). All the regulatory sites allosterically potentiate the binding of the others and thus act synergistically to increase the NMDA receptor response following activation (Mayer et al., 1992). A σ -opiate site has also been discovered: this is thought to bind neuropeptide Y *in vivo* (Roman et al., 1991).

Metabotropic glutamate receptors (mGluRs)

The metabotropic glutamate receptors (mGluRs) comprise a group of at least seven sub-types along with alternatively spliced variants that have been cloned, sequenced and expressed in oocytes where their pharmacological properties have been identified (Tanabe et al., 1993).

Different classes of mGluRs have been described according to the second messenger system employed. mGluRs1a, b and mGluR5 are linked to a phospholipase, PLC, whilst mGluRs2, 3, 4, 6, and 7 are negatively linked to an adenylate cyclase.

mGluR coupling to phosphoinositide hydrolysis

mGluR1 (α and β) and mGluR5 were found to activate phosphoinositide hydrolysis and Ca^{2+} mobilisation after stimulation with glutamate, ibotenate and quisqualate (Abe et al., 1992). This activation was shown to involve G-protein coupling (Sugiyama et al., 1987), and thus it could be assumed that mGluRs are linked directly to PLC via a G-protein (Anwyl, 1991).

Presynaptic mGluRs exist. The application of quisqualate to rat cerebral cortex synaptosomes mobilises intracellular calcium stores (Adamson et al., 1990). Also, quisqualate and 1S,3R-ACPD increase the calcium-dependent release of glutamate from synaptosomes (Herrero et al., 1992a). The release requires the presence of arachidonic acid (Herrero et al., 1992b): the arachidonic acid is probably produced via the activation of postsynaptic ionotropic receptors and serves as a retrograde messenger (Nicholls, 1992).

mGluR coupling to cyclic AMP formation

mGluRs2, 3, 4, 6 and 7 are all negatively linked to adenylate cyclase and inhibit the accumulation of cAMP (Tanabe et al., 1992). In the hippocampus of the rat, a mGluR has been found that is negatively linked to adenylate cyclase

via a G-protein (Desai et al., 1992). The forskolin-stimulated formation of cAMP is inhibited by t-ACPD, and is picrotoxin- (PTX) sensitive (Manzoni et al., 1992).

Presynaptic mGluRs may be present on glutamatergic neurons and may thus inhibit further glutamate release (Schoepp, 1994). L-AP3, a mGluR receptor antagonist inhibits the the mGluR-mediated release of glutamate from synaptosomes (Herrero et al., 1992a and 1992b). In addition these receptor subtypes may also inhibit the release of transmitters at inhibitory synapses: Hayashi et al. (1993) found that application of the selective mGluR2 agonist DCG-IV reduced the GABA-mediated inhibitory postsynaptic currents. mGluR7 is also expressed presynaptically and is negatively linked to adenylate cyclase (Saugstad et al., 1993). mGluR6 is restricted to the retina, and is activated by L-AP4 to reduce adenylate cyclase activity (Nakajima et al., 1993).

GluR1a has been found to enhance cAMP accumulation as well as activate an adenylate cyclase (Aramori and Nakanishi, 1992). Adenosine deaminase abolished 1S,3R-ACPD-stimulated cAMP accumulation in rat hippocampal slices whilst adenosine uptake blockers enhanced the response, suggesting that cAMP stimulation by endogenous levels of adenosine is potentiated by mGluR activation (Winder and Conn, 1993). The adenosine receptor that mediates the cAMP increase is the A₂ receptor (Cartmell et al., 1993).

mGluR coupling to other second messenger systems

Glaum and Miller (1993) found that 1S,3R-ACPD increases AMPA-induced currents and suppresses GABA_A currents via the activation of guanylate cyclase. This action may be mediated by nitric oxide, as 1S,3R-ACPD increases cGMP levels through a NO-dependent mechanism in rat cerebellar slices (Okada, 1992).

Inhibitory Amino Acid Receptors

GABA Receptors

GABA is the main inhibitory transmitter in the vertebrate brain, with between 20 and 50% of synapses being GABAergic (Young and Chu, 1990). The majority of these neurons are thought to be involved in local circuitry as interneurons, although groups of projection neurons are known (Paredes and Agmo, 1992). There are at least two, perhaps three, distinct subtypes of GABA receptor (Stephenson, 1988). The most studied of these is the GABA_A receptor of the vertebrate CNS (Olsen and Vantor, 1986).

The GABA_A receptor

The GABA_A receptor is present both pre- and postsynaptically, although it is predominantly postsynaptic (Paredes and Agmo, 1992). It is ionotropic and conducts Cl⁻ when an agonist is bound. Activation of the GABA_A receptor channel moves the membrane potential towards the Cl⁻ equilibrium potential, and produces inhibition of neural activity as it antagonises any excitatory effect due to hyperpolarisation of the membrane or a reduction in membrane resistance (Bormann, 1988). GABA_A receptors mediate fast IPSPs .

The GABA_A receptor has binding sites for benzodiazepines (BDZ), barbiturates, steroids and GABA (Nicholls, 1994). BDZs increase the probability of the Cl⁻ channel opening and hence increase the GABA_A current, but has no effect on the time of channel opening, whilst the barbiturates and steroids increase the length of time of channel opening without directly affecting the conductance (Study and Barker, 1981). Other specific agonists for the receptor include muscimol and THIP, whilst bicuculline reduces GABA-induced membrane currents by inhibiting Cl⁻ channel activation (Bormann, 1988). The level of intracellular Ca²⁺ reduces the sensitivity of the receptor

(Inoue et al., 1986). In addition, studies using SCN^- and Cl^- have demonstrated that the GABA_A receptor channel has at least two binding sites for anions and the rate of ion transport is limited by the binding of these anions (Bormann, 1988).

The GABA_A receptor can be phosphorylated as there are consensus sites for PKA, PKC or tyrosine kinases. Indeed, a basal level of phosphorylation appears necessary to maintain GABA_A receptor function (Chen et al., 1990). PKA-dependent phosphorylation can inhibit, potentiate or have no effect on the GABA_A current, depending on the subunit composition (Swope et al., 1992).

The GABA_B Receptor

The GABA_B receptor has yet to be cloned, but it is metabotropic and is located both pre- and postsynaptically. The GABA_B receptor does not bind THIP and is insensitive to bicuculline (Paredes and Agmo, 1992). The main agonist used to study the receptor is baclofen. Baclofen-induced hyperpolarisation is antagonised by phaclofen, although phaclofen has low affinity for GABA_B receptors (Paredes and Agmo, 1992). GABA_B receptors mediate the late IPSP.

GABA_B receptor activation reduces the duration of Ca^{2+} -dependent action potentials and affects neurotransmitter release by this mechanism. This suggests that there could be either a direct coupling of the receptor to a G-protein or that a second messenger system is involved such as cAMP or PKC (Paredes and Agmo, 1992). Incubation of cells containing GABA_B receptors with pertussis toxin abolishes the GABA-induced inhibition of Ca^{2+} currents (Holz et al., 1986). This G protein coupling is not linked to adenylate cyclase as intracellular application of GTP, but not cAMP, reduces the Ca^{2+} current (Dolphin and Scott, 1987). However, PLC is also implicated in the inhibition of Ca^{2+} channels as the effect of GABA is mimicked by a diacylglycerol

analogue which activates PKC (Rane and Dunlap, 1986), although GABA_B agonists have no effect on phosphatidylinositol turnover (Brown et al., 1984). GABA_B receptors are thought to activate phospholipase A₂ activity (Bormann, 1988), which in turn stimulates the production of arachidonic acid activity and this in turn activates PKC (Routenberg, 1988). Arachidonic acid has been described as a retrograde messenger, and therefore allows the possibility that it interacts with presynaptic PKC to stimulate GABA_B receptor activity and thus increase IPSPs.

In addition to their action on Ca²⁺ channels, GABA_B receptors also act to increase the K⁺ current in hippocampal pyramidal cells without affecting the conductance of Ca²⁺ (Borman, 1988). This suggests that there may be two spatially distinct GABA_B receptor types, the peripheral type affecting the conductance of Ca²⁺, and the CNS type that affects K⁺ conductance (Paredes and Agmo, 1992). The opening of K⁺ channels by GABA_B is inhibited by pertussis toxin, suggesting mediation via a G protein (Bormann, 1988). The channel itself may be an inwardly rectifying channel, as has been activated by serotonin (Gage, 1992). It has been suggested that GABA_B agonists may cause an increase in K⁺ conductance by activating PLA₂ and liberating arachidonic acid.

Amino acid uptake systems

The synaptic actions of amino acid transmitters are terminated by an uptake system from the synaptic cleft into the presynaptic cells. The exact subcellular sites of uptake appear to vary both with the amino acid and the CNS tissue preparation that is employed. For example, after injection into the CNS *in vivo*, or incubation with tissue slices or homogenate *in vitro*, the inhibitory amino acids glycine and GABA are accumulated mainly in neuronal tissues (synaptosomes and vesicles) with some labelling of glial cells, whilst glutamate and aspartate have major uptake sites in glial cells, both *in vivo* and in slices: these findings can be correlated with the compartmentalisation of these amino acids in brain tissue (Wheeler, 1987).

The selectivity of the transport system was demonstrated by Fyske and Fonnum (1990), who found that the regional distribution of vesicular GABA and glutamate uptake was related to the distribution of GABAergic and glutamatergic terminals respectively. A varying ratio of uptake was found indicating they are taken up into different vesicle preparations. More recently the transporters have been cloned, and it is apparent that the glutamate transporters belong to a family distinct from that comprising the biogenic amine, GABA, glycine, proline and taurine transporters, which all cotransport Na^+ , Cl^- and the amino acid (Nicholls, 1994). The glutamate transporter is thought to exchange 2Na^+ and a glutamate for the expulsion of K^+ and $\text{OH}^-/\text{HCO}_3^-$ (Bouvier et al., 1992). The stoichiometry of the system allows sub-micromolar, and therefore non-neurotoxic, levels of glutamate outside the neuron whilst the cytoplasm may contain millimolar concentrations. The prediction is that there would be a minimum of $0.6\mu\text{M}$ glutamate externally, high enough levels to activate presynaptic metabotropic receptors and thereby reduce further glutamate release (Bouvier et al., 1992). In addition they suggest that there may be some activation of postsynaptic NMDA receptors at this concentration.

Chapter 2: Synaptic Plasticity, Learning and Memory, with reference to the Domestic Chick *Gallus domesticus*

Introduction

Learning can be defined as "an experience dependent generation of enduring internal representations, and/or experience-dependent lasting modifications in such representations. These internal representations may be neuronally encoded structural versions of the world which could potentially guide behaviour, with brain states corresponding to behavioural states. Memory is the retention of such representations over time." (Dudai, 1989). Thus, learning is the process of acquiring information and memory is the persistence of learning in a state that can be accessed and used at a later time (Squire, 1987). It is now known that learning is accompanied by biochemical, physiological and ultimately morphological changes.

Kandel and Spencer (in Squire, 1987) propounded that "...analysis of the plastic properties of neurons is a prerequisite for the neurophysiological study of learning." Memory is thought to involve a persistent change in the relationship between neurons through biochemical events that may lead to structural modifications.

Plasticity within the nervous system

Neurons are not static. Morphological and functional changes occur during development. Changes may also occur, particularly at the level of the synapse, during the cell's mature life. Post-synaptic potentials (PSPs) increase and decrease in intensity according to recent pre-synaptic activity. At some synapses PSPs grow during repetitive stimulation to several times the size of a single event. When this growth occurs within a second, and decays equally rapidly, it is called facilitation. A more gradual rise of the PSP (in minutes) is called potentiation; its slow decay after stimulation is post-tetanic potentiation (PTP). Augmentation, i.e. enhanced synaptic transmission, may also occur. Other synapses may experience fatigue or depression, whereby sustained pre-synaptic activity results in a progressive decline of the PSP amplitude.

Most synapses display a mixture of these processes. During a train of action potentials, synaptic transmission may rise due to facilitation, in which successive spikes evoke PSPs of increasing amplitude. This facilitation is pre-synaptic in origin, reflecting increasing number of transmitter quanta released per spike. A synaptic depression will tend to overwhelm the facilitation caused by a reduction in the number of transmitter quanta released due to a depletion of the releaseable neurotransmitter store (Zucker, 1973).

If the depression is not too severe, augmentation and potentiation leads to a partial recovery of transmission during the tetanus. Such potentiations are thought to be caused by intracellular accumulation of calcium and sodium. Finally PTP decays, and PSPs return to the same amplitude as that elicited by an isolated pre-synaptic spike.

Learning-related Synaptic Plasticity

In these ways neurons may change their integrative, communicative and, hence, computational and representational properties with time. These changes will initially depend on neurotransmitter release, modulation of ion channels, availability or activity of enzymes and regulatory proteins, alteration of availability or sensitivity of receptors and modification of cellular components.

Experience-dependent synaptic modifications were proposed as being a possible mechanism for learning and memory by Tanzi (1893). In his textbook of mental diseases he talks of the formation of mnemonic impressions:-

"...molecular vibrations become more intense and diffuse themselves, momentarily altering the form of the dendrites, and thus, if the conditions are favourable, new expansions and collaterals originate and become permanent",
also

"..neurons associated in the action ultimately become unified in a stable functional solidarity" and the result "...is attained by a progressive process of functional hypertrophy, which leads to more or less permanent increase of the neurodendrons that connect the nervous elements..".

The conditions of these synaptic modifications were suggested by Hebb (1949) in a statement known as Hebb's postulate of learning:

"When an axon of cell A is near enough to excite cell B, or repeatedly, or consistently takes part in firing it, some growth process or metabolic change takes place in one or both cells, such that A's efficiency, as one of the cells firing B, is increased."

A Hebbian synapse would, therefore, be one in which the post-synaptic neuron receives two synapses, one of which is capable of causing the post-

synaptic cell to fire whilst the other is initially too weak to do so . If however, weak inputs occur in close temporal proximity with those from the strong synapse, they will be strengthened so as to be capable of causing the post-synaptic cell to fire in their own right. The Hebbian mechanism described is therefore essentially heterosynaptic, with learning occurring as a postsynaptic phenomenon.

The Hebbian formulation appears to translate the known rules of associative learning into a cellular model, as the two inputs could be regarded as representing the unconditioned and conditioning stimulus, and the changed response represented by the conditioned response.

Long-term potentiation

One neurophysiological phenomenon which can display the properties of a Hebbian synapse is long-term potentiation (LTP). LTP is a use-dependent and persistent increase in synaptic strength that can be induced by brief trains of high-frequency stimulation. LTP was first described in the rabbit hippocampus (Bliss and Lomo, 1973) and was subsequently demonstrated to occur in numerous excitatory synapses of the central (including the cortex of mammals) and peripheral nervous systems of vertebrates and invertebrates (Bliss and Collingridge, 1993).

NMDA receptor-dependent LTP

LTP demonstrates input specificity in that it is limited to the synapses that have been activated, and other synapses that are not active at the time of the tetanus do not share in the potentiation (see Bliss and Collingridge, 1993).

It is an associative process in that a weak input can be potentiated if it is active at the same time as is a strong tetanus to a separate but convergent input. This associativity provides an analogue of classical conditioning and is an implicit property of the Hebb synapse (see Bliss and Collingridge, 1993).

The LTP demonstrates cooperativity, or a threshold, that results from the presence of a voltage-dependent block on the channels responsible for the control of Ca^{2+} influx. The threshold for inducing LTP is a complex function of the intensity and pattern of tetanic stimulation; between 'weak' trains which produce only PTP and 'strong' trains that induce LTP, lies an intermediate range of activation which engages short-term potentiation (STP) (see Bliss and Collingridge, 1993).

LTP is thought to consist of two phases: that of its induction, and the maintenance of the potentiation (also known as expression) (Bliss and Collingridge, 1993).

LTP is induced by the repeated stimulation of afferents leading to the synapse under study (Teyler and Grover, 1992). The stimulation may be via a tetanic stimulation of the afferents, by altering the chemical environment of the synapse or by behavioural techniques involving the training of the animal on certain tasks.

In pyramidal cells of the rat hippocampus, stimulation of the input pathways generates both excitatory post-synaptic potentials (EPSPs) and inhibitory post-synaptic potentials (IPSPs) in the target cells due to the existence of inhibitory interneurons (Massicote and Baudry, 1989). The IPSPs generally truncate EPSPs and prevent their temporal summation during the course of bursts (3-4 pulses) of high-frequency (100-400 Hz) repeated at the "theta" frequency (5-7 Hz) : the major hippocampal EEG pattern when the rat is engaged in exploring a novel environment (Eichenbaum et al., 1987).

The IPSPs are produced by GABA. It has been shown that GABA_A receptor-mediated IPSP_A prevents significant activation of the NMDA receptor system during low frequency transmission (Dingeldine et al., 1986). If IPSP_A is blocked by bicuculline, NMDA receptors contribute a component to the EPSP. The importance of the hyperpolarisation produced by the IPSP_A has been

demonstrated in two ways: activation of an NMDA-receptor mediated synaptic component can be demonstrated in the presence of intact synaptic inhibition if the cell is strongly depolarised by current injection or if the hyperpolarisation is limited by voltage-clamp techniques (Collingridge et al., 1988). Therefore a major role of IPSP_A is to prevent activation of the NMDA receptor system during low frequency transmission.

The IPSPs recorded from the CA1 region of the hippocampus exhibit a fast IPSP from the GABA_A receptor component and a slow IPSP governed by the activation of GABA_B receptors. The fast IPSP usually prevents the temporal summation of EPSPs following a brief burst of stimulation. The fast IPSPs are, however, reduced some 200 msec after the initial stimulation to the same inputs (Larson et al., 1986). This is due to the presence of GABA_B autoreceptors which inhibit the release of GABA from the interneurons (Davies et al., 1991). The inhibition is thought to "prime" the target cell so that a second stimulation of the same, or indeed a different, input into the cell results in EPSP summation if a burst occurs 200 milliseconds later (Larson and Lynch, 1986). Therefore GABA-mediated responses can modulate activity of the NMDA receptor channel complex, and this is a potentially important mechanism for the regulation of neuronal plasticity.

LTP induction requires Ca²⁺ influx into the postsynaptic cell. Perfusing slices with a reduced Ca²⁺ medium results in non-sustainable LTP (Dunwiddie and Lynch, 1979). Intracellular injections of calcium chelators such as EGTA prevents induction of LTP in CA1 pyramidal cells (Lynch et al., 1983); this effect suggests a postsynaptic site for the action of Ca²⁺, in addition to a presynaptic site. As the ion channel that forms part of the NMDA receptor has a significant permeability to Ca²⁺, it is thought that Ca²⁺ entering via this route has a role in the induction of LTP. Indeed, the tetanic stimulation that elevated Ca²⁺ levels, as found by Muller and Connor (1991), was largely dependent on NMDA receptors. The Ca²⁺ flow is regulated by a voltage-

sensitive Mg^{2+} block of the NMDA receptor-gated Ca^{2+} channel whose relief requires a sufficient level of postsynaptic depolarisation (Ascher and Nowak, 1988). Thus Ca^{2+} influx through the channel requires a coincident postsynaptic depolarisation and the activation of NMDA receptors. Both these events are produced by strong, high frequency stimulation of presynaptic fibres, which causes sufficient activation of non-NMDA receptor channels to depolarise the postsynaptic cell, removing the Mg^{2+} blockade of the NMDA receptor channels and allowing Ca^{2+} to enter the cell.

Ca^{2+} influx is thought to lead to the phosphorylation of proteins via a group of Ca^{2+} -dependent enzymes. Protein kinase C (PKC) has been suggested to be involved: injection of PKC induces an LTP-like potentiation in the hippocampal slice (Hu et al., 1987) as does the application of phorbol esters, compounds that activate PKC (Muller et al., 1988). In addition, inhibitors of PKC, such as H-7, also reversibly inhibit LTP (Malinow et al., 1988). A modulatory role for PKC may be via the phosphorylation of NMDA receptors (Massicotte and Baudry, 1991). There is evidence that the calcium-calmodulin-dependent protein kinase type II (CaM II kinase) is involved in LTP in the hippocampus. Inhibitors of the kinase inhibit LTP formation (Ito et al., 1991). The inhibitor KN-62 is effective when present during tetanus, but not if applied following the tetanus, and has no effect on LTP in the mossy-fibre pathway. These data suggest that the enzyme is activated by Ca^{2+} entering as a result of NMDA receptor activation (Bashir and Collingridge, 1992).

Maintenance

Once induced, LTP may be maintained depending on the amplitude of the postsynaptic depolarisation, which is related to the amount of calcium influx. The increase in the postsynaptic response generated at potentiated synapses could be due to (1) presynaptic modifications which result in an increase in the amount of glutamate released, (2) postsynaptic modifications involving a change in the number or sensitivity of receptors, (3) morphological modifications or (4) an extrasynaptic change, such as a reduction in the uptake of transmitter (Bliss and Collingridge, 1993).

Increased transmitter release

Initial evidence for the involvement of amino acids in LTP comes from the work of Dolphin et al. (1982). Their investigations involved perfusing the end zone of perforant path fibres in rat hippocampus in a stimulating environment. They were able to demonstrate an increase in newly synthesised (³H) glutamate that was associated with LTP. Subsequent work by Bliss et al. (1986) showed an increase in the concentration of both glutamate and aspartate following LTP, and their evidence also suggested that LTP may be associated with a decrease in the release of GABA. Lynch et al. (1985), however, did not find a significant release of aspartate after LTP. Davies et al. (1989) applied glutamate agonists ionophoretically to the postsynaptic cell and found no change in receptor responsiveness soon after the induction of LTP, but an increase during later stages of LTP, suggesting that maintenance is initially presynaptic, and only later postsynaptic. Despite the evidence expressed above there are uncertainties as regards the nature and localisation of the pool of the sampled glutamate. More recently, however, Errington et al. (1994) were able to demonstrate an increase in glutamate release following the induction of LTP *in vivo*, and this increase was associated with perforant path terminals and could be blocked by perfusion with AP-5. As well as the increased release into the extracellular compartment in intact animals, there was also an increase in the ability of slices and synaptosomes from the potentiated tissue to release glutamate in response to depolarisation: Richter-Levin et al. (1995) demonstrated a persistent increase in the concentration of glutamate in the dentate gyrus of a rat *in vitro*, that had LTP induced in this region *in vivo*.

Retrograde messengers

If the induction of LTP requires post-synaptic activation, and maintenance involves a pre-synaptic increase in transmitter release, some

message may be sent from the post-synaptic to the weak pre-synaptic cell that strengthens subsequent signals. This hypothetical signal has been termed a retrograde messenger. If this retrograde messenger diffuses widely, it is possible that facilitation would occur at synapses onto other, non-associated cells, as is found in *Aplysia* (Hawkins and Kandel, 1983). This result has been observed in the visual cortex (Boltz et al., 1990) where it is found that induction of LTP in any given cell in the CA1 region in the hippocampus (by a Hebbian mechanism) leads to the expression of LTP in neighbouring post-synaptic cells through a non-Hebbian step in which the post-synaptic cell does not fire, but in which others may be induced to do so.

Bliss et al. (1989) suggested that arachidonic acid or a related metabolite is released by the post-synaptic cell to serve this function. Increased Ca^{2+} concentrations stimulate the activity of phospholipase A_2 producing hydrolysis of phospholipids and the release of arachidonic acid. In turn arachidonic acid (ArA) was found to produce a slowly developing increase in EPSPs when applied exogenously in the hippocampus *in vitro* or *in vivo* (Bliss et al., 1990). The PLA_2 inhibitor nordihydroguaiaretic acid (NDGA) was found to prevent the formation of LTP (Williams and Bliss, 1989). The release of ArA was found to be elevated 3 hours after the induction of LTP and to be PLC dependent (Clements et al., 1991). It has been found that the mGluR receptors linked to a PLC metabolises PIP_2 into IP_3 and diacylglycerol (DAG). The latter can be split by a DAG lipase to release ArA.

More recently nitric oxide (NO) has been proposed as a retrograde messenger in both the dentate gyrus and CA1 pyramidal cells (Bredt and Snyder, 1992; Schuman and Madison, 1991). Inhibitors of nitric oxide synthase (NOS) such as L-nitro-arginine prevent LTP formation in hippocampal slices (Schuman and Madison, 1991). Moreover, sodium nitroprusside and hydroxylamine, both of which release NO, induce an LTP-like increase in EPSPs which occlude tetanus-induced LTP. Haemoglobin, a scavenger of free

NO, blocks the induction of LTP, implying that NO is released extracellularly. A major problem with the hypothesis of NO as a messenger is that NOS may not be present in hippocampal cells and an enzyme with similar properties, NADPH diaphorase, is only found in scattered neurons in the CA1 (Vincent and Kimura, 1992).

Postsynaptic modifications

The increases in the postsynaptic currents found with LTP may be explained by modifications to AMPA receptors. Quantitative autoradiography has shown that there is an increase in the binding of AMPA agonists following LTP induction in the perforant path (Tocco et al., 1992). LTP may involve changes in the ratio of expression of the 'flip' and 'flop' forms of the AMPA receptor or a change in the relative expression of the different subunits of the receptor (Bliss and Collingridge, 1993). In addition there are changes in the decay of the synaptic currents (related to the opening time of the receptor population; Magelby and Stevens, 1972) with the induction of LTP. Increasing the size of the responses by stimulating more synapses or enhancing release did not affect the decay, as would be expected for a variable dependent on the voltage-independent AMPA receptor ionophore (Baudry and Lynch, 1992).

Structural modifications

Morphological changes in spines have been found to occur in association with LTP in the dentate gyrus (Desmond and Levy, 1990). In the CA1 of the hippocampus, LTP was shown to be associated with an increase in the number of synapses on dendritic shafts and a modification of the balance of shaft and spine synapses (Chang and Greenough, 1984). Whether the increase in synapses results from the transformation of already existing spines or the formation of new contacts has not been proven, but the formation of new contacts could account for the differential effects of the potentiation on AMPA vs. NMDA-receptor mediated response components only if the additional synapses were deficient in NMDA receptors (Baudry and Lynch, 1992).

Non-NMDA receptor-dependent LTP

In contrast to the CA1 region, LTP in the mossy-fibre pathway to CA3 is non-Hebbian and not associative. When weak stimulation of the mossy-fibre pathway is associated with strong stimulation of another input into CA3, the mossy-fibre input does not undergo LTP (Brown et al., 1990). Moreover whereas LTP in the dentate gyrus and CA1 is restricted to the tetanised pathway, specific, strong stimulation of the mossy fibres can produce heterosynaptic LTP of other inputs to the CA3 (Brown et al., 1990). In this model calcium influx is through voltage-dependent calcium channels not via NMDA receptors (Gamble and Koch, 1987). Indeed, noradrenaline is thought to be the major neurotransmitter in this system, as β -receptor antagonists block LTP induction in the CA3 pathway (Johnston et al., 1988). In addition mossy-fibre LTP has been shown to be blocked by AP3 and induced by trans-ACPD.

The involvement of LTP in learning

Evidence implicating LTP in memory storage comes from studies on spatial and context memory, olfactory memory in rodents, trace conditioning in rabbits and passive avoidance learning in chicks (see later).

Barnes (1979) subjected rats to a spatial memory task. She implanted recording electrodes into the dentate gyrus of these animals and stimulated the perforant path. A correlation was found in these animals between the ability to retain experimentally induced LTP and memory for the task. Further evidence implicating LTP in hippocampal spatial memory comes from the work of Morris and his associates (Morris et al., 1986, 1990). Chronic intraventricular infusion of APV in the hippocampus or lateral ventricle impaired the memory for location of a hidden platform in a water maze. There was also substantial correlation between impairment in the task and impairment in the induction of LTP (Morris et al., 1990). AP-5 impaired

spatial learning in a dose-dependent manner correlated with its impairment of LTP *in vivo* and at doses that are equivalent to those that impair LTP *in vitro* (Davis et al., 1992). In contrast APV did not affect performance in a visual discrimination task (Morris et al., 1986).

Enhanced population spike amplitudes were recorded in rats trained to retrieve food in an operant conditioning situation compared to those animals that were not trained (Skelton et al., 1987). This effect was still detectable at least 10 days after the end of training. Sharp et al. (1985) measured hippocampal field potentials of rats with implanted electrodes into the perforant path and a recording electrode in the dentate gyrus, and transferred them from a rearing cage into a room with a variety of different boxes and ramps: a 'complex' environment. They found that LTP-like changes were found initially in this complex environment, which then subsided, but reappeared on transfer to another complex environment.

Data from Richter-Levin et al. (1995) showed that there was an increase in glutamate release in the dentate gyrus from intact animals that had undergone LTP. A similar increase in glutamate was observed in rats that had been trained on a variety of learning tasks including the Morris water maze and a classical conditioning task, suggesting that similar presynaptic mechanisms are involved in LTP and these learning tasks and that LTP itself may be a substrate for learning.

Two categories of LTP have been described: NMDA receptor-dependent LTP, which includes associative LTP, and NMDA receptor-independent LTP which includes mossy-fibre LTP and paired-pulse facilitation (Bliss and Collingridge, 1993).

Long-term depression

Like LTP, Long-term Depression (LTD) provides a means for regulating the strength of synaptic connections in the mammalian brain. In LTD this is via a prolonged inhibition of synaptic transmission. Associative LTD was first described in the cerebellum where Purkinje cells (the output of the cerebellar cortex) receive two major excitatory inputs: one from up to 80,000 parallel fibres, and the other from a single climbing fibre (CF). Synaptic transmission at both inputs occurs via glutamate receptors. Upon repetitive stimulation of both inputs, Ito et al. (1982) found a long-lived associative depression of synaptic transmission between the parallel fibres (PFs) and Purkinje cells (PCs). Stimulation of either CFs or PFs alone does not produce LTD. LTD has been described in the neocortex and in the hippocampus (Artola and Singer, 1994). Both a heterosynaptic LTD, in which there is a depression of nonstimulated input systems, and homosynaptic LTD, where there is a use-dependent, long-lasting depression affecting the activated synapses themselves, have also been described (Artola and Singer, 1994).

Induction of LTD requires a critical level of postsynaptic depolarisation. LTD induction can be blocked by the coactivation of GABAergic neurons (Artola et al., 1992) and by directly hyperpolarising the postsynaptic cell (Artola et al., 1990). The concomitant activity of the CFs required for the induction of LTD in PFs can be substituted by the direct depolarisation of PCs (Crepel and Krupa, 1988). These data suggest that CF activation is primarily required to obtain a sufficiently strong depolarisation of PCs during the interval at which PFs are active (Artola and Singer, 1994).

Evidence suggests that the PFs release glutamate as the major transmitter, and as transmission is blocked by CNQX (Hirano, 1990c) it is thought that the site of action is the AMPA receptor (Ito, 1994). As it has been shown that pairing glutamate application with CF activation leads to a strong

and lasting decrease of the sensitivity to the applied amino acid (Ito et al., 1982), it was suggested that LTD is due to a sustained desensitisation of AMPA facilitated under the influence of CF synapses (Ito, 1994). Ito (1989) suggests that it is the combined activation of the AMPA receptor channel with the elevation of Ca^{2+} (due to the activation of the ACPD-sensitive mGluR) that is responsible for long term desensitisation. Because activation of mGluRs may lead to an increase in IP₃ and therefore release Ca^{2+} from internal stores, there may be a direct increase in intracellular Ca^{2+} concentration, or the reduction in K^+ conductances may contribute to the depolarisation and increase the activation of voltage-gated Ca^{2+} conductances (see Chapter 1). NMDA receptor involvement may not be necessary for LTD, since LTD can be induced in the presence of AP5 (Artola et al., 1990). However, more recent work by Gean and Lin (1993) showed that the pairing of a low frequency synaptic stimulation with postsynaptic depolarisation induced an LTD of the NMDA receptor EPSP and this LTD is blocked by APV. From this they suggested that LTD required an increase in postsynaptic Ca^{2+} at least in part contributed to by the synaptic activation of NMDA receptors during concomitant postsynaptic depolarisation. The expression of the NMDA-EPSP LTD is at a higher threshold than that for the AMPA EPSP. In addition, Dudek and Bear (1992) also found that NMDA receptor antagonists prevented LTD in the hippocampus CA1 region, and proposed that the depression was triggered by prolonged NMDA receptor activation below the threshold that induces synaptic potentiation. In contrast to this was the result that in slices of visual cortex the same tetanic stimulus which induces LTP will produce LTD when the NMDA receptors are blocked (Artola et al., 1990).

The rise in internal Ca^{2+} may activate a nitric oxide synthetase, as blockade of NO synthesis by L-NMMA prevents LTD induction (Ito and Karachot, 1990). Sodium nitroprusside induces LTD when combined with PF activation (Shibuki and Okada, 1991). Despite this evidence for the involvement of NO in LTD it has been found that LTD can still be induced by

combined application of AMPA and depolarisation, even in the presence of L-NMMA (Linden and Connor, 1992), and there is no evidence to support the presence of a NO synthase in CFs or in PFs (Ito, 1994).

From the above, it can be suggested that it is the amplitude of the surge in Ca^{2+} that differentiates between LTP and LTD: LTD requiring less Ca^{2+} than LTP. The mechanism for this may be due to the Ca^{2+} -dependent enzymes having differing affinities for Ca^{2+} (Lisman, 1989). Low levels of Ca^{2+} will lead to the activation of phosphatases whilst higher levels activate kinases. Alternatively, the mechanism may be due to the site of the Ca^{2+} increase: with NMDA receptor-gated increase at greatest effect in the immediate vicinity of postsynaptic receptors; close to the endoplasmic reticulum with mGluR activation; and at the membrane when voltage-dependent Ca^{2+} channels are activated (Artola and Singer, 1994).

Amino acids and learning

Direct evidence that excitatory amino acids are involved in learning and subsequent memory formation have mainly come from pharmacological applications of specific competitive antagonists for their receptor types, before, during and after a learning task: i.e. such that the learning and/or recall of the task is impaired.

As has already been described, Morris (1989) carried out experiments in which D-AP5 causes a dose-related impairment of both spatial learning and hippocampal LTP *in vivo*, and an impairment in the storage of new information without effect upon the recall of previously established memories; the effects of D-AP5 are not due to a sensorimotor disturbance. Also Riedel et al. (1994) showed that pretraining application of D-AP5 impairs the acquisition and retention of a Y-maze task employing spatial alternation learning via a footshock reinforcement. Walker and Gold (1991) have shown that the NMDA antagonist NPC 12626 (which is small enough to diffuse across

the blood-brain barrier) inhibits LTP and impairs learning and recall of an inhibitory avoidance task in mice. Melan et al. (1991) have shown that an active avoidance task for mice is impaired by the NMDA antagonist γ -LGLA, and Ungerer et al. (1991) found that it also blocked a post-training improvement in the performance of rats in a Y-shaped-maze avoidance learning task. MK-801 (dizocilpine), an antagonist of NMDA, significantly impaired the acquisition of a delayed alternation task in a T-maze (Hauber, 1993). From the data described there does seem to be some discrepancy as to whether the recall of the learning task is affected by the administration of NMDA antagonists, with the type of learning task affecting the outcome: the recall of spatial tasks is not affected, but the recall in avoidance and alternation tasks is affected by NMDA antagonists.

The glycine site of the NMDA receptor has also been implicated in learning tasks in mammals. Milacemide, a glycine agonist, has been found to enhance the performance of rodents in avoidance tasks, in the Morris water maze, and also improve the performance of humans in word retrieval tasks (Finkelstein et al., 1994).

Metabotropic glutamate receptors (mGluRs) have also been found to be involved in learning and memory. The effect of tADA (trans-azetidine-2,4-dicarboxylic acid), a mGluR agonist, applied intracerebroventricularly pretraining in rats was to cause amnesia for the spatial learning task 24 hours later (Riedel et al., 1995). Mice that have the gene encoding the mGluR1 receptor deleted show an impaired conditioned eyeblink response and associative LTD (Aiba et al., 1994a) and also show an impairment in an associative learning task and LTP (Aiba et al., 1994b). MCPG disrupted the performance of rats in this task (Richter-Levin et al., 1994), and also impaired retention in a Y-maze task (Riedel et al., 1994).

Drugs that attenuate the autodesensitisation of AMPA receptors are found to increase the excitatory synaptic strength and also to improve the

performance of rats in both the water maze task and in passive avoidance tasks (Zivkovic et al., 1995). The AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzoquinoline impairs the performance of rats in the water maze; this impairment is attenuated by the agonist 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4 benzothiadiazine S,S dioxide (IDRA 21) when administered orally.

In addition, the inhibitory neurotransmitter, GABA, has been found to produce time and dose-dependent effects on the retention performance of rats and mice in appetitive and aversely motivated tasks (Castellano et al., 1989).

Benzodiazepines induce anterograde (*sic*) amnesia via effects mediated through the GABA_A complex by enhancing GABA-induced synaptic inhibition. The memory impairing effects of benzodiazepines may preferentially involve the basolateral nucleus of the amygdala. Silva and Tomaz (1995) carried out pre-training injections of diazepam into the central and basolateral nuclei of rats. When trained on an inhibitory avoidance stepdown task 15 minutes post injection, diazepam induced anterograde amnesia in those rats that had the drug administered to the basolateral, but not the central, amygdaloid nucleus.

The full allosteric GABA_A modulator triazolam produced dose-related decreases in the acquisition and performance of conditional discriminations in monkeys (Auta et al., 1995). Triazolam also eliminated the retention of the discrimination task. However, when combined with other partial allosteric GABA_A agonists there is a partial attenuation of the effects of triazolam when administered alone, suggesting that the disruptive effects of benzodiazepines on learning and memory may be a function of the effect of these compounds at different GABA_A receptor subtypes.

Muscimol was found to induce state-dependent learning in the Morris water maze, with an increased latency to reach the submerged platform when

it was present and with less time spent in the area that had previously had the platform when it had been removed (Nakagawa et al., 1995).

Picrotoxin and bicuculline enhance retention for passive avoidance (Brioni and McGaugh, 1988) and active avoidance (Bovet et al., 1966) tasks and also in spatial learning (Brioni and McGaugh, 1988). Breen and McGaugh injected picrotoxin intraperitoneally, post-training. This was found to enhance rats' learning of an appetively-motivated multiple T-maze task (Breen and McGaugh, 1961).

Diazepam was also found to impair the retention of spatial information in rats trained on the Morris maze (Brioni and Arolfo, 1992). There was no effect of diazepam on retrieval or cue learning.

The effects of stimulation of GABA_B receptors on learning suggests that agonists may interfere with memory consolidation as post-training baclofen disrupts the retention of the passive avoidance task (Swartzwelder et al., 1987).

From the data described it appears that GABA antagonists enhance retention in different learning tasks with GABA agonists inhibiting the retention.

Adenosine

The purine adenosine is a key modulator of neuronal activity in the central nervous system (for a review see Stone, 1989). It acts via presynaptic mechanisms (either by a reduction of calcium influx or the decrease or suppression of intracellular calcium availability) to inhibit transmitter release, and post-synaptically to hyperpolarise neurons; this is achieved by the activation of potassium conductances or by enhancing chloride conductances (Akhondzadeh and Stone, 1994).

The receptors mediating these effects were initially classified into A₁ and A₂, with the A₁ receptor decreasing cyclic AMP (cAMP) production and the A₂ receptor increasing levels of this second messenger. Because receptor-mediated effects can occur independently of changes in cAMP levels, the current receptor classification is now based on the relative orders of potency of several selective ligands (Kirk and Richardson, 1995). These criteria, together with the advances in molecular cloning, have led to the currently accepted classification of adenosine receptors into A₁, A_{2a}, A_{2b}, and A₃ subtypes (Fredholm et al., 1994). All adenosine receptor subtypes have seven hydrophobic regions: a characteristic of members of the G-protein coupled receptor superfamily (Meng et al., 1994).

The release of adenosine and other purines from both peripheral and central nervous tissue is well established (reviewed in Snyder, 1985). ATP may be released initially and then converted to adenosine, or the conversion may have taken place just prior to release. Since extracellular ATP is rapidly degraded to 5'-AMP and then to adenosine by the enzyme ecto-5'-nucleotidase it was considered that much of the extracellular adenosine was derived from the hydrolysis of released ATP (McIlwain, 1972). However, work by Lloyd et al. (1993) showed that 70-85% of released adenosine is formed intracellularly prior to its release.

Adenosine inhibits the release of acetylcholine (Spignoli et al., 1984), noradrenaline and dopamine (Ebstein and Daly, 1982) from the mammalian brain. In addition the effect of adenosine and its analogues on the release of endogenous amino acids has also been investigated in mammalian brain: adenosine (300 μ M) was found to inhibit the evoked potentials and the release of aspartate and glutamate from the CA1 region of the hippocampus following stimulation of the stratum radiatum, as did the receptor antagonists 1-phenylisopropyladenosine (PIA) and cyclohexyladenosine (CHA: both at 1 μ M) (Corradetti et al., 1984). Dolphin and Archer (1983) demonstrated an inhibition of potassium-induced glutamate (but not GABA) release from slices of dentate gyrus of the rat following superfusion with the non-selective agonist 2-chloroadenosine (2-CADO). The effect of an A₂ agonist was to increase glutamate and aspartate release from ischaemic rat cerebral cortex (O'Regan et al., 1992). The A₁ antagonist 8-phenyltheophylline (5 μ M) increased the release of excitatory amino acids, when applied during low-frequency stimulation, and antagonised the CHA-induced inhibition of the release of these amino acids (Corradetti et al., 1985). At low (1 μ M) concentrations of adenosine, EPSCs are facilitated whilst higher concentrations inhibit the EPSC in the rat hippocampus (Garaschuk et al., 1992). Low concentrations of adenosine produce an increase in the amplitude of the EPSP due to an increase in glutamate release (Okada et al., 1992). Quantal analysis showed that application of CHA (A₁ agonist) reduced both the EPSP and *m*, the quantal content, suggesting a pre-synaptic effect of adenosine on amino acid release (Lupica et al., 1992). Yamamoto et al. (1993) described a suppression of the EPSP in the CA3 region. In this way adenosine may contribute to the extent of EPSP summation via actions on the release of amino acid transmitters. Activation of excitatory, A₂, adenosine receptors is required in order to enhance excitatory amino acid release directly. Alternatively, A₁ (inhibitory) receptor activation may serve to inhibit the release of GABA from inhibitory interneurons.

Differential effects of adenosine A_1 receptor activation have also been found to occur according to whether mGluR activation is present. Thus, A_1 receptor activation may enhance IP_3 formation induced by mGluR activation and also enhance Ca^{2+} mobilisation from internal stores (Ogata et al., 1994). However, in the absence of mGluR activation, A_1 receptor activation may lead to hyperpolarisation, depressing the depolarisation-induced increases in Ca^{2+} . It has been demonstrated in the rat hippocampus that there is a synergistic interaction between arachidonic acid and mGluRs, such that PKC activation and inositol phospholipid metabolism were enhanced when both agents were present (McGahon and Lynch, 1994). This suggests possible mechanisms for adenosine interactions with mGluRs, as both presynaptic receptors, mGluR subtype and A_{2b} receptor, can enhance inositol phospholipid metabolism, adenylate cyclase activity and PKC activity. This would suggest a potential excitatory or inhibitory feedback, as A_{2b} receptor activation has been demonstrated to enhance glutamate release (Simpson et al., 1992), which could interact with presynaptic mGluRs to enhance or diminish further glutamate release.

Adenosine also inhibits GABA release from slices of cerebral cortex (Hollins and Stone, 1980) and hippocampus (O'Regan et al., 1992) via adenosine A_1 receptor agonists. Higher concentrations of these agonists, which would also activate low affinity A_{2b} receptors, did not affect GABA release. In addition, application of the specific A_2 receptor agonist CGS21680 inhibited release of GABA. Therefore, adenosine acting at A_1 and high-affinity A_{2a} receptors appears to block GABA release, whilst co-activation of A_{2b} (low-affinity) and A_1 receptors results in the loss of the inhibitory effect of adenosine. These findings were substantiated by Kirk and Richardson (1994) who described A_{2a} receptors present on GABAergic striatal nerve terminals that acted to inhibit the release of GABA. The mechanism of this inhibition was examined by Akhondzadeh and Stone (1994). They described how

adenosine was able to potentiate the ability of muscimol (GABA_A agonist) to inhibit evoked potentials. The chloride channel blocker DIDS was able to reduce the inhibitory action of both muscimol and adenosine. This suggests that the inhibitory action of adenosine was mediated by enhancing chloride fluxes and that this can lead to a potentiation of responses to GABA_A receptor activation. There is a potentiation of the effects of adenosine with muscimol suggesting that two distinct chloride channels are activated. Kirk and Richardson (1995) found that the inhibition of GABA release by adenosine is mediated by A_{2a} inhibition of PKC activity.

The actions of adenosine are very dependent on the location of the receptors, and the receptor types: e.g. it has been demonstrated by Cunha et al. (1994) that ACh release in the hippocampus is differentially regulated, such that in the CA1 area only A₁ receptors modulate the release, in CA3 both A₁ and A_{2a} receptors modulate the ACh release, but in the dentate gyrus both receptor types are present but are not activated by endogenous adenosine.

Adenosine has been implicated as having a role in long term potentiation (LTP). Dolphin (1983) found that a high intensity, high frequency train of electrical stimulation, when applied to the perforant path of the hippocampus, did not produce LTP when applied in the presence of the adenosine agonist, 2-chloroadenosine (2-CADO). The EPSPs produced by the stimulation were reduced during 2-CADO perfusion. Specific activation of A₁ receptors attenuated LTP (DeMendonça and Ribeiro, 1990) whilst A₁ antagonists augment LTP (Hitchcock et al., 1992). The inhibitory effect of adenosine was indirect, via a decreased transmitter release at individual excitatory synapses (Asztely et al. 1994; and Mitchell et al., 1993) or in unison with GABA by a change in chloride conductance (Akhondzadeh and Stone, 1994).

The involvement of adenosine in memory formation has been demonstrated using pharmacological manipulations following training on a

behavioural task e.g. Normile and Barraco (1991) described a dose-dependent impairment of memory for the retention of an inhibitory avoidance task following pre-training injections of N⁶-cyclopentyladenosine (CPA), a selective A₁ agonist. This impairment could be blocked by the selective A₁ receptor antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine). The acquisition of a passive avoidance task by mice was inhibited by CHA and R-PIA (N⁶-phenylisopropyladenosine), both A₁ agonists (Zarrindast and Shafaghi, 1994). Low doses of A₁ antagonists blocked the CHA and R-PIA-mediated amnesia.

Avian learning

The following sections give a brief outline of models and paradigms of avian learning, and their associated learning and memory-related changes in synaptic plasticity in the avian brain.

Two paradigms, food storing and song learning, are presented briefly, followed by two, more in depth, accounts of models employing the chick as the experimental animal.

Song learning

Ontogenesis of song

Song learning is a form of behaviour that is established and refined by learning in sensitive periods (see imprinting section). In general, song learning is restricted to one or two periods during the first year of life (Slater, 1983). Species such as the zebra finch *Taeniopygia guttata* and chaffinch *Fringilla coelebs* are "critical period" or "age-limited" learners: they develop an initial sub-song, consisting of a rambling series of notes given at low volume, then discrete passages of song resembling the adult, the plastic song, and finally full or mature song is "crystallised" by the following spring (Nottebohm, 1991). In a few other species (e.g. the canary *Serinus canaria* and the nightingale *Luscinia megarhynchos*) the male continues to produce new songs into adulthood, and these species have been termed "open-ended" or "age-independent" learners. The song is used to communicate: to females the song may signal the species, the location and fitness of a potential mate; to a male it may describe the owner of the song as a neighbour or a stranger (Konishi, 1985).

Neural substrates for song learning

The song system of birds consists of several nuclei including the high vocal centre (HVC) and the robust nucleus of the archistriatum (RA: present in the archistriatum). The RA sends afferents to a nucleus (nXII ts) that innervates the vocal organ, the syrinx. The RA itself receives inputs from the HVC and the magnocellular nucleus of the anterior neostriatum (MAN) (Konishi, 1985). Intracellular recording has shown that neurons in HVC and RA receive an auditory input, and the auditory neurons of HVC send their axons to another nucleus, area X (Katz and Gurney, 1981). Lesions of HVC or RA produce a deterioration of adult song (Nottebohm et al., 1976). The HVC, RA and nXIIts are necessary for the acquisition and expression of learned song (Nottebohm, 1991) whilst area X and MAN are required for song learning but not the production of the song (Nottebohm, 1991). Sex differences occur, such that the male zebra finch and canary have larger (in volume) nuclei involved in song learning, although the sex differences could be eliminated following injection of testosterone (Nottebohm, 1991). The volumes of the nuclei of the males undergo seasonal changes in size which relate to the level of testosterone, the size of the testis and the extent of the song repertoire (Nottebohm et al., 1981). During development song nuclei grow rapidly and then regress through cell death and loss of connections. It is these processes that produce the sexual differences, as regression and cell death is much greater in females (Konishi and Akutagawa, 1985).

Wallhauser-Franke et al. (1995) showed that comparing social, song-experienced male zebra finches with those that had been isolated showed that they had 41% fewer spines in the lateral MAN. This loss in connectivity is also demonstrated in auditory imprinting (see below). The concept is explained as an elimination of connections inappropriate to the memory. Wallhauser-Franke et al. (1995) suggest the following mechanism for song acquisition: "proliferating spines of the ... lMAN ... may provide a substrate

for encoding the range of song learning shown by the species. Many sounds will not activate this network, providing the physical basis for the constraints on song learning.....any appropriate song stimulus will activate only a subset of these connections. Spines that are activated would become stabilised and would induce mechanisms that eliminate unstabilised spines." In addition, these 'unwanted' connections may provide a method whereby the correct connection is always made; if certain connections fail there will be others to replace them.

In the canary, a species in the age-independent group, there is continued neurogenesis in the HVc and RA nuclei (Bulley et al., 1990). These neurons are generated, migrate, and become incorporated into existing circuits of the adult brain (Goldman and Nottebohm, 1983). As there is no change in the size of the HVc from year to year, it is suggested that some RA-projecting neurons are discarded and replaced by new ones, thus indicating neuroplasticity in a major motor pathway in birds (Bulley et al., 1990).

Food storing

Some birds, including the corvids (crows) and the parids (chickadees and titmice) store food in scattered hoards and recover the cache hours, days or even months later. This may be an important part of their foraging behaviours, especially in species faced with long, hard winters. The retrieval of the food caches involves the retention of memories for the site where the food is stored (Shettleworth, 1991).

Research into the neural basis of food storing behaviours has focused on the hippocampal formation. This region is thought to be a homologue of the mammalian hippocampus by some authors (Shettleworth., 1991). Lesions to the hippocampus in birds results in an inability to retrieve their caches above chance levels (Sherry and Vaccarino, 1989). Post-storing lesions in this area also produced a deficit in the birds' ability to find the store (Clayton and

Krebs, 1994). Also, there is an impairment of both spatial and working memory following lesioning (Sherry and Vaccarino, 1989). Further evidence that suggests a role for the hippocampus in food storing comes from data that show the hippocampus of food-storing birds has a relatively greater volume (compared to the rest of the telencephalon) as compared to their non-storing congeners (Krebs, 1990). The hippocampal volume has been shown to vary seasonally, with the maximum relative volume found during October in Black-capped chickadees: the month at which food storing activity is at its peak (Smulders et al., 1995).

In addition to the involvement of the hippocampus, Szekely et al. (1992) found that there was an increased immunoreactivity to the immediate early gene *fos*, in the areas of the intermediate medial hyperstriatum ventrale (IMHV), archistriatum and lobus parolfactorius (LPO) in the marsh tit, following food storing.

The domestic chick, *Gallus domesticus*, as a model system for learning and memory-related synaptic plasticity

The day-old chick provides an excellent model for memory formation with important advantages over other vertebrate models and paradigms. The precocial chick has a well developed central nervous system with a well-organised behavioural repertoire of predictable behaviours. In contrast, the mammalian CNS is developmentally much less advanced until several weeks after birth. The chick's blood-brain barrier is, however, not well developed until weeks after hatching, allowing the rapid diffusion of injected agents into the brain. Furthermore, the chick's skull is unossified which facilitates intracerebral injections into specific brain areas and electrophysiological recordings from these areas. Another advantage of using the day-old chick is that a single-trial learning event leads to demonstrable changes, "perhaps due to the lack of masking effects by the neural correlates of previous experiences which create neural 'noise'". (Lowndes, 1992).

The chick does have some disadvantages as a model for memory formation, however. In comparison to the mammalian brain, chick neuroanatomy is not as well understood, although work in progress is rapidly making this a less valid argument against the chick as an experimental subject. Perhaps more importantly, the chick, as a precocial animal, will be learning a great number of different facts about its environment, and very quickly. The chick's visual system shows some asymmetry until some days after hatching, which appears to be related to the different exposures of the two eyes to light whilst the chick is still in the egg (Rogers, 1991). Also, the chick is a rapidly developing organism, where all the learning-induced changes in brain biochemistry and neuroanatomy (see below) are being super-imposed "upon a highly dynamic and plastic situation." (Rose, 1991). However, the use of suitable controls, both for the behaviour and the biochemistry, may allow direct comparisons between groups to demonstrate changes that are specific to the task, and the learning and memory related plasticity associated with the task.

The chick brain

The two regions of the chick brain that are examined for changes in transmitter release in this study are the intermediate medial hyperstriatum ventrale (IMHV) and the lobus parolfactorius (LPO).

The IMHV is regarded as being homologous to the mammalian striate cortex and receives inputs from different sensory systems (Rose, 1991). Bradley et al. (1985) described the afferent inputs into the IMHV using retrograde tracers. The results showed that there is a complete range of information coming into the IMHV, such that visual, auditory and somatosensory systems have afferents to this region. The auditory connections include that from a region in the neostriatum (Field L, the auditory receiving area) which receives a projection from the thalamic

auditory nucleus. Also somatosensory and visual inputs from the neostriatum have been shown. The IMHV also receives afferents from the hippocampus and the archistriatum.

The efferent connections from the IMHV include possible visual inputs into the archistriatum, which in turn have projections to the hippocampus. This may exist in a circuit: the IMHV projects to the archistriatum posterior, which projects to the medial part of the hippocampus, which in turn projects to the nucleus septalis lateralis, which projects to the IMHV.

The LPO, together with the palaeostriatum augmentatum (PA), palaeostriatum primitivum (PP) and nucleus accumbens comprise the palaeostriatal complex of the chick. They are thought to be equivalent to mammalian striatum and are involved in the integration of motor responses, and may also play a role in the processing of information involved in taste and smell (Rehkamper and Zilles, 1991).

There is no evidence for a direct connection between the IMHV and the LPO. However, two routes may link them. These are the pathways to the archistriatum and to the PA. The archistriatum also projects to the LPO in the chick (Davies et al., 1991). More recent evidence suggests that the ventral archistriatum might act as the bridge between the LPO and IMHV, as both the LPO (Szeleky et al., 1994) and the IMHV (Csillag et al., 1994) project to here.

Chick learning paradigms

Two main paradigms are presently used to follow learning and memory in the chick: these are passive avoidance learning and imprinting. These two paradigms exploit 'innate' behavioural responses shown by chicks shortly after hatching: pecking at small objects or approaching imprinting objects. These tasks, and the biochemical consequences of learning these tasks, are described below.

Imprinting

Imprinting is a learning process through which the subsequent social preferences of animals become restricted to a particular stimulus (Bolhuis and Horn, 1992). The biological function of imprinting is probably to enable the animal to recognise close kin. A distinction is made between filial and sexual imprinting: filial imprinting is involved in enabling the young animal to form an attachment to, and a preference for, the parent and other members of its own species; whilst sexual imprinting is involved in the formation of mating preferences enabling the animal to mate with an individual that is neither too closely nor too distantly related (Bateson, 1979).

Imprinting of a stimulus is most readily obtained if learning occurs during a 'sensitive period' i.e. in a restricted time span of an individual's life. Filial imprinting occurs just before the stage when, for its own safety, the young animal needs to discriminate between its parents and other members of its own species. In precocious species, such as *Gallus domesticus*, this happens shortly after birth or hatching. Changes in preferences may however occur; since birds learn the characteristics of their siblings at an early age and the plumage of the siblings changes with age, it seems likely that they update the representation of the sibling (Bateson, 1979).

Sexual imprinting

Sexual preferences develop as a result of a long period of exposure to, and social interaction with, the parents as well as siblings. The amount of social interaction with the parents and the number of siblings that the young bird is reared with may affect later sexual preferences. In zebra finches (*Taeniopygia castanotis*) sexual imprinting occurs until at least 100 days post-hatch (Immelmann et al., 1991). A stage of acquisition, and a second, later, stage of stabilisation of the object may occur, the latter if the male has courted a female of the same species as its parents; and if the courtship female is

replaced by another suitable object a new preference may emerge, especially if a longer exposure to this new courtship object is obtained (Kruijt and Meeuwissen, 1991). The fact that the information learned in early development has to be verified in a sexual context suggests that sexual imprinting may be very similar to song learning, but less similar to filial imprinting (Immelmann et al., 1991).

Auditory imprinting

Auditory stimuli are thought to play an important role in the formation of filial imprinting; for example the sounds most effective in eliciting pursuit of a moving visual object are conspecific maternal calls (Gottlieb, 1971). In addition young birds learn the characteristics of auditory stimuli played to them shortly after hatching (Gottlieb, 1988); indeed several days before hatching a vocal contact between the chick in the egg and the parent may be established (Gottlieb, 1965). The paradigm consists of imprinting chicks on rhythmic tone bursts of usually either 1.8 kHz or 2.5 kHz, the rhythm corresponding to the maternal contact call or "iambus". The chicks are tested in a Y-maze with loudspeakers at both branches, one playing back the imprinting stimulus, the other a novel stimulus.

Braun (1980) and Maier and Scheich (1983) showed that Guinea fowl chicks imprinted on rhythmic auditory stimuli, and were able to recognise them and orient towards the sound source without visual cues. The sensitive period of auditory imprinting starts in embryo and ends during the 4th day post-hatch according to Maier and Scheich (1983). These authors were able to demonstrate differential 2-DG uptake in the chick forebrain with three regions showing increases: the HAD (hyperstriatum accessorium and dorsale), the LNH (lateral neostriatum and hyperstriatum) and the MNH (medial neostriatum and hyperstriatum ventrale). The HAD and LNH were later demonstrated to be connected to the visual aspects of behaviour while the MNH may serve auditory or vocal-motor functions (Scheich, 1987). There

may be an additional non-auditory input from the limbic system and from the reticular formation, perhaps activating the three areas together, as the labelling of auditory and visual areas are often concomitant. Subsequently it was suggested that a tonic (motivational) activation of MNH afferents from the dorsomedial thalamic nucleus may prevail after hatching, and, together with the first acceptable input arriving through the auditory MNH afferents, may lead to a critical level of activity to produce permanent changes in the MNH. Golgi analysis showed a 47% lower density of spines on a large neuron type (Type I MNH neuron) in auditory-imprinted chicks (Wallhäußer and Scheich, 1987): this reduction was correlated with a reduction of synapses, the imprinting process causing a selection amongst initial synaptic contacts leading to selective stabilisation of the inputs representing the imprinted stimulus until at least day 20 (Wallhäußer and Scheich, 1987). With two inputs to the MNH cooperativity may be induced. The main features of LTP are also found in the MNH: the EPSP and population spike amplitudes are potentiated after tetanus, the spike latency is reduced and a very large depolarisation of the membrane potential is found (Wang et al., 1994). This depolarisation and subsequent potentiation is NMDA receptor linked.

Visual imprinting

Visual imprinting has been extensively employed as a learning paradigm in precocial birds. In many of the studies the following procedures were used (see McCabe et al. 1982). After hatching, chicks are reared in individual compartments in a dark incubator until they are approx. 24 h old. The chicks are then placed, individually, in running wheels c. 50 cm from the imprinting stimulus, the whole apparatus being contained within a black box. The chicks are then exposed to the stimulus for between 1 h to 4 h. A chick's preference is then measured by either a sequential or a simultaneous choice test. The sequential test involves placing the chick in a running wheel and exposing it to the familiar stimulus and a novel stimulus in succession and in

a balanced order. The ratio of approach counts to the familiar object, to the total number of approach counts made in the test, provides a score for the chick's preference. In the simultaneous choice test the training and novel objects are presented at the same time and the chick expresses its choice by attempting to approach one of them (Bateson and Wainwright, 1972).

Training was found to be associated with an increase in the incorporation of radioactive lysine into protein, and of radioactive uracil into RNA, in the forebrain roof (Bateson et al., 1969, 1972). The uracil incorporation into RNA was found to be restricted to a part of the hyperstriatum ventrale, the IMHV (Horn et al., 1979). Bilateral lesioning of the IMHV before training prevented the acquisition of a preference and impaired the retention of an acquired preference (McCabe et al., 1981, 1982).

Electrical stimulation of the IMHV at a specific frequency leads to a subsequent preference for that frequency when it is delivered from an external light source (McCabe et al., 1979). Stimulation of visual areas linked to the IMHV did not produce a preference (Bradley et al., 1985).

Morphologically, imprinting was found to be associated with an increase in the mean length of the post-synaptic density (PSD) in synapses of the left IMHV. The effects were restricted to the axospinous synapses. These axospinous synapses are most commonly of the asymmetrical, excitatory form. An increase in glutamate binding was also found in trained chicks. McCabe and Horn (1988) described a significant increase (59%) in the NMDA-sensitive binding in the left, but not right, IMHV of imprinted chicks compared to controls. This increase in binding is thought to represent a real increase in the number of NMDA receptors. A positive correlation between a preference score for the object and NMDA binding in the left IMHV was found. The time course suggests that the magnitude of increase in NMDA binding is also affected by the length of time that elapses after training; significant differences were not found till 6-8.5 h post-training (Horn and McCabe, 1990, McCabe and

Horn, 1991). In right IMHV-lesioned chicks, injection of a NMDA-receptor blocker, AP-5, into the left IMHV significantly impaired imprinting (McCabe et al., 1992). A correlation has also been found between a behavioural measure of imprinting and the spontaneous mean firing rate of neurons in the left IMHV of chicks that were anaesthetised after training (Bradford and McCabe, 1992).

Acetylcholine has been implicated with an increase in cholinergic receptor binding in trained chicks (Bradley and Horn, 1981). The noradrenergic system is also implicated, with a depletion agent DSP4 impairing training onto a red box but not onto a stuffed junglefowl; this also suggests that the different stimuli require different neuronal mechanisms (Davies et al., 1985).

Brown and Horn (1990) studied changes in protein synthesis following imprinting; they found a decrease of a ca. 50 kDa protein and a concurrent increase in the synthesis of a 80 kDa protein. An increase in the phosphorylation of the protein kinase C substrate MARCKS has also been found.

The evidence suggests that the left IMHV serves as a long term store. The right IMHV appears to serve as a transfer store, slowly passing information out to another storage system, S'. If the right IMHV is destroyed immediately after training, then there is no evidence of storage in S', and retention subsequently depends crucially on the left IMHV (McCabe, 1991). In addition Johnston et al. (1992) described an increased affinity (but not an increase in number) of glutamate receptors in the left lobus parolfactorius (LPO); perhaps this is S'.

Passive-avoidance learning

A day-old chick will readily peck at small objects such as arthropods. If the object is noxious, the chick will learn not to peck at similar objects again and will, in this way, learn to distinguish food from other items such as their own faeces. This natural behaviour has been successfully transferred to the laboratory in the form of a one trial passive avoidance training task (Cherkin, 1969). Cherkin (1969) used this paradigm to demonstrate retrograde amnesia with flurothyl in order to measure a consolidation time for the task.

In the test, day-old chicks are placed in pens under controlled illumination. They are trained by being presented with a small chrome bead dipped either in water (W) or a bitter-tasting aversant methyl-anthranilate (MeA), which, when pecked and tasted, causes the chick to exhibit an apparent "disgust" response. Chicks pecking at the bead are tested at various times after training. On presenting chicks that had pecked at the bead covered with MeA with another, uncoated, chrome bead, in at least 75% of cases the chicks will avoid pecking. More than 80% of the chicks presented with the bead covered in water will subsequently peck at a dry bead (Rose, 1991).

The main advantage of passive avoidance learning over the other avian paradigms mentioned above, is that memory formation is initiated after a single event. Thus, the precision of timing that is possible with a one-trial event can be unequivocally timed and observed. Other models involving the formation of a long-term memory have to be examined following several training sessions, or a continual exposure to the training stimulus, in order that a memory can be formed (e.g. the imprinting studies described in the preceding section). With the bead training, events that are associated with the training experience itself can be more readily dissociated from the extensive biochemical processes occurring during memory formation phases when the training stimulus is no longer present (Rose, 1991). This is a powerful and

precise tool for investigating time-dependent processes that are involved in the different phases of memory formation (see below).

Disadvantages of the passive avoidance model do, however, also exist. The chicks, trained on the bead covered with MeA, that subsequently do not peck the dry chrome bead when presented at the testing stage, may not peck for a number of reasons that may not be associated with their ability to recall the task. For example, an emotional state of arousal or fear at the time of testing will interfere with the chick's innate pecking behaviours. However, the use of an adequate number of experimental animals and also the experimenter's alertness to the emotional state of the chick, or group of chicks, being tested will diminish or avoid this complication.

Stages of memory formation for the passive avoidance task

Using pharmacological agents that could disrupt memory formation, Gibbs and Ng (1977) proposed a three stage model for memory formation in the chick, for the passive avoidance task. Each of the three stages was found to be dependent on different neurochemical mechanisms. The early stage of memory formation (STM) lasts about 10 minutes and depends on hyperpolarisation due to potassium conductance: this stage can be disrupted by depolarising agents such as glutamate and LiCl. A second stage, intermediate term memory (ITM), lasts from about 10 to 30 minutes and can be disrupted by injection of sodium/potassium ATPase inhibitors such as ouabain. Long term memory (LTM) occurs after 30 minutes and requires the synthesis of proteins shortly after training. Each stage is considered to be sequential, but inhibition of the later stages does not interfere with the earlier ones.

Location of activity

Kossut and Rose (1984) demonstrated that the chick brain utilises the intermediate medial hyperstriatum ventrale (IMHV), the palaeostriatum augmentatum (PA) and the lobus parolfactorius (LPO) during the learning of the task, as shown by increased radioactive 2-deoxyglucose (2-DG) uptake 30 minutes following training. 2-DG is taken up by metabolically active cells along with the natural substrate, glucose. 2-DG cannot be metabolised by glycolysis and is therefore accumulated by active cells. The addition of a radiolabel (^{14}C) to the 2-DG makes it possible for autoradiography to identify areas of accumulated 2-DG. These results are taken as indicators of specific brain regions that are metabolically active during learning.

Further studies investigating asymmetries between the hemispheres have shown the left hemisphere, specifically the left IMHV, to be more active than the right, following injections of 2-DG 10 minutes, but not 30 minutes, after training (Rose and Csillag, 1985), and to be more sensitive to amnesic agents (Serrano et al., 1992).

The glucose energy is required for a series of biochemical, physiological and morphological events that follow the task, many of which have been described (see below).

Lesion studies

Studies of the behaviour of chicks following the lesioning of specific regions of the brain have proved illuminating. Pre-training bilateral lesions of the IMHV produced amnesia for the task when the chicks were tested 3 hours, or more, later (Davies et al., 1988). The chicks still showed the normal behaviour associated with pecking the bead including shaking of the head etc. (i.e. the disgust response). Lesioning the right IMHV before training did not produce amnesia (Patterson et al., 1990). These results were interpreted to

mean that the left IMHV is required for the acquisition of the task. To examine whether the left IMHV was required for the retention of the task, lesions were carried out, 1 hour or later after training, of both IMHVs. When subsequently tested 1 or 6 hours later, the chicks no longer showed amnesia for the task (Patterson et al., 1990). From this it could be concluded that after the acquisition of the task, by the left IMHV, the IMHV is no longer necessary in the retention of the memory. In this way, the memory, or memories, must be either relocated or further distributed to other sites for storage (perhaps the S' described in the imprinting paradigm: see above).

As noted in the section on imprinting, the storage site, region S', may well be the LPO. Bilateral lesions of the LPO pre-training did not result in amnesia for the task, however lesioning the LPOs 1 hour after training did produce amnesia in chicks when tested 24 hours afterwards (Gilbert et al., 1991). These results suggested that long term memory for the task is dependent on the LPO. Only bilateral lesions proved effective in eliminating recall of the task; this might mean that either LPO is sufficient for the recall of memory, and that the redistribution of the memory is to both LPOs (Gilbert et al., 1991). Pre-training LPO lesions, followed by post-training IMHV lesions, produced varied results depending on which hemisphere's IMHV was lesioned. Bilateral lesions and lesions to the right IMHV rendered the chicks amnesic. Lesions to the left IMHV, after training, did not produce amnesia. From these results it was considered that a 'flow' of memory occurs from the left IMHV to the right IMHV, then to both LPOs. This was confirmed in that pre-training lesions to the right IMHV, followed by post-training lesions to both LPOs, did not result in amnesia, suggesting that the memory trace remained in the left IMHV under these circumstances. However, pre-training right IMHV lesions, followed by post-training lesions to the left IMHV, which according to the model should produce amnesia, did not. This would suggest that there is no simple memory trace model, and that the brain acts as a fully interacting, functional system with a distribution of the memory/memories

throughout the brain.

The connections between the LPO and the IMHV, which is thought to allow a flow of information from one to the other, are thought to converge on the archistriatum (ARCH). Bilateral, pre-training lesions of the ARCH have been found to render chicks amnesic for the task (Lowndes and Davies, 1994).

In addition, the avian hippocampus may also play a role in the formation of memory for the task. Sandi et al. (1992) looked at the effect of lesions of the left hippocampus on the recall of the task. These lesions produced amnesia when carried out pre-training, but not when given 1 hour after training. The left hippocampus was therefore assumed to be required for the acquisition of the task (Sandi et al., 1992) indeed, the IMHV has been found to receive afferents from the hippocampus (Bradley et al., 1985).

Electrophysiology

Mason and Rose (1987) discovered electrophysiological correlates to LTP in the chick brain. They recorded spontaneous multi-unit activity within forebrain structures. There was an overall increase, with almost a four fold increase in the IMHV, of high-frequency, large-amplitude spikes. The increase was not due to the training procedure, as demonstrated by the fact that electroshock-amnesic chicks had no such multi-unit firing (Mason and Rose, 1988).

Also reported in this 1988 paper was work that showed bursting in the IMHV was abolished by 2-APV (a NMDA-receptor competitive antagonist), and the LTP-like effect was also abolished.

Bradley et al. (1991) have carried out experiments on potentiation in chick forebrain slices. They found that long lasting changes in the synaptic efficiency could be obtained in the IMHV when there was a sufficiently large, late post-synaptic response during the conditioning. Such changes were not as

great in either magnitude or duration as LTP, although this may be due to the preparation. It was also suggested that as the late postsynaptic response lasts longer than a response to a single stimulus, it may be being produced by NMDA receptors.

Further data from our laboratories have described the time course of the increases in bursting behaviour from the IMHV (Gigg et al., 1993) and the LPO (Gigg et al., 1994). In the IMHV, significant increases in the bursting activities were recorded at 3-4, 5-6 and 6-7 hours post-testing (testing was carried out 1 hour after training) in MeA-trained chicks (Gigg et al., 1993). There was also a lateralised effect of this bursting, such that bursting in the right IMHV was significantly higher than in the left IMHV of MeA-trained chicks 6-7 hours post-testing. This study did not look at any time before 2 hours after training, which would have been useful in order to compare this data with the receptor studies and the transmitter release data from the present study. For the LPO, the data were lumped into 4 hour periods, from which it was shown that there was a highly significant increase in bursting activity between 4 and 7 hours post-testing comparing MeA-trained chicks to the water 'controls' (Gigg et al., 1994). These data were shown to be specific for the memory of the task in that electro-shock, which induced amnesia in half of the chicks (which then pecked the test bead), showed that there was a significant increase in bursting in the MeA-trained chicks that avoided the bead compared to those that pecked the test bead and had been made amnesic by the electroshock.

The increases in bursting activity that occur in the IMHV and the LPO also describe a double wave of events. The increases are apparent in the left and right IMHVs initially but are then transposed into the right IMHV and both LPOs. This is in line with the lesion data and suggests a redistribution of the memory or memories from the left IMHV to the right IMHV and both LPOs.

Receptor activity

Excitatory amino acids and their receptors have been implicated in passive avoidance learning. NMDA-receptor binding following the task was described by Stewart et al. (1992). At 30 minutes NMDA receptor binding activity increased by 39% in the left IMHV of MeA birds compared to W trained birds, but declined to control levels by 3 hours. The left LPO also showed increased binding at this time although in the lateral neostriatum there was a decrease. Injections of MK-801 (a non-competitive NMDA antagonist) at various times between 1 hour before and 5 minutes after training produced amnesia for the task 3 or 24 hours after training (Burchuladze and Rose, 1992). The binding of MK801 was increased 30 minutes after training in the left and right IMHV and the left LPO (Stewart et al., 1992). The increases in receptor binding in the left LPO and left IMHV were demonstrated to be specific to the avoidance task itself, as chicks that had been trained to avoid the MeA bead and then rendered amnesic by electroshock did not show these changes but those chicks given electroshock that were subsequently able to recall the task showed equivalent increases in receptor binding at 30 minutes post-training as did those trained on the MeA bead which were not given the electroshock treatment (Steele et al., 1995). In addition, Steele et al. (1993) reported that an antagonist of the NMDA glycine binding site inhibited the retention of memory for the task.

Steele and Stewart (1995) demonstrated that AMPA receptors are also involved in the learning task. They showed an increased affinity (measured as a decrease in the K_D value) of ^3H -AMPA 6.5 hours following the task in the left and right IMHV₃ and right palaeostriatum augmentatum (PA). Injections of CNQX 4.5 and 5.5 hours after training into the IMHVs produced amnesia for the task when the chicks were tested at 6.5 hours, indicating that the increased affinity of AMPA receptors, in the IMHV at least, is associated with longer-term memory for the passive avoidance task.

In our laboratories Holscher carried out a series of experiments that suggested a role for mGluRs in memory formation in the chick (Holscher, 1994). Amnesia, caused by the mGluR antagonist MCPG, was not apparent until 1 hour following training. This suggests a role for mGluRs in the chick, at a time at which protein synthesis may be occurring. This is in agreement with a study by Rickard and Ng (1995) who found that MCPG induced amnesia during the long-term stage of memory formation but did not affect the earlier stages.

There have also been some autoradiographic and biochemical studies of GABA receptors in the chick brain. Ramirez et al. (1983) studied the development of GABA in the chick optic tectum and Meza et al. (1985) provided evidence for a role for GABA in the chick vestibule. Stewart and Bourne (1987) demonstrated that there were temporal changes in GABA binding, with the highest level of binding present one day post-hatch. Quantitative analysis was used to investigate the distribution of high-affinity GABA receptors (Stewart et al., 1988). The highest levels of GABA-immunoreactivity (^3H -muscimol binding) were present in the cerebellum, HV, PA and LPO of 3 week old chicks (Stewart et al., 1988). GABA receptors have also been identified as being increased in number and activity following training on the one-trial passive avoidance task (Martijena and Arce, 1994). ^3H -flunitrazepam binding in synaptosomal preparations from forebrains was examined in one-day old chicks. An increase in the B-max (indicating an increase in receptor number) was found 30 minutes after the training task (Martijena and Arce, 1994).

In addition to amino acid receptors, other receptor types have been identified as being involved in memory formation in the chick. The δ -opioid receptor was found to show increased binding, 30 minutes after the training task, in the right PA and both LPOs (Csillag et al., 1993). Also, an increased binding to muscarinic acetylcholine (ACh) receptors, and a decreased level of

binding to nicotinic ACh receptors, was found 30 minutes after training (Rose et al., 1980; Aleksidze et al. 1981).

An important event in biochemical signalling processes is the influx of Ca^{2+} into neurons through voltage-gated channels (see also LTP). In the chick Ca^{2+} entry has been described following passive avoidance learning. The results described by Clements and Rose (1995) showed an increased uptake of $^{45}\text{Ca}^{2+}$ into prisms of IMHV 30 minutes after training. This uptake was inhibited, in the left IMHV only, by the N-type Ca^{2+} -channel blocker ω -conotoxin.

The increased Ca^{2+} -uptake, and effects of channel blockers on the retention of the task, suggest that neurotransmitter activity may be changed following passive avoidance learning. Rusakov et al. (1993) found a spatial rearrangement of vesicles in the synapses in the left IMHV of MeA-trained chicks 30 minutes after passive avoidance training. In their study two spatially separate pools of vesicles were identified. A rearrangement of the vesicles occurred with a greater number of vesicles near the active zone, suggesting an altered functional state in the synapses examined (the data described above will be discussed in greater detail in Chapters 4, 5 and 8).

Protein kinases

The increased effectiveness of the presynaptic neuron may be described in terms of the enhanced phosphorylation of membrane proteins resulting in the opening of Ca^{2+} channels and triggering intracellular second messenger systems which may lead to an increase in transmitter release.

The enzymes that phosphorylate membrane proteins have been described (see section on LTP). These proteins include protein kinase C and A, both of which may be substrates for arachidonic acid and NO activity.

Ali et al. (1988a) demonstrated a decrease in the *in vitro*

phosphorylation of a 52 kDa presynaptic plasma membrane phosphoprotein after the training task. Bilateral injections of melittin showed that this protein is not required for the short-term phases of memory (minutes to 1 hour after training) but is required for long-term memory formation (Ali et al., 1988b). This phosphoprotein, considered to be B-50, is a presynaptic substrate of protein kinase C (PKC), and is phosphorylated on depolarisation of the synaptic membrane under conditions that release neurotransmitters (Rodknicht and Wofchuk, 1992). An increase in the membrane:cytosol ratio of PKC in the left IMHV is also found, which is interpreted as indicating translocation of the enzyme (Burchuladze et al., 1990). The translocation of the α and β forms of the PKC to the membrane is dependent on Ca^{2+} concentration, and the phosphorylation of B-50 is regulated by the ratio of soluble to bound PKC. Therefore presynaptic Ca^{2+} influx, or mobilisation from internal stores, results in PKC translocation followed by B-50 phosphorylation, and the activation of synapsin which should result in vesicular transmitter release.

In addition to PKC, cAMP-dependent protein kinase (PKA) may be involved in memory formation in the chick. A significant increase in whole forebrain levels of cAMP has been observed between 30 and 60 minutes following passive avoidance training in the chick (Brown, 1984). The use of PKA inhibitors showed that PKA was involved in the formation of long-term memory in the chick (Zhao et al., 1995).

Retrograde messengers

Retrograde messengers (see section on LTP) have also been reported to be involved in the training task, suggesting a mechanism for the strengthening of synapses.

Phospholipase A_2 (PLA $_2$) inhibitors produced amnesia for the passive avoidance task (Hölscher and Rose, 1994). Arachidonic acid is synthesised via

PLA₂. Therefore, it can be assumed that arachidonic acid is necessary for memory formation of the passive avoidance task.

Hölscher and Rose (1992, 1993) also demonstrated that nitric oxide (NO) production is necessary for the formation of memory in the passive avoidance task. N-nitro-L-arginine (an inhibitor of NO synthase) injected into the IMHV, left or right, before the training task produced amnesia from 30 minutes to 24 hours later. This suggested that NO production is of importance in the first few minutes after the initiation of memory formation rather than in the later stages.

It is therefore thought that NO is but the first wave carrier of information and forms a first wave of retrograde signalling, reaching a peak of synthesis by 30-60 minutes after which NO production starts to decrease and arachidonic acid release, the presumed second wave, is increased. These retrograde messengers are thought to signal to the presynaptic neuron to increase its effectiveness "vis-a-vis the postsynaptic neuron at a particular synapse or set of synapses." (Rose, 1992).

Immediate early genes

Training also results in the activation of the cell's general protein synthetic machinery. A general molecular biological mechanism in the switching on of protein synthesis involves the expression of a family of immediate early genes (IEGs). These genes "switch-on" certain late genes, which enable or direct protein synthesis. Under quiescent conditions, these immediate early genes are not expressed. Following stimulation during a novel or stressful situation, the IEGs are activated. IEG activation may occur following a rise of intracellular Ca²⁺ associated with NMDA receptor activation.

Anokhin and Rose (1991) demonstrated that mRNA encoding the IEGs *c-jun* and *c-fos* was increased 30 minutes after training in both the left

and the right IMHVs and LPOs. In addition to the passive avoidance task, a pebble floor discrimination task was carried out in order to prove a specific learning-related effect of the increases in IEG production. This is an appetitive task in which chicks learn to discriminate between food and non-food items. Three groups were used such that one of the groups was learning the task for the first time on the second trial (no learning experience occurred on the first trial), the other two groups were repeating the previous trial's experience. From these experiments the highest IEG expression occurred in the group with the novel experience in the second trial: the conclusion from this is that learning a behaviour, as opposed to repeating an already learned behaviour, increases IEG expression.

The level of *c-fos* mRNA was increased by 2 to 2.5 fold in the left and right IMHV and the LPO, 30 minutes after the training task (Anokhin et al., 1991). This increase was not thought to be due to neural activity in visual processing, as dark-reared chicks, and chicks maintained in familiar visual environments, showed low levels of *c-fos* expression.

The expression of the proteins, *fos* and *jun*, was increased following the training task at 1 hour in both IMHVs and at 2 hours in the right LPO (Freeman, 1994).

Protein synthesis

Two waves of neuronal activity are thought to occur. This activity has been described in terms of the synthesis of glycoproteins. Glycoprotein synthesis has been found to be essential for memory in the chick. A precursor for glycoprotein synthesis is fucose. When radiolabelled fucose was injected into the chick brain, an increased incorporation was found hours following the passive avoidance task (Rose, 1989). The incorporation of fucose into glycoproteins was inhibited by 2-deoxy-galactose (2-Dgal). Intracranial injection of 2-Dgal produces amnesia at 24 hours when injected between 2

hours pre-, and 2 hours post-training (Bullock et al., 1990). The increased incorporation of fucose was found to be specific for memory formation, as chicks that were electroshocked 10 minutes post-training (a time which does not produce amnesia) still showed increased fucose incorporation compared to untrained but shocked, and trained and immediately shocked, chicks (Rose and Harding, 1984).

Crowe et al. (1994) found that the effects of 2-Dgal preceded the effects of anisomycin (a protein synthesis inhibitor). This suggested that the initial phases of memory formation involved the post-translational modification of already present proteins.

The glycoproteins that are increased during the second wave are described as learning-associated glycoproteins (LAGs) (Rose, 1995). These LAGs include a presynaptic 50kDa, and postsynaptic 33, 100-120, and 150-180kDa proteins found in the LPO (Bullock et al., 1992). These glycoproteins are thought to include forms of cell adhesion molecules (N-CAMs). N-CAMs are known to be involved in synaptic recognition, selection and subsequent stabilisation during development (Edelman, 1985). There are both 120 and 180kDa forms of N-CAM; the smaller form is thought to be an embryonic form and is converted into the mature 180kDa, low sialylated form. Antibodies to N-CAM are thought to interfere with this process. When injected 30 minutes pre-training, anti-N-CAM was found to produce amnesia between 5.5 and 8 hours after training (Scholey et al., 1994). This time period is thought to represent the time for a second wave of neuronal activity. This second wave is postulated to take place only if the learning event was sufficiently strong (Rose, 1994). This is substantiated by the fact that if chicks are trained on a weak aversive substance, such as 10%MeA or quinine, the avoidance response on testing declines from 6 to 9 hours after the training event (Rose, 1994).

N-CAM is enriched at a sub-cellular site in the chick (Rusakov et al.,

1995b) and is rearranged following training (Rusakov et al., 1994).

The second time window shows the effects of anisomycin precede the effects of 2-Dgal (Freeman et al., 1995). This implies that the second wave of glycoprotein synthesis takes place on newly synthesised proteins.

In addition to these cell-cell interactive proteins, tubulin, a structural component of axonal transport, has been shown to be increased up to 24 hours following training (Scholey et al., 1992).

Morphological changes

The changes in glycoprotein and tubulin synthesis may eventually be incorporated into structural modifications. For example, the density of asymmetrical spine synapses increased by c.40% at 1 hour post-training in the right IMHV of MeA-trained chicks, although this was reduced to a basal level 24 hours post-training (Doubell and Stewart, 1993). These changes were not seen in the left IMHV at this time. The average post-synaptic density was smaller (57%) in these chicks, suggesting that the new synapses were formed by the splitting of existing synapses: synapse formation followed by selective elimination has been suggested as a mechanism for learning (Changeux and Danchin, 1976).

In the left IMHV the thickness of pre- and post-synaptic electron densities was found to be increased 30 minutes after training (Rusakov et al., 1995a). In addition the synaptic apposition zone profiles were increased in length. Both suggest an increase in synaptic efficacy associated with learning the task.

The numerical density of synapses is also increased, initially in the left hemisphere (30%) 24 hours after training, and by 48 hours the increase is present in both LPOs. Also increased by 24 hours is the density of synaptic vesicles and an increase in spine density, both in the left LPO (Stewart et al.,

1984). As synaptic number changes do not occur until 24 hours and after in the LPO, the increase observed is likely to be due to synaptogenesis, rather than due to splitting of synapses as suggested to occur at perforated synapses in the mammalian brain following a novel experience (Stewart et al., 1992). In keeping with this, changes in synaptic density have been found to be reduced following the injection of anisomycin 30 minutes before training (Sojka et al., 1995). This would suggest that *de novo* protein synthesis is involved in the increases seen following training.

At 48 hours there is an increase in synaptic density height (similar to PSD length) and dendritic spine volume in the LPO. Spine density changes are found 24 hours after training in multipolar projection neurons of the left LPO (Lowndes, 1992).

Aims

No direct evidence for enhanced glutamate release or the release of other amino acids in the passive avoidance task has so far been demonstrated. It had been assumed that glutamate release would be increased but at what times after the task? Would these increases, if present, coincide with changes in receptor binding and other biochemical changes? The research project described in this thesis aims to describe the time course of amino acid transmitter release from the IMHVs and LPOs of day-old chicks following the passive avoidance task. Both hemispheres will be examined to determine if lateralisation of release occurs. In addition adenosine, a potential modulator of amino acid release, will be analysed for its time course of release, effect on amino acid release, and effect on the retention of the task.

Chapter 3: The development of a method of High Performance Liquid Chromatography (HPLC) for analysis of metabolic amino acids in the chick brain

General Principles

Chromatography is a separation technique in which mixtures are resolved by the differential migration of their constituents during passage over a chromatographic substrate. The separation process is determined by the distribution of the components between two immiscible phases, which is described by the partition or distribution coefficient (K_d). For a compound distributing itself between equal volumes of two immiscible solvents A and B, the value for this coefficient is a constant at a given temperature and is given by the expression:

$$K_d = [\text{solute}] \text{ in A} / [\text{solute}] \text{ in B}$$

The distribution of a compound can also be described in terms of its distribution between two phases. One is a stationary phase; this may be solid, gel, liquid or a solid/liquid mixture which is immobilised. The second mobile phase is a liquid or a gas and flows over the stationary phase. The choice of phases is made so that the compounds to be separated will have different distribution coefficients. The separation (resolution) also depends on the distribution of the sample through the chromatographic column, which is dependent on the number of stages that a separation of compounds can occur throughout the column, i.e. the number of theoretical plates (N) that the

compound must pass through. The resolving power of the system increases with the number of theoretical plates per unit length; as this is related to the surface area of the stationary phase it follows that the smaller the particle size the better the resolution. Unfortunately, the smaller the particle size the greater the resistance to eluant flow.

This is where high performance liquid chromatography (HPLC) comes into its own. By employing stainless steel tubing, high pressure pumps and a solid column packed with stationary phase, a much greater resolution is achieved with a reduced time of analysis.

Amino Acid Analysis

The fundamental problem with the analysis of amino acids is their detection. Most amino acids are very weak chromophores in the UV-visible region and possess no native fluorescence. To overcome this difficulty, some derivative of the amino acid that is a good chromophore or that fluoresces is needed. Both postcolumn derivatisation and precolumn derivatisation techniques exist. In the present work precolumn derivatisation was used.

Precolumn derivatisation, followed by the separation of the amino acid derivatives by reverse phase (polar solvent, non-polar mobile phase) chromatography, is fast becoming the preferred method of analysis. The advantages of this approach include short analysis times, high sensitivity, and elimination of costly and sometimes cumbersome postcolumn reaction systems. These advances have been spurred by rapid improvements in liquid chromatography equipment and the widespread introduction of high efficiency chromatographic packings. Today's small particle (3-5 μ m) alkyl-bonded silica materials make it feasible to separate complex mixtures of derivatised amino acids in as short a period of time as 10 minutes. Coupled with high sensitivity detectors, these materials allow for estimation of

picomolar quantities.

The ideal derivatisation protocol will have the following characteristics: (1) uniform reactivity and quantitative reaction yields, (2) mild reaction conditions and short reaction times and (3) stable products.

HPLC of amino acids after precolumn derivatisation was first described by Bayer et al. (1976) who used dansyl chloride as the derivatising agent and resolved about 17 amino acids in both normal and reversed phase systems. Dansyl amino acid analysis suffers from variable yields of lysine, low yields of all amino acids in the presence of salts and incomplete separation of several amino acids (Ebert, 1989).

Precolumn labelling with *o*-phthalaldehyde (OPA) produces fluorescent amino acid derivatives (isoindoles) which are well resolved in several reversed-phase systems (Cohen and Strydom, 1988). The reagent itself does not fluoresce, and therefore produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with a rapid reaction time with the amino acids to produce the derivative, makes it amenable to automated derivatisation and analysis (Cohen and Strydom, 1988). The principal limitations of this method are the inability to derivatise secondary amines such as those found in proline and hydroxyproline, and the poor stability of the reaction products. However, a significant improvement of the method came when the OPA-derivates are coupled to electrochemical detection (e.g. Allison et al., 1984). OPA derivatisation can be coupled with β -mercaptoethanol to produce derivatives that undergo oxidation at the anode at moderate potentials. The electrochemical properties of the amino acids are less susceptible to change than their fluorescent properties, thus making the derivatives much more stable. Authors have used the OPA/ β -mercaptoethanol procedure to analyse postmortem human brain (Ellinson et al., 1987), rat brain homogenate and human cerebrospinal fluid (Canevari et al., 1992).

Heinrikson and Meredith (1984) introduced a substantial improvement for UV analysis of amino acids with the publication of a method which utilised the Edman reagent, phenylisothiocyanate (PITC); not to synthesise phenylthioldantoin (PTH) amino acids, but to produce phenylthiocarbamyl (PTC) derivatives in a reaction which proceeds quantitatively within minutes at room temperature (Figure 3.1).

This methodology has been adapted for a wide range of applications including the analysis of food and animal feed samples (e.g. Lanneluc-Sanson et al., 1986), purified acid-hydrolysed proteins and peptides (e.g. Strydom et al., 1985), and analysis of free amino acids present in biological fluids (Cohen et al., 1986). Most of the remaining part of this chapter will describe adaptations to the published method, of PITC-derivatisation and subsequent chromatography, in order to tailor it to amino acid analysis from chick brain slices.

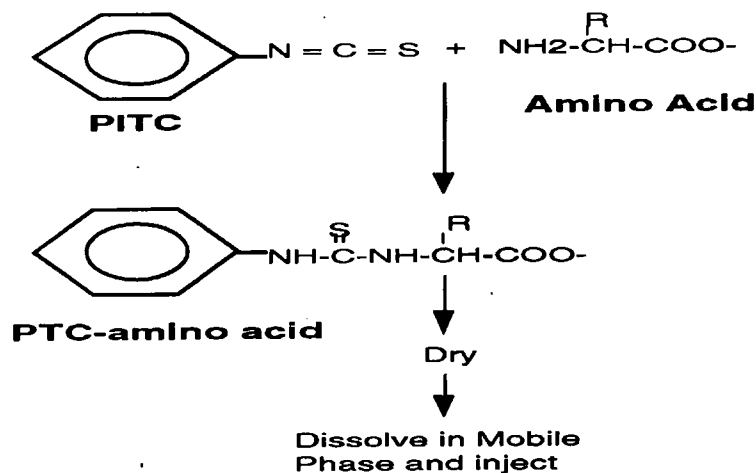


Figure 3.1. Production of PTC-amino acids with phenylisothiocyanate (PITC) using a modification of the Edman degradation reaction.

Sample preparation and derivatisation

A Waters PICO-TAG workstation was used in conjunction with a vacuum pump for pre-column derivatisation of samples.

One hundred microlitres of the medium from the release experiments (Chapters 4, 5 and 6) were taken and placed into a marked and numbered vial suitable for the WISP auto-sampler. Eight such vials were put into a 'reacti-vial', placed into the PICO-TAG workstation and vacuum dried to 65 millitorr.

The second step involved redrying the solution to obtain the required pH level for the subsequent reaction. Fifty microlitres of a re-drying solution (methanol:tetraethylammonium (TEA):water, 2:1:1) were added to each sample vial and again vacuum dried to 65 millitorr. Originally ethanol was used in place of the methanol as this was part of the standard procedure; however, as salts were present in the samples from the buffer solutions used and these can affect the reaction yield of glutamate and aspartate, methanol was used in its stead as this largely eliminates the deleterious effects of high salt concentrations on yield.

Derivatisation was carried out using 50 μ l of a derivatising solution (methanol:water:TEA:PITC, 7:1:1:1) added to each sample tube. This was left to stand for 20 minutes at room temperature for the derivatisation to take place. The reacti-vial was put into the PICO-TAG system and again vacuum dried to 65 millitorr to remove all traces of PITC.

The derivatised sample was then re-suspended in 100 μ l of sample diluent (710mg/l Na₂HPO₄, 5% ACN at pH 7.40 with 10% H₃PO₄) and a fraction injected into the HPLC.

It was possible to store the samples in a -40°C freezer at any stage after vacuum drying, for future analysis.

The Hardware

The HPLC system used was a Waters PICO-TAG amino acid analysis apparatus: consisting of a model 600 multisolvent delivery system, a model 441B UV detector set at 254nm and a model 712 Waters intelligent sample processor (WISP). A Novapak C-18 column (15cm x 3.5mm, Waters) was maintained at 37°C using the Waters 600 controller and a column heater. A Waters 740 data module was used for plotting chromatograms and for peak analysis, a discussion of which will follow.

A gradient elution was carried out for the analysis of amino acids. In the analysis of mixtures it is not always possible to obtain a satisfactory separation using only one solvent (an isocratic elution). There is often insufficient retention of the early emerging compounds and excessive retention times of late-eluting peaks. This can be solved by a gradual change in the composition of the mobile phase during the course of the separation: a gradient elution. Gradients can be either stepwise or continuous. Throughout the separation the solvent composition and flow rate can be described in terms of an appropriate gradient curve shape that can be selected to control the transition between two time points (Figure 3.2).

The published method on which the adjustments were made was that of Rogers et al. (1987). The separation of PTC amino acids was generated with a gradient using sodium acetate buffer and acetonitrile as the predominant organic solvent. Two mobile phases were used. Eluent A was composed of 6% acetonitrile, 14mM sodium acetate, 0.05% TEA in Milli-Q water (double-distilled, deionised), and adjusted to pH 6.6 at room temperature with glacial acetic acid. Eluent B consisted of 60% acetonitrile and 40% Milli-Q water. Initial conditions of the gradient were 100% eluent A @ 1.1ml/min. At 1 min eluent B was stepped to 5% and at 10 mins to 6%. Eluent B was then increased to 100% via a linear program, and the flow rate

flushed with eluent B at this flow rate for at least 4 minutes to eliminate hydrophobic impurities present in eluent A, or in the sample. Re-equilibration time took a further 10 minutes, resulting in a turn-around time of 35 minutes.

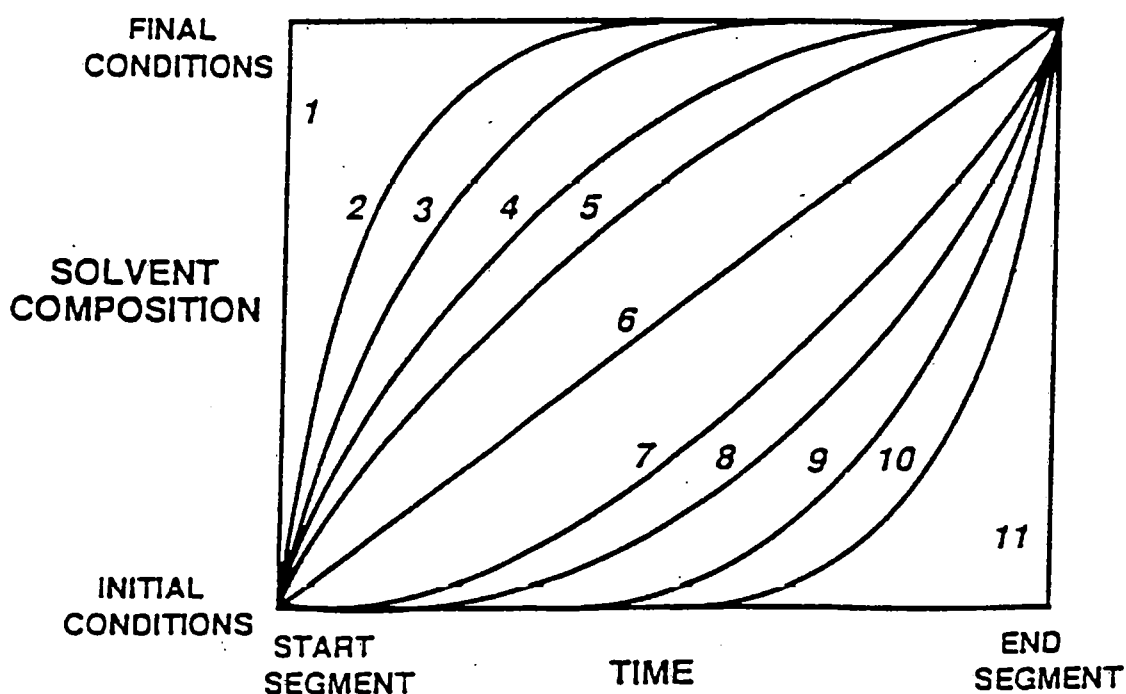


Figure 3.2. Gradient curve shapes used to describe the rate of eluent flow and mobile phase composition between two identified time-points. This diagram and Figures 3.3, 3.4, 3.5, 3.6 and 3.8 were taken and adapted, with permission, from a Millipore "Liquid Chromatography School" document.

Methods Development

Even within the published methods there was still scope for improvement. The methodologies of both the sample preparation and the chromatography itself were developed, producing more effective separations. This was one of the key laboratory activities that enabled the work described in this thesis to be carried out, and therefore this will be discussed in some detail. The parameters that can be varied are described below; the method developments that were carried out are also outlined.

In order for two peaks from solutes of different chemical types to be separated, their bands of elution must be sufficiently apart during their passage through the column. In addition, band widths must remain narrow if compounds are to be eluted as discrete peaks. The resolving power of the system depends on three parameters: selectivity, efficiency, and capacity, each of which can be manipulated. The desired method of chromatography is one that achieves a satisfactory resolution of solutes in the minimum amount of time. Resolution (R_s) describes the degree of separation of one component from another and is defined as the difference in retention volumes (V_{R_s}) of the two solutes divided by their average peak width (w). The equation below describes the resolution of the separation.

$$R_s = (V_{R2} - V_{R1}) / 0.5 \times (w_1 + w_2)$$

Figure 3.3 (below) describes how the resolution can be measured from a chromatogram. The values of V and w can be measured in volume, time or chart length (from the point of injection) as long as the unit is kept constant to allow subsequent comparisons.

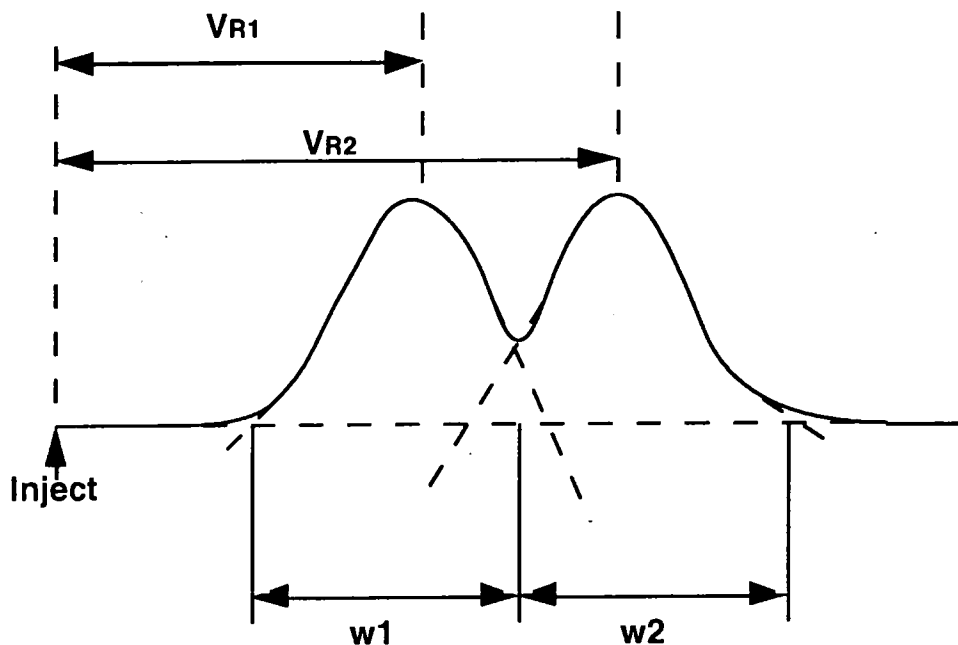


Figure 3.3. The measurement of Resolution (R_s). VR_1 and VR_2 are the retention volumes of the first and second eluting components respectively and w_1 and w_2 are the peak widths of the first and second eluting components respectively. See text for further explanation.

Developing a Separation

In order to produce a method from scratch or to develop an existing method, certain chromatographic parameters can be used and adjusted so as to yield a method tailored to the particular assay that one requires. A flow diagram that represents the steps in developing a viable method is described.

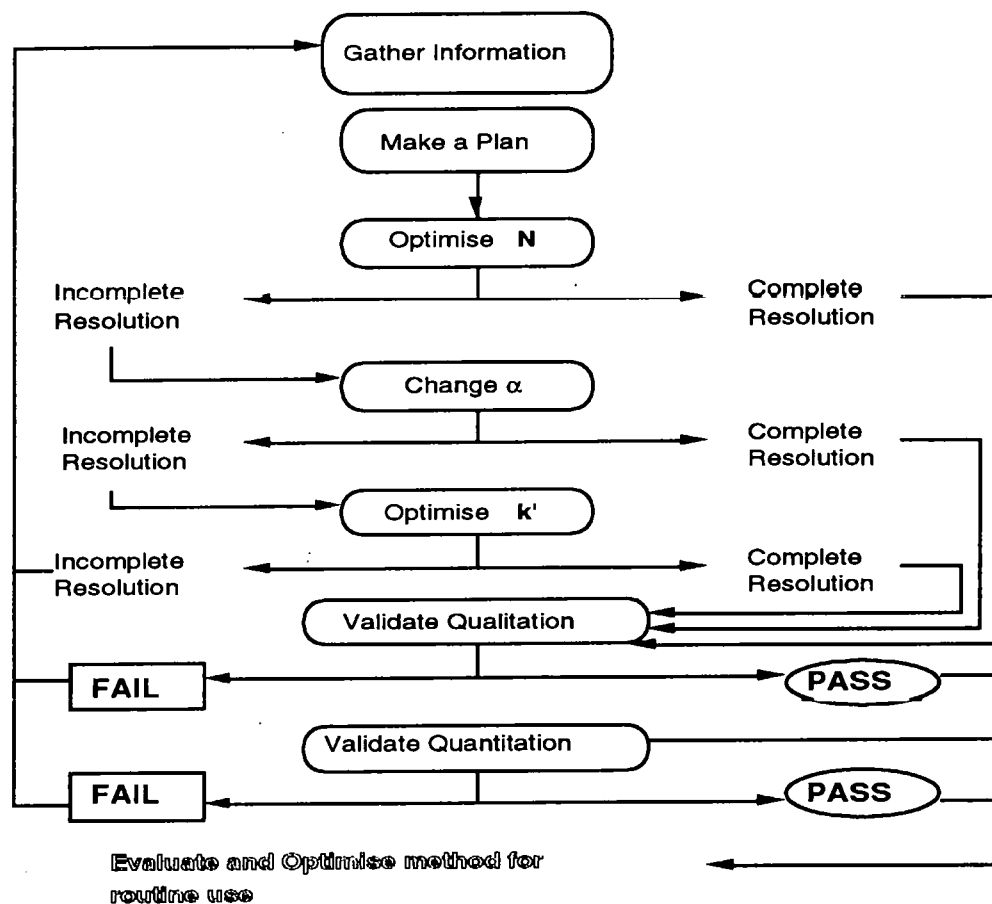


Figure 3.4. This flow chart represents a step by step method development strategy. The factors N , α and k' are explained in detail below and following the description of each are the procedures carried out in order to improve the technique.

For a desired degree of resolution three conditions have to be met: 1) the peaks have to be retained on the column (as described by the capacity factor, k'); 2) the peaks have to be separated from each other (the selectivity factor, α); and 3) the column must have a minimum number of plates/number of separations that occur in the column (column efficiency, N).

shown diagrammatically in Figure 3.5.

α , k' , N — HOW THEY CONTROL RESOLUTION

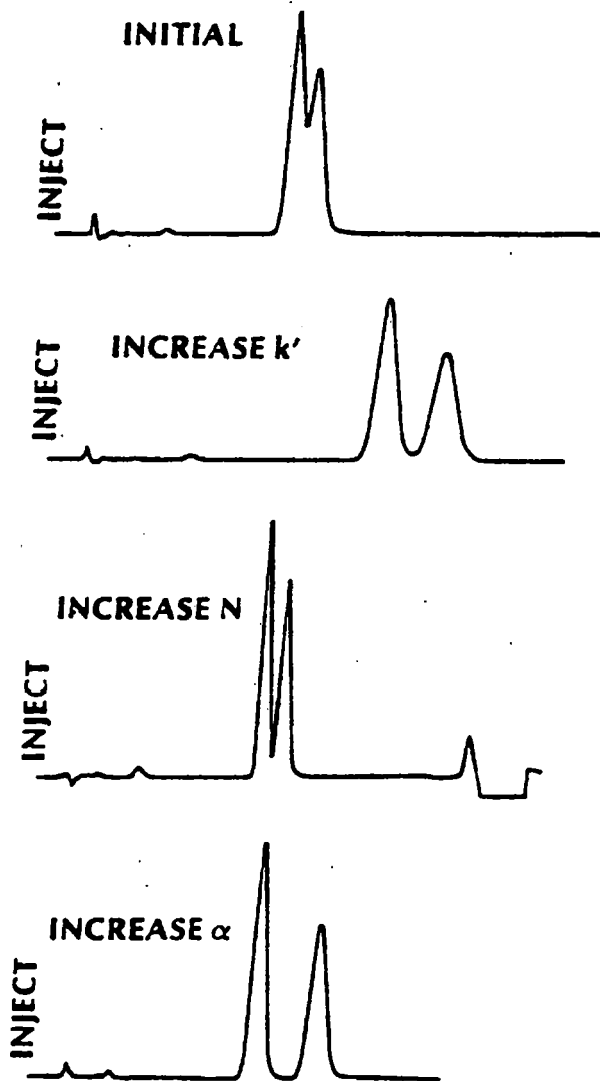


Figure 3.5. The control of resolution of a separation using capacity (k'), selectivity (α), and column efficiency (N).

A decrease in the k' values of the original bands will cause a decrease in

Figure 3.5. The control of resolution of a separation using capacity (k'), selectivity (α) and column efficiency (N).

A decrease in the k' values of the original bands will cause a decrease in resolution and a concomitant increase in analysis time and a decrease in peak heights. Improved efficiency (N) results in an improved separation of the two bands and narrower peak widths. If selectivity is increased, the resolution will be improved without changes in analysis time or peak heights.

Column Efficiency (N)

The dispersion of the compound through the column can be measured by the number or height of theoretical plates of the column (N), which determines the extent of bandspreading in a peak, and can be given by the following expression:

$$N = 25 (VR / W5\sigma)^2$$

where $W5\sigma$ is the width of the peak, see Figure 3.6. (Other width values can be used but $W5\sigma$ shows up the tailing of peaks which other measures may miss).

In addition the plate height (H) can be measured

$$H = L / N \quad \text{where } L = \text{the column length.}$$

Commercially produced hplc columns have more than 50 000 plates m^{-1} when packed with $5\mu m$ particles. In this way a 15cm column as used in the present chromatography would have a plate number N of about 7500 and a plate height of $2\mu m$. An acceptable chromatogram will probably be produced with c. 5000 plates or so.

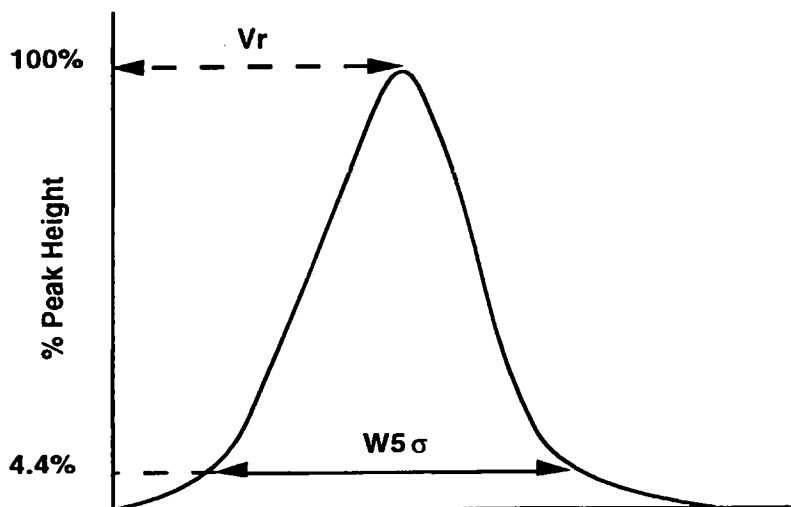


Figure 3.6. Method for determining column efficiency using the 5 sigma method (see text for details).

Optimising N

Initially, a run of amino acid standards (Pierce, UK Ltd) was carried out using the method of Rogers et al. (1987) (see above). The results were less than satisfactory with band broadening occurring, causing peaks to run into each other and not achieving a separation. The appearance of band broadening suggested a deficiency of the resolving power of the set-up rather than something intrinsic in the methodology. As the column was at least 4 or 5 years old, and had had unknown previous usage, it was decided to determine the column's efficiency using the equation outlined above. A 5σ method was employed, using the band width @ 4.4% peak height. Therefore $N = 25 (V_R/W)^2$. The N value was calculated at 3454 plates. This is a somewhat low number (5000 plus is usually considered necessary for reasonable separations: Millipore technical advice to me) and it was decided to replace this column with a new C-18 Novapack column. On receiving this N was again calculated using the 5σ method and this time was found to be 10805 plates. The amino acid standards were run using the same gradient set-up, and as expected the new column gave considerably better results: band

broadening was considerably reduced and the separation of most amino acids was achieved.

The separation was not complete, however, as co-eluting and close eluting peaks were found in the incubation media.

In order to improve resolution by changing N , flow rate may in addition be changed. The most practical way is to decrease the flow rate which will be accompanied by a concomitant increase in separation time. However, by increasing the flow rate during times where no close eluting peaks are present but decreasing flow rate when close eluting peaks are present, total run time or turn-around time was kept down. Thus the initial flow rate was reduced from 1.1 ml/min to 1ml/min and then to 0.75ml/min of eluent A. This increased the separation of the early-eluting peaks, to the extent where most peaks were visible, but co-elution, particularly of glycine and glutamine, was still occurring even at 0.5 mls/min. Therefore, as no better separation could be achieved using just a change in N the next stage was evaluated i.e. a change in k' , the capacity factor.

Capacity (k')

A peak on a chromatogram is identified by some measure of retention. The degree of retention of a particular compound in a mixture is often expressed quantitatively in terms of the retention time (t_R) or the retention volume (V_R). A useful quantity to identify a peak is the capacity factor (k') which describes where the peaks elute relative to t_0 or V_0 (the retention volume or time of a non-retained solute).

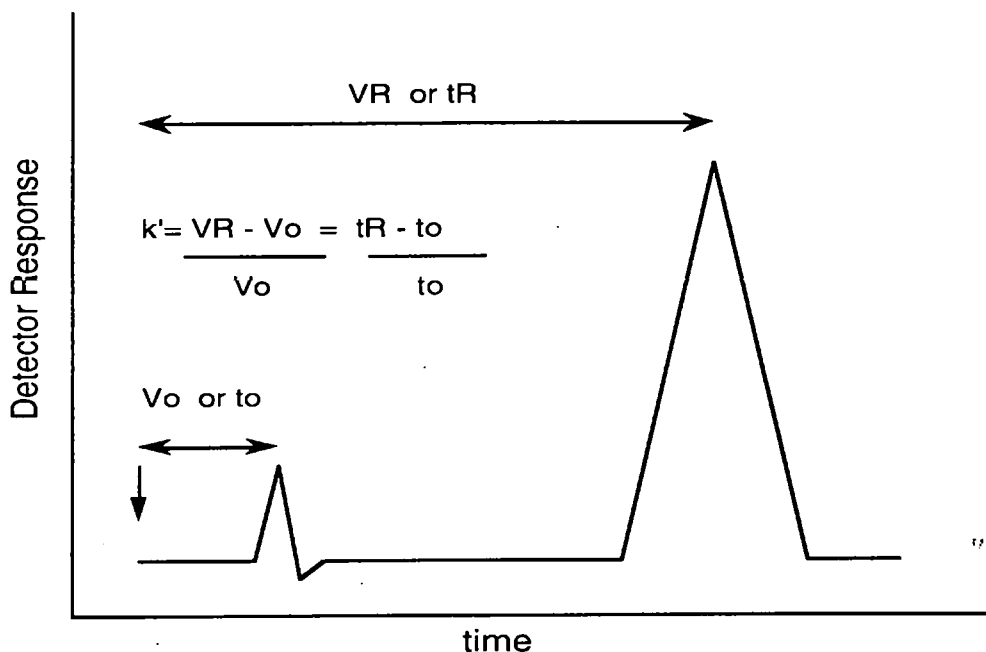


Figure 3.7. Measurements of capacity factor, k' . V_0 is the void volume and is the measure of the retention of an unretained compound. V_R is the retention volume of the solute. t_0 and t_R are the void times and retention times respectively. See text for further details.

The ratio, the capacity factor (k') is given the following expression:

$$k' = (V_R - V_0) / V_0$$

For low values of k' resolution increases very rapidly with increasing k' . At high values of k' there is a much diminished return of resolution for increasing k' . The optimal values of k' are in the range of $1 < k' < 10$.

To achieve an optimal k' value the retention time of a component can be altered by a change in the chemical nature of the two chromatographic phases, such that the solute concentration ratio is changed. This is usually carried out by altering the solvent strength of interaction with the solute: an increase in mobile phase polarity will lead to an increase in the k' values, as the strongest solvent is the least polar in a reverse phase separation. To determine the effect of a solvent on the chromatography, a 'scale of polarity'

table can be consulted. This shows the adsorption energies (the Snyder parameter) and a summation of the different intermolecular interactions (the solvent polarity parameter) present in the solvent (see Table 3.1).

Alternatively, instead of changing the actual solvents, the volumes of the solvents in the mixture may be changed, as the given solvent polarity parameter for a mixture is roughly equal to the volume fraction of each solvent multiplied by the solvent polarity P' such that

$$P' = \phi_a P_a + \phi_b P_b$$

where ϕ_a and ϕ_b are the fraction volumes of solvents A and B in the mixture and P_a and P_b refer to the P' values of the pure solvents.

Thus the values of k' are characteristic of individual solutes. By choosing a certain combination of the mobile and stationary phases, the k' values will be different for each component in a given mixture.

Solvent	Snyder Parameter	Solvent Parameter P'
trichloromethane	0.40	4.4
acetonitrile	0.65	6.2
methanol	0.95	6.6
water	1.00	10.2

Table 3.1. An elutropic series of commonly used solvents. The Snyder parameter refers to the adsorption energies of the solvents and the solvent parameter refers to the intermolecular interactions present in the solvents (e.g. hydrogen-bonding, Van der Waal forces, Coulombic forces, dipole interactions). The higher the parameter number the more polar the solvent.

Optimising k'

From table 3.1 it can be seen that the solvent parameter for eluent A in the Rogers method is 0.94×10.2 (94% water) and 0.06×6.2 (6% ACN) = 9.96. Eluent B is therefore 0.6×6.2 and $0.4 \times 10.2 = 7.8$. Thus there is a decrease in P value from eluent A to B. In this way the more polar and smaller amino acids elute at the beginning of the run, with the larger amino acids that will have a smaller amount of charge per unit mass eluting at the end of the series. In order to increase the k' values it was decided to increase the water content of the mobile phases. Eluent A's acetonitrile content was dropped to 4%, so that the solvent parameter was now 10.04, and the separation was carried out again. There was a much improved separation of the early-eluting peaks, but glutamine and glycine still appeared to be co-eluting. Therefore eluent B's water content was increased to 50%, and in a subsequent run to 60%, to see if there was any increase in the glycine/glutamine separation. There was no noticeable improvement in the separation. It was decided at this juncture to try a different approach, and to effect a change in the selectivity factor, α .

Selectivity or separation factor (α)

The selectivity factor (α) is a measure of the separation of two adjacent components. It is the ratio of capacity factors in the mixture such that

$$\alpha = k'_2 / k'_1 = (V_{R2} - V_0) / (V_{R1} - V_0)$$

(k'_2 is the capacity of the component with the longer retention time).

If $\alpha = 1$ the bands are not resolved as the capacities are equivalent. If $\alpha > 1$ the points of maximum concentrations of the two peaks are not coincident. However, if the bands are not contained in a small volume of mobile phase, poor separation may result in spite of favourable α values, as α

does not take into account any overlapping of the peak areas. The value of α should be as large as possible given all factors being equal. This is a powerful tool for improving resolution as R_s can be increased at the same time as decreasing the separation time t .

The separation factor may be varied by one or more of the following mechanisms (the following list is in decreasing order of effect on the resolution)

- a change of the mobile phase solvent;
- a change of the mobile phase pH; and
- a change of the stationary phase.

Changing the mobile phase solvent will give variable and sometimes quite unexpected results and although a powerful tool, may not be practicable on occasion (e.g. miscibility of solvent, solubility of the sample).

The second most popular means of varying the selectivity is by changing the pH of the solutes. In practice the pH of the mobile phase is varied, and the resulting changes in the capacity, k' , and α are measured from the chromatogram; this technique is restricted to samples of ionisable acids or bases allowing changes in the pK values. When a change of pH gives optimal α values but k' values are not between 1 and 10 then a change of solvent strength by varying the proportions of components in the mobile phase can be carried out (see previous section).

A change of stationary phase is usually impractical but if used requires further adjustments to be made to the capacity k' values.

Optimising α

α is directly related to the solute-solvent interactions. The change in the thermodynamics of the system can be brought about by varying the phase ratio (V_s/V_m), by using different phase gradients, by changing the solvent composition and pH, and by means of secondary equilibria. The most effective and easiest option of maintaining k' values whilst changing α , is a change in the gradient run. By retaining a low level of organic solvent (acetonitrile) for a longer period, and by reducing the rate of total acetonitrile content in the mobile phase over time, it is possible to vary the phase ratio so that more subtle distinctions between the polarities of the different PTC-amino acids are used. Thus by reducing the total concentration of eluent B in the system for as long as possible, and by reducing the initial flow rate, much improved separations could now be carried out. In addition the level of TEA in eluent A was increased to 0.1%, because Ebert had found this to be effective at separating ammonia from threonine and phenylalanine (Ebert, 1986).

The pH of eluent A was also varied to see if there was an increase in resolution of the system. Runs with the eluents at pH 6.0, 6.8 and also at pH 7.0 were carried out. These suggested that a pH of 6.8 was probably optimal, although there was little difference in the separation of any of the amino acids between the three pH values.

Chromatographic conditions

The new chromatographic conditions are described in full below, and are shown in Figure 3.8.

Eluent A was now composed of 4% acetonitrile, 14mM sodium acetate, 0.1% TEA in Milli-Q water, and adjusted to pH 6.8 at room temperature with glacial acetic acid. Eluent B still consisted of 60% acetonitrile and 40% Milli-Q water. Initial conditions of the gradient were 100% eluent A @ 1.5ml/min. After 1 minute eluent B was stepped to 2% but down to 1.1ml/min, and at 10 minutes eluent B was stepped to 5%. Eluent B was then increased to 50% by 12 minutes via a linear program, and to 100% by 14 minutes. At 14.5 minutes the flow rate was increased to 1.5 mls/minutes. The column was flushed with eluent B at this flow rate, until 19 minutes, to eliminate hydrophobic impurities present in eluent A, or in the sample. From 18 to 19 minutes, a linear program pushed eluent A to 100%, and from 19 to 27 minutes the flow rate was dropped to 1.1ml/min. Injections of a new sample, or a repeat injection, could proceed from ca. 30 minutes after the first. If no further injections occurred then the program would run down to 0.10mls/min of 100% eluent B by 45 minutes.

A)

TIME (mins)	Flow Rate (ml/min)	%A	%B	Curve
0	1.50	100	0	*
1.00	1.10	98	2	11
10.00	1.10	95	5	11
12.00	1.10	50	50	6
14.00	1.10	0	100	6
14.50	1.50	0	100	6
18.00	1.50	0	100	6
19.00	1.50	100	0	6
27.00	1.10	100	0	6
35.00	1.10	100	0	6
45.00	0.10	0	100	6

B)

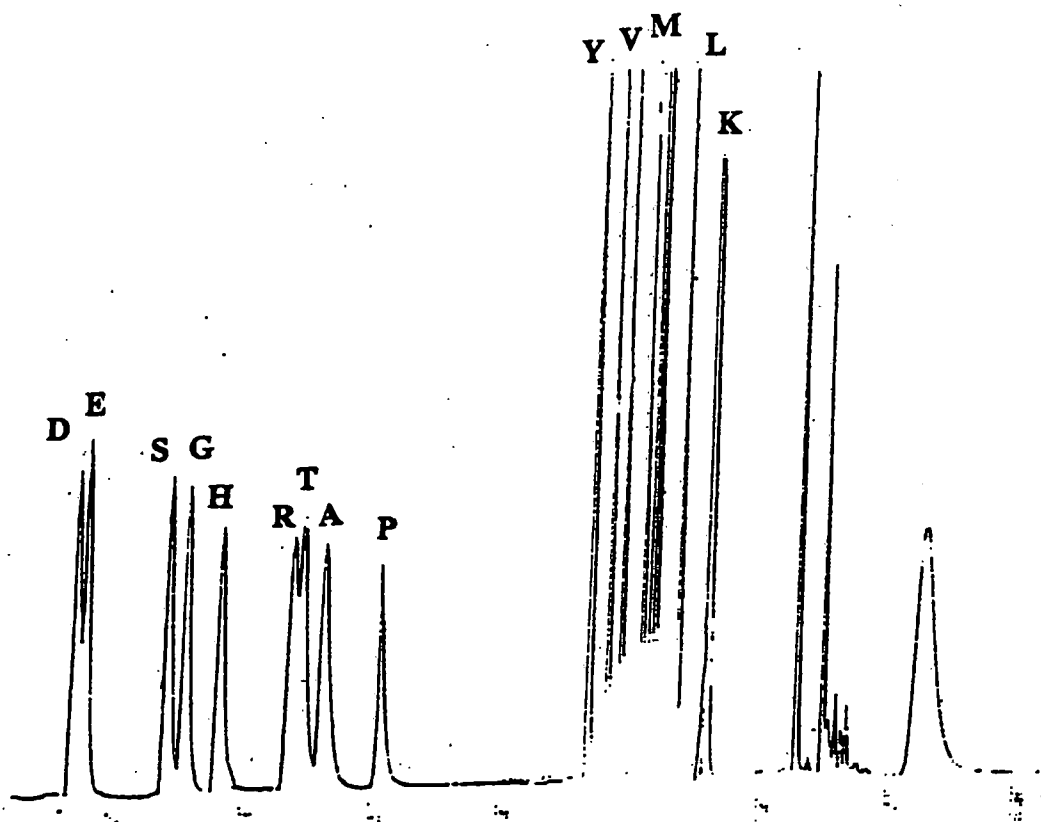


Figure 3.8. A) Table showing the gradient run parameters used in the present investigation; and B) a chromatogram of Pierce amino acid standards. The amino acids are: D=aspartate, E=glutamate, S=serine, G=glycine, H=histidine, R=arginine, T=threonine, A=alanine, P=proline, Y=lysine, V=valine, M=methionine, L=leucine and K=hydroxyproline (GABA was not contained within the Pierce standards).

Qualitative Chromatography

Qualitative chromatography refers to identifying the components of the chromatogram. One method is to refer retention times or volumes to standard values, i.e. comparing the retention volumes of the standard Pierce amino acids to those of the sample chromatogram. This technique may not provide convincing accuracy, and identification is best confirmed by "spiking" the peaks of the sample with additional amino acid. Therefore if there is a peak on the chromatogram that elutes at the same time as the glutamate in the standard sample, to identify the peak further one could add a glutamate standard to the sample and re-inject. If the peak was indeed glutamate it would now be considerably larger than before due to the added standard. Ideally, the spiked sample should be run changing several chromatographic parameters; in all cases the standard and the sample peak should elute at the same time as the amino acid (see Figure 3.9).

Spiking of samples was carried out at regular intervals during analyses, particularly at times following the replacement of mobile phases. Renewal of the mobile phase tended to cause significant changes in the HPLC analysis due to slight changes in pH (due mainly to faulty pH-meter readings) and possibly changes in ACN content.

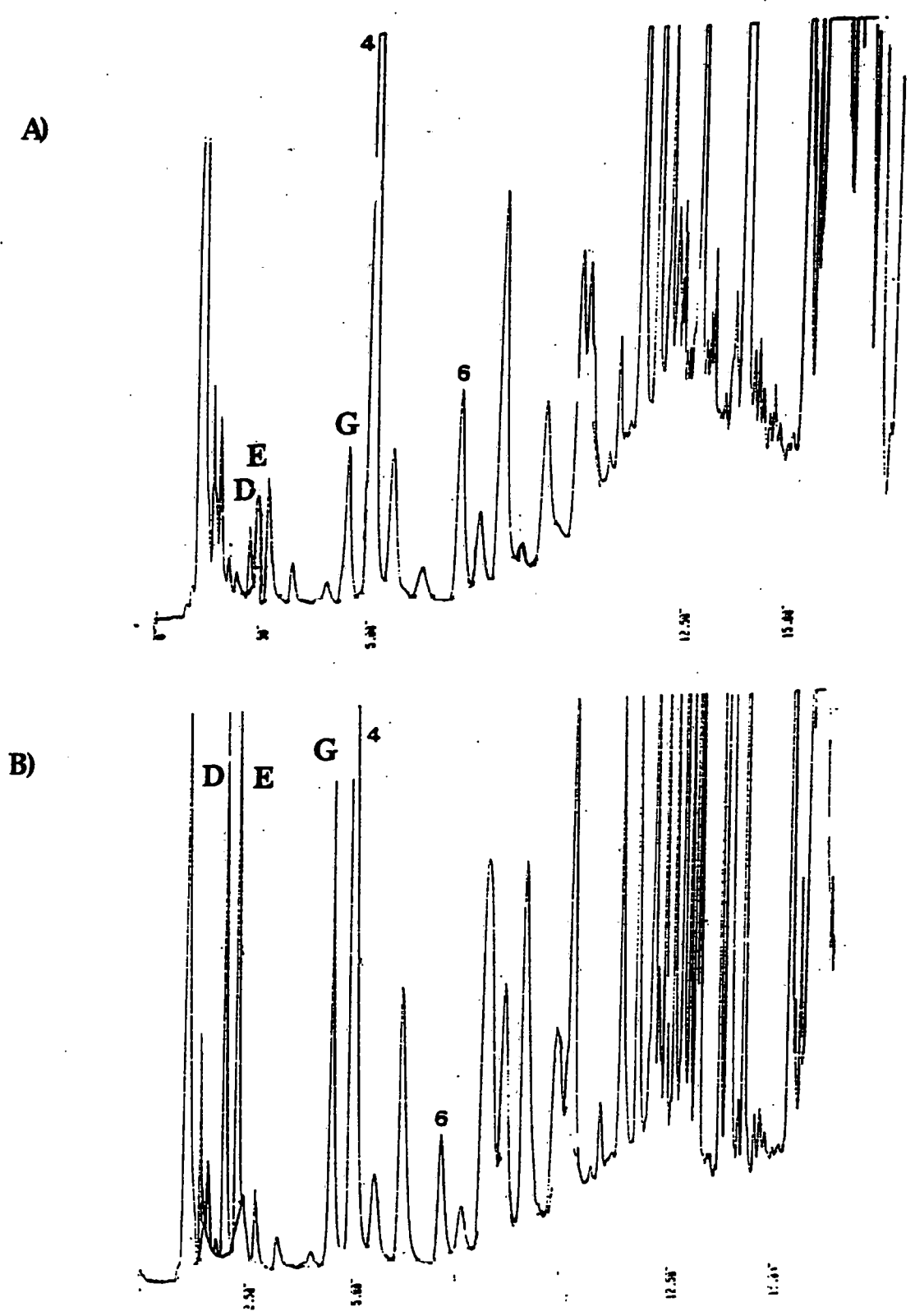


Figure 3.9. Chromatogram of a sample medium (A) without, and (B) with, amino acid standards added to the derivatised sample to demonstrate coelution of the peaks. Peaks D, E and G as defined in Figure 3.8B; 4=glutamine, 6= γ -aminobutyric acid.

Quantitative Chromatography

Quantitative analysis of chromatograms was carried out using a Waters 740 Data-module. Both peak height and peak areas could be determined by mathematical integration. Peak height measurements are more accurate than peak areas as they are less subject to interference by adjacent overlapping peaks. However peak height is more prone to variations in the experimental conditions, as a slight increase in elution time will affect the height. Peak areas are not affected by elution times, although aberrations in the chromatogram such as tailing, shoulder and leading peaks and baseline drift may severely alter the area calculation. Fortunately the Waters Data module has a variety of commands that enable these peaks to be integrated accurately (see Figure 3.10).

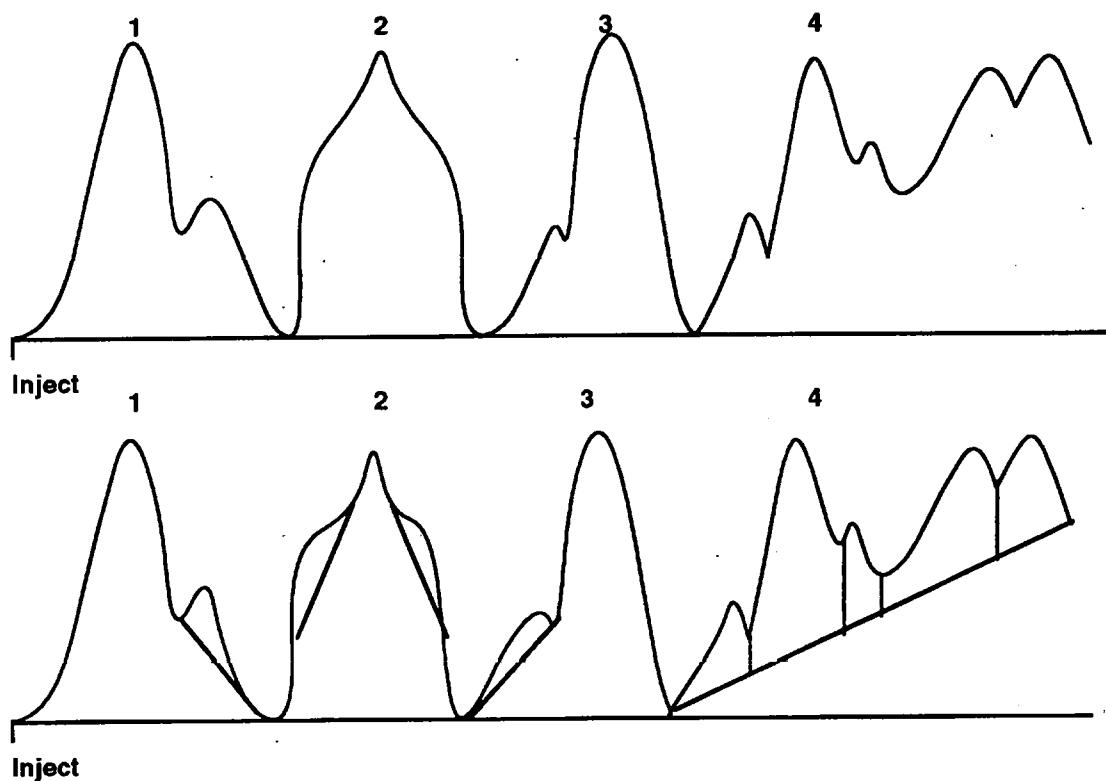


Figure 3.10. a). The diagram above shows an example chromatogram with tailing (1), shoulder (2) and leading (3) peaks and baseline drift (4) before integration. b) Shows the sample chromatogram after integration.

The quantity of compound present was printed out as either area or height in the number of absorbance units adjacent to the retention time of the compound allowing identification of all peaks.

Calibration Methods

External Standardisation

This method involves the construction of a calibration plot using standards made up from a known stock of the amino acid. Pierce produce a solution containing a range of amino acids of known concentration which was diluted with 0.1 N HCl to produce a suitable range of concentrations. A fixed volume of each standard was then injected, and the resulting chromatogram integrated to produce peak heights and areas for each injection. A calibration plot of the resultant peak height or area vs. standard concentration is carried out for each amino acid. In general the calibration plot is almost linear and extrapolates through the origin, although at the highest concentrations a tailing-off becomes apparent. A possible problem with standardisation curves is that, even when measuring peak areas, there may be a significant variance from the curve due to changes in the sample injection volume as even automatic sampling volumes may vary. The Waters 712 automatic sampler can be programmed for increased accuracy when carrying out injections, which was generally employed when injecting small sample volumes (5 to 20 μ l) and standards to minimise the possible variance. In addition, standards were injected after every change in mobile phase and before any prolonged group of injections (although this was not on a daily basis).

Internal Standardisation

An additional method of determining relative concentrations between differing chromatograms is by the addition of an equivalent concentration of a compound, that is not itself contained in the sample, to each sample under investigation. From this a ratio of each sample peak to the internal standard peak can be found. In addition, separate chromatograms can be compared when the levels of internal standard should be equal but for some reason (e.g. injection volume may have changed) are different.

The criteria for a suitable internal standard include: that it should be of similar structure to the component of interest, it should be completely resolved from other components in the sample and that it should be stable and of high purity.

For amino acid analysis the internal standard should be a non-physiological amino acid. Both l-homoarginine and l-norleucine were used in this thesis as the internal standards. All data in the release experiments were determined relative to the internal standard used.

By using standardisation techniques it was possible to calculate the amount of amino acid present in the injected sample and to extrapolate this to find the total amount of amino acid present in the original sample. For example if 50 μ l of the total 100 μ l of the sample diluant is injected, and the derivatised sample was 50 μ l of the medium (from 1000 μ l original incubation medium), then this is equivalent to analysing 25 μ l of the original (50 x 50/100 μ l). The total amino acid released from the tissue into the medium is 40 times (i.e. 1000/25) the level found in the chromatogram.

GABA analysis

The pH is of particular importance as regards the separation of GABA. If the pH is too low the GABA peak is poorly resolved from that of citrulline (Cohen and Strydom, 1988). The current methodology, with a pH of 6.8, allows a good separation of GABA (see Figure 3.9).

A problem encountered with the analysis of GABA when using a different derivatisation procedure, most notably OPA-derivatisation, is the lack of stability of the derivatised amino acid. OPA produces derivatives that are chemically unstable and this technique, therefore, requires rapid production of the derivative and reproducible timing to obtain accurate results (Reynolds and Pearson, 1993). According to Lasley et al. (1984) the speed of derivatisation process was vital for GABA and glycine derivatives since both exhibited a half-life of 4 minutes only, which would inevitably result in the degradation of the derivative on the column. PITC-derivatives of GABA on the other hand, are very stable and show similar characteristics to the other amino acid derivatives and will produce reproducible results even 1 week following storage in a freezer (Cohen and Strydom, 1988).

In addition, problems have been found in the yield of the amino acid following filtration with certain kinds of membrane. The present methodology did not employ the use of any filtration with centrifugation being the only method for removing unwanted particulates, and the amounts of GABA found following the chromatography used in the present study were in general agreement with others (e.g. Ghisjen et al., 1992, Levi and Morisi, 1979) in both the actual levels and levels relative to the other amino acids.

Adenosine analysis

During the course of the thesis it was found necessary and appropriate to analyse the levels of the nucleoside adenosine from the chick brain media. The methodology used for amino acid analysis, as described above, allowed satisfactory detection of adenosine. However, problems were found due to some co-elution of the purine with derivatised amino acids, specifically glutamine and glycine. Therefore, it was decided to use a different chromatography.

Nucleosides can be separated using reversed-phase systems employed with a methanol/buffer gradient (Hartwick and Brown, 1976). Since they lack the charged phosphates of the nucleotides, nucleosides such as adenosine can be separated on C18 packings, such as the Novapak system used for the analysis of amino acids in the present study.

Nucleosides are strong chromophores, absorbing light in the u.v. spectrum between 240 and 270nm (Perrett, 1986). The u.v. detector used for the analysis of amino acids had a wavelength that coincided with this frequency at 254nm and could therefore be readily applied to adenosine analysis.

A suitable chromatography was described by Chen et al. (1992) and was used as follows. A mobile phase of 0.01M NaH_2PO_4 with 6% methanol (pH 6.1) was prepared and de-gassed with helium. The eluant was pumped at a flow rate of 0.8 ml/min and was used as an isocratic system (i.e. no change in buffer or flow rate was carried out in the analysis). No sample preparation was carried out other than centrifugation (8000rpm for 4 minutes) of the medium. The limit of sensitivity of the system for adenosine was 5 picomoles. Figure 3.11. shows a typical chromatogram of an adenosine standard using this methodology. A peak that had a retention time equivalent to the adenosine standard was found to elute at ca. 6.8 minutes after injection in to the HPLC.

Subsequent 'spiking' of the sample with adenosine standard demonstrated that this peak was indeed adenosine. Quantification was carried out using an external standardisation procedure (calibration of adenosine standards).

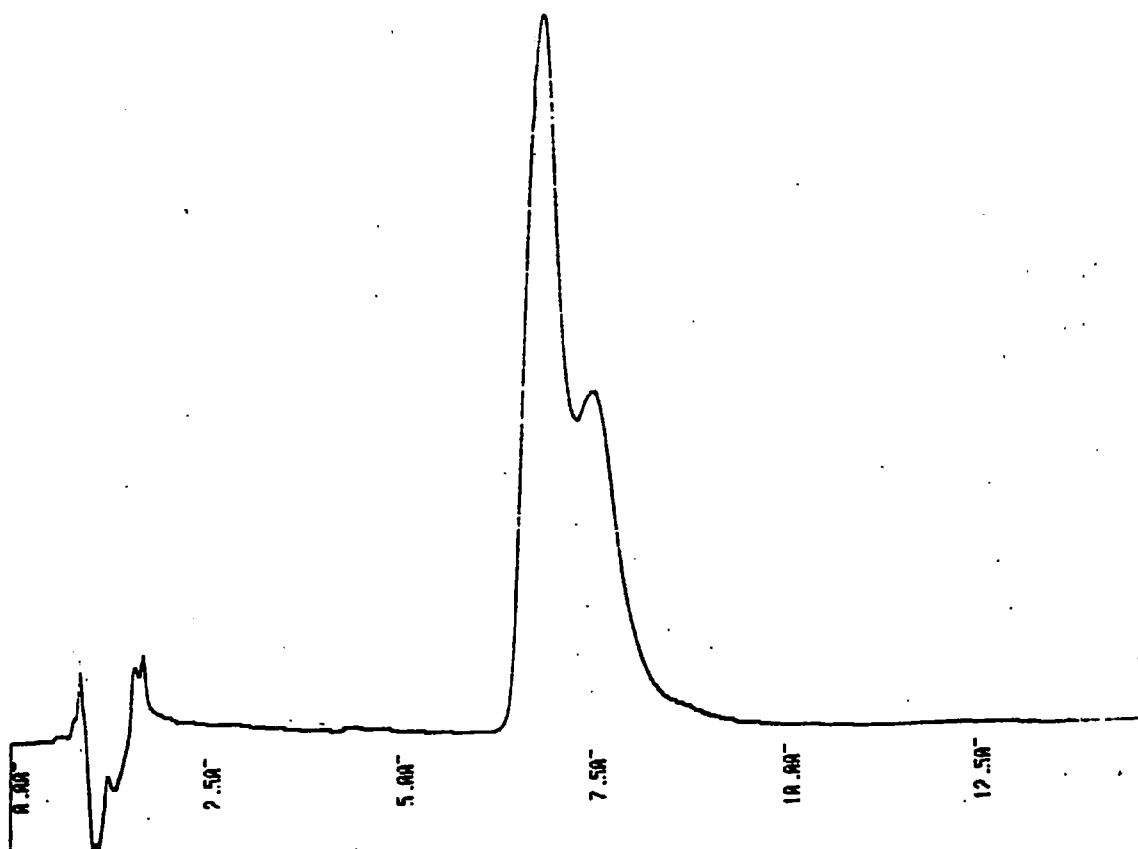


Figure 3.11. Chromatogram of an adenosine standard using the method for adenosine analysis described in the text.

Chapter 4: The Release and Uptake of Amino acid Transmitters from the IMHV of the day-old Chick following a Passive Avoidance Task

Introduction

The IMHV of the day-old chick, as has been outlined in the Chapter 2, is a site of neuronal activity and change following the passive avoidance task. In this region there are a range of synaptic events associated in the biochemical cascade following passive avoidance learning. Amongst the early necessary steps towards the formation of memory and its recall is that of neurotransmitter release. It was decided to look at the release of putative amino acid transmitters from areas of the chick brain thought to be involved in learning and memory, namely the IMHV (Chapter 4) and lobus parolfactorius (LPO; Chapter 5). A role for the amino acid neurotransmitters, glutamate and GABA, and their receptors has been suggested in passive avoidance training (e.g. Bullock et al., 1993; and Clements and Bourne, 1995).

The experiments were carried out as a longitudinal study in order to look at the time course of release of amino acids from the chick IMHV. A time course approach was taken as previous evidence from other biochemical studies had suggested that the biochemical events may occur in 'waves' (see Chapter 2), and a study at specific time points would allow comparison and correlation with other events at the time points taken.

Materials and Methods

Ross Chunky chicks of both sexes were held in a communal incubator on a 12-hr light/12-hr dark cycle at 38-40°C until they were 24 (\pm 6) hrs old. The chicks were then removed and placed in pairs in metal pens (size: 20x25x20 cm) each illuminated with a 25-W red light. They were allowed to acclimatise to this experimental environment at 28-30°C. Sixty minutes later the chicks were exposed to a pre-training trial with a small white bead (2mm in diameter) attached to a perspex rod. The bead was introduced into the pen for up to 20 seconds. During this time the bead was presented to each chick, and whether or not the chick pecked at the bead was recorded.

This pre-training trial was followed by two similar pre-training trials, each approximately five minutes apart. Chicks that did not peck on at least two of the three presentations were not trained. The training trial commenced at least 10 minutes after the third presentation. A chrome bead (4mm in diameter) was presented to each chick for 10 seconds. This bead was, according to the test conditions, dipped in either water (denoted W) or methylanthranilate solution (MeA). Again the pecking response of the chicks was noted. Those that peck at the MeA bead generally exhibited a strong disgust response; they usually closed their eyes, shook their heads and wiped their beaks on the ground in an effort to remove remaining MeA. Those that did not peck on training (generally < 10%) were eliminated from the experiment and did not contribute to the data or analyses at the end of the experiment.

A test for recall of the task was carried out after training, at times between 10 minutes and 24 hours. The test consisted of a 10 second presentation of a dry chrome bead identical to that used in training. It was noted whether the chick pecked or not, and the latency to first peck. The number of pecks made at the bead was also noted in some cases. Chicks that

did not peck were called "avoid" and chicks that did were called "peck". This classification is useful for behavioural pharmacology, where due to the large number of chicks that have to be used and the statistics that have to be carried out, they are by necessity classified into just these two groups; however for the biochemical work the MeA chicks that avoided the chrome test bead were used only if they performed a "head shake" or "beak-wipe" response as exhibited when they trained on the MeA bead itself. This eliminated those chicks that, although they avoided the bead, may not actually have learned the task: many groups of chicks became extremely anxious due to noise or other disturbance over the experimental period and would avoid the bead without perhaps having learned the task.

Dissection procedure

All chicks were killed swiftly by decapitation using heavy scissors. The feathers and tissue on the crown were then trimmed away with a lighter pair of scissors to expose the skull. One blade of the scissors, which was directed upwards, was inserted beneath the skull just above a suture line near the orbital socket. A cut was made along this suture line anteriorly to the right orbital socket, then posteriorly to the foramen magnum and back to the left orbital so that an oval of bone could be removed with tweezers to expose the brain. Next a spatula was inserted anteriorly between the eye sockets and the brain itself and shifted under the forebrain. The forebrain, including optic tecta, could be extracted from the skull. For dissection of specific forebrain areas the brain was placed in a specialised brain mould (Figure 4.1). The areas required were selected by slicing the brain in the mould at points A and B (for IMHV) and for points B and C (for LPO). The brain area was then dissected out by scalpel and placed into a vial containing pre-incubation medium until used for the experiment.

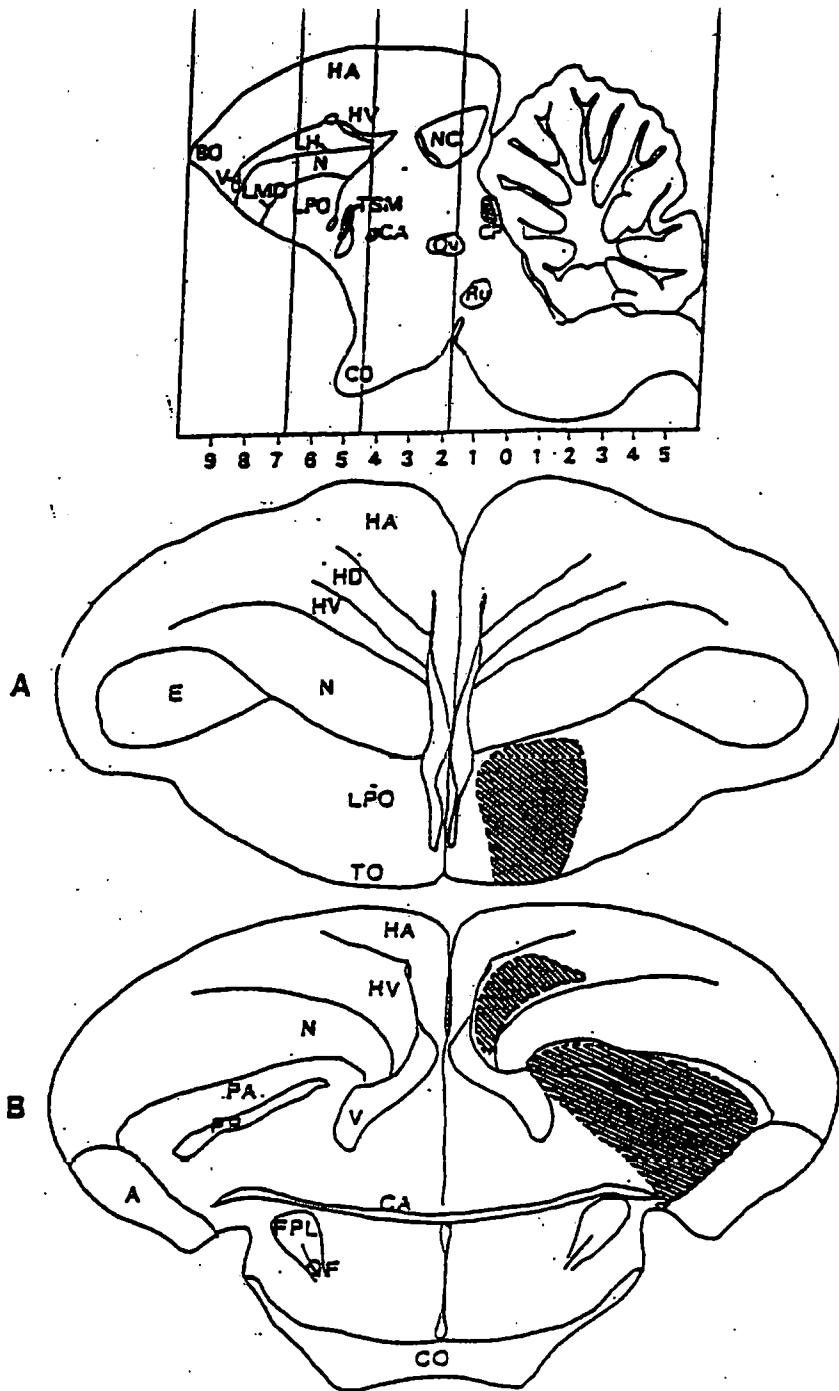


Figure 4.1. Schematic drawings of coronal slices of day-old chick brains prepared using a brain mould. The position and angle of cuts are shown in the sagittal scheme. The first slice, A (taken at level "a") was used for dissecting LPO and the second, B (taken at level "b") was used for dissecting PA and MHV (after Bullock et al., 1987).

Release procedure

Tissues were prepared on a McIlwain chopper, with slices produced by a single cut of 350 μ m. The tissue was collected in 95% O₂-5% CO₂ gassed Krebs-Henseleit buffer. Composition of buffer in mM: NaCl 118, KCl 4.7, CaCl₂·2H₂O 1.3, MgSO₄·7H₂O 1.2, NaHCO₃ 25, glucose 10 and KH₂PO₄ 1.2, at pH 7.4 and a temperature of 37°C.

The slices were then pre-incubated in this gassed medium for 60 minutes whilst being shaken at 100 strokes per minute in a water bath: the pre-incubation buffer was replaced at 20 and 40 minutes with fresh Krebs-Henseleit: the IMHVs were combined from 3 or 4 chicks according to the treatment and the hemisphere, such that 3 or 4 left IMHVs of MeA-trained chicks were incubated together, left IMHVs of water-trained chicks were also incubated together etc.. Following preincubation, the slices were then separated such that roughly equal quantities of the combined tissue were placed in vials containing 1ml of one of four media, measuring: the total basal release (Krebs-Henseleit buffer); calcium-independent basal release (a Krebs-Henseleit buffer with the CaCl₂ replaced by 2mM EGTA), the calcium-independent potassium stimulated release (a Krebs-Henseleit buffer with 50mM KCl and an equimolar reduction in NaCl concentration and 2mM EGTA); and the total stimulated release (a Krebs-Henseleit buffer containing CaCl₂ and 50mM KCl).

The tissues were incubated in 1ml of these media for 2 minutes, following which 200 μ l of the media from each vial were collected and frozen immediately, with protein determinations taken of the tissue.

Protein Determinations

A Biorad-protein assay (Bradford, 1976) was used to measure protein levels in the tissues assayed. For this 100mg Coomassie brilliant blue G-250 was dissolved in 50ml of 95% ethanol. To this 100ml of 85% (w/v) phosphoric acid was added. This solution was then diluted to 1 litre with distilled water to give a final concentration of the reagents: Coomassie blue (0.01%), ethanol (47%) and phosphoric acid (8.5%), all v/v.

Samples were prepared by centrifugation (8,000 rpm for 5 minutes), followed by the removal of the supernatant and subsequent addition of a known amount of distilled water to dilute the sample such that the 10 μ l taken for analysis would contain a level of protein within a range of prepared standards. Following addition of water, the sample was homogenised and then sonicated at medium power for 20 seconds; the sample was now ready for protein analysis. The microprotein assay consisted of the following: 30 μ l 0.1M NaOH was transferred to each well on a micro protein plate. To this was added 10 μ l of the standard solution or the prepared sample. Finally 250 μ l of the Bradford dye was added to each well and after 10-15 minutes the absorbance at 595nm was measured and compared with a range of standards of bovine serum albumin. All analyses were carried out on the HPLC following pre-column derivatisation with phenylisothiocyanate (see Chapter 3). Both hemispheres were measured to provide data on any lateralisation effects.

Statistical treatment of data

HPLC analysis was carried out on each sample at least 4 times in order to determine the variance in the sample, and between each sample. This provided data for nested-ANOVAs (carried out on Statview). The statistical significance of the differences between the release values from MeA-trained and water-trained chicks was calculated. Data were expressed as means \pm S.E.M. values of n experiments. The significance level was set at $p < 0.05$.

Experiment 4.1. The time course of the release of amino acids from the intermediate medial hyperstriatum ventrale (IMHV) of the day-old chick following a one-trial passive avoidance task

Results

Basal release of amino acids

The basal release of glutamate, aspartate, glycine and GABA from the left and right IMHVs of day-old chicks, as measured following a 2 minute incubation in Krebs-Henseleit buffer only, was determined 10, 30 minutes, 1, 3 and 6.5 hours following training on the passive avoidance task (n= for MeA-trained chicks 8,7,6,6,9 at these times respectively), and compared with that for birds trained on the water bead (n= 7,7,7,7,8 respectively).

No differences were found in the basal release of any of the four amino acids measured between the combined data of the two groups (MeA v W; ANOVA, all $p > 0.2$). The results shown in Figure 4.2 are the combined data of left and right IMHVs.

Calcium dependency of release

The calcium-independent (K^+ -stimulated Ca^{2+} -free minus normal K^+ , Ca^{2+} -free medium) data are described in Figures 4.3 for left IMHV and in Figure 4.4. for the right IMHV. The numbers of chicks used at each time point and for each hemisphere are as follows: for the left IMHV n= for MeA, 7,8,7,9,6; for water-trained, 8,7,9,11,7; at 10 and 30 minutes and 1, 3 and 6.5 hours post-training respectively: in the right IMHV, n= for MeA, 7,7,8,8,8; for water-trained, 7,7,9,8,7 at these times respectively. No differences were noted between the groups in the calcium-independent component of release of glutamate, aspartate or GABA in the left or right IMHVs ($p > 0.20$). There was, however, a significant increase in glycine release in the left IMHV of MeA-trained birds 30 minutes after training ($F=5.924$ $p=0.032$ $df=13$).

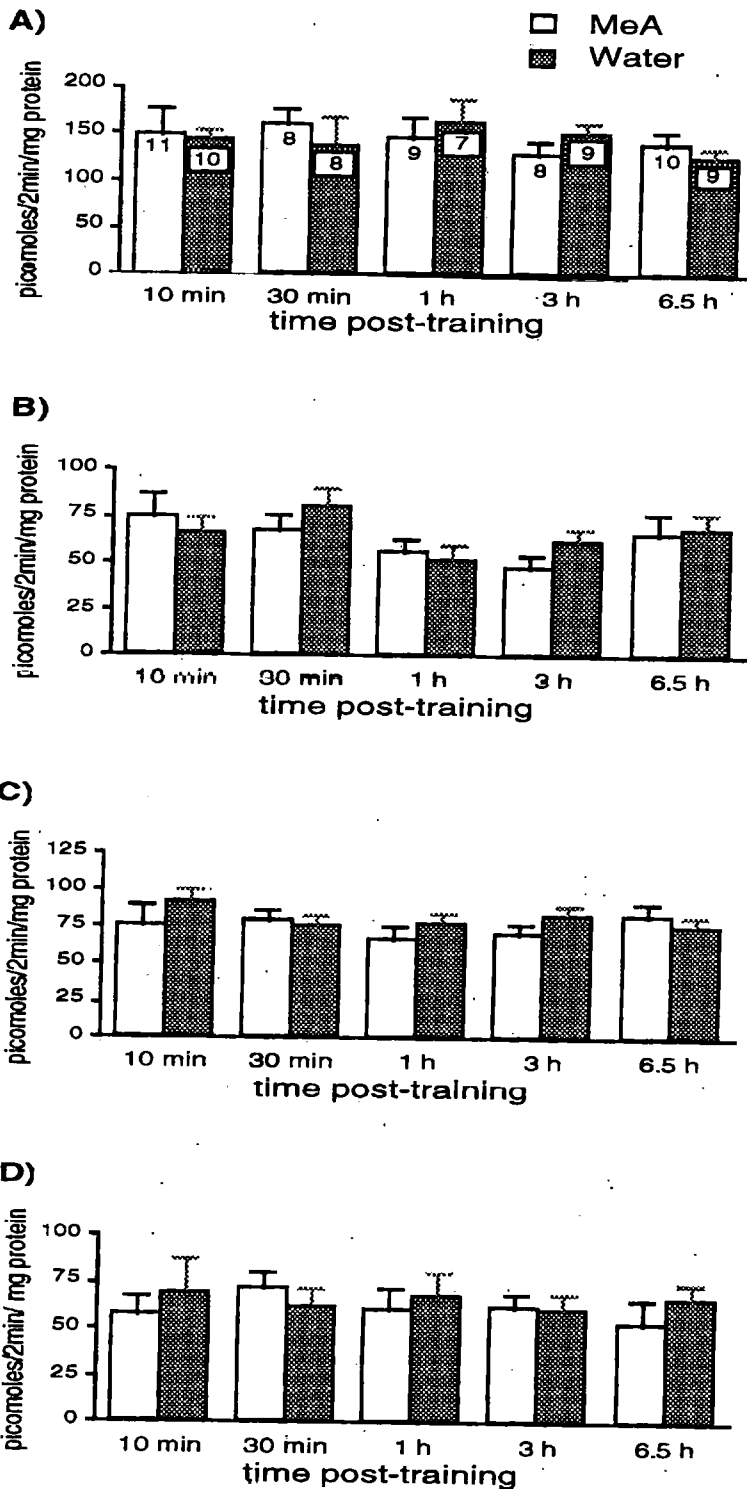


Figure 4.2. The effect of a passive avoidance task on the basal release of (A) glutamate, (B) aspartate, (C) GABA and (D) glycine from the combined left and right IMHVs of day-old chicks, 10 and 30 minutes, 1, 3 and 6.5 hours after training: picomoles amino acid released per 2 minutes per mg protein. The sample sizes for each group are the same as those indicated in the bars showing glutamate release. Error bars are means \pm S.E.M.

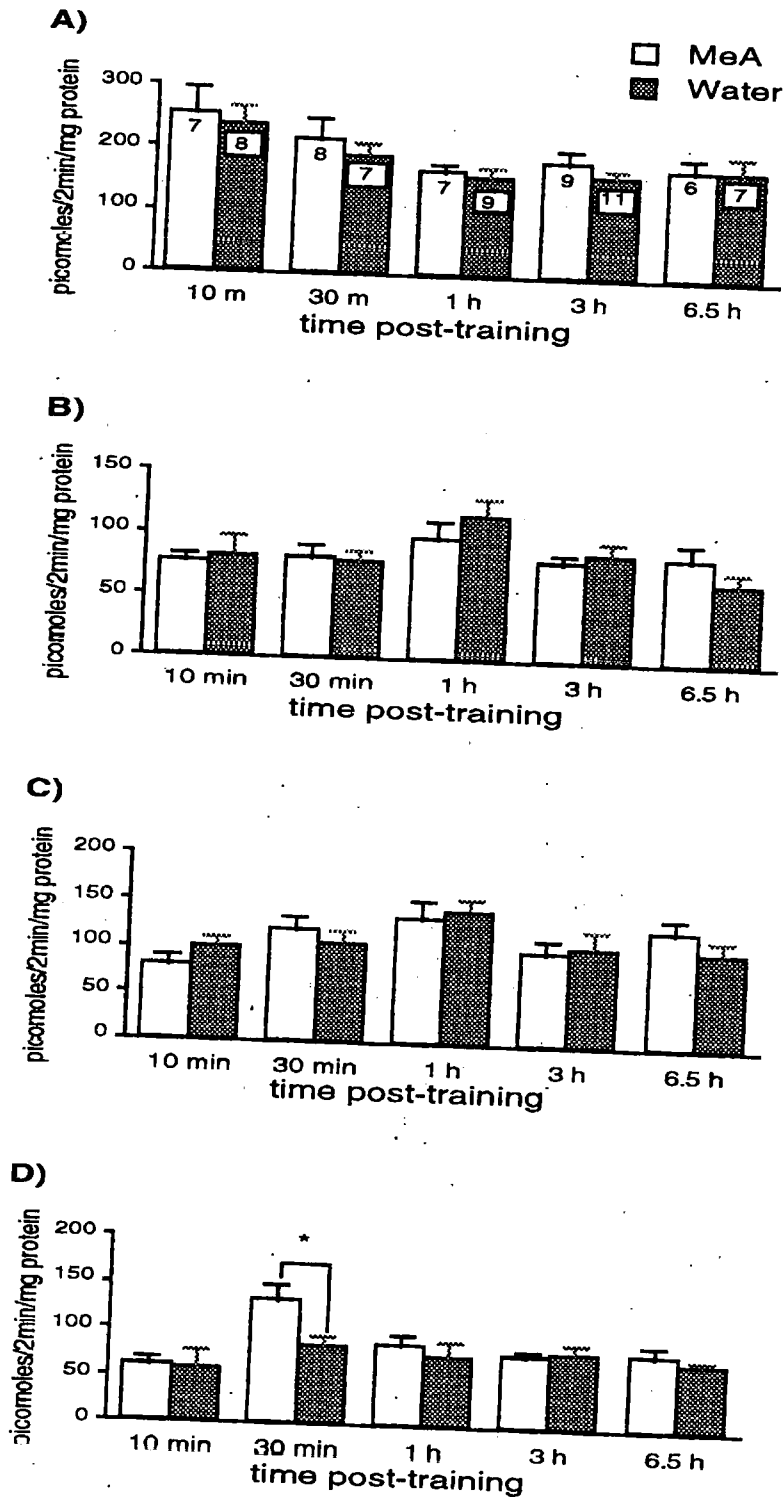


Figure 4.3. The effect of a passive avoidance task on the calcium-independent release of (A) glutamate, (B) aspartate, (C) GABA and (D) glycine from the left IMHVs of day-old chicks, 10 and 30 minutes, 1, 3 and 6.5 hours after training: picomoles amino acid released per 2 minutes per mg protein. The sample sizes for each group are the same as those indicated in the bars showing glutamate release. An asterisk * indicates a significant difference between MeA and water-trained chicks ($p < 0.05$, ANOVA). Error bars are means \pm S.E.M.

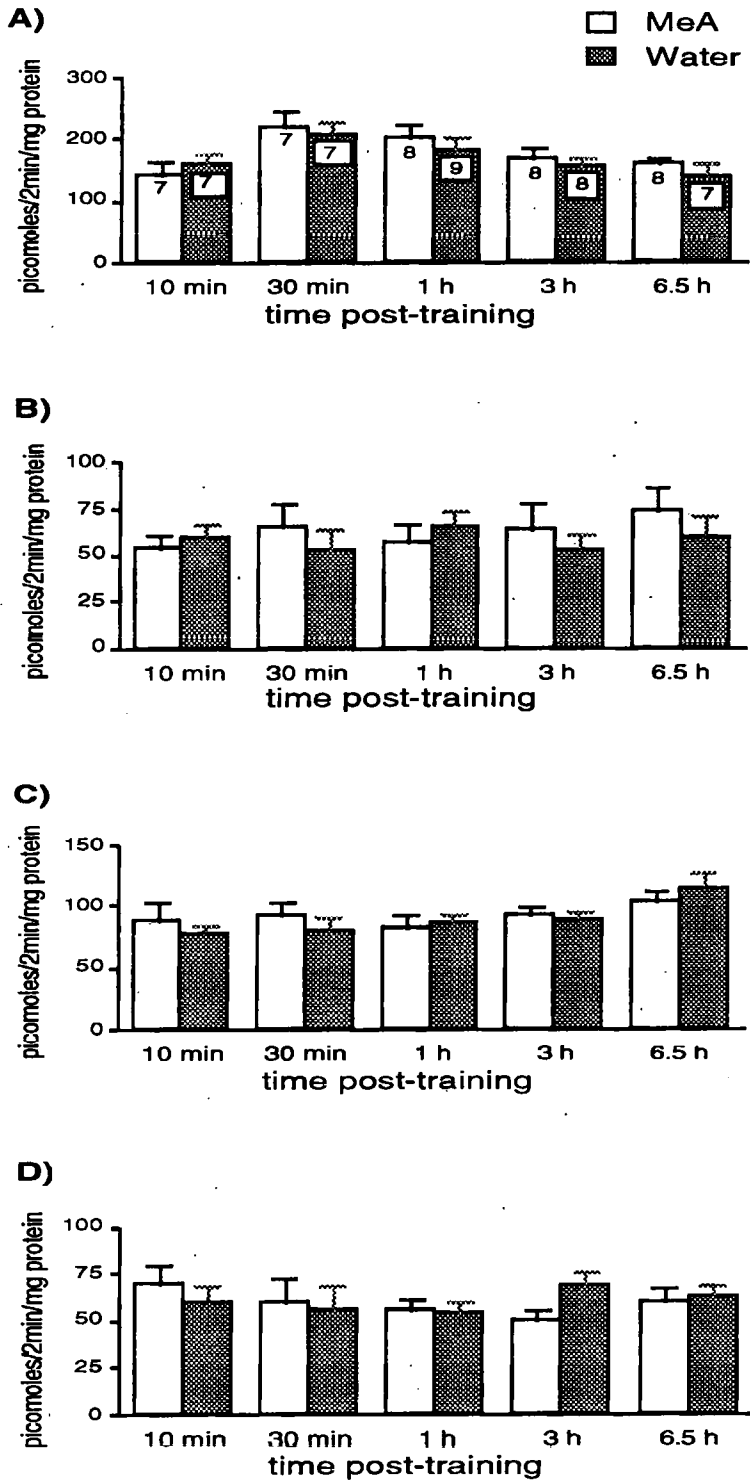


Figure 4.4. The effect of a passive avoidance task on the calcium-independent release of (A) glutamate, (B) aspartate, (C) GABA and (D) glycine from the right IMHVs of day-old chicks, 10 and 30 minutes, 1, 3 and 6.5 hours after training: picomoles amino acid released per 2 minutes per mg protein. The sample sizes for each group are the same as those indicated in the bars showing glutamate release. Error bars are means \pm S.E.M.

The K⁺-induced Ca²⁺-dependent release component was estimated by subtracting the Ca²⁺-independent release values from the total K⁺-induced release in the presence of Ca²⁺; the final figure being the calcium-dependent release.

Thirty minutes after training there was a significant increase in the calcium-dependent release of glutamate in the left IMHV of MeA-trained compared to water-trained chicks (Figure 4.5, F=5.78 p=0.033 df=13). The elevated glutamate release was still apparent at 1 hour in the left IMHV (F=7.05 p=0.019 df=14). The 3 and 6.5 hour samples did not show any differences in release of the amino acid as compared to the controls in the left IMHV (p > 0.05).

The right IMHVs of MeA-trained chicks showed increased calcium-dependent increases in glutamate release at 30 minutes (F=4.891 p=0.046 df=12), 3 hours (F=8.448 p=0.013 df=14) and at 6.5 hours (F=10.294 p=0.0075 df=12) after training, compared to the water controls, but not at 10 minutes or at one hour post-training.

Significant differences were also found between the hemispheres: 1 hour post-training there was an increase in the release of glutamate in the left compared to the right IMHVs of MeA-trained chicks (F=8.433 p=0.013 df=13), and at 3 hours the right hemisphere showed an increase in the release of glutamate compared to the left (F=5.039 p=0.0441 df=14).

Calcium-dependent aspartate release showed an increase in MeA-trained chicks at the 1 hour point in the left IMHV (Figure 4.6, F=7.121 p=0.018 df=13). Aspartate release was significantly higher in the right IMHV than the left of MeA-trained birds 3 hours after training (F=6.433 p=0.0211 df=15). Also at 3 hours there was an increase in aspartate release in trained birds compared to water controls in the right IMHV (F=6.016 p=0.0269 df=14). No differences were found in water-trained chicks (p>0.05).

One hour after training there was also an increase in **GABA** release from the left IMHV of MeA-trained chicks compared to W-trained birds (Figure 4.7; $F=15.577$ $p=0.0017$ $df=13$). This increase was also evident when comparing the left and right IMHVs of MeA-trained chicks with a significantly greater release (186 %) in the left at one hour ($F=15.590$ $p=0.0017$ $df=13$). GABA release was also increased in MeA birds at 6.5 hours in the right IMHV as compared to the water controls ($F=8.478$ $p=0.0121$ $df=13$).

Calcium-dependent release of **glycine** was also measured; no changes at any time in the left or right IMHV of either group were demonstrated ($p > 0.05$; see Figure 4.8).

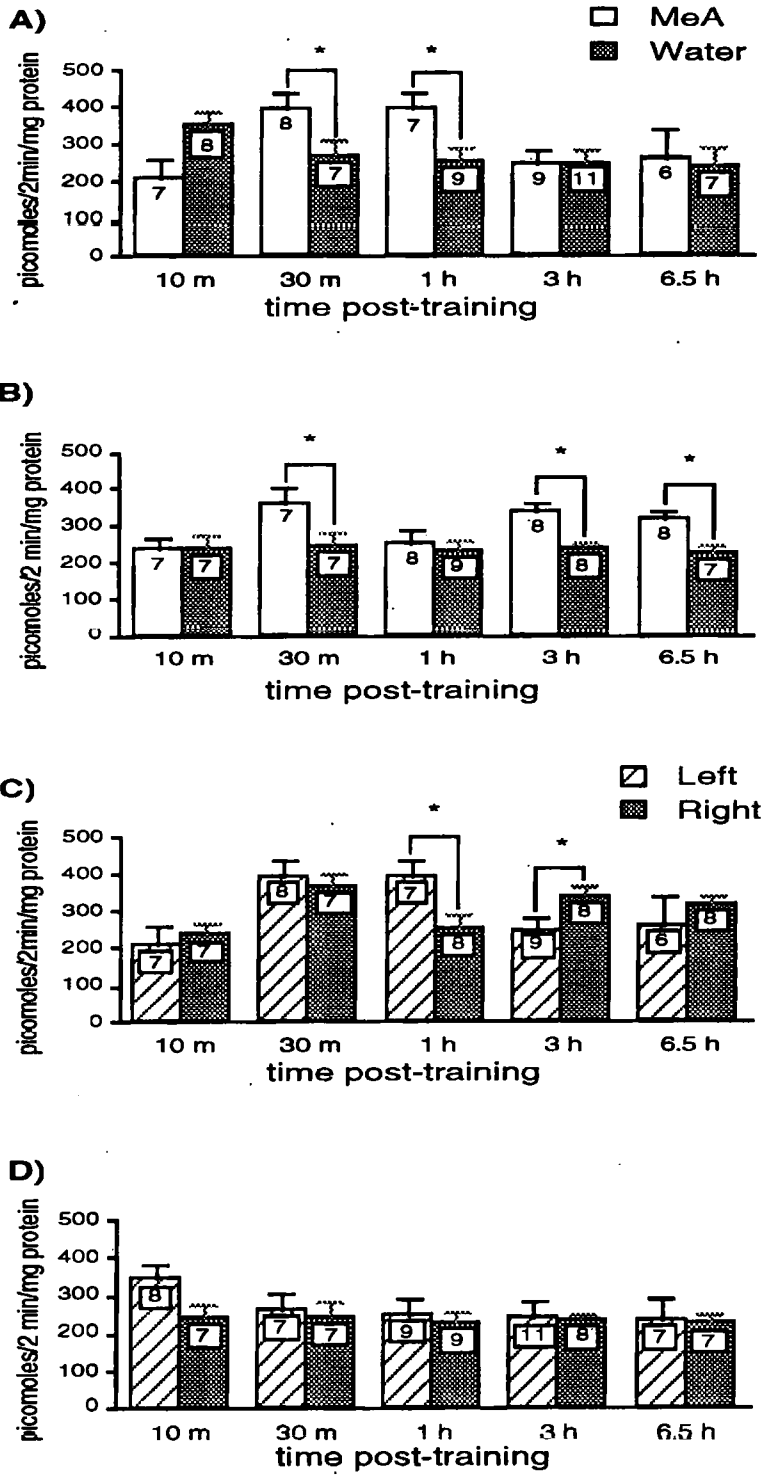


Figure 4.5. The effect of a passive avoidance task on the calcium-dependent release of glutamate from the left (A) and right (B) IMHVs of MeA-trained and water controls; and a comparison between the release from the left and right IMHVs of MeA-trained (C) and water control (D) chicks over time: picomoles amino acid released per 2 minutes per mg protein. The sample sizes for each group are indicated in the bars. An asterisk * indicates a significant difference between MeA and water-trained or left v. right IMHV ($P < 0.05$, ANOVA). Error bars are means \pm S.E.M.

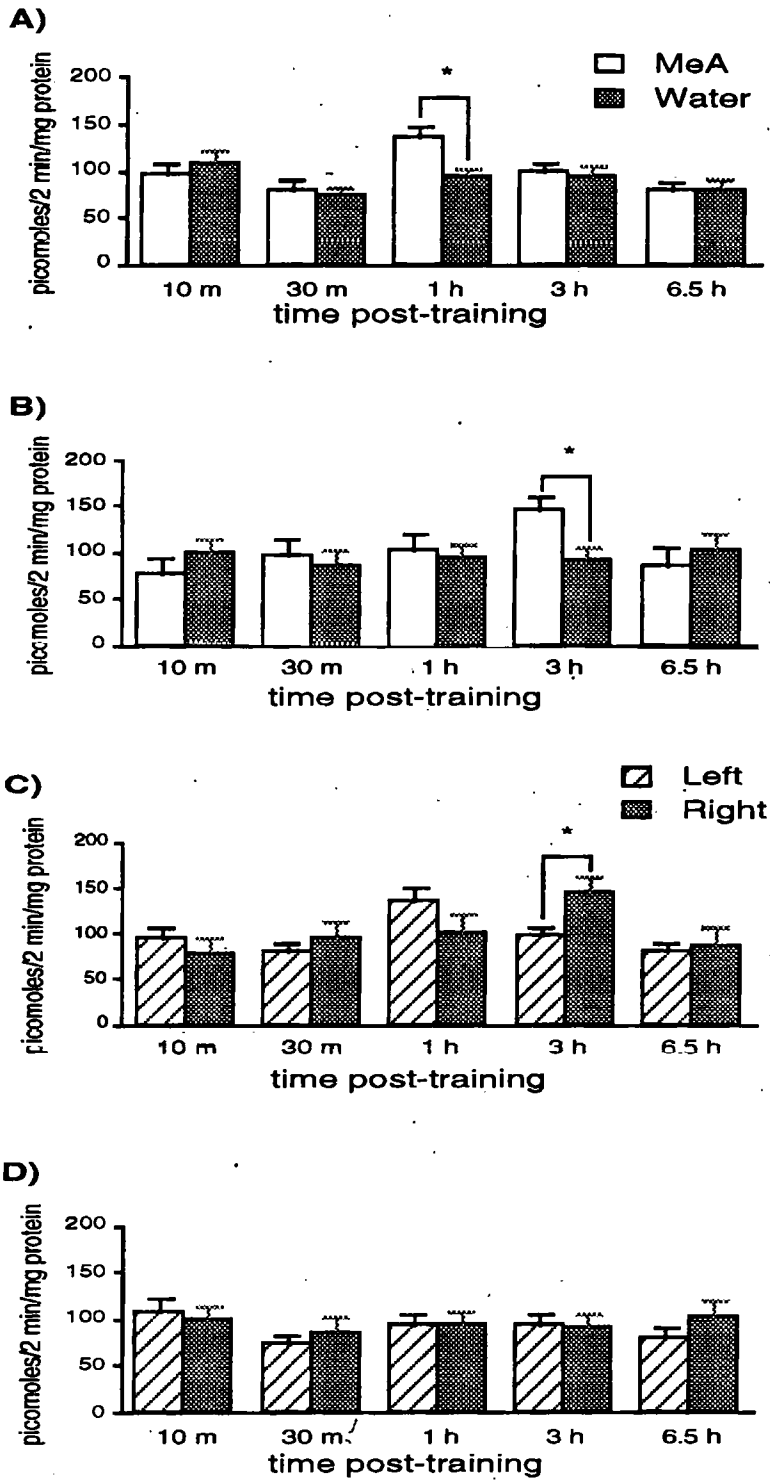


Figure 4.6. The effect of a passive avoidance task on the calcium-dependent release of aspartate from the left (A) and right (B) IMHVs of MeA-trained and water controls; and a comparison between the release from the left and right IMHVs of MeA-trained (C) and water control (D) chicks over time: picomoles amino acid released per 2 minutes per mg protein. The sample sizes for each group are as Figure 4.5. An asterisk * indicates a significant difference between MeA and water-trained or left v. right IMHV ($P < 0.05$, ANOVA). Error bars are means \pm S.E.M.

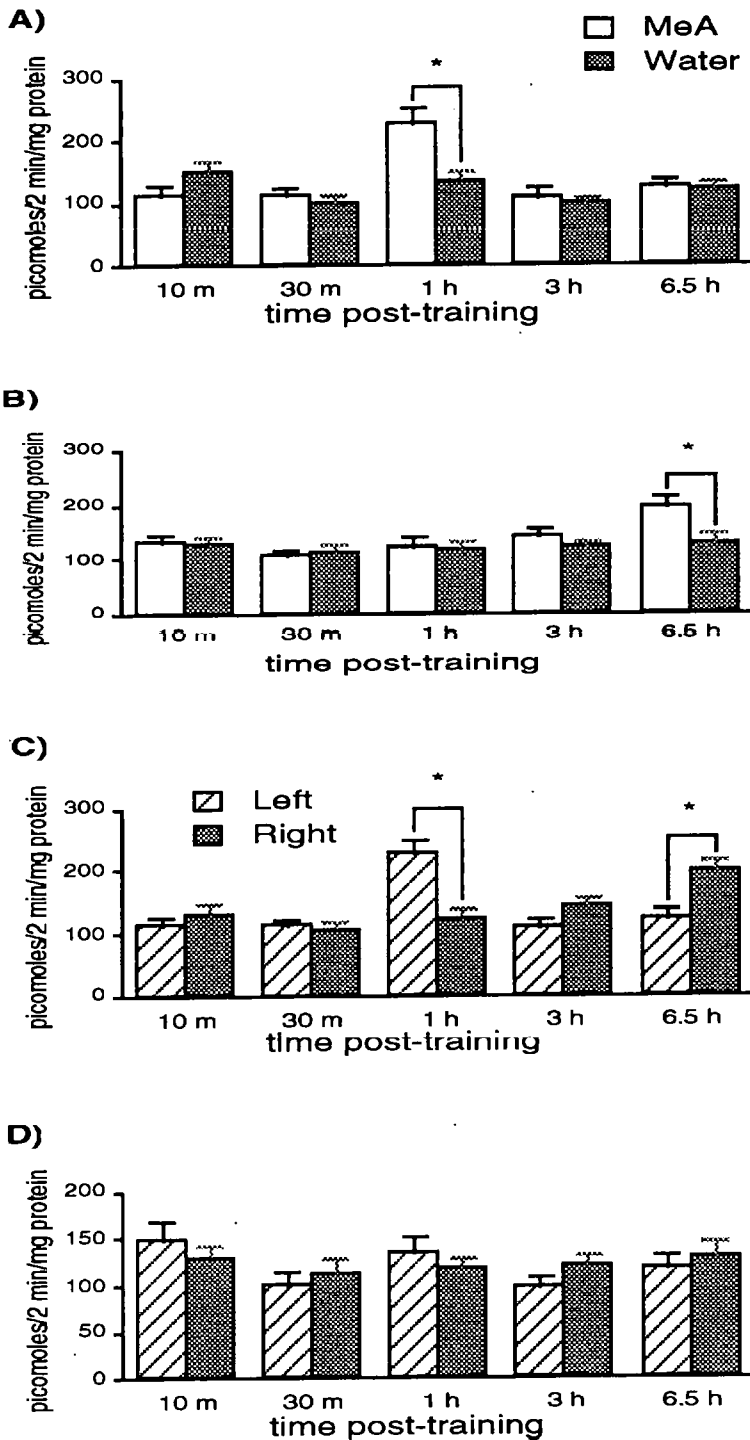


Figure 4.7. The effect of a passive avoidance task on the calcium-dependent release of GABA from the left (A) and right (B) IMHVs of MeA-trained and water controls; and a comparison between the release from the left and right IMHVs of MeA-trained (C) and water control (D) chicks over time: picomoles amino acid released per 2 minutes per mg protein. The sample sizes for each group are as Figure 4.5. An asterisk * indicates a significant difference between MeA and water-trained or left v. right IMHV ($p < 0.05$, ANOVA). Error bars are means \pm S.E.M.

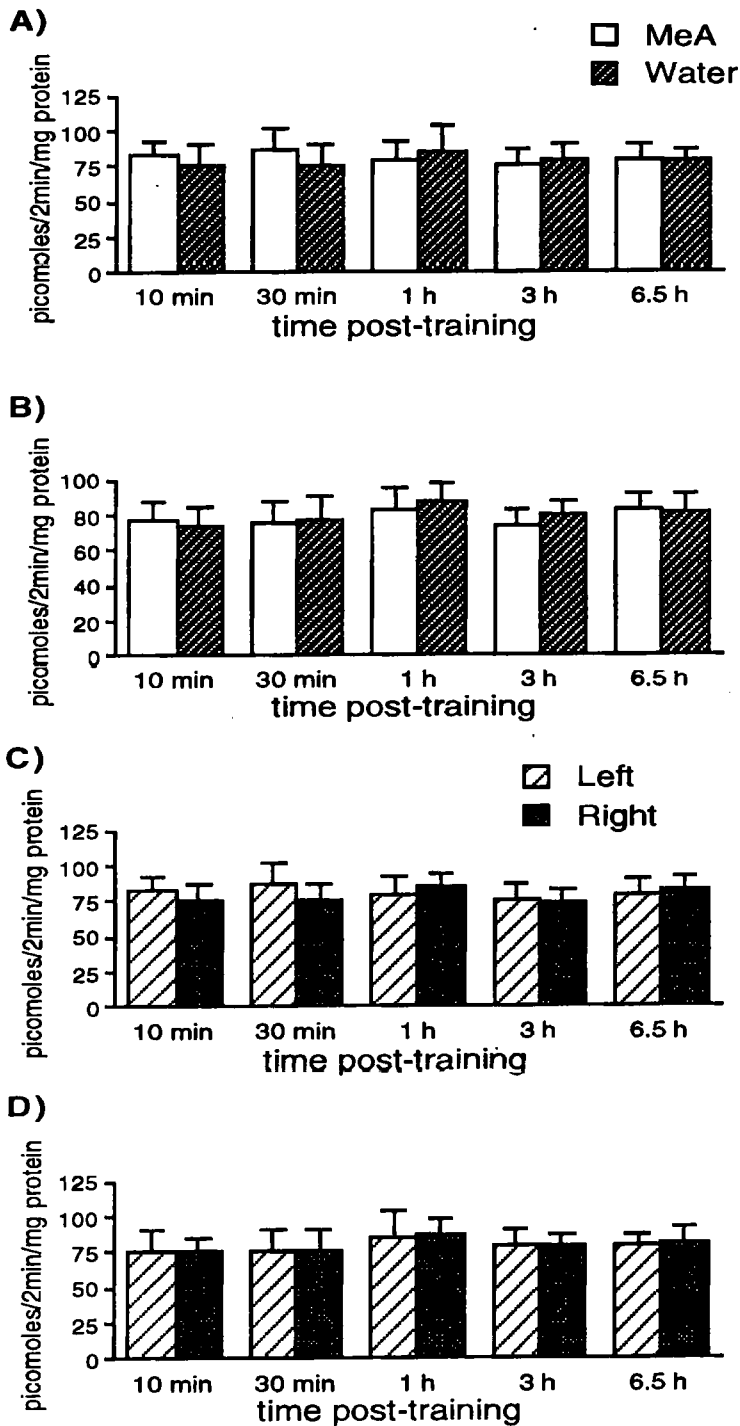


Figure 4.8. The effect of a passive avoidance task on the calcium-dependent release of glycine from the left (A) and right (B) IMHVs of MeA-trained and water controls; and a comparison between the release from the left and right IMHVs of MeA-trained (C) and water control (D) chicks over time: picomoles amino acid released per 2 minutes per mg protein. The sample sizes for each group are as Figure 4.5. Error bars are means \pm S.E.M.

Experiment 4.2. The uptake of glutamate in the IMHV of the day-old chick following a passive avoidance task

Introduction

In section 4.1 the release of glutamate was found to be increased 30 minutes after training in the left and right IMHVs of MeA chicks and one hour after training in the left IMHV. It was decided to look at the uptake of glutamate at these time points and also at 15 minutes post-training. Uptake, as has already been described in Chapter 1, is an essential requirement to terminate the effect of amino acid transmitters and, in the case of glutamate, to keep the extracellular concentration below neurotoxic levels (Bouvier et al., 1992). It is proposed that as the release was increased following training, a concomitant increase in the uptake might occur to compensate for this. Increased glutamate uptake would, therefore, be hypothesised to occur in the left IMHV at 30 and 60 minutes and at 30 minutes in the right IMHV, and the following experiments were carried out to test this.

The present study used slices of chick IMHV, allowing both the glial and neuronal uptake systems to contribute, but did not allow any analysis of their relative efficacies.

Methods

Following dissection, prisms of chick IMHVs were incubated in 4.5 ml of an oxygenated Krebs-Henseleit buffer at 37°C in a water bath with a shaking rate of 100 strokes per min. After 10 minutes incubation, 0.5 ml of a solution of Krebs-Henseleit-buffered medium containing ³H-labelled glutamate (250nM) was added to the medium (final volume 5 ml). Five minutes later, the uptake reaction was stopped by dilution with 5ml ice-cold Krebs-Henseleit buffer and the medium filtered through Whatman No. 54 (cellulose acetate) filter paper under vacuum. The prisms were then digested

with 0.2 ml NaOH (1M), and 5ml of a scintillation fluid (Packard emulsifier) was added. A count of disintegrations per minute (dpm) was then determined using a Beckman LS 7500 liquid scintillation counter and the Bradford method was used to estimate the amount of protein.

Uptake of glutamate was determined in the left IMHV at 15, 30 and 60 minutes after training (n= for MeA-trained, 7,8,7; for water-trained, 7,8,7 respectively) and in the right IMHV at these times (n= for MeA-trained, 8,9,7; for water-trained, 7,8,8 respectively).

Statistical analysis was carried out using a two-tailed Student's t test (Statview). Data were expressed as means \pm S.E.M. values of n experiments. The significant level was set at $p < 0.05$.

Results

The results are shown in Figure 4.9. The left and right IMHVs of both MeA-trained chicks that avoided the test bead and water controls which pecked the test bead were used at 15 minutes, 30 minutes and one hour following training. An increase in dpm/mg protein, and therefore an increase in the level of uptake, was found 30 minutes ($t=3.719$ $p=0.0012$ $df=14$) and one hour ($t=5.653$ $p=0.00005$ $df=12$) after training in the left IMHVs of MeA-trained chicks. No differences in glutamate uptake were found 15 minutes after training.

The uptake of glutamate from the right IMHV was increased in MeA-trained chicks 30 minutes following training ($t=4.751$ $p=0.00015$ $df=15$). No changes were seen 15 minutes or one hour after training (both $p>0.60$).

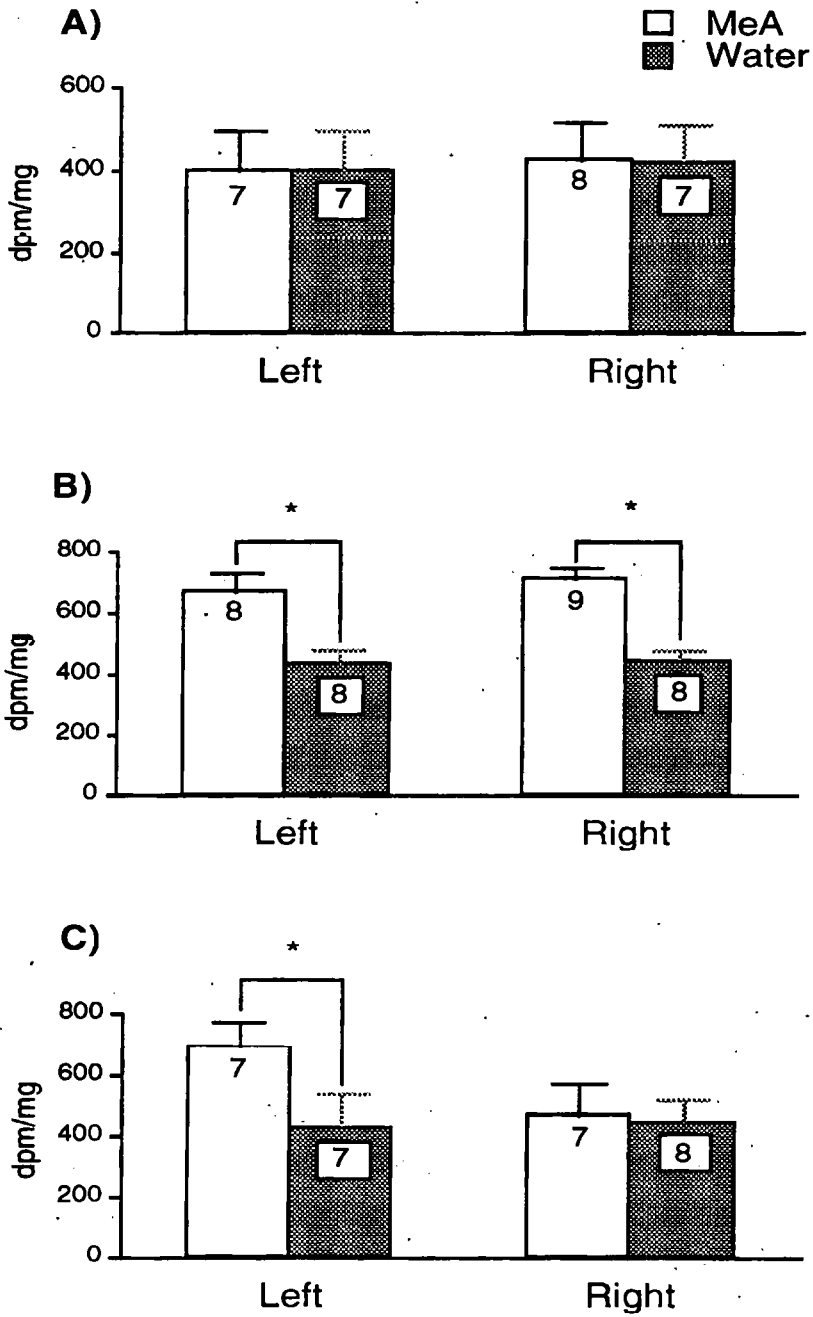


Figure 4.9. The effect of a passive avoidance task on glutamate uptake (A) 15 and (B) 30 minutes and (C) 1 hour after training, in the left and right IMHVs: dpm per 5 minutes per mg protein. The sample size for each group is indicated in the bars. An asterisk * indicates a significant difference between dpm levels in MeA-trained and water-trained chicks ($p < 0.05$, t-tests). Error bars are means \pm S.E.M.

Discussion

This study shows the effect of passive avoidance learning on transmitter release and uptake from slices of the IMHV of day-old chicks.

No changes in transmitter release were found 10 minutes after passive avoidance training in either hemisphere. By 30 minutes, a rise occurred in the K^+ -induced Ca^{2+} -dependent release of glutamate in both IMHVs. This increase appeared specific in its calcium dependency as no change in the basal release or Ca^{2+} -independent release was observed. The enhanced release of glutamate was still present in the left IMHV at one hour post-training but not in the right. At this time aspartate and GABA release were also enhanced in the left IMHV. Beyond one hour, no differences between MeA-trained and water-trained chicks were apparent in the left IMHV. Changes in the release were now removed to the right IMHV: at 3 hours glutamate and aspartate release were increased, at 6.5 hours glutamate and GABA release were now enhanced.

Glutamate uptake was also found to be increased at the same times, and in the same hemispheres, as these early increases in release. The results described in Experiment 4.2 demonstrate an increased uptake of the neurotransmitter glutamate from the left IMHVs of chicks both 30 minutes and one hour following the training task. The results show a 54% increase at 30 minutes and 74% after one hour. This increase had not become apparent at 15 minutes, and the time course of the experiments does not allow assessment of the persistence of the response. In the right IMHV there is an increase (59%) in glutamate uptake at 30 minutes, but not at 15 or 60 minutes, following the task. All these increases are consistent with the increased neuronal activity shown in the release study at the equivalent time points and hemispheres. As has already been stated (Chapter 1) the reuptake system is the mechanism by which synaptically released excitatory amino acids are inactivated and kept below toxic levels in the extracellular space. The

reuptake also allows rapid neuronal recycling of glutamate so that internal stores can be replenished. In this way, activity at the synapse can be maintained. The data from the uptake experiments supports the release data demonstrating a significant increase in glutamate transmitter activity.

The K^+ -induced Ca^{2+} -dependent component of release is thought to represent the release of endogenous amino acid from the vesicular transmitter pool (reviewed in Nicholls and Sihra, 1986). Therefore, the increase in this component of release observed in this study may indicate a long-term enhanced recruitment of the vesicular transmitter pool of amino acids 30 minutes to 6.5 hours after training. Rusakov et al. (1993) found a spatial rearrangement of vesicles in synapses in the left IMHV of MeA-trained chicks 30 minutes after passive avoidance training. In their study, two spatially separate pools of vesicles were identified. Thirty minutes after training they found that a rearrangement of these two vesicle pools had occurred, with a greater number of vesicles near the active zone, suggesting an altered state of activation in the synapses examined. They did not, however, differentiate between synapses containing asymmetrical (excitatory) or symmetrical (inhibitory) vesicles. Therefore, there is a possibility that the two pools may correspond to two different populations of synapses, and that the changes following training may reflect relative changes in the sizes of these populations (Rusakov et al., 1993). This is interesting in the light of the increases of glutamate release found at 30 minutes post-training in the present study. Does the increase in active zones correlate with an increase in vesicular glutamate release? This seems possible, although there is no specific evidence from the ultrastructural study to corroborate this. More recently Rusakov et al. (1995) reported that an increase in the width and thickness of synaptic apposition zones (SAZs) occurred in IMHVs 30 minutes after the task. They also demonstrated that there was an enlarged pool of synaptic vesicles adjacent to the SAZ in the left IMHV. They suggested that this may reflect enhanced synaptic vesicle exocytosis, and therefore an increased release of transmitter.

These data support the release studies presented in this chapter and suggest morphological correlates of the increased transmitter release. The arrangement of synaptic vesicles reported in the right IMHV showed an increased number of vesicles in both proximal and distal populations (Rusakov et al., 1995). This was thought to correspond with a mobilisation of vesicles from outside the active terminals: this observation might explain the increased glutamate release in the right IMHV at 30 minutes.

A long lasting change in presynaptic activity is suggested by the increased release. Such a persistent increase in glutamate release could lead to modifications of excitatory amino acid receptors. As noted in Chapter 2, Stewart et al. (1992) demonstrated increases in the binding to NMDA-sensitive glutamate receptors in chick forebrain 30 minutes after training in the left, but not the right, IMHV of MeA-trained birds compared to chicks trained on the water bead. The increased binding may be due, either to an increase in the number of receptors due to neosynthesis or the freeing of previously occluded receptors. The latter mechanism is suggested, as no increase in the number of synapses has been found up to 1 hr after training (Doubell and Stewart, 1993).

The absence of an elevated glutamate release at an early time (10 minutes) after training is in agreement with a study by Bullock et al. (1993) which showed no differences between the glutamate-stimulated inositol phosphate accumulation in MeA-trained and water-trained chicks 5 minutes after the training task. This result suggested that NMDA receptors might not be active at this time; however, it does not rule out the activation of other, excitatory amino acid receptors.

Bullock et al's (1993) study did describe NMDA receptor activation 30 minutes following the task. A decreased level of IP accumulation was found following the task suggesting the involvement of the NMDA receptor in learning the task. IP turnover is reduced because it is the activation of the NMDA receptor which is thought to reduce the availability of glutamate for

interaction with metabotropic receptors. By injecting MK801 (a non-competitive NMDA antagonist) prior to the training, the glutamate-stimulated decrease in IPs is abolished (Bullock et al., 1993), again indicating a role for this receptor type in memory formation.

The results show an increase in the release of glutamate from the right IMHV 6.5 hours after training. Previously it has been shown that 6.5 h post-training there is a bilateral decrease in the number of NMDA receptors in both left and right IMHVs of the chick (Steele, 1995). This was suggested to be due to a decrease in the number of synapses in a selective stabilisation, leading to an increased synaptic specificity.

In contrast to the data for NMDA receptor activation there is an increase (in the affinity) of AMPA receptors at 6.5 hours, but not at the earlier times of 30 minutes and 3 hours, after training (Steele and Stewart, 1995). This correlates well with the evidence that these receptors have been found to be active at time points from 3-4 hours (Tocco et al., 1991) and up until 48 hours (Tocco et al., 1992) after training on a nictitating membrane classical conditioning paradigm in the rabbit. Steele and Stewart (1995) showed that injections of an AMPA receptor antagonist CNQX 5.5 hours post-training, resulted in amnesia for the task when chicks were tested 1 hour later. This again suggests that the increase in glutamate shown at 6.5 hours in the present study may be mediated by AMPA receptor activation. However, the increase demonstrated in the present study occurred in the right IMHV only, Steele and Stewart's (1995) results indicated that both left and right IMHVs were required for the maintenance of longer term memory, and that AMPA receptors played a role in both hemispheres. This disparity is difficult to explain, but may reflect a loss in synapse number in the left IMHV of MeA-trained chicks such that, although the remaining synapses showed increased efficacy, the total glutamate release was no longer significantly enhanced compared to water-trained chicks that had retained a larger number of

synapses. The results from the autoradiographical and behavioural pharmacological experiments appear to suggest that AMPA receptors are not involved in the early stages of memory formation but are involved during the long term formation of the memory for the passive avoidance task. In contrast, Rickard et al. (1994) showed that antagonism of non-NMDA receptors as early as 90 minutes post-training produced amnesia for the task. The task employed by this group did differ from the paradigm used in our laboratories, in that coloured beads were presented to the chicks; also the antagonist, DNQX, shows low selectivity for AMPA receptors and may be activating kainate receptors and not AMPA receptors (Steele, 1995).

Metabotropic glutamate receptors are thought to play a role in the initiation of PKC activity. B-50 or GAP-43 is a presynaptic substrate of protein kinase C (PKC), and is phosphorylated on depolarisation of the synaptic membrane under conditions that release neurotransmitters: phosphorylation will release calmodulin and may increase its availability for the calcium/calmodulin-dependent kinase II-catalysed phosphorylation of synapsin I (Rodknight and Wofchuk, 1992). The 30 minute time point demonstrating enhanced glutamate release coincides with an increase in the membrane:cytosol ratio of PKC in the left IMHV (interpreted as indicating translocation of the enzyme; Burchuladze et al., 1990). The translocation of the α and β forms of the PKC to the membrane is dependent on Ca^{2+} concentration, and the phosphorylation of B-50 is regulated by the ratio of soluble to bound PKC. Therefore, presynaptic Ca^{2+} influx, or mobilisation from internal stores, results in PKC translocation followed by B-50 phosphorylation and the activation of synapsin resulting in vesicular transmitter release. Why then is no amnesia apparent at 30 minutes, a time point at which there is a demonstrated increase in glutamate release from the left and right IMHVs in this study? This suggests that PKC-regulated phosphorylation of the B-50 substrate is not involved in the initial stages of memory formation, but is an enabling mechanism for a long term memory

formation, occurring in parallel with the shorter term memory phases. Thus, although the transmitter release at 30 minutes is important for memory processes, the phosphorylation of B-50 is necessary for subsequent events.

The results described by Clements and Rose (1995) showed an increased uptake of $^{45}\text{Ca}^{2+}$ into prisms of IMHV 30 minutes, but not at 5 minutes or 24 hours, post-training. This uptake was inhibited by the N-type Ca^{2+} -channel blocker ω -conotoxin in the left, but not the right, IMHV (Clements and Rose, 1995). These results demonstrate, therefore, an increase in Ca^{2+} -uptake at a time (30 minutes) showing an increase in NMDA receptors, increased translocation of PKC and increased glutamate release (this study). The fact that the NMDA receptor subtypes that are increased in expression in the left and right IMHVs are different (cNMDA-right; nNMDA-left: Steele, 1995) may, possibly, explain the differences in the Ca^{2+} -channels that appear to be activated in the left and right IMHVs (N-type in the left; not N-type, or N-type and others in the right) as described by the ω -conotoxin selectivity. Further to this, the type of subtype activated may also affect the time course of release: as glutamate has a higher affinity for the nNMDA subtype, this might lead to a higher level of excitation in the neuronal environment of the left IMHV which persists for some time, whilst the cNMDA activity in the right IMHV does not: the results in the present experiment (4.1) shows enhanced glutamate release after 1 hour in the left IMHV but not the right.

Thirty minutes after training there is an elevation in a tubulin-enriched fraction of proteins as determined by colchicine binding in the anterior forebrain roof (Mileusnic et al., 1980). Microtubules are necessary for the axonal transport of materials from the neuronal cell body to the synapses in the form of secretory vesicles from Golgi bodies containing glycoproteins and, more importantly as regards this study, neurotransmitters and the enzymes required for neurotransmitter metabolism in the nerve terminal. Increased

tubulin production in the chick forebrain might lead to an increase in microtubule formation and allows a stabilised or increased transmitter release at synapses. It is not known, however, whether the increase in tubulin at 30 minutes could account for the increased glutamate release *per se* at this time, or whether the tubulin and subsequent microtubule formation is required for the replacement or replenishment of neurotransmitters and their enzymes used in the early stages of memory formation.

The role of aspartate as a neurotransmitter has been debated and still remains uncertain (see Chapter 1). Aspartate is a potent agonist at NMDA receptors, as has been demonstrated by altering the glutamate/aspartate ratio which altered the short and long components of the joint EPSP: the long, NMDA, component was significantly increased when the ratio favoured aspartate. The increase in aspartate release might therefore reflect NMDA receptor activation in these areas, although no increases in NMDA receptor binding have been found at the time points in question (1 hour in the left and 3 hours in the right IMHV). The discussion in Chapter 8 puts forward the proposal that aspartate is being released with glutamate following peptidase action on released N-acetylaspartylglutamate.

In the present study GABA release was enhanced in the left hemisphere at 1 hour, and in the right IMHV 6.5 hours, after training in MeA-trained chicks. The increases in GABA release that were found in this study are not unprecedented. Following LTP in rat hippocampal slices, Ghijsen et al.(1992) also found an increase in the *in vitro* release of this inhibitory transmitter. The timing of the increases suggests that GABA release may produce sufficient inhibition to reduce glutamate release after 1 hour, such that there is a pulse of glutamate release which is turned-off 60 minutes post-training. Alternatively, the increase in GABA release may inhibit further GABA release by action on pre-synaptic autoreceptors. This would lead to there being no longer the necessity for increased glutamate release, as the GABA released would serve to

prevent further inhibition. The neuronal set-up may also now be refined either due to initial synapse elimination, or to the selection and stabilising of only a certain number of synapses such that increased glutamate release is not demonstrably higher than control chicks as it would be confined to the synapses "representing" the memory. The increase in GABA release found by Ghijsen et al. (1992) could be explained in that it may allow a reduction in the GABA-mediated inhibition in the hippocampus by action on GABA_B-receptors localised on GABA-ergic terminals (e.g. Davies et al., 1991). Another possibility was that a sustained increase in GABA release may lead to postsynaptic GABA receptor desensitisation, resulting in a decrease of inhibition (see Chapter 8).

One might expect from this evidence an initial decrease in the release of GABA following training, to enable NMDA activation to allow sufficient EPSP summation to produce a biochemical cascade. GABA release would only have to be reduced for a very short period to allow NMDA receptor activation. From the time points sampled, however, no decrease was found in GABA release; thus a time before 10 minutes or between 10 and 30 minutes may show a decrease in GABA release and may have been overlooked due to there being no continual sampling. Indeed, a recent study from our laboratories (Clements and Bourne, 1995) demonstrated that the retention of the passive avoidance task could be modulated by GABA. They showed that pretraining injections of muscimol produced amnesia for the task as early as 10 minutes post-training. This suggests that GABA-receptor mediated events are important at a very early time following training. These events might or might not include an increase in the release of the transmitter. In addition, Martijena and Arce (1994) showed an increase in the binding of a GABA_A receptor agonist following passive avoidance training in the day-old chick. This increase was apparent at 30 minutes post-training, but not at 10 or 60 minutes. These results are not consistent with either the release data or the pharmacological data. The technique used in Martijena and Arce's study

involved binding of the analogue to homogenates of whole forebrain. This may suggest that increases in GABA_A receptor number do not occur in most areas of the forebrain at 10 and 60 minutes, but does not exclude the possibility that receptor numbers are increased in the IMHV at these times, and also it does not preclude an increased number of GABA_B receptors at any time.

The increase in glycine release at 30 minutes after training could be expected: the binding of glutamate to the NMDA receptor is allosterically potentiated by glycine (Bliss and Collingridge, 1993, also see Chapter 1), and evidence now suggests that glycine may play an important modulatory role in NMDA-receptor activation and LTP induction (Thompson et al., 1992). Moreover, Steele et al. (1993) have recently reported from our laboratories that an antagonist of the NMDA glycine binding site, 7-chlorokynurenate (7-ClK), inhibits the retention of memory for the task. 7-ClK produced a block on the recall of the memory, 30 minutes, 1 and 3 hours after training, when injected before, but not after, training into the left IMHV of day-old chicks. Thus glycine, acting at the NMDA receptor, is clearly implicated in the passive avoidance task. The increase in glycine efflux at 30 minutes will act to potentiate the receptors: an increased number of NMDA receptors would call for an increase in synaptic glycine to bind to them. No effect of 7-ClK was found when injected into the right IMHV (Steele et al., 1993). This may be because the number of cNMDA (antagonist preferring) receptors was found to be increased in the right, in the left the number of nNMDA (agonist preferring) receptors was increased in number. It could be suggested that the two receptor subtypes are differently sensitive to glycine, thus explaining the effect of 7-ClK in the different hemispheres.

Vesicular glycine release is unlikely as glycine is described as being a neurotransmitter of the lower part of the CNS (e.g. spinal cord and medulla; Aprison and Nadi, 1978). The K⁺-induced Ca²⁺-independent component of the release of glycine more likely reflects the activity of the respective uptake

carriers, acting in a reversed mode to expel glycine due to the severe (50mM) K^+ depolarisation applied to the prisms (Verhage et al., 1989). Therefore the increased K^+ -induced Ca^{2+} -independent glycine release from MeA-trained chicks may indicate a reversal of the uptake carrier for this amino acid.

A possible consequence/role of the increases in amino acid release in synaptic transmission efficacy may be in neuronal "bursting" activity in the IMHV. [Bursting is defined here as "a high frequency train of action potentials superimposed on slow membrane depolarisations". (Schneiderman and MacDonald, 1991)]. Following passive avoidance learning in the chick, Gigg et al. (1993 and 1994) and Mason and Rose (1987 and 1988) found that elevated bursting in the chick was associated with the memory formation. There is a significant increase in neuronal bursting at 3 hours post-testing (4 hours post-training) in MeA-trained birds compared to water-trained birds (Gigg et al., 1993). It is thought that excitatory amino acid transmitters play an important role in the initiation and spread of synchronous bursting because EAAs can produce seizures (Neuman et al., 1989) and antagonists of EAAs act as anticonvulsants (Meldrum, 1984). The generation of bursting is dependent on the activation of the NMDA glutamate receptor by cellular depolarisation produced by excitatory input or by disinhibition. The depolarisation releases the Mg^{2+} voltage-dependent block of NMDA-activated calcium channels. Non-NMDA receptors are largely responsible for the bursts in the presence of Mg^{2+} and provide the initial depolarisation which triggers bursts by activation of NMDA and other voltage-dependent channels. NMDA receptors play a dominant role only when the extracellular Mg^{2+} is lowered sufficiently to relieve the calcium-channel blockade. It is therefore surprising that there is no increase in number or affinity of the NMDA receptor subtype in the IMHV at this time point, although an increase was found at 30 minutes (Stewart et al., 1992). Bursting in the presence of Mg^{2+} may be initiated by non-NMDA receptors, such as AMPA receptors, which may explain this anomaly as AMPA receptors show increased affinity following the task (although not until 6.5

hours post-training; Steele and Stewart, 1995). In the present work an increase in the release of glutamate is found only in the right IMHV after 3 hours, whilst the electrophysiological data described by Gigg et al. (1993) does not show a lateralised effect at this time with a bilateral increase in bursting. Changes in bursting behaviour implies a change in the function and connectivity of synapses. Evidence that no increase in bursting behaviour is observed at the earlier times suggests that increased glutamate release and an increased number of NMDA receptors are enabling events that precede bursting in neurons, which may be concomitant with an increased synaptic efficacy.

Between seven and eight hours after training, neuronal bursting in the IMHV of MeA-trained chicks is at its greatest. This bursting is lateralised, with the right IMHV showing a significant increase in bursting compared to the left (Gigg et al., 1993). The burst-frequency at this time point is within the theta range, which points to LTP-like biochemical activity in the IMHV.

The data presented here also show evidence of a lateralised effect at (approximately) this time (6.5 hours), with no increase in glutamate release from the left IMHV but significantly higher levels of release found in the right IMHV of MeA-trained chicks compared to both water control chicks and the left IMHVs of the MeA birds. This implicates glutamate in bursting behaviour, which is also lateralised at 6.5 hours. A role for GABA also seems likely as evidenced by increased release of this amino acid at 6.5 hours (see Chapter 8).

Bursting and glutamate release are coincident with the second wave of glycoprotein synthesis. A second wave of glycoprotein activity occurs approximately 5.5 to 8 hours following training, as demonstrated using injections of the metabolic inhibitor 2-deoxygalactose (Scholey et al., 1993). This second wave is anisomycin-sensitive, suggesting that it involves *de novo* synthesis of proteins, which almost certainly include the cell-adhesion

molecules NCAM and L1 (Scholey et al., 1993 and Scholey et al., 1994). The increase in glutamate release at these times may be appropriate to reinforce the connections between cells and to make sure that specific and selective stabilisation of synapses occurs at the vital time when the long term memories have been established.

As noted above, it is thought that inhibitory mechanisms limit excitation within networks of neurons (Schneiderman and MacDonald, 1991). Synchronous bursting may therefore require a decrease in the inhibitory tone produced by the release of GABA at usual physiological levels. In line with this, picrotoxin and bicuculline (both GABAergic antagonists) induce spontaneous bursting by blocking GABA_A-mediated IPSPs, thus allowing excitatory activity to spread through neural circuits (Schneiderman and MacDonald, 1991). Also, following repetitive stimulation of hippocampal slices (CA1) there is a decrease in the GABA-mediated inhibition (Kamphuis et al., 1990). The decrease in GABAergic inhibition may lead to an enhancement of NMDA responses. The desensitisation of GABA receptors may be enhanced by an increase in GABA release: the prolonged activation of the receptor-linked Cl⁻ channel by the endogenous agonist may lead to depression caused by intracellular chloride accumulation, following repetitive IPSPs, leading to a change in the chloride gradient and equilibrium potential (Tehrani and Barnes, 1988). In the hippocampus it has been found that there is a decrease in the paired pulse depression in the CA1 but there is also an increase in the exocytotic release of GABA (e.g. Kamphuis et al., 1990).

GABA_B-mediated events have also been implicated in bursting. GABA_B receptor-mediated events may produce significant suppression of inhibitory output to enhance signal transmission in the hippocampus (Mott et al., 1993). The GABA_B receptor-mediated depression of the IPSC occurs only during the synchronised activation of a network (Otis and Mody, 1992). Synchronised neural activity has been found to occur in the theta rhythm

found in exploring rats (the theta rhythm, 3-12 Hz is thought to be important in certain learning tasks: Winson, 1978), and also in hippocampal sharp waves, which are thought to be involved in memory consolidation (Buzsaki, 1989); and the generation of this synchronised activity is thought to involve a suppression of inhibition (Buzsaki, 1989). The disinhibition of the GABA_B receptors is such that the transmission of the signal is enhanced only between 2.5 and 10 Hz (Mott et al., 1993). Thus, there is a filtering of signals allowing selective enhancement during repetitive activation at frequencies in the range of the theta rhythm. LTP is induced by repetitive, synchronised activity (McNaughton et al., 1978), and as such the production of the theta rhythm or sharp waves may induce LTP (Buzsaki, 1989). Following on from this it has been found that the induction of LTP is dependent on GABA_B receptor-mediated disinhibition which relieves the Mg²⁺ block of the NMDA receptors and allows the expression of the EPSP_{NMDA} and LTP. The slow IPSC produced by GABA_B receptor activation would be able to counter the slow EPSCs produced by NMDA receptors, and thus potentially inhibit NMDA receptor-mediated events (Ling and Benardo, 1994). Davies et al. (1991) suggest that an autoreceptor system is in operation, so that the fatigue of the IPSPs required for EPSP summation is due to GABA feeding back and inhibiting its own release through an action on GABA_B receptors. Stimulation of GABA_B receptors inhibits the major excitatory and inhibitory afferent systems within the neostriatum, and is proposed as a local feedback mechanism with presynaptic receptors inhibiting either excitation or inhibition: a similar mechanism is proposed for the hippocampus (Nisenbaum et al., 1992). The slow IPSCs produced by GABA_B-receptor activation are decreased by blockade of ionotropic glutamate receptor antagonists and GABA_A receptors, suggesting that both glutamate and GABA_A-mediated events participate in the recruitment of GABA_B IPSCs. Moreover, GABA_A-mediated events have been shown to cause excitation of interneurons, which in turn trigger GABA_B IPSCs (Michelson and Wong, 1991). The depression of the fast GABA_A IPSCs will

reduce the GABA_B IPSC.

The lateralisation effects seen in this study, as alluded to above, are not unprecedented. Left/right asymmetries have been found previously in the chick IMHV: IP accumulation was reduced in the left not right IMHV (Bullock et al., 1993), increased NMDA binding in left IMHV only (Stewart et al., 1992) etc. (see Chapter 2 for further examples). These hemispheric differences may be accounted for by having two modes of analysis of an experience. The mode of analysis fed by the left eye of the chick is suggested to record detailed and specific properties of the stimulus and is mainly responsible for spatial orientation, whilst the right eye feeds a system used to select cues which allow the stimulus to be categorised and to be responded to appropriately (including the identification of food items) (Andrew, 1991). "When both hemispheres are involved in learning the task the differences between them in the predominant type of analysis may result in different structures being active in learning and memory formation." This suggests that the IMHV, changes of which are predominantly found in the left, may be important in processes involving the selection of cues and their association with the subsequent reinforcement (Andrew, 1991). These differences in visual learning ability may be due in part to a structural asymmetry in the visual projections from the thalamus to the visual area of the brain, the Wulst (Rogers, 1991). The left side of the thalamus has a larger number of projections to the right hyperstriatum than does the right side to the left hyperstriatum. This demonstrates that the right eye connects to both hyperstriata, the left connects almost only to the right, as the thalamus receives projections from the contralateral eye only (Rogers and Sink, 1987). Therefore transmitter release and activation of NMDA receptors occurs predominantly in the right eye system initially, suggesting a categorisation and the adoption of a necessary response (avoiding the bead), whilst at a later time point the more detailed specifics about the bead may be internalised. Lesion studies from our laboratories have suggested that if the chick uses a number of different

categorisation or classification cues then lesions of one specific area may eliminate one of these cues but still allow for the recall and use of the others, assuming that different brain regions 'hold' different cues. According to Vallortigara et al. (1990) such categories include colour, shape, position and possibly pattern and brightness. Patterson and Rose (1992) suggest that the processing and storing of the association of colour and aversion occurs in the left IMHV, lesions of which will produce a deficit in learning such that chicks will avoid all beads of the similar shape and colour of the aversive bead and will not be able to discriminate between colours. This would explain why there are alterations in the biochemistry of the left IMHV although post training lesions in this region are not amnesic, as these cellular modifications will be involved with forming a representation of the colour-specific aspect of the bead (Patterson and Rose, 1992). Representations, based on the different classifications described above, are multiple and may be held in widely different brain regions following dispersal from perhaps the left IMHV, as suggested from most lesion data. However, difficulties in the explanation arise as there is no effect of left IMHV lesions under monocular conditions as described by Sandi et al. (1993). This could be interpreted as the right IMHV being involved in learning the task, or at least having the capacity to take over some of the left IMHV's processes. This is in agreement with the release data in that the transient increase in right IMHV activation (30 minutes only) may reflect an involvement of the right in the learning process or a priming effect, necessary if additional essential information were to arrive for processing in the left IMHV, allowing the area to prioritise the new information whilst still being able to hold the details of the bead in the right IMHV.

Chapter 5: The time course of the release of Amino Acids from the Lobus Parolfactorius (LPO) of the day-old Chick following Passive Avoidance Training

Introduction

In Chapter 4 an increase in the release of amino acid transmitters from the IMHV of the day-old chick was demonstrated following the one-trial training task. It has been shown previously that there is also increased neuronal activity in the LPO (see Chapter 2). This region has been shown to be metabolically active following training (Rose and Csillag, 1985). This activity has since been shown to include an increase in the number of a glutamate receptor subtype (Steele, 1995), increased neuronal bursting (Gigg et al., 1994), and an increase in synaptic membrane glycoproteins (Bullock et al., 1992).

The LPO is one of the nuclei of the palaeostriatal complex, a basal forebrain structure. The complex is thought to control motor function, spatial orientation and attentional behaviours (Brauth et al., 1978). The palaeostriatum augmentatum-lobus parolfactorius-nucleus accumbens region of the avian brain is considered homologous with the mammalian striatal complex (caudate nucleus, putamen and nucleus accumbens; Reiner et al., 1983). The LPO has been suggested to be involved in the integration of emotional behaviours (Kuenzel and Blaehser, 1989).

The experiments described in this section describe the timecourse, and

the lateralisation, of glutamate and GABA release from the LPO following the training task. The results found in this study are then related with those that have previously demonstrated changes in this region following training.

Materials and Methods

The procedures were carried out as described in Chapter 4. These can be summarised as follows: pairs of day-old chicks were housed in pens and trained either on a bitter-tasting MeA-covered bead or on one that had been dipped in water. Chicks that had trained successfully were tested at various times after training with a similar but dry bead. MeA-trained birds that pecked the dry bead and water-trained chicks that avoided the test bead were not used any further in the experiment as this result suggested that they did not train successfully or did not remember their training. Immediately after testing, the chicks were decapitated and their forebrains were removed. The left and right LPOs were then dissected out, and combined as described in Chapter 4. The tissue regions were prepared either as slices or prisms and placed in 95% O₂-5% CO₂ gassed Krebs-Henseleit buffer. Following preincubation, the tissues were separated such that roughly equal quantities were placed in one of four media, measuring: the total basal release; calcium-independent basal release; the calcium-independent potassium stimulated release and the total stimulated release. The tissues were incubated in these media for 2 minutes and the media collected and frozen immediately, with protein determinations taken of the tissue.

All analyses were carried out following pre-column derivatisation with phenylisothiocyanate on the HPLC (see Chapter 3). Both hemispheres were measured to provide data on any lateralisation effects.

Statistical treatment of data

The statistical significance of the differences between the release values from MeA-trained and water-trained chicks was calculated using a nested-ANOVA as described in Chapter 4. Data were expressed as mean \pm S.E.M. values of n experiments.

Results

Basal release of amino acids

The basal release of glutamate and GABA from the left and right LPOs of day-old chicks is described in Figures 5.1 and 5.2. No differences in the levels of release were found at any of the time points or in either hemisphere (ANOVA, $p > 0.2$). The sample sizes are $n=7$ for all conditions i.e. 7 groups of combined LPOs (3 or 4) for each group.

Calcium-independent release

The calcium-independent data are described in Figures 5.1C and D (glutamate), and 5.2C and D (GABA). No differences were noted between the groups in the calcium-independent component of release of either glutamate or GABA in either the left or right LPOs ($p > 0.2$).

Calcium-dependent release

The calcium-dependent release was derived from the total stimulated release from which the calcium-independent release was subtracted.

Thirty minutes after training there was a significant increase in the calcium-dependent release of glutamate in the left LPO of MeA-trained compared to water control chicks ($F=10.80$, $p=0.0155$, $df=12$, Figure 5.3A). There was no difference between the trained and control chicks at 3 hours. By 6.5

hours there was an increase in glutamate release from the left LPO of MeA-trained chicks ($F=14.073$, $p=0.0028$, $df=12$). An increase was also apparent at 24 hours ($F=11.12$, $p=0.0082$, $df=12$).

The right LPO showed no differences in calcium-dependent glutamate release between the two groups, at either 30 minutes or 3 hours post-training ($p > 0.2$, Figure 5.3B). By 6.5 hours however, there was a significant increase in release shown by MeA-trained birds ($F=10.412$, $p=0.0128$, $df=12$). As in the left LPO, the increase was also apparent 24 hours after training ($F=9.45$, $p=0.0100$, $df=12$).

The increase in glutamate release seen at 30 minutes post-training in the left LPO of MeA-trained chicks is greater than that found in the right LPO at this time ($F=14.157$, $p=0.0027$, $df=12$, Figure 5.3C). No other differences between the hemispheres of trained birds were found ($p > 0.15$). Also, no changes were seen in the chicks trained on a water bead ($p > 0.3$, Figure 5.3D).

GABA release was also assessed for its calcium dependency (see Figure 5.4). Increased GABA release was found 30 minutes after the training task in the left LPO of MeA-trained chicks ($F=6.263$, $p=0.028$, $df=12$, Figure 5.4A). Again no changes were found at 3 hours post-training, but by 6.5 hours, and also at 24 hours, an increase in GABA release was found in the left LPO in MeA-trained birds ($F=4.899$ and 4.944 respectively, $p=0.0477$ and 0.0464 respectively, $df=12$).

GABA release was increased in the right LPO at the same times as glutamate (Figure 5.4B). Hence, increases were found at 6.5 hours ($F=5.01$, $p=0.045$, $df=12$) and 24 hours ($F=6.04$, $p=0.0289$, $df=12$). Also, the training-induced increase in GABA was lateralised only at the 30 minute time point of MeA-trained birds, when there was an increase in the left LPO compared to the right ($F=4.944$, $p=0.0463$, $df=12$, Figure 5.4C). No lateralisation in release was seen in water control birds at any time point (Figure 5.4D).

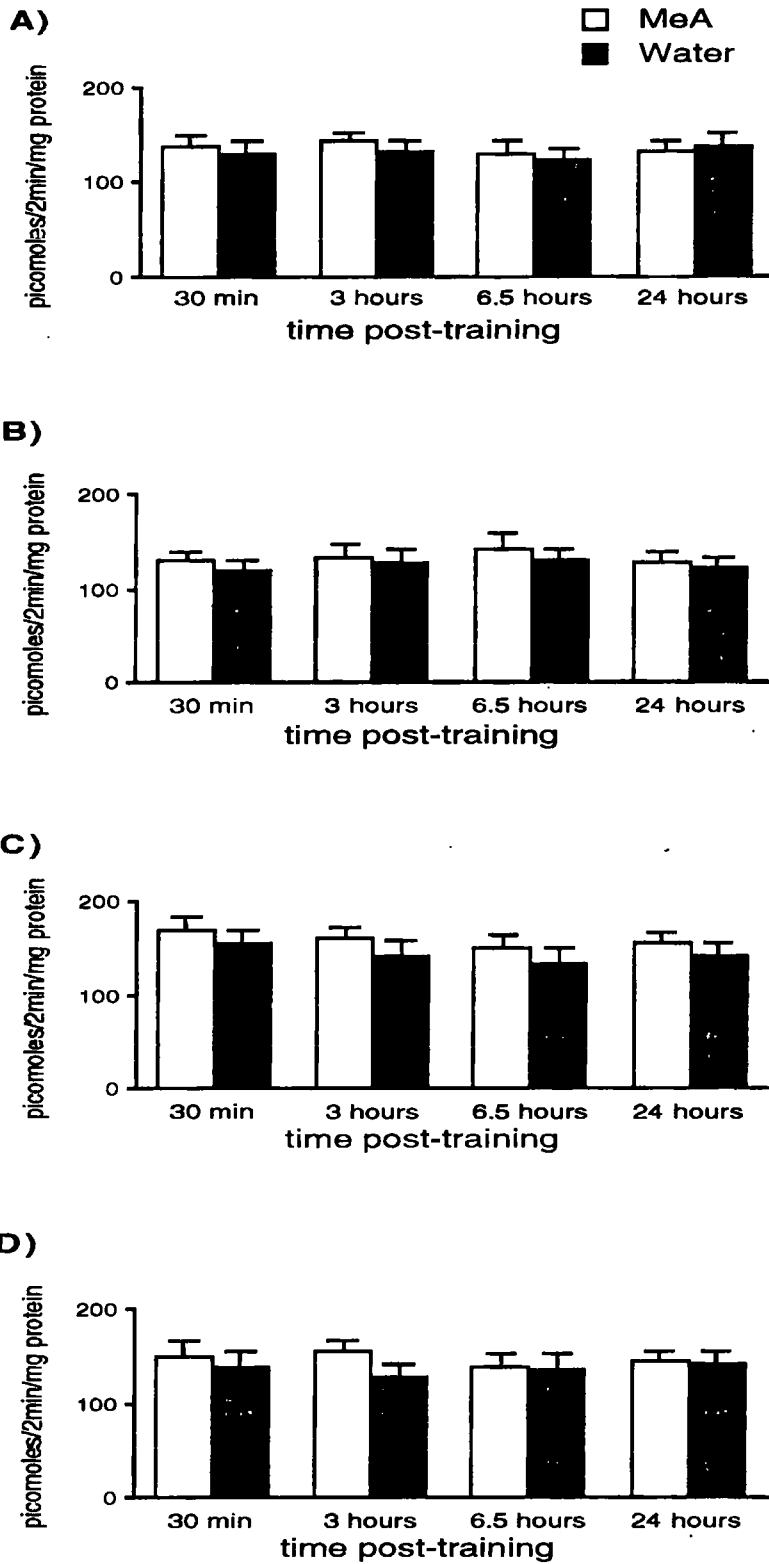


Figure 5.1 The effect of a passive avoidance task on the basal release, and calcium-independent release, of glutamate from the LPO of the day-old chick. Basal release was measured from A) the left and B) the right LPO, and calcium-independent release was also measured from C) the left and D) the right LPO. The sample size, *n*, is 7 for all conditions. Error bars are means \pm S.E.M.

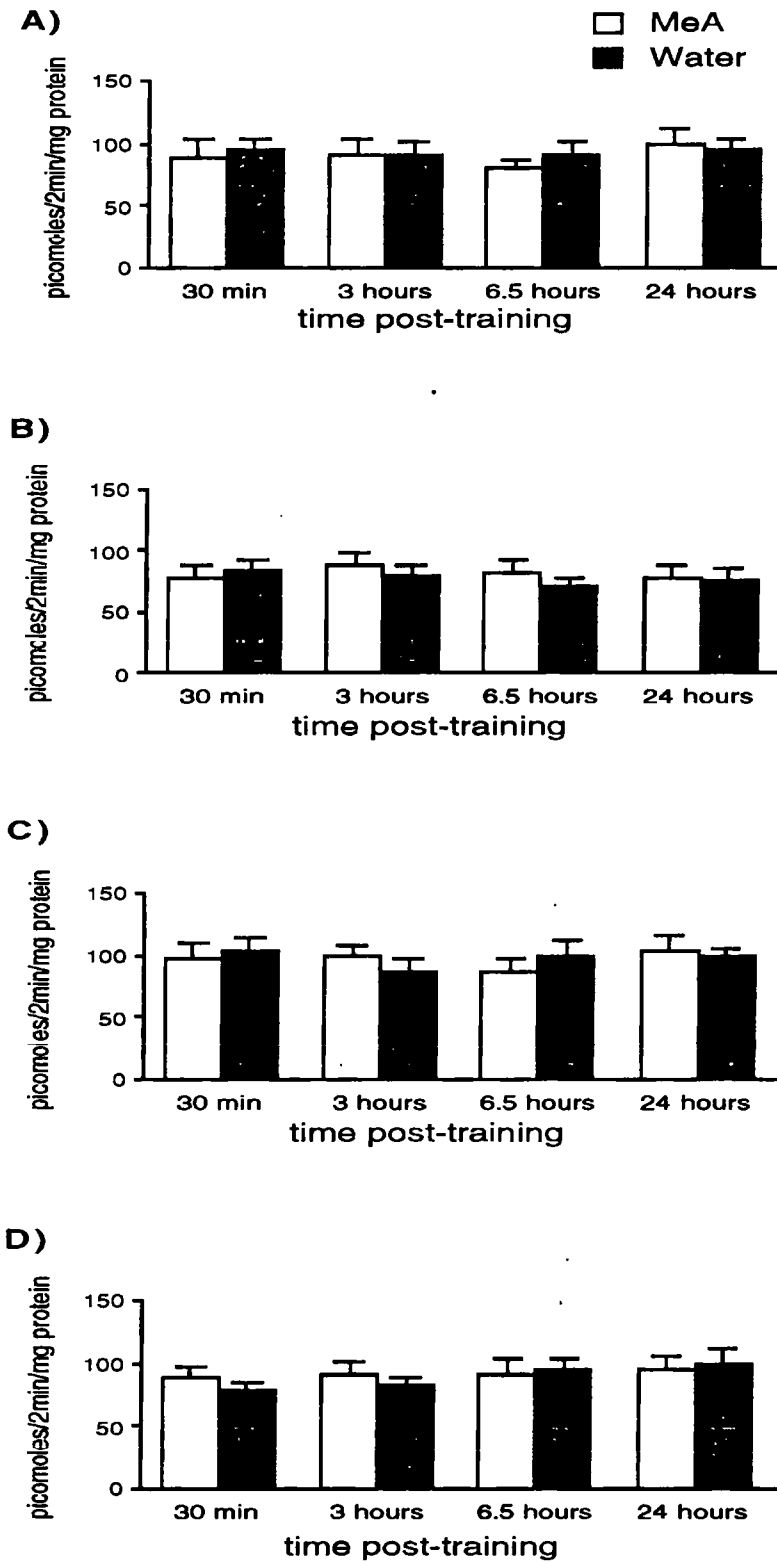


Figure 5.2. The effect of a passive avoidance task on the basal release, and calcium-independent release, of GABA from the LPO of the day-old chick. Basal release was measured from A) the left and B) the right LPO, and calcium-independent release was also measured from C) the left and D) the right LPO. The sample size, n , is 7 for all conditions. Error bars are means \pm S.E.M.

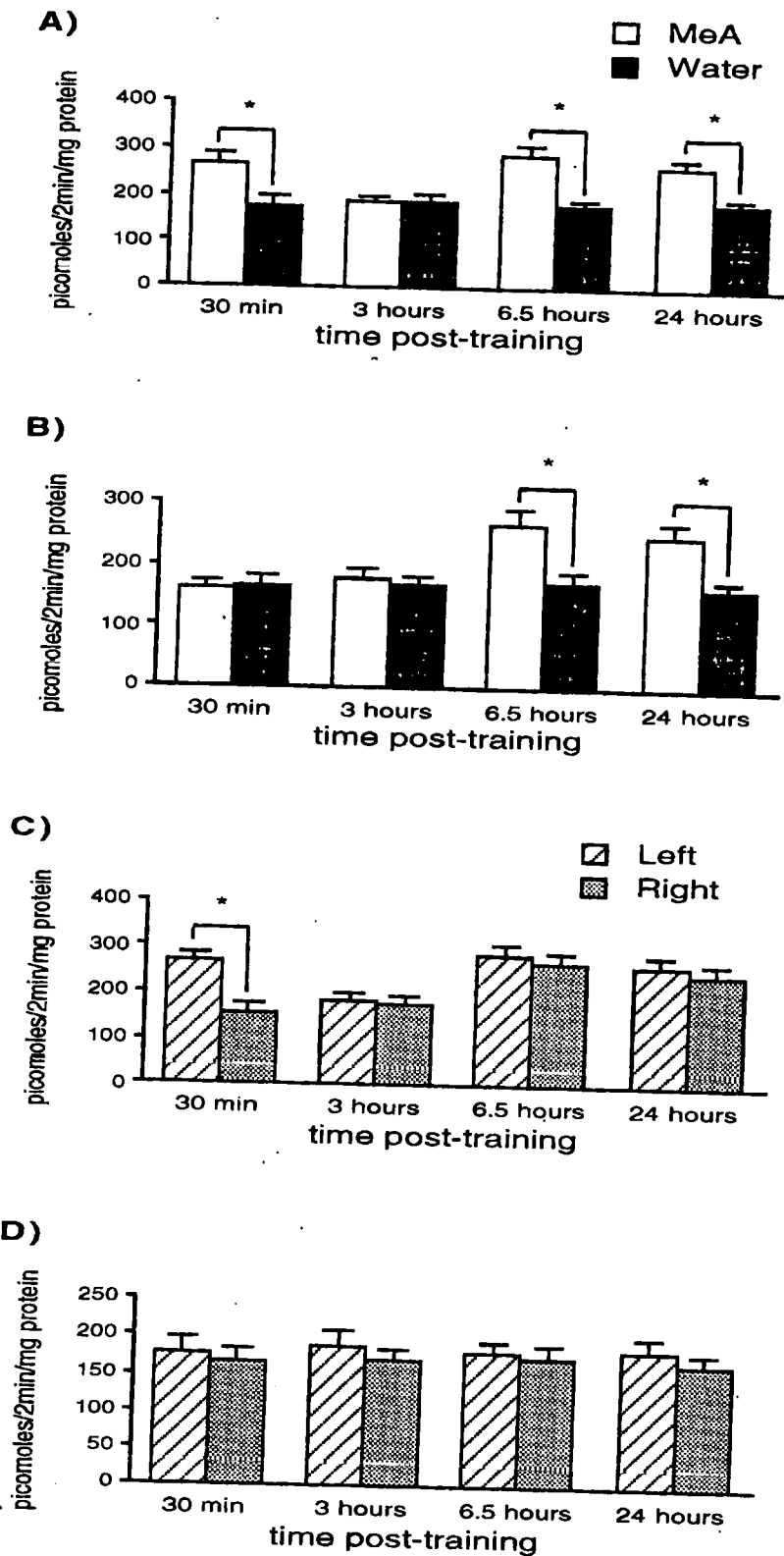


Figure 5.3. The calcium-dependent release of glutamate from (A) the left and (B) the right LPO of MeA-trained and water control chicks. The lateralisation of the increases of glutamate in the LPO is shown in (C) MeA-trained chicks and (D) water controls. The sample size, n , is 7 in all conditions. An asterisk * indicates a significant difference from control levels ($p < 0.05$, ANOVA). Error bars are means \pm S.E.M..

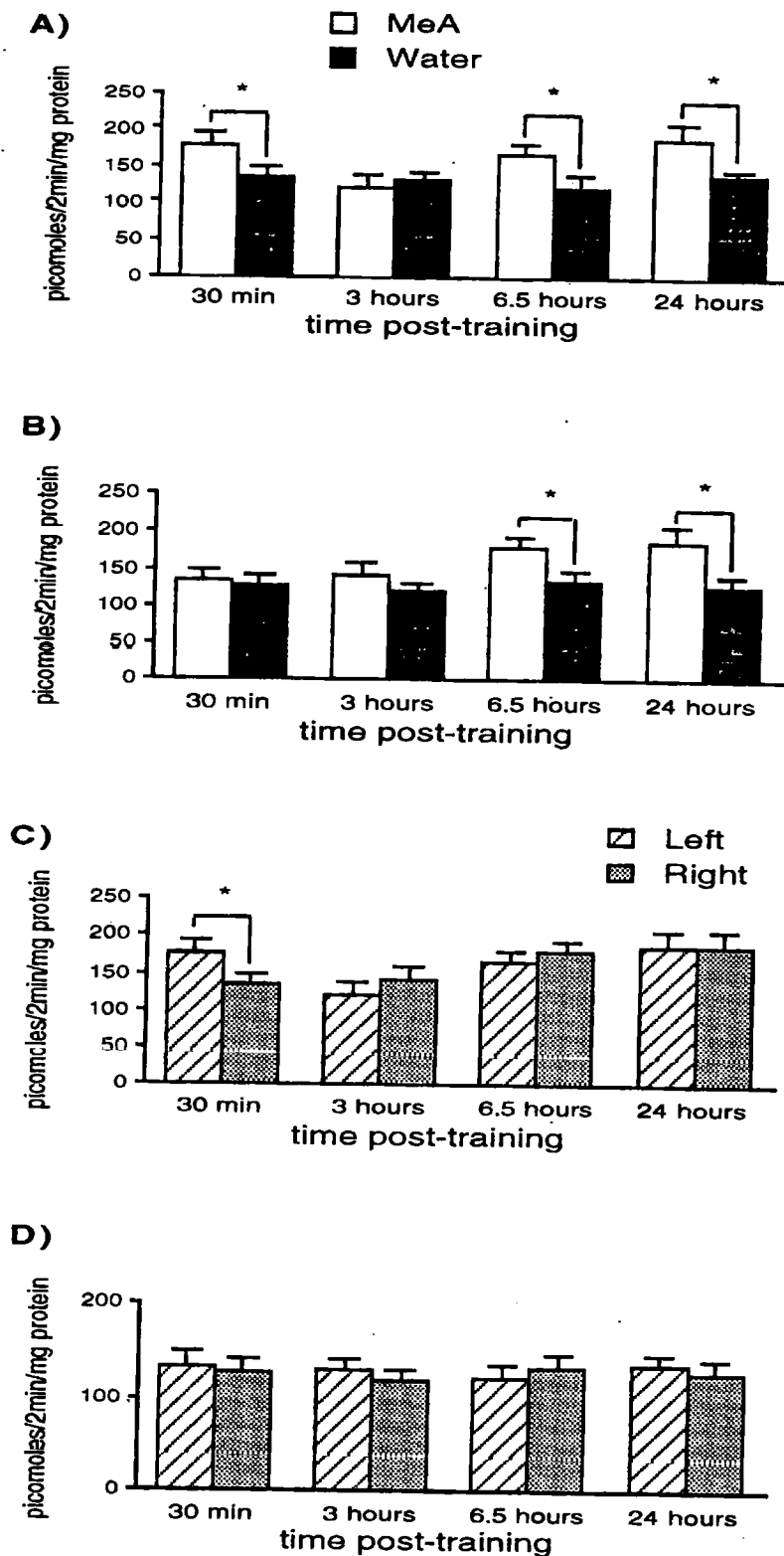


Figure 5.4. The calcium-dependent release of GABA from (A) the left and (B) the right LPO of MeA-trained and water control chicks. The lateralisation of the increases of glutamate in the LPO is shown in (C) MeA-trained chicks and (D) water controls. The sample size, *n*, is 7 in all conditions. An asterisk * indicates a significant difference from control levels ($p < 0.05$, ANOVA). Error bars are means \pm S.E.M.

Discussion

These results indicate training-induced increases in the calcium-dependent release of glutamate and GABA from slices of LPOs from the day-old chick. The increases occurred at specific times post-training in both hemispheres (i.e. at 6.5 and 24 hours), and were shown to be lateralised to the left LPO 30 minutes after the task. Glutamate and GABA were increased at the same times and in the same hemispheres of the chick LPO as one another.

The data show that the different hemispheres, and the different structures (LPO and IMHV), undergo changes at specific times post-training, suggesting that they are linked to stages and/or processes in the acquisition and/or retention of the memories for the task. The left IMHV appears to be active during a "time window" of roughly 30-60 minutes post-training, after which time it appears to fall silent, as far as any changes in amino acid transmitter release are concerned. The left LPO is also active at around this time (30 minutes only, was examined in this study), but it undergoes a "second wave" of activity which is seen at 6.5 and 24 hours. The right hemisphere shows a more synchronous increase in activity; both the IMHV and LPO are active at 30 minutes (as in the left hemisphere), but the right IMHV is also active after this time, unlike the left IMHV, with an increase in glutamate release found at 3 hours (before increased activity is found in the LPO), and activity continuing at 6.5 hours (at which time the LPO becomes active in amino acid transmitter release again).

In general, the increased activity in the LPO is temporally correlated with changes in the IMHV, suggesting that one structure is influencing the other. Indeed, it has been found that there is a connection from the IMHV to the ventral archistriatum (Csillag et al., 1994), to which the IMHV has an efferent projection, and that there is a substantial projection from the ventral archistriatum to the LPO (Szekely et al., 1994). Thus, the data presented by

Gilbert et al. (1991) suggests that following passive avoidance learning, information is transferred from the IMHV to long-term storage in the LPO via the archistriatum. A possible mode of action for the LPO would be the inhibition of pecking behaviour, as the LPO may play a role in the regulation of motor output.

Morphological changes were not found until 24 hours post-training in the LPO (Stewart et al., 1992). All the changes seen at this time suggest a facilitation of neurotransmission in the LPO. The numerical density of synapses is increased bilaterally 24 hours after training (Stewart et al., 1987). Also increased by 24 hours is the density of synaptic vesicles and an increase in spine density, both in the left LPO. The latter might be related to an enlargement of the surface area of the neurons' receptive field and therefore the facilitation of the transmission of the impulse to the cell body. The former is likely to be related to the enhancement of transmission; the high density of vesicles would enhance the electrical field potential in the vicinity of the synaptic membrane (Jack et al., 1975). In the *robustus archistrialis* of canaries there is an increase in synaptic vesicle number associated with the acquisition of a new behaviour (DeVoogd et al., 1985). Spine density changes are found 24 hours after training in multipolar projection neurons of the left LPO (Lowndes, 1992).

Recent results described by Rusakov et al. (1995) showed that the synaptic apposition zones (SAZs) in the left LPO became rounder, or more regular in shape, and the distances between the "transmission zones" increases. They interpret the data as indicating the sprouting of synapses: transmitter release may be enhanced (as seen in the present study) in order to establish these new synapses, or, the transmitter release may be enhanced because of new connections. In the right LPO the distances between the SAZs are decreased. These data are related to the increase in the expression of α -tubulin in the right, but not the left, LPO 24 hours after training (Scholey et al., 1992).

As synaptic number changes do not occur until 24 hours and after in the LPO, the increase observed is likely to be due to synaptogenesis, rather than due to splitting of synapses as suggested to occur at synapses in the mammalian brain following a novel experience (Stewart et al., 1992).

Dendritic spines have been suggested to contain individual calcium compartments, and are thus thought of as the basic functional units of neuronal integration, with a single spine able to use its internal calcium concentration to register the temporal coincidence of the input and the output of the neuron (Yuste & Denk, 1995). The chemical compartmentalisation is thought to be as a result of the spine geometry, such that diffusional exchange between the spine head and the dendritic shaft is limited (Yuste & Denk, 1995). The Type II Ca²⁺/calmodulin-dependent protein kinase (Type II CaM kinase) is concentrated in regions of the mammalian brain where long term plasticity is found (Erondu & Kennedy, 1985). The CaM kinase is involved in regulating neurotransmitter release (Llinas et al., 1985). Therefore, it is possible that the increase in spine density seen in the LPO at 24 hours allows an upregulation of the CaM kinase, which acts to increase the level of transmitter release from the neurons by phosphorylating the tail region of synapsin I (the phosphorylation of which leads to a reduction in the strength of its association with the surface membrane of synaptic vesicles, Rodknight & Wofchuk, 1992). Calcium influx is required initially, but CaM kinase II has the ability to then be able to autophosphorylate its substrates independent of calcium levels (Miller & Kennedy, 1986). There is therefore the possibility that once phosphorylated as a result of an influx of calcium, the CaM kinase remains activated for long periods of time, and by phosphorylating new enzyme copies maintains the cascade beyond the time course of protein turnover. Kinases could potentially enhance synaptic responses by a direct or indirect action on AMPA receptors (Greengard et al., 1991). In addition, PLA₂ activity is subjected to inhibition by CaM II kinase (Piomelli, 1991), forming a biochemical link between the kinase

and AMPA receptors. The GluR1 subunit of the AMPA channel is heavily phosphorylated by CaM II kinase, and this phosphorylation increases the current through these channels. This is suggested to serve as a mechanism by which information stored by CaM II kinase in the PSD can affect synaptic strength (Lisman, 1994).

As stated above, the increases in morphological attributes appear to be confined mainly to the left LPO at 24 hours after training. However, in this study, transmitter release was found to be raised at the same times in the right LPO as in the left at the later times, 6.5 and 24 hours, looked at. (Stewart et al. [1987] did find an increase in synaptic number at this time in the right LPO, but this increase was not considered as statistically significant by Hunter[1991]). In addition, the increases in release of both glutamate and GABA were of the same order in both hemispheres at these times. If this is the case, and transmitter release is required at some point in order that morphological plasticity can be expressed, why are there no equivalent structural changes found in the right LPO as in the left at 24 hours? The answer may lie in the fact that no increase in activity, as determined by amino acid transmitter release, occurs 30 minutes after training in the right LPO as is seen in the left. The increases seen at 30 minutes may possibly act as an initial trigger or "first wave" of neuronal activity. It has been found that 1 hour after training, there is an increase in the immunoreactivity of α -tubulin (49%) in the left LPO, but not the right. At 6 hours after training there is now an increase in the immunoreactivity to α -tubulin in both hemispheres. Lowndes (1992) suggests that if levels of tubulin are related to an increased potential for dendritic plasticity, it is possible that the hemispheric asymmetries in spine density may be related to the timing of glycoprotein synthesis. The changes seen in the left LPO may be initiated in the first wave of synthesis and increased by the second wave. The spine changes seen in the right may be related to the second wave of biochemical activity only, and are thus produced to a lesser degree, e.g. a smaller increase in spine density. Thus, the data found in the study of tubulin

synthesis ties in very well with the transmitter release results found here; whether an increase in transmitter release precedes the increase in tubulin production or *vice versa* cannot be said, as tubulin synthesis was not analysed in the LPO before the 1 hour time point.

At 30 minutes, in addition to an increase in the release of transmitters, there is an increase in the number of NMDA receptors in the left LPO, but not the right, of MeA-trained chicks (Steele et al., 1995). This increase is not seen at any subsequent time. This again suggests a distinct and discrete increase in activity in this region which is related to synaptic plasticity and precedes the structural changes seen later.

Bullock et al. (1992) found an increase in the levels of a presynaptic 50kDa and postsynaptic 33, 100-120, and 150-180kDa protein species in the LPO at 24 hours post-training. The former may well be the B50 protein alluded to in the last chapter. The post-synaptic proteins may well include two forms of NCAM, the highly sialylated embryonic form (120kDa) and the low sialylated, mature 180kDa form. When antibodies to NCAM are injected at 6 hours post-training, they produced amnesia for the task (Scholey et al., 1993). This is similar to that found by Doyle and Regan (1993) in rats trained on a step-down passive avoidance task. They suggested that there is a transient overproduction of synapses followed by selection and stabilisation in which there is a conversion of the 120kDa form of the NCAM into the 180kDa form; the NCAM antibodies presumably interfere with this conversion process.

No changes in receptor number (NMDA or AMPA) were found at either 3 or 6.5 hours after training (Steele et al., 1995). In the present study, increases in both glutamate and GABA release are found at 6.5 hours. In addition, in the study of neuronal bursting in the LPO carried out by Gigg et al. (1993), increased bursting was found 5 to 8 hours after training. This increase was not found to be lateralised to either hemisphere; indeed a non-significant trend for increased bursting in the right LPO was seen in both water-control and MeA-

trained chicks. Steele (1995) suggested that the lack of any changes in receptor binding in the LPO, other than at 30 minutes, may be due to the fact that processes involved in the second wave of memory formation in the LPO involve a different receptor mechanism, e.g. the metabotropic glutamate receptors. Also there are moderate amounts of GABAergic fibres in the chick brain (Dietl et al., 1988b), and as has been stated before in this thesis, GABA and GABAergic interneurons are a very powerful component in synaptic plasticity, allowing inhibition via direct effects on excitatory synapses, and excitation by modulation of its own release via autoreceptors and inhibition of other GABAergic neurons. Hence, the increases in GABA release allow further possibilities for memory formation and subsequent morphological changes. The LPO is also rich in cholinergic (Horn, 1985 & Dietl et al., 1988a) and dopaminergic (Dietl & Palacios, 1988) fibres; the release of either of these two transmitters is not examined in this thesis, but may be important for reducing the inhibitory tone in the LPO.

Chapter 6: Adenosine release from the IMHV of the day-old chick, and its effects on the release of amino acids

Introduction

A candidate that may play a vital role in modulating the release of amino acid neurotransmitters following the task, is the purine adenosine (see Chapter 1).

Adenosine has been found to decrease, or increase, glutamate release depending on the receptor type activated and the area of the brain involved e.g. it has been demonstrated by Cunha et al. (1994) that ACh release in the hippocampus is differentially regulated, such that in the CA1 area only A₁ receptors modulate the release, in CA3 both A₁ and A_{2a} receptors modulate the ACh release, but in the dentate gyrus both receptor types are present but are not activated by endogenous adenosine. Adenosine release and its actions at different receptor subtypes would prove a powerful mechanism to increase or decrease the likelihood of a learning experience being assimilated, assuming the release of glutamate (demonstrated in Chapters 4 and 5) and receptor activation (see Steele, 1995) are involved in memory formation.

This chapter describes the release of adenosine following passive avoidance training; its effects on the release of amino acids; and the NMDA and KCl stimulation of its release.

Experiment 6.1. The release of adenosine from the IMHV of the day-old chick following a passive avoidance task

Introduction

An increase in amino acid transmitter release was demonstrated at various time points following the training task in the IMHV and LPO of the day-old chick (Chapters 4 and 5). Previous work has also demonstrated that amino acids, specifically glutamate, and their receptors are required in the acquisition of passive avoidance learning in the chick, as well as other learning tasks (see Chapter 2). Adenosine may play an important role in the modulation of synaptic transmission, specifically amino acid transmitter release, and thus in the acquisition, retention or recall of the task itself. Therefore, it was decided to examine the release of adenosine from the IMHV following a one-trial passive avoidance task, at similar times to those analysed for amino acid transmitter release.

Materials and Methods

The methodology has been described in Chapter 4. In short: pairs of day-old chicks were housed in pens and trained (following 3 pre-training trials) either on a bitter-tasting methylantranilate- (MeA) covered bead or on one that had been dipped in water (W). Chicks that had trained successfully were tested at various times after training with a similar but dry bead. Immediately after testing, the chicks were decapitated and their forebrains removed. The left and right IMHVs were then dissected out, prepared as slices (350mM) and placed in 95% O₂-5% CO₂ gassed Krebs-Henseleit buffer: the IMHVs were combined from 3 or 4 chicks according to the treatment and the hemisphere. Following preincubation (60 minutes), the tissues were separated such that roughly equal quantities were placed in one of four media, measuring: the total basal release; calcium-independent basal release; the calcium-independent potassium stimulated release; and the total stimulated release of

adenosine. The tissues were incubated for 2 minutes, after which the media were collected and frozen immediately for HPLC analysis, and the IMHV slices were taken for protein determinations. Adenosine could be measured using the same chromatographic conditions as used for amino acid analysis.

Statistical treatment of data

The statistical significance of the differences between the release values from MeA-trained chicks and their W-trained chicks were calculated using a nested-ANOVA as described in Chapter 4. Data were expressed as mean \pm S.E.M. values of n experiments.

Results

Basal release of adenosine

The basal release of adenosine was examined, 30 minutes, and 1, 3 and 6.5 hours, following training on the passive avoidance task, in the left and right IMHVs of day-old chicks following incubation in a normal Krebs-Henseleit buffer (the number of samples, n= 7 at all time points for both hemispheres and treatments).

No differences were found between the MeA-trained chicks and the water controls ($p > 0.3$). Indeed, the values of released adenosine changed very little from one time point, treatment and hemisphere to another (see Figure 6.1). The average level of basal release was 18 ± 2.7 pM per milligram in the 2 minutes that the incubations lasted.

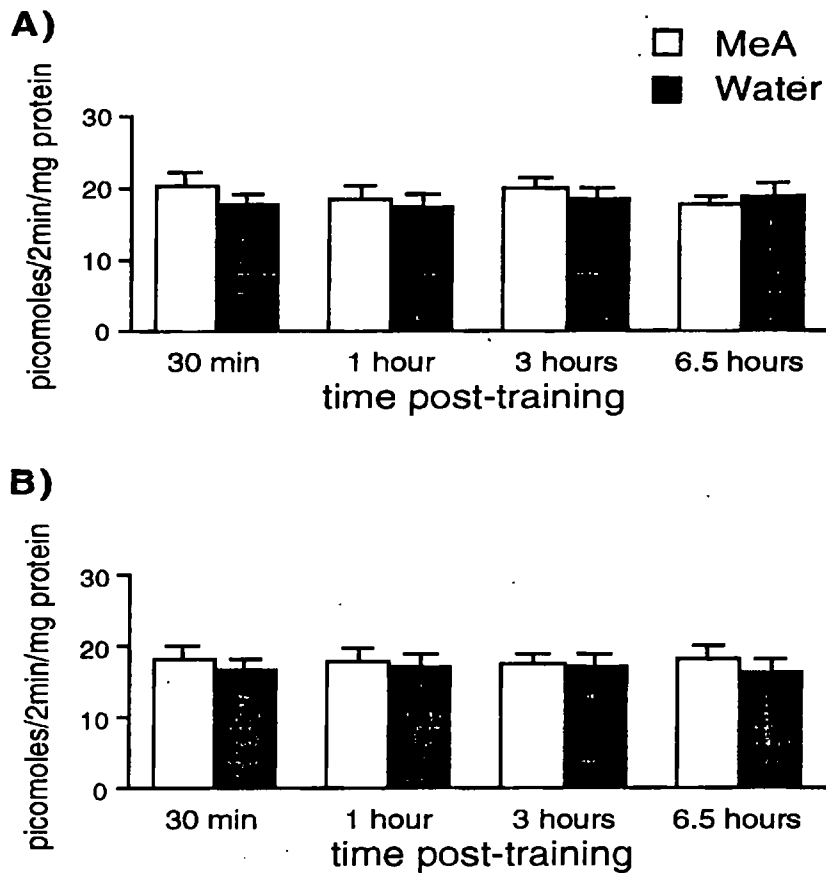


Figure 6.1. The effect of a passive avoidance task on the basal release of adenosine from, **A)** the left and, **B)** the right IMHVs of day-old chicks, 30 minutes, 1, 3 and 6.5 hours following training on a MeA-covered bead (MeA) or a water bead (W) (n=7 at each time point). Error bars are means \pm S.E.M..

Potassium-stimulated release

Figure 6.2 shows the total potassium-stimulated release (50mM KCl with 1.3mM CaCl₂: Medium C) of adenosine from slice preparations of the left and right IMHVs of MeA-trained chicks and water controls.

In the left IMHV there was an increase in adenosine release 30 minutes after training compared to the controls ($t=2.546$, $p=0.0257$, $df=12$). There was also an increase in release from the left IMHVs of MeA-trained chicks seen at the 1 hour time point ($t=2.458$, $p=0.032$, $df=12$). At the later times sampled, 3 and 6.5 hours, there were no differences between the two groups ($p > 0.4$).

The right IMHV also shows a training-induced increase in adenosine release 30 minutes after training ($t=3.803$, $p=0.0025$, $df=12$). No difference was found between MeA and water birds after 1 hour ($t=0.525$, $p=0.609$, $df=12$), but by 3 hours post-training, the right IMHVs of MeA-trained chicks showed enhanced adenosine release ($t=2.331$, $p=0.038$, $df=12$) and an increase was also found at the last time point analysed, 6.5 hours ($t=3.385$, $p=0.0054$, $df=12$).

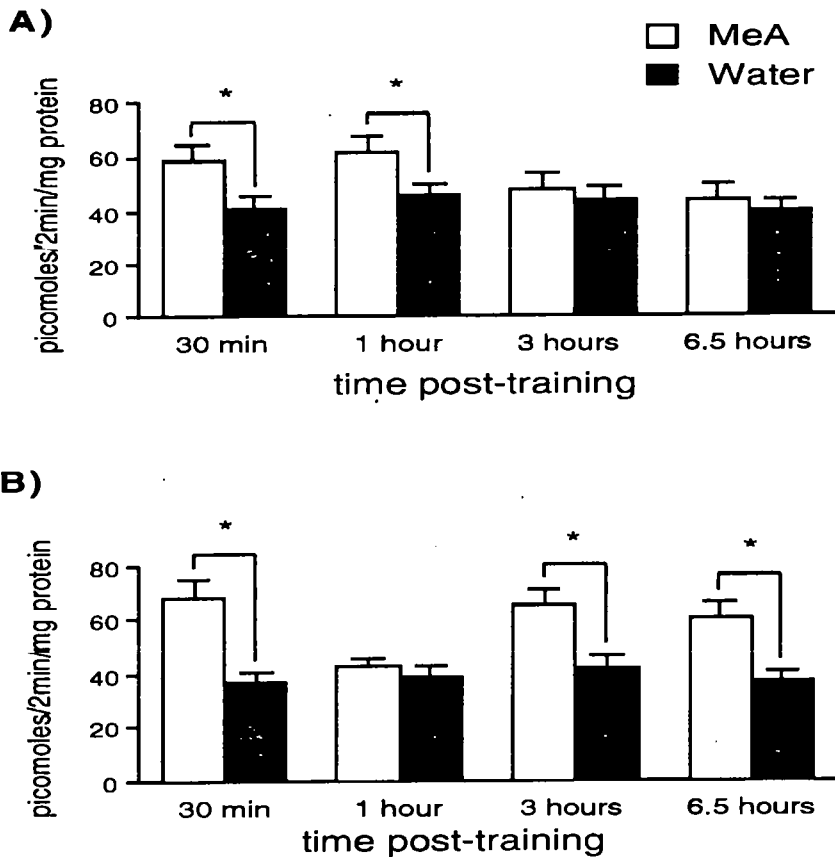


Figure 6.2. The effect of a passive avoidance task on the total potassium-stimulated (50mM KCl; 1.3mM CaCl₂) release of adenosine from, **A)** the left and, **B)** the right IMHVs of day-old chicks, 30 minutes, 1, 3 and 6.5 hours following training on a MeA-covered bead (MeA) or a water bead (W) (n=7 at each time point). An asterisk * indicates a significant difference between the two groups ($p < 0.05$, t-test). Error bars are means \pm S.E.M..

Calcium-dependency of release

The calcium-independent release of adenosine from the IMHVs of day-old chicks, following training on a passive avoidance task, is shown in Figure 6.3. Thirty minutes after training there were increases in adenosine release in both left and right IMHVs of MeA-trained chicks compared to W-trained birds (t 's=2.400 and 2.487 respectively, p 's=0.0335 and 0.0286 respectively, both df 's=12). An increase in release was found in the left IMHV 1 hour post-training ($t=2.187$ $p=0.0492$, $df=12$), but not the right ($p>0.2$). At 3 and 6.5 hours the right IMHV of MeA chicks showed increased release compared to W-chicks ($t=2.774$ and 3.368 , $p=0.00168$ and 0.0056 , df 's=12).

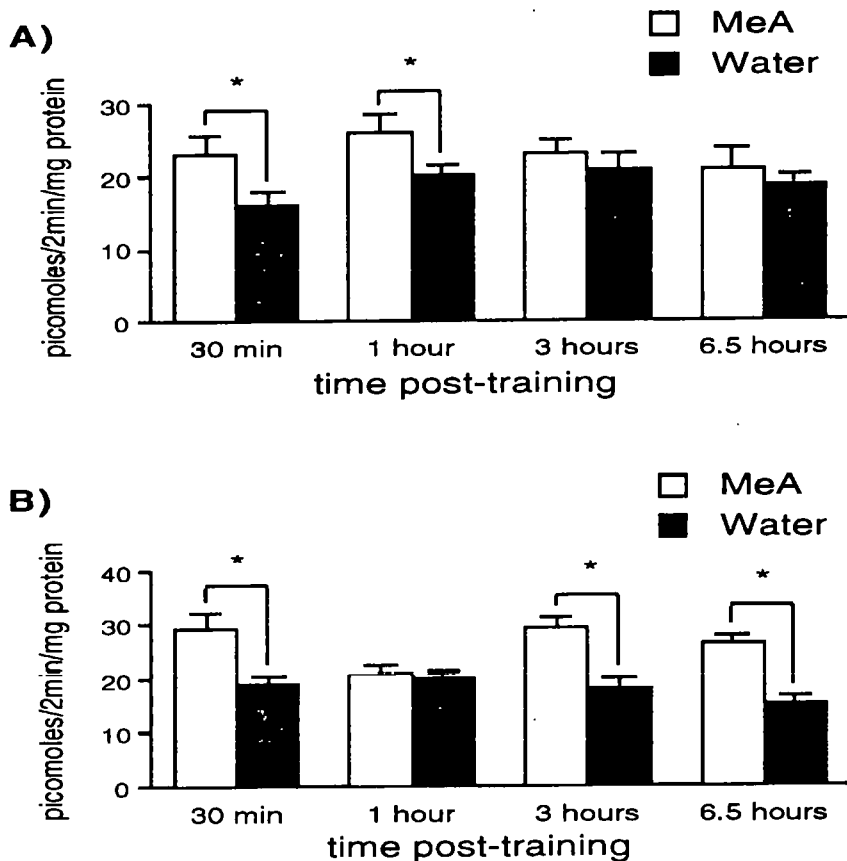


Figure 6.3. The effect of a passive avoidance task on the calcium-independent release of adenosine from **A)** the left IMHV and **B)** the right IMHVs of day-old chicks, 30 minutes, and 1, 3 and 6.5 hours after training on a MeA-covered bead (MeA) or a water bead (W). The sample size for each group is 7. An asterisk * indicates a significant difference between the two groups ($p < 0.05$, t-test). Error bars are means \pm S.E.M..

The calcium-dependent release of adenosine is described in Figure 6.4. As can be seen, increases in calcium-dependent release occurred at similar times following the task to those which showed increased calcium-independent release. Thirty minutes after training there were significant increases in the release of adenosine in both the left ($t=0.0224$, $p=2.62$, $df=12$) and the right ($t=4.196$, $p=0.0012$, $df=12$) IMHVs. One hour following the task there was an increase in calcium-dependent adenosine release from the left IMHV only ($t=2.382$, $p=0.0346$, $df=12$). Increases were again found in the right IMHV at 3 hours and 6.5 hours post-training (t 's=2.71 and 2.23, p 's=0.019 and 0.0221 respectively, both df 's=12)

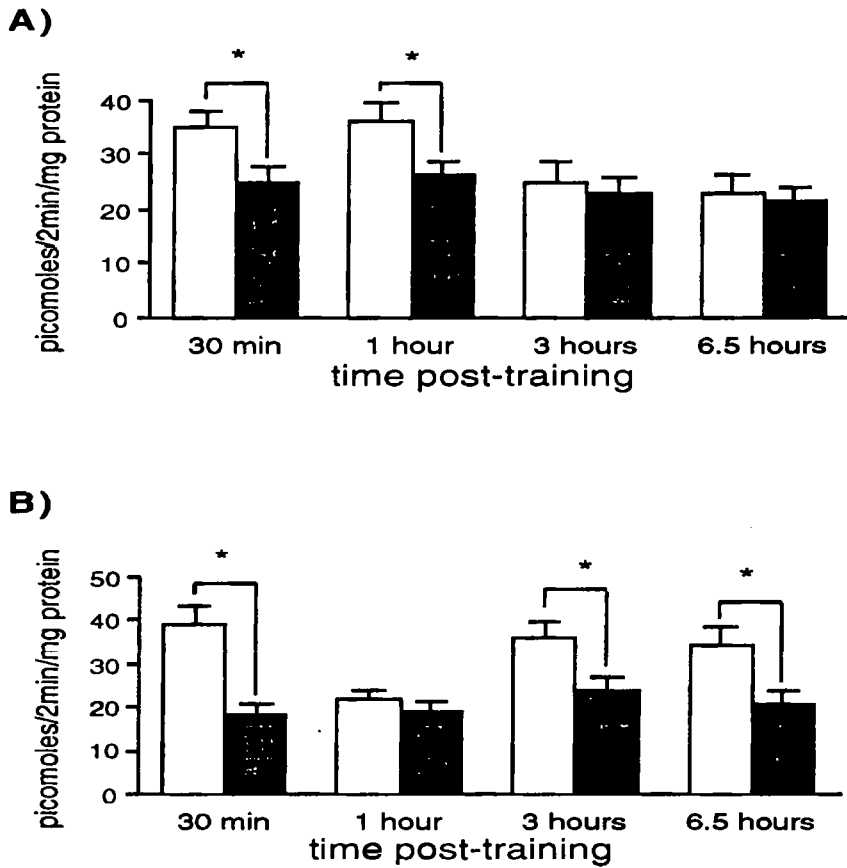


Figure 6.4. The effect of a passive avoidance task on the calcium-dependent release of adenosine from **A)** the left and **B)** the right IMHV of day-old chicks, 30 minutes, and 1, 3 and 6.5 hours after training on a MeA-covered bead (MeA) or a water bead (W). The sample size for each group is 7. An asterisk * indicates a significant difference between the two groups ($p < 0.05$, t-test). Error bars are means \pm S.E.M..

Experiment 6.2. The effect of adenosine analogues on the release of amino acids from slices of the IMHVs of day-old chicks

Introduction

This section describes a series of experiments that were carried out to determine if adenosine has an effect on the release of amino acids in the chick IMHV. Experiment 6.1 showed concomitant increases in the release of adenosine with glutamate, but no conclusions as to the effect of the enhanced purine release on the release of any of the amino acids can be drawn without first demonstrating an effect in the chick slice preparation.

The effect of adenosine and its analogues on the release of endogenous amino acids has been investigated extensively in mammalian systems: adenosine (300mM) was found to inhibit the evoked potentials and the release of aspartate and glutamate from the CA1 region of the hippocampus following stimulation of the stratum radiatum (CA3/CA2 region), as also did the receptor agonists 1-phenylisopropyladenosine (PIA) and cyclohexyladenosine (CHA @ 1mM) (Corradetti et al., 1984): calcium-dependent stimulation of this region had previously been shown to produce an increase in the release and synthesis of these amino acids (Corradetti et al., 1983b). Dolphin and Archer (1983) also demonstrated inhibition of potassium-induced glutamate release (but not GABA) from slices of the dentate gyrus of rat, following superfusion with the non-selective agonist 2-chloroadenosine (2-CADO). The effect of an A₂ agonist was to increase glutamate and aspartate release from ischaemic rat cerebral cortex, although it did not alter basal release (O'Regan et al., 1992). The A₁ antagonist 8-phenyltheophylline (5 mM) increased the release of aspartate and glutamate, when applied during low-frequency stimulation, and antagonised the CHA-induced inhibition of the release of these amino acids (Corradetti et al., 1984).

In addition, histochemical (Goodman et al., 1983) and neurochemical

(Lee et al., 1983) studies have suggested that a large percentage of adenosine receptors are located on the terminals of neurons releasing excitatory amino acids, and regional differences in the density of the receptors may confer differential sensitivity to adenosine modulation.

The following experiments investigated the effect of adenosine and adenosine analogues, on the release of the four amino acids investigated in the last two chapters:

Materials and Methods

Release procedure

To determine the effect of adenosine and adenosine analogues on the release of amino acids from chick IMHV slices, two batches of tissue were prepared; both derived from the left IMHVs of untrained day-old chicks. Slices (350µM) were placed in a pre-incubation buffer for 60 minutes, with replacement of buffer as described in Chapter 4. After this period the slices were divided into two randomly assigned groups; 'control' slices were incubated in Krebs-Henseleit buffer; 'test' slices were incubated in a Krebs-Henseleit buffer containing the drug at the desired concentration. After 2 minutes the incubations were halted by placing the incubation vials on ice, and the media were removed to storage (at -40°C) for subsequent analysis by HPLC, with the slices collected for subsequent protein estimations.

Results

The effect of adenosine on the calcium-dependent release of amino acids

The concentration of adenosine applied to the incubation buffer should determine which receptor systems are activated in the slices; lower concentrations will activate A₁ inhibitory receptors and higher concentrations probably activating both A₁ and A₂ types, producing opposing effects on transmitter release. Therefore it was deemed necessary to vary the concentration of the adenosine applied to the preparation in order to describe its effects fully : three concentrations of adenosine were used in the present study, 10, 100 and 500mM, which were considered (from the literature) to cover a range of possible responses.

Adenosine at 10mM had no effect on the release of any of the amino acids measured (Figure 6.5, $p > 0.1$). The higher concentration of 100mM selectively inhibited the release of glutamate from chick IMHV slices ($F=9.972$, $p=0.0081$, $df=12$). The release of aspartate, GABA and glycine were all unaffected by this concentration of adenosine ($p > 0.25$). No differences were found for any of the amino acids following 500mM adenosine treatment, although there appeared to be a slight tendency (non-significant, $p=0.11$) for a decrease in the release of glutamate.

The calcium-independent, and basal, release of the amino acids was not affected by the addition of adenosine to the incubation medium (data not shown graphically; all p values > 0.2).

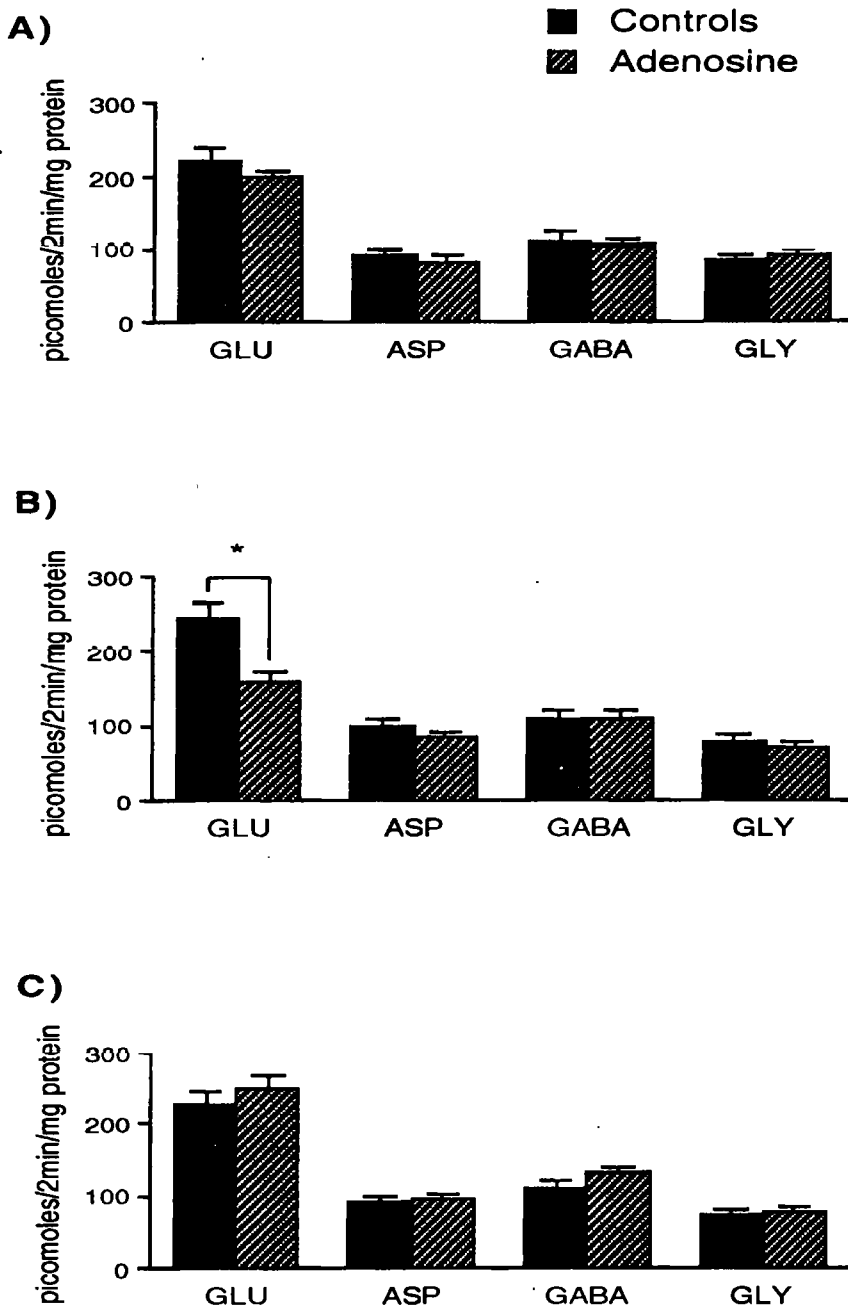


Figure 6.5. The effect of adenosine on the calcium-dependent release of amino acids from slices of the IMHV of the day-old chick. Media containing adenosine were added to slices of chick IMHV following a pre-incubation in a Krebs-Henseleit buffer at the following concentrations; **A)** 10mM; **B)** 100mM; and **C)** 500mM. Following a 2 minute incubation the media were removed and analysed by HPLC for amino acid content. Protein estimations were carried out on the slices and values of picomoles release per minute were computed. Values are means \pm S.E.M. for 7 experiments at each concentration. An asterisk * indicates a significant difference between the two groups ($p < 0.05$, ANOVA). GLU =glutamate, GABA =g-aminobutyric acid, ASP =aspartate and GLY =glycine

The effect of the specific adenosine A₁ agonist, cyclohexyladenosine (CHA), on the release of amino acids

The methodology was similar to that used for determining the effect of adenosine on amino acid release, but with CHA (10mM) replacing the added adenosine. CHA, at this concentration, would be expected to activate A₁ receptors only, and therefore was expected, on the basis of results obtained in mammals, to inhibit amino acid release.

The results are described in Figure 6.6 A (sample size, n=7). A selective decrease in the release of glutamate, and not of the other amino acids analysed (aspartate, GABA or glycine), was indicated (F=7.93, p=0.0159, df=12) from slices of chick IMHV treated with CHA .

The effect of the adenosine A₁ receptor-selective antagonist cyclopentyltheophylline (CPT) on the basal and CHA-affected release of amino acids from the IMHV of the day-old chick

CPT is a selective antagonist for the A₁ receptor site. Its affinity (K_d) is approximately ten times that of CHA (CHA=1.2nM, CPT=11 nM). These experiments were intended to determine whether the actions of CHA found in the last experiment were strictly of A₁ receptor-regulated events or whether the agonist was having non-receptor linked indirect effects on amino acid release. To determine which was applicable, the competitive antagonist CPT was used in order to attenuate the inhibitory effect of CHA. If the CHA-induced decrease in glutamate release was abolished with CPT it would be possible to say that the A₁ receptor system was being inhibited.

In the initial experiment CPT (100mM) was added to the incubation media in place of adenosine. The concentration used allows for the lower affinity of this molecule compared to CHA. The results are shown in Figure 6.6B (sample size, n=7 for all conditions). CPT (100mM) was found to increase

the calcium-dependent release of glutamate ($F=5.11$, $p=0.046$, $df=12$). None of the other amino acids measured were altered in their release ($p > 0.1$).

The second experiment involved the joint addition of CPT (100mM) and CHA (10mM), concentrations that allowed for competitive interactions at the receptor site. The results are shown in Figure 6.6C (sample size, $n=7$ for all conditions). No change in the release of glutamate ($F=3.72$, $p=0.071$, $df=12$) or aspartate, GABA and glycine from the control levels was now found.

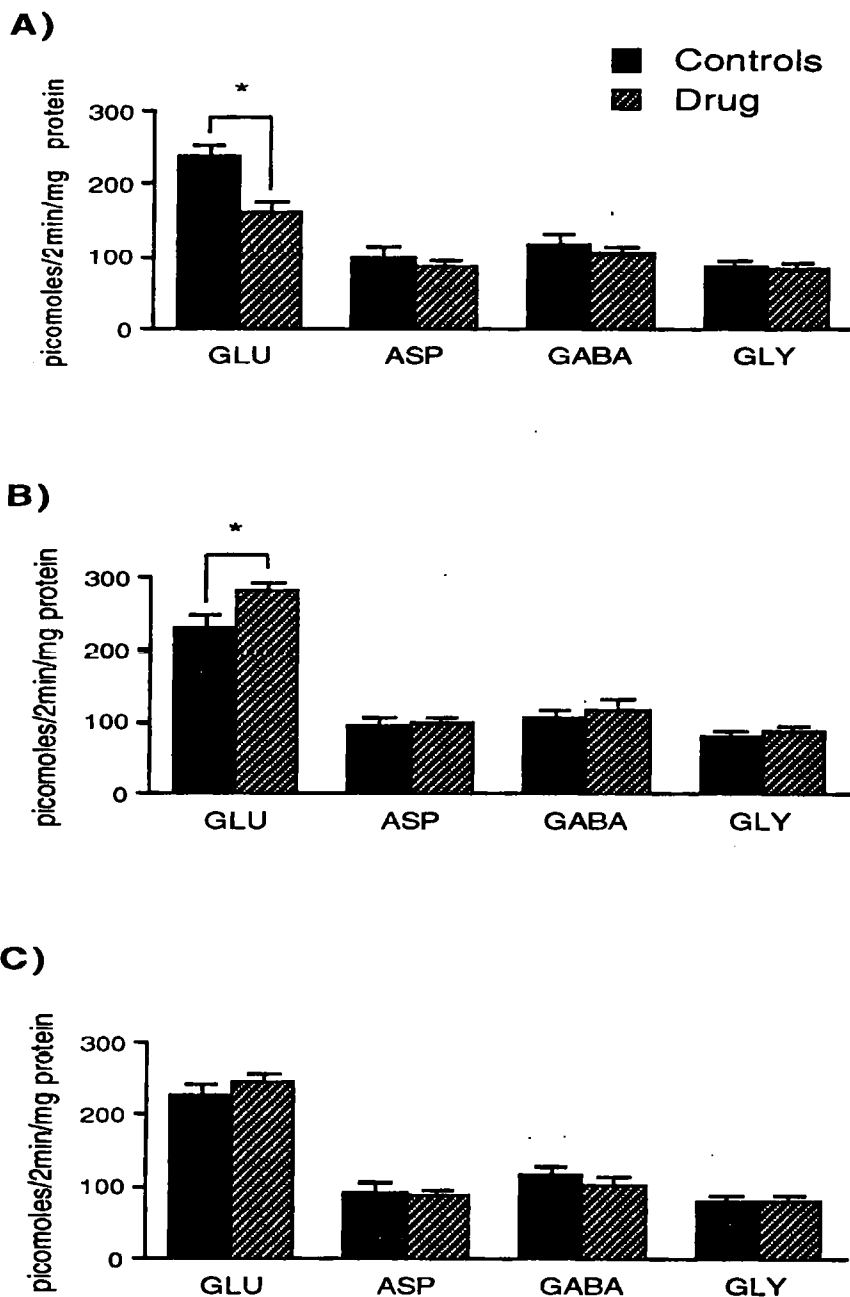


Figure 6.6. The effect of adenosine A₁ receptor activation on the calcium-dependent release of amino acids. **A)** The specific A₁ agonist cyclohexyladenosine (CHA; 10mM, n=7); **B)** the A₁ antagonist cyclopentyltheophylline (CPT; 100mM, n=7) ; and **C)** both CHA (10mM) and CPT (100mM) (n=7), were added to slices of chick IMHV following pre-incubation in a Krebs-Henseleit buffer. Following 2 minutes incubation, the media were removed and analysed by HPLC for the amino acid content. Protein estimations were carried out on the slices, and values of picomoles release per minute were computed. Values are means \pm S.E.M.. An asterisk * indicates a significant difference from control levels ($p < 0.05$, ANOVA). GLU =glutamate, GABA =g-aminobutyric acid, ASP =aspartate and GLY =glycine.

The effect of the adenosine A₂ receptor agonist, CGS 21680, on the calcium-dependent release of amino acids

The highly specific A₂ receptor agonist, 2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamido adenosine hydrochloride (CGS 21680) (affinity for A₂=19nM; A₂/A₁=0.03), was added to the incubation media, and samples were taken for analysis after 2 minutes. Two concentrations of the drug were used, because O'Regan et al. (1992a and 1992b) have previously demonstrated concentration-dependent effects of the CGS compound.

The results of the amino acid analyses are shown in Figures 6.7A and B (sample size, n=7 for all conditions). There is a selective, and significant, increase in the calcium-dependent release of glutamate (F=5.111, p=0.0433, df=12) with 5mM CGS 21680. This increase was greater than that with the A₁ antagonist CPT (133% and 120% respectively). The lower concentration (10nM) produced a significant decrease in the release of GABA (F=5.6, p=0.037, df=12). It had no effect on the release of the other amino acids (p > 0.35).

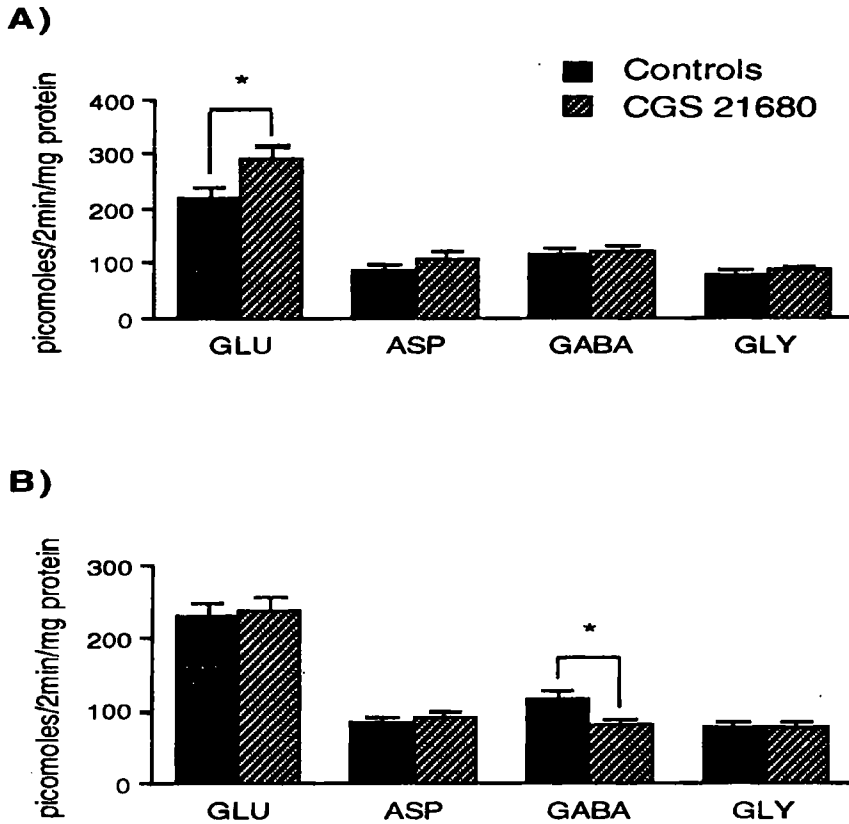


Figure 6.7. The effect of the adenosine A_7 receptor agonist, CGS21680, on the calcium-dependent release of amino acids. **A)** 5mM or **B)** 10nM CGS 21680 was added to slices of chick IMHV following pre-incubation in a Krebs-Henseleit buffer. Following 2 minutes incubation, the media were removed and analysed by HPLC for the amino acid content ($n=7$). Protein estimations were carried out on the slices and values of picomoles release per minute were computed. Values are means \pm S.E.M.. An asterisk * indicates significant difference from control levels ($p < 0.05$, ANOVA). GLU =glutamate, GABA =g-aminobutyric acid, ASP =aspartate and GLY =glycine.

Experiment 6.3. Endogenous release of adenosine evoked by KCl and NMDA

Introduction

Adenosine release from depolarised brain slices has been well characterised (Pull and McIlwain 1977). Release can be obtained with electrical stimuli, potassium, ouabain or veratridine. Also glutamate has been found to release radiolabelled adenosine derivatives and adenosine itself from slices (Hoehn and White, 1990) *in vivo* (Perkins and Stone, 1983), and from synaptosomal preparations (Hoehn and White, 1990). It has also been demonstrated that potassium depolarisation releases endogenous adenosine from cortical slices (Hollins and Stone, 1980; Hoehn and White, 1990), and from synaptosomal preparations (Hoehn and White, 1990; White and Macdonald, 1990).

We have shown simultaneous increases in adenosine release with the increases in glutamate found following passive avoidance training in the IMHVs of day-old chicks (see Section 6.1). This investigation was carried out in order to determine whether the increases in adenosine release were mediated by the NMDA glutamate receptor and to determine the effects of potassium stimulation on release.

Materials and methods

Release procedure

Slices (350mM) were prepared (see Chapter 4) from the IMHVs of untrained day-old chicks and placed in a pre-incubation buffer for 60 minutes, during which time the buffer was replaced twice. As described previously (Chapter 4) the IMHVs of 3 or 4 chicks were combined in a vial. Following the first replacement of buffer, the combined IMHVs were separated into two roughly equal portions. One portion was used for the experimental treatment, the other as the control: this allowed paired t-tests (using the statistical package Statview) to be carried out on the data. Two minutes after the second replacement, a sample (100ml of 1ml) of the 'pre-stimulation' buffer was taken (see Figure 6.8). Following the next 20 minutes pre-incubation, the buffer was then replaced with an incubation medium (Krebs-Henseleit buffer, oxygenated and at 37°C: see Chapter 4) containing either the desired concentration of NMDA (200mM), D-AP5 (200mM) or 50 mM KCl. Controls were incubated in normal Krebs-Henseleit buffer only. After a 2 minute incubation, a sample of the medium was removed (the 'incubation' sample, sample 2). Eight minutes later the remaining media was replaced with a normal Krebs-Henseleit buffer to remove the NMDA/D-AP5 or 50mM KCl, from which a sample was again taken 2 minutes after the replacement of the buffer (sample 3). This was repeated two more times (samples 4 and 5) following buffer renewal (with a Krebs-Henseleit buffer) after which the slices were removed and prepared for protein estimations (Bradford method, see Chapter 4). These post-stimulation samples were taken because of the post-stimulation effects that had been found by Hollins and Stone (1980), discussed at the end of this section.

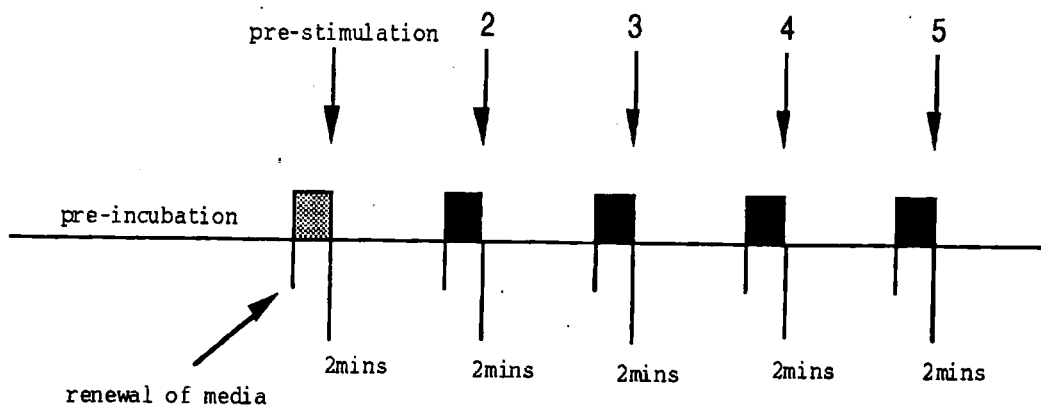


Figure 6.8. Diagram showing the time course over which sampling of the reaction media took place. The horizontal line represents time, with the 'pre-stimulation' and numbers (2-5) indicating the sampling points. Samples (100ml) were removed 2 minutes after renewal of each incubation buffer, and buffer renewal was every 10 minutes. (See release procedure for further details).

HPLC analysis

Adenosine was analysed using the procedure described in Chapter 3.

Results

Endogenous release of adenosine evoked by NMDA and KCl

Samples of the media were taken 2 minutes after the second replacement of the pre-incubation medium as a level of pre-stimulation release of adenosine (sample 1), after 2 minutes of replacing the buffer with medium containing 200mM NMDA (the stimulated release; sample 2: sample size, n=8 for both NMDA-treated and controls) and subsequently following the next three replacements of Krebs-Henseleit buffer, each time after 2 minutes of incubation in the medium (samples 3-5) (see Figure 6.7).

Adenosine release was significantly higher than control levels following NMDA-stimulation of the slices at collection points 3 and 4 (see Figure 6.9 and Table 6.1). There was almost a 250% increase in the level of release in sample 3. This result was highly significant (paired t-test, $t=7.618$, $p=0.0001$, all $df's=13$). The 4th sample also produced significantly higher levels of adenosine ($t=4.8431$, $p=0.0004$). The final sample, taken 22 minutes following stimulation with NMDA, did not show elevated adenosine release, nor did the 2nd sample ($p > 0.05$).

The K^+ -stimulated release of adenosine is presented in Figure 6.10 and table 6.2. KCl (50mM) was used to stimulate the slice preparation (sample size n=8 for KCl-stimulated slices and controls). The results show an increase in the release of K^+ -stimulated adenosine release compared to controls at collection periods 3 ($t=2.621$, $p=0.0223$, $df=13$) and 4 ($t=2.311$, $p=0.03$, $df=13$). No differences occurred between the other samples ($p > 0.2$).

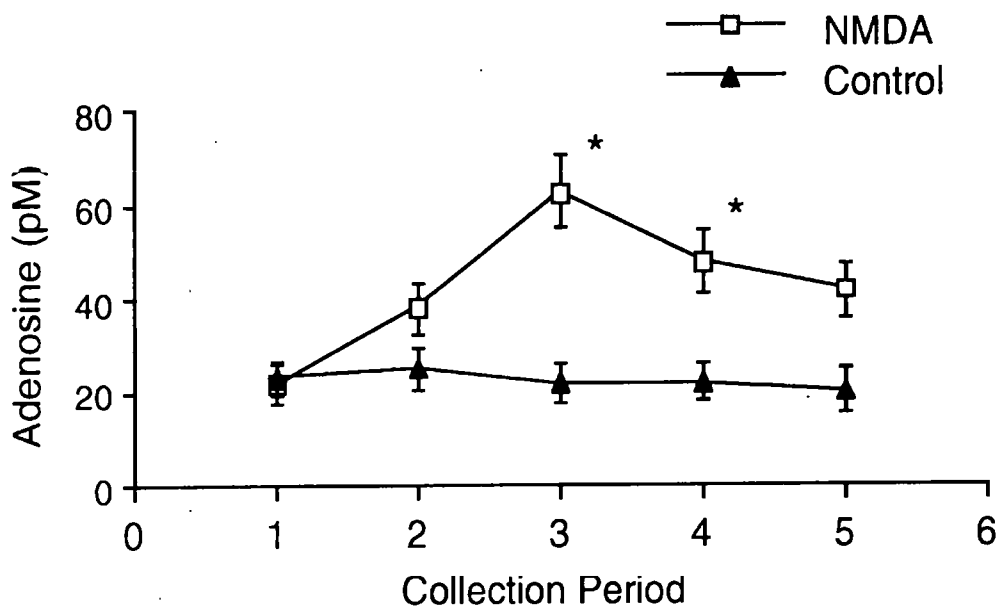


Figure 6.9. The effect of NMDA (200mM) on the release of adenosine from slices of the IMHV of day-old chicks. Adenosine release was measured (picomoles/ 2 min collection period/ mg protein) at 5 collection points (n=8): 1) pre-stimulation, 2) during stimulation with 200mM NMDA and 3-5) post-stimulation (see text for further details). An asterisk * indicates a significant difference between stimulated and control values (p < 0.05, paired t-test).

Release protocol	collection periods				
	1	2	3	4	5
NMDA	22±4.3	38±5.8	62.6±7.7*	48±6.8*	41.7±5.7
Control	23±3.8	25±4.4	21.8±4.0	22±4.1	20±5.1

Table 6.1. The release of adenosine (picomoles/ 2 min / mg protein) at 5 collection points (n=8): 1) pre-stimulation, 2) during stimulation with 200mM NMDA (or normal KHB buffer for controls) and 3-5) post-stimulation (see text for further details). An asterisk * indicates p < 0.05, paired t-test.

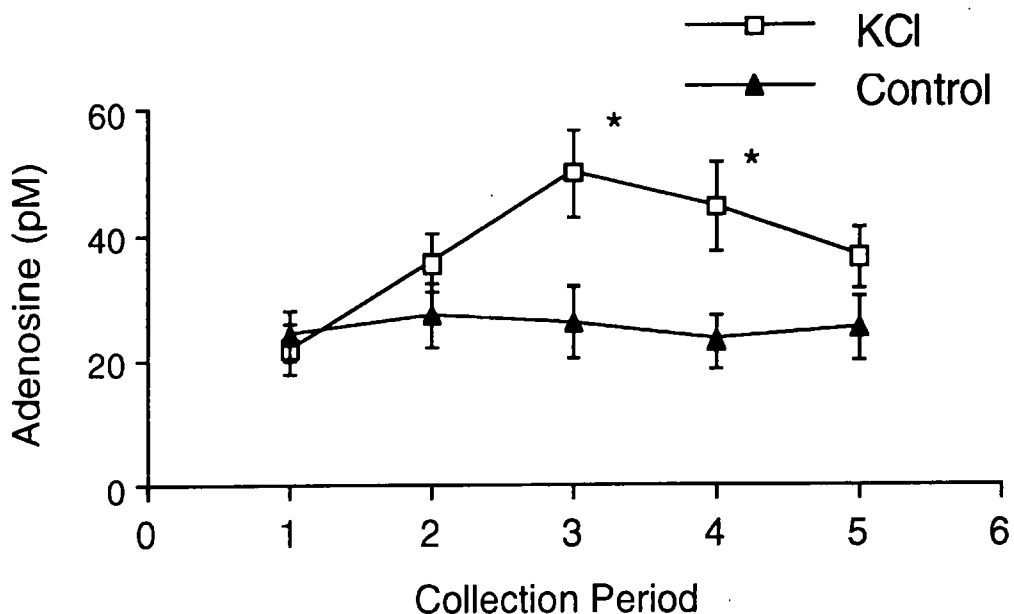


Figure 6.10. The effect of 50mM KCl on the release of adenosine from slices of the IMHV of day-old chicks. Adenosine was measured (picomoles/ 2 min / mg protein) at 5 collection points (n=8): 1) pre-stimulation, 2) during stimulation with 50mM KCl (or normal KHB buffer for controls) and 3-5) post-stimulation (see text for further details). An asterisk * indicates a significant difference between stimulated and control values

Release protocol	collection periods				
	1	2	3	4	5
KCl	22±4.1	35.7±4.9	49.8±7.0*	44.5±7.1*	36.2±4.9
Control	24±4.	27.3±5.1	26±5.7	23±4.3	25±5.0

Table 6.2. The release of adenosine (picomoles/ 2 min / mg protein) at 5 collection points (n=8): 1) pre-stimulation, 2) during stimulation with 50mM KCl (or normal KHB buffer for controls) and 3-5) post-stimulation (see text for further details). An asterisk * indicates $p < 0.05$, paired t-test.

NMDA-receptor mediated release of adenosine

To determine whether the NMDA-evoked release of adenosine was mediated specifically by NMDA receptors, the competitive NMDA receptor antagonist, 2-amino-5-phosphopentanoic acid (D-AP5, 200mM), was used to antagonise the effects of NMDA and KCl stimulation. The experiment was similar to that described above, although chick IMHVs were now combined three ways, with D-AP5 being present at the same time as the NMDA or 50mM KCl buffer: ANOVAs were carried out on the data.

The results are presented in figure 6.11 and table 6.3 (n=8 for both AP-5-treated samples and controls). The effect of D-AP5 was to significantly reduce the NMDA-evoked release of adenosine during periods 3 ($t=5.9647$, $p=0.0001$, $df=12$) and 4 ($t=3.672$, $p's=0.0032$, $df=12$: this is comparing these data with those of NMDA and KCl stimulations shown previously). There was now no difference in the release of adenosine, at any of the sampling points, as compared to unstimulated, Krebs-Henseleit buffer-only controls (ANOVA, $p > 0.50$).

The 50mM KCl-evoked release of adenosine was decreased by the addition of D-AP5 ($t=2.379$, $p=0.0348$, $df=12$), although there were no differences after this time ($p > 0.3$). Again, there were no differences as compared to control values (ANOVA, $p > 0.4$).

The experiment was repeated, with only the first post-stimulation media (collection point 3) being taken for analysis in this instance. Both the stimulation media were used and both had AP-5 added to them in two separate experiments: combined IMHVs were divided into two sets in each experiment, one undergoing AP-5 inhibition the other stimulated by KCl (one experiment), the other undergoing AP-5 inhibition the other stimulated by NMDA (a separate experiment). The results are shown in Figure 6.12. There were significant differences between both AP-5-containing media and their controls: KCl (paired t-test, $t=3.842$, $p=0.0130$, $df=13$); NMDA ($t=6.783$, $p=0.0003$, $df=13$) (sample sizes, $n=8$ in all groups).

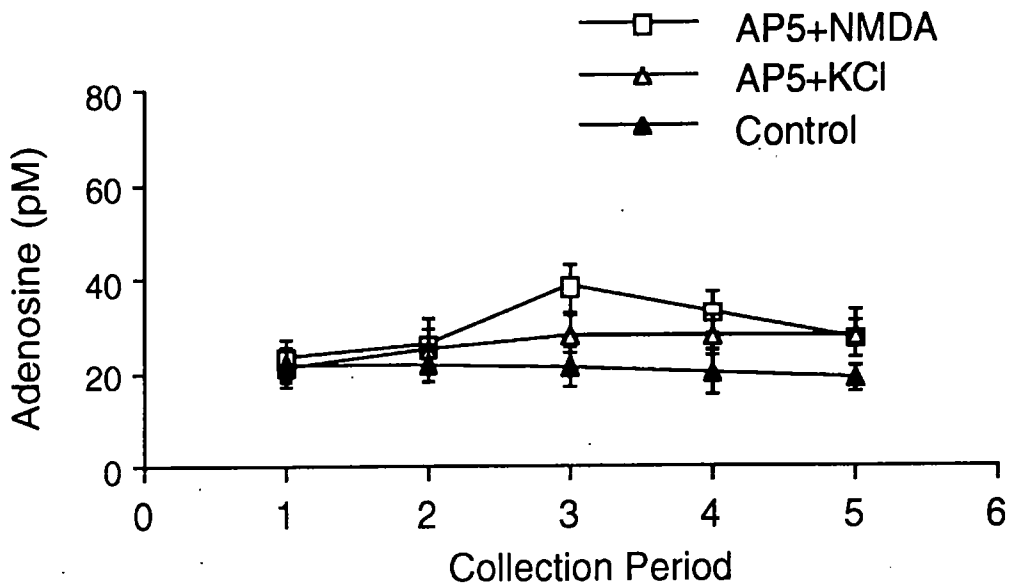


Figure 6.11. The effect of the NMDA antagonist AP-5 on the release of NMDA-, and K-stimulated, adenosine from slices of chick IMHV. Adenosine release was measured (picomoles/ 2 min / mg protein) at 5 collection points: 1) pre-stimulation, 2) during stimulation with either 200mM AP-5 and 50mM KCl or 200mM AP-5 and 200mM NMDA (or normal KHB buffer for controls) and 3-5) post-stimulation.

Release protocol	collection periods				
	1	2	3	4	5
NMDA+ AP5	23±4.3	26±5.8	38±5.3	33±4.3	27±4.2
KCl + AP5	21±3.8	25±4.4	28±4.1	28±4.1	28±5.1
Control	22±3.4	22±4.0	21±4.2	20±4.8	19±3.1

Table 6.3. The release of adenosine (picomoles/2 min/mg protein) at 5 collection points: 1) pre-stimulation, 2) during stimulation with either 200mM AP-5 and 50mM KCl or 200mM AP-5 and 200mM NMDA (or normal KHB buffer for controls) and 3-5) post-stimulation (see text for further details).

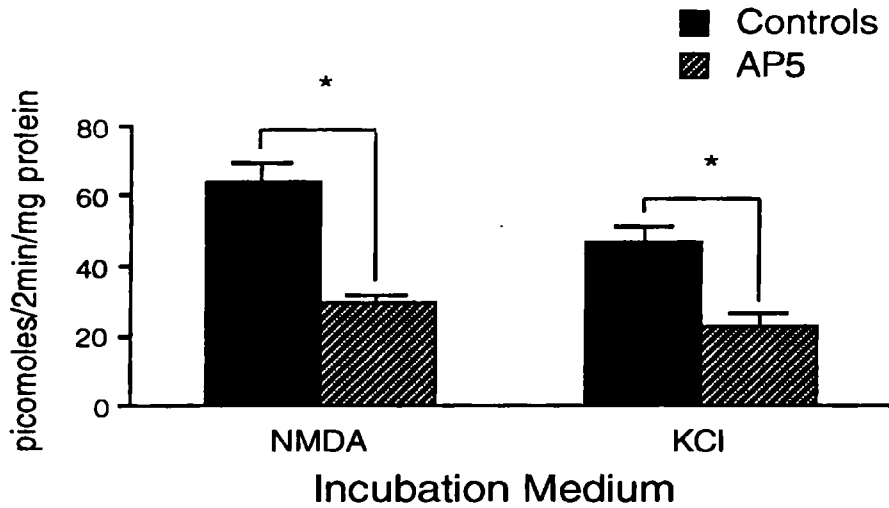


Figure 6.12. The effect of the NMDA antagonist, AP-5, on the release of NMDA-, and K⁺-stimulated, adenosine from slices of the IMHV of day-old chicks. AP-5 (200mM) was added to Krebs-Henseleit media containing either NMDA (200mM) or a 50mM KCl-Krebs-Henseleit media (all sample sizes n=8). Slices of chick IMHVs were incubated for 10 minutes in the stimulation media. Samples were taken 2 minutes after replacing the stimulation media with a Krebs-Henseleit buffer. Values are the means \pm S.E.M. from seven experiments. An asterisk * indicates a significant difference between stimulated and control levels ($p < 0.05$, paired t-tests).

Discussion

Calcium-dependent and Ca^{2+} -independent adenosine release was increased in both left and right IMHVs of day-old chicks following training on a passive avoidance task (Experiment 6.1); the time points coincided markedly with concomitant increases in the release of glutamate as found in Chapter 4. Hence, 30 minutes and 1 hour following training on the task, there were calcium-dependent increases in adenosine release from the left IMHV, and there were increases in adenosine release 30 minutes, 3 and 6.5 hours after the training task in the right IMHV, as per glutamate release.

Calcium-independent release of the purine from IMHV slices was also found. This suggests that two mechanisms may be in operation following stimulation with high levels of KCl: a vesicular or transmitter-like release, and the operation of the bidirectional nucleoside transporter.

The following conclusions may be drawn from the results described in Experiment 6.2. Firstly, that adenosine acts in a concentration-independent manner to inhibit, selectively, the calcium-dependent release of the excitatory amino acid glutamate. The relatively low dose of 10mM produced no significant effects on release, nor did the highest concentration (500mM) used. The intermediate dose of 100mM, however, did selectively inhibit the release of glutamate from the slice preparation. Previously it has been found that, in the rat, free adenosine content in brain tissue was between 5 and 10mM (Rubio et al., 1978). In the hippocampus a significant depression (25 %) on population spikes recorded from CA1 neurons was found in the presence of only 2.5mM adenosine, with 100% depression found when the slices were superfused with 20mM adenosine (Schubert and Mitzdorf, 1979). In addition EPSPs were also reduced in a concentration-dependent manner. These results suggest that endogenous adenosine levels may tonically inhibit synaptic activity. Corradetti et al. (1984) demonstrated a decrease in the release of

aspartate and glutamate from rat hippocampal slices following the inclusion of adenosine in the incubation medium. In their study adenosine inhibited the amplitude of both the CA1 EPSPs and the population spike in the pyramidal cell layer; effects were found at concentrations as low as 10-20mM, with complete inhibition of the evoked potentials between 100-300mM. Okada et al. (1992) also found that 100mM adenosine significantly reduced the release of glutamate. In marked contrast, however, was their finding that a small concentration (0.1mM) produced excitatory effects in the CA3 region of the hippocampus, and they describe a biphasic, concentration dependent effect in the hippocampus, with excitatory effects with concentrations ranging from 10nM to 1mM and inhibitory effects at 10mM to 1mM. From this, it is suggested here, that the different effects of adenosine, and its receptor-subtype agonists and antagonists, which have been found, appear to depend upon the tissue source being used. For example, Okada et al. (1992) have described excitatory, but not inhibitory, actions of both receptor subtype agonists in the superior colliculus of the rat, whilst they found both excitatory and inhibitory effects in the hippocampal slice preparation. From these results, they have suggested that two broad receptor types occur in the brain; inhibitory (A_I) and excitatory (A_E) receptors (as defined by Ishikawa et al., 1994). The two receptor subtypes would also have differing affinities for the endogenous purine, suggested to be a high affinity (A_E) and a low affinity (A_I) receptor type. Hence, it was thought that the superior colliculus would lack the inhibitory receptor subtype as adenosine inhibitory effects were not noted but that the hippocampus would have both types of receptor.

The selective inhibition of calcium-dependent glutamate release was found to be mediated by the addition of adenosine to the IMHV slice preparation. In Experiment 6.2, an A_1 receptor agonist, cyclohexyladenosine (CHA), inhibited glutamate release. This effect was blocked when an equivalent concentration of the A_1 antagonist CPT was used to compete for receptor binding. This demonstrates that the adenosine A_1 receptor acts to

inhibit glutamate release in this preparation. CPT alone produced an increase in the release of glutamate, suggesting that endogenous adenosine levels of adenosine in the IMHV slice are at levels at which there is a 'natural' inhibition of glutamate release which is being controlled by adenosine A₁ receptors.

At concentrations which would probably activate only A₁ receptors, Simpson et al. (1992) showed that the adenosine analogues, CPA and NECA, significantly depressed ischaemia-evoked aspartate and glutamate release. At higher concentrations, concentrations at which both the inhibitory A₁, and excitatory A₂, receptor subtypes would be activated, no inhibition of release was now seen. This is similar to the results found in the chick slice preparation with the application of adenosine only; the higher (500mM) concentration of adenosine probably activates both receptor subtypes and the inhibitory effects are not found at this concentration. Ameri and Jurna (1991) have suggested that A₂-mediated effects will prevail over the effects mediated by A₁ receptors. Also Burke and Nadler (1988) found that higher concentrations of NECA were less potent than expected (Ebstein and Daly, 1982), again suggesting that its actions at A₂ receptors may offset its inhibitory actions.

The specific activation of the A₂ excitatory adenosine receptors with the agonist CGS 21680 produced an increase in the release of glutamate from chick IMHV slices when the agonist is applied at a relatively high concentration (5mM). The lower concentration (10nM) did not produce this effect but instead inhibited the release of GABA from the chick IMHV slice preparation. In the rat brain, two adenosine receptor sites are labelled by CGS 21680, the higher affinity site representing the A_{2a} receptor. As it is the lower concentration (10nM), only, of the agonist that inhibits the release of GABA, it is the higher affinity site, i.e. the A_{2a} receptor, that is implicated in the selective inhibition of the amino acid. The effect of adenosine analogues on

GABA release remains unclear; in the ischaemic rat preparation the selective activation of either A₁ or A_{2a} receptors resulted in an inhibition of the release (O'Regan et al., 1992a). When, however, the drugs were applied at higher concentrations, i.e. at a dose that would activate both A₁ and A₂ receptors, there was no change in GABA release (O'Regan et al., 1992). The inhibitory effect of adenosine may be linked to the activation of DIDS-sensitive chloride channels on hippocampal CA1 pyramidal neurons, which in turn can lead to a potentiation of responses to GABA_A receptor activation (Akhondzadeh and Stone, 1994). O'Regan et al. (1992) suggested that it was the lower affinity excitatory A_{2b} receptor that may mediate the increase in the excitatory amino acids.

The interaction of adenosine on GABA release was initially investigated by Hollins and Stone (1980). They described an inhibition of GABA release, from slices of cerebral cortex, following the application of adenosine. However, Dolphin and Archer (1983) and Limberger et al. (1986) did not find any inhibition of release of GABA by 2-CADO. This latter study measured release of GABA synthesised from glutamine: the GABA is, therefore, much more likely to be released from vesicles as a neurotransmitter. Opposed to this, O'Regan et al. (1992) did demonstrate adenosine inhibition of GABA release. The application of adenosine receptor agonists at concentrations allowing the specific activation of A₁ receptors inhibited GABA release. Higher concentrations of these agonists, which would also activate A_{2b} receptors, did not affect GABA release. In the present study, application of CGS 21680, the specific A₂ receptor agonist, inhibited the release of GABA at concentrations implicating the high affinity A_{2a} receptor. Therefore it could be concluded that A₁ and high-affinity A_{2a} receptors block both inhibitory amino acid release, whilst the co-activation of A_{2b} (low-affinity) and A₁ receptors results in the loss of the inhibitory effect of adenosine. These findings are in agreement with those found by Kirk and Richardson (1994) who described A_{2a} receptors present on GABAergic striatal

nerve terminals that acted to inhibit the release of GABA (this action may be mediated by A_{2a} inhibition of PKC activity; Kirk and Richardson, 1995). The mechanism of this inhibition has been examined by Akhondzadeh and Stone (1994). They showed that adenosine was able to potentiate the ability of muscimol ($GABA_A$ agonist) to inhibit evoked potentials. The chloride channel blocker DIDS reduced the inhibitory action of both muscimol and adenosine. This suggested that the inhibitory action of adenosine was mediated in postsynaptic neurones by enhancing chloride fluxes and that this can lead to a potentiation of responses to $GABA_A$ receptor activation. The fact that they found a potentiation of the effects of adenosine with muscimol, also suggests that two distinct chloride channels may be being activated.

The results in Experiment 6.3 described the NMDA and KCl-evoked release of adenosine from the *in vitro* slice preparation of the chick IMHV. The K^+ -evoked release data found in this study are in agreement with those noted by Hollins and Stone (1980), who found that increasing the potassium concentration to 54mM did not immediately evoke an increase in radiolabelled adenosine, which they took to indicate that adenosine was not released in a transmitter-like manner, however, there was an increase immediately following potassium stimulation which possibly suggests that another pool of adenosine is being released initially, perhaps being followed by the radiolabelled pool, or that the release is indeed delayed following stimulation. K^+ -evoked release of adenosine from cortical slices has been shown to be partially mediated, indirectly, by the release of an excitatory amino acid, which in turn acts at NMDA receptors to promote adenosine release (Hoehn and White, 1990). The results described in experiment 6.3 would tend to agree with this, as the action of AP-5 is not selective just for the NMDA-evoked adenosine release, but also inhibits the K^+ -stimulated release of adenosine, suggesting that the K^+ -stimulation involves NMDA receptor interactions to produce the release of the purine.

Potassium depolarisation is weaker in eliciting release than ouabain or veratridine (Shimizu et al.,1970); this would suggest that purine release is associated with the influx of sodium ions or of calcium ions moving specifically through sodium channels. Indeed a Na⁺-dependent transport system may exist in central neurons which may release adenosine when the Na⁺-electrochemical gradient is reversed. However, much of the release has been suggested to arise from intracellular adenosine exiting cells via a bidirectional nucleoside transporter which acts by facilitated-diffusion when the intracellular concentration exceeds the extracellular level (White and MacDonald, 1991).

NMDA application to the incubation media increased the release of adenosine in a similar manner to K⁺-stimulation. The NMDA-evoked release of adenosine was abolished when the selective NMDA-antagonist AP-5 was applied with NMDA to the slices. The fact that AP-5 blocked NMDA-evoked adenosine release indicates the release was mediated through NMDA receptors. This is in agreement with studies in other preparations: Chen et al. (1992) showed the release of endogenous adenosine from the hippocampus of the rat to be mediated by NMDA receptors. In rat cortical slices, at least 50% of the glutamate-evoked release of adenosine was determined to be regulated by the NMDA receptor system, although a further 66% of the remaining release was diminished by a non-NMDA receptor antagonist, suggesting that both NMDA and non-NMDA receptors were involved in the release in this preparation (Hoehn and White, 1990). Studies with non-competitive antagonists such as Mg²⁺ and MK-801 suggest that only a small fraction of the available NMDA receptors must be activated for adenosine release from cortical slices to be maximal (Hoehn et al., 1990). Therefore it seems unlikely that adenosine is released to act as a neuroprotective device in cases of over-stimulation but more likely as a fine tuning control, inhibiting the further release of glutamate and/or diminishing post-synaptic responses providing

another inhibitory threshold (as well as the Mg^{2+} -block of the NMDA receptor) which must be overcome in order for NMDA receptor-mediated processes to be carried out. Thus adenosine may maintain processes such as synaptic plasticity, learning and memory (Hoehn and White, 1990). Adenosine release during low levels of EAA receptor activation could inhibit the further release of glutamate if the adenosine was released in nanomolar quantities, or actually stimulate further glutamate release, if the adenosine released was at a micromolar level. Released adenosine may provide, at least initially, an inhibitory threshold which must be overcome in order for NMDA-mediated transmission to be able to proceed; and once over this threshold, adenosine actions at A_2 receptors may allow a positive feedback system to evolve such that maximal adenosine release will produce effects that override the A_1 inhibitory system and stimulate the release of excitatory (and inhibitory?) transmitters.

NMDA has been found to cause a reduction in the sensitivity of adenosine: if AP-5 is present before removing the Mg^{2+} , thus preventing any transient activation of NMDA receptors, the adenosine responses remain unaffected (Bartrup and Stone, 1990). These results may be explained as being due to the activation of excitatory adenosine receptors, which would have actions similar to suppressing the inhibitory action of adenosine.

Since NMDA receptors show an increased number and activity shortly after the training task (Steele, 1995), their activation might increase adenosine levels in the active synapses to a level that activates A_2 receptors, which according to the results presented in Experiment 6.2, would lead to either a decrease in GABA release (and thus a reduction in the inhibitory tone of the neuronal environment) or might increase glutamate release and thereby increase the excitation in the neural population subjected to NMDA-receptor activation. This would allow an explanation for the results described in Experiments 6.1 and Experiment 4.1 showing a concomitant increase in

glutamate release and adenosine release 30 minutes after training in both IMHVs.

The proposed neuroprotective effect of adenosine may, yet, also explain the results. It has been shown that glutamate and aspartate enhance the release of adenosine from rat hippocampal synaptosomes (Poli et al., 1991). As adenosine decreases the evoked release of these amino acids a feedback would exist, whereby adenosine acting presynaptically will reduce excitatory amino acid release, and post-synaptically it may inhibit the NMDA receptor-mediated glutamate response.

Chapter 7: The Effect of Adenosine Receptor Agonists and Antagonists on Memory Formation following a Passive Avoidance Task

Introduction

Increases in the release of glutamate, GABA and aspartate occurred in the IMHVs and LPOs of the chicks at specific times following training on a one-trial passive avoidance task (Chapters 4 and 5). Training-induced increases of adenosine were also found that coincided temporally with the increased glutamate release (Chapter 6). In addition adenosine analogues were shown to inhibit or elevate the release of glutamate or GABA from chick IMHV, depending on the adenosine receptor subtype activated, and it was also shown that NMDA stimulated the release of endogenous adenosine from the slice preparation.

The following experiments were carried out to demonstrate whether adenosine has an effect on the acquisition or retention of the learning task.

Materials and Methods

Training procedure

The training procedure was as described previously (Chapter 4).

Statistical treatment of data

The statistical significance of the differences between treated and control chicks was calculated using the χ^2 test (Statview).

Drug concentrations and injection procedure

For administration of intra-cranial (*i.c.*) injections, animals were placed in a stereotaxic plexiglass headholder (as described in Davis *et al.* 1979) which contains boreholes that guide the injection cannula to defined stereotaxic coordinates and which allows fast and precise injections into the IMHVs. The chemical under test was injected into the forebrain using a Hamilton syringe, fitted with a plastic sleeve as a stop for the depth coordinate (4mm) for the IMHV. Since chicks have an unossified skull, such injections took only a few seconds for each bird, and no anaesthetic was required.

2-chloroadenosine (2-CADO), cyclohexyladenosine (CHA) and cyclopentyltheophylline (CPT), all from Sigma (Poole, England), and CGS 21680 from Research Biochemicals Int., were dissolved by sonication in saline (0.9%) prior to injection. Solutions were freshly prepared on the day of use. For *i.c.* injections, 5 μ l of a stock solution was injected in each hemisphere in each experiment. For the dose-response tests, fresh solutions of the appropriate concentrations were prepared. Sterile saline solution (0.9%) was injected into the IMHVs of control chicks.

For all drugs used in the study, the dosage that would prove effective at inhibiting memory formation, whilst at the same time not interfering with any other of the chicks' behaviours, was determined. Chicks were pre-trained

in the usual manner (see Chapter 4); those chicks that did not peck the bead were discarded from the experiment. Chicks were then injected bilaterally (5ml per hemisphere) with a range of concentrations of the drug being tested. The chicks were then tested 30 minutes or 1 hour later with a dry chrome bead. The behaviour of the chicks was then noted: they should peck the bead and show no side effects on their locomotor activity. If the chicks did not show normal behaviours, then the dose which the chicks had been given was considered to be too great for the effect of the drug on memory formation to be distinguished. In general, the maximum dose used for behavioural experiments was half of the highest dose that did not cause an inhibition of pecking or other behaviours.

Experiment 7.1. The effect of the non-selective adenosine analogue 2-chloroadenosine (2-CADO) on recall of a one-trial passive avoidance task

Introduction

2-CADO is a non-selective A₁ and A₂ receptor agonist (Bruns et al., 1986). The behavioural investigations using this substance were carried out prior to the *in vitro* slice work on adenosine release, and therefore represented a 'look and see what happens' approach. It was hypothesised that 2-CADO would produce amnesia for the task by inhibiting transmitter (glutamate) release, but at the time of the study no direct evidence of an effect on amino acid release had been found (this was provided subsequently as described in Chapter 6).

Methods

Dose dependency of 2-CADO

To determine the dose-response for 2-chloroadenosine (2-CADO), four concentrations of the drug were used: 20, 50 and 200 μ M and 2mM. Thirty minutes before the training procedure, each group of chicks received a bilateral injection (5 μ l) of one of the four concentrations of 2-CADO in 0.9% sterile saline, whilst a control group of chicks received bilateral injections (5ml per hemisphere) of 0.9% saline only (n = for 2-CADO, 15, 17, 17, 15 for each dose; for saline, 21, 19, 20, 21 respectively). Chicks were tested for recall of the task 1 hour later.

Time-dependency of 2-CADO

To determine the time of the onset of amnesia following 2-CADO injections, four groups of chicks received a bilateral 5 μ l injection of 50 μ M 2-CADO in 0.9% sterile saline 30 minutes before training, whilst a control group

of chicks received bilateral injections (5 μ l per hemisphere) of 0.9% saline only. Groups of chicks were tested for recall of the task at one of four time points later, each group being tested at one time point only. Thus one group of chicks was tested at 15 minutes and other groups at 30 minutes, 45 minutes or at 1 hour (n = for 2-CADO, 13, 12, 15, 15; for saline, 14, 15, 15, 12 respectively).

To determine whether the onset of amnesia described in this experiment was due to a time point in the acquisition of the task, as opposed to a diffusional effect or the fact that the drug may take a certain number of minutes to act, two further experiments were carried out in which the time of injection was varied. In the first instance, the time of injection was increased to 1 hour pre-training: bilateral injections of 5 μ l of either 50 μ M 2-CADO or 5 μ l 0.9% saline, with testing at 15, 30, 45 and 60 minutes post-training (n = for 2-CADO, 14, 12, 15, 13; for saline, 11, 12, 12, 11 respectively). A second experiment was carried out with injections 5 minutes pre-training. Again these were bilateral injections, of either 50 μ M 2-CADO or 0.9% saline, with the chicks tested at 15, 30, 45 and 60 minutes post-training (n = for 2-CADO, 14, 14, 13, 15; for saline, 13, 15, 11, 12 respectively).

Results

The lowest concentration used, 20 μ M, failed to produce amnesia ($\chi^2=1.71$, $p=0.2206$; data presented graphically in Figure 7.1). The next highest concentration (50 μ M) produced amnesia 1 hour after training ($\chi^2=7.65$, $p=0.0057$), as did 200 μ M ($\chi^2=12.67$, $p=0.0004$) and 2mM ($\chi^2=6.05$, $p=0.0161$).

As shown in Figure 7.2A amnesia had not set in by 15 minutes ($\chi^2=0.84$, $p=0.3586$), but by 30 minutes after training it had ($\chi^2=11.40$, $p=0.0009$), with 30 minutes pre-training injections of 2-CADO. Those chicks injected either at 1 hour or at 5 minutes pre-training (Figure 7.2B and C) also showed amnesia when tested from 30 ($p<0.05$), but not at 15 ($\chi^2=0.77$, $p=0.43$) minutes.

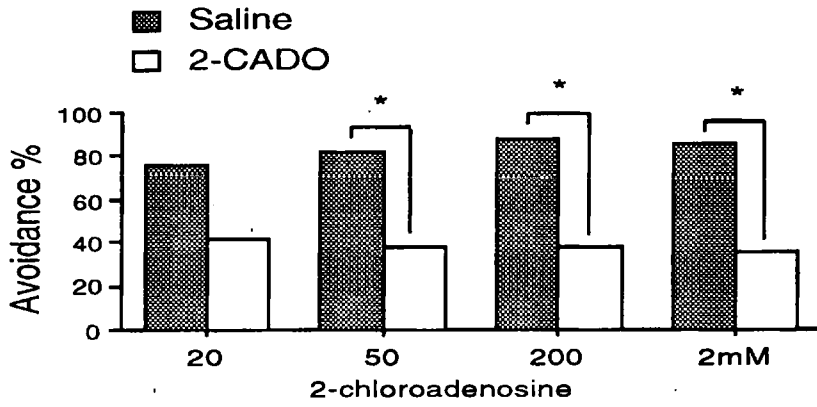


Figure 7.1. Dose-response effect of 2-chloroadenosine (2-CADO) on recall for the passive avoidance task. % avoidance of chicks injected (i.c.) bilaterally (5 μ l per hemisphere) 30 minutes pre-training, with 20, 50, 200 μ M and 2mM 2-CADO or 0.9% sterile saline only (controls). The chicks were tested 1 hour after training. An asterisk * indicates significant differences between saline- and drug-injected chicks (χ^2 , $p < 0.05$).

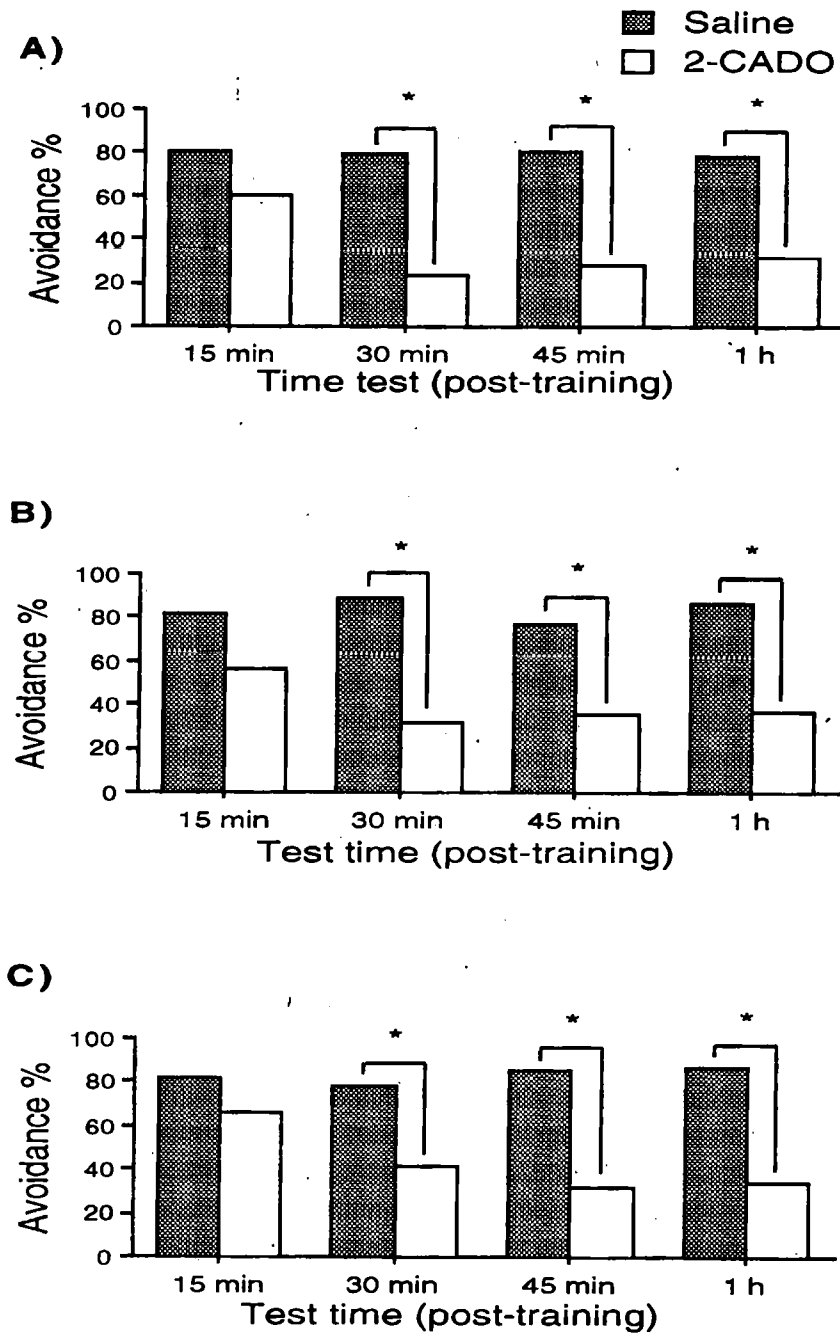


Figure 7.2. Time-dependency of 2-chloroadenosine (2-CADO) on the retention of the passive avoidance task. % avoidance of chicks injected (i.c.) bilaterally with 50 μ M 2-CADO (5 μ l per hemisphere), (A) 30 minutes, (B) 1 hour or (C) 5 minutes pre-training, and tested 15 minutes to 1 hour after training. An asterisk * indicates significant differences between saline-, and drug-injected chicks (χ^2 , $p < 0.05$).

Experiment 7.2. The effect of the adenosine A₁ agonist cyclohexyladenosine (CHA) on recall of the passive avoidance task

Introduction

CHA is a member of the N⁶-cycloalkyladenosines which is a group of highly selective A₁ agonists in the mammalian brain (Moos et al., 1985; affinity for A₁ = 1.2nM for A₂ = 500nM). The effects of CHA on the release of amino acids from the chick IMHV slice preparation have been described in Chapter 6. The decrease in glutamate release observed may be highly detrimental to the acquisition of the task, as it has been shown previously that glutamate release is increased following the task (Chapter 4 and 5); that antagonists of glutamate NMDA receptors produce amnesia for the task (e.g. Burchuladze and Rose, 1992); and that there is an increase in the number and affinity of glutamate receptors following training (Steele, 1995). Injections of an agonist for the A₁ receptor may, therefore, be expected to produce amnesia for the task.

Methods

Dose dependency of CHA

To determine the dose-response for CHA, four concentrations of the drug were used: 2, 10, 50 and 100µM. Thirty minutes before the training procedure, each group of chicks received a bilateral 5µl injection of one of the four concentrations of CHA in 0.9% sterile saline, whilst a control group of chicks received bilateral injections (5µl per hemisphere) of 0.9% saline only (n = for CHA, 13, 15, 15, 12; for saline, 18, 16, 17, 15 respectively). Chicks were tested for recall of the task 1 hour later.

Time-dependency of CHA

To determine the time of the onset of amnesia following CHA injections, groups of chicks received a bilateral 5ml injection of 10mM 2-CHA in 0.9% sterile saline either 1 hour, 30, 15 or 5 minutes before training, whilst a control group of chicks received bilateral injections (5µl per hemisphere) of 0.9% saline only. Groups of chicks were tested for recall of the task 1 hour post-training.

Results

The dose dependency of CHA is described graphically in Figure 7.3. The lowest concentration used, 2µM, did not produce amnesia when injected 30 minutes pre-training and tested 1 hour post-training ($\chi^2=1.01$, $p=0.28$). CHA at 10, 50 and 100µM all produced amnesia for the task (χ^2 , all $p<0.05$).

In Figure 7.4 10µM CHA was found to produce amnesia when injected at any time between 5 minutes and 1 hour before training, and tested at 1 hour post-training (χ^2 , all $p<0.05$).

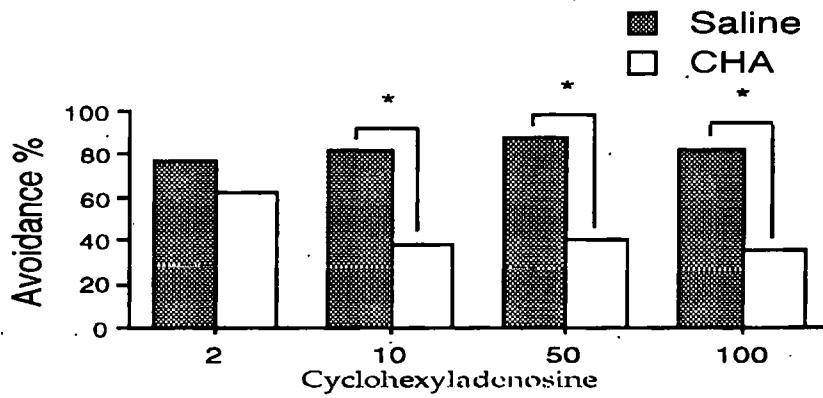


Figure 7.3. Dose response effect of cyclohexyladenosine (CHA) on recall for the passive avoidance task. % avoidance of chicks injected (i.c.) bilaterally (5μl per hemisphere) 30 minutes pre-training, with 2, 10, 50 or 100μM CHA or 0.9% sterile saline only (controls). The chicks were tested 1 hour after training. An asterisk * indicates significant differences between saline- and drug-injected chicks (χ^2 , $p < 0.05$).

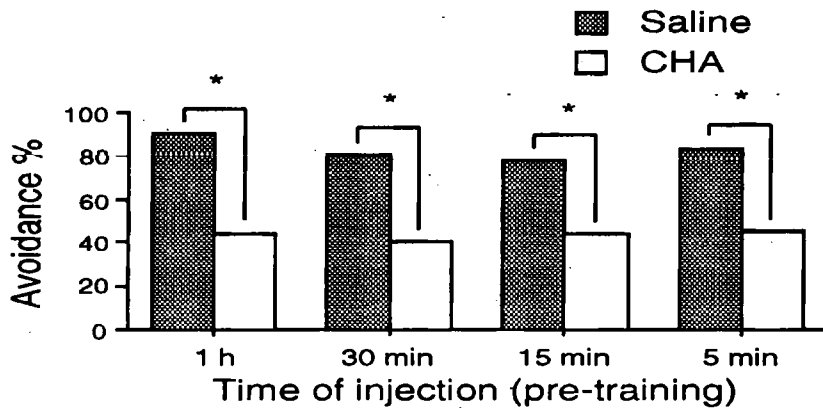


Figure 7.4. Time-dependency of CHA on the retention of the passive avoidance task. % avoidance of chicks injected (i.c.) bilaterally (5μl per hemisphere), 1 hour, 30, 15 or 5 minutes pre-training, and tested 1 hour after training. An asterisk * indicates significant differences between saline- and drug-injected chicks (χ^2 , $p < 0.05$).

Experiment 7.3. Is the effect of CHA lateralised to either hemisphere?

Introduction

The left IMHV has been shown to be of major importance for the acquisition of the one-trial passive avoidance task by the day-old chick in various studies. Lesioning the IMHV, for example, did not produce amnesia if the right IMHV only was lesioned. If the left IMHV was lesioned alone before training, amnesia occurred (Patterson and Rose 1992, and see Chapter 2).

Methods

CHA (5 μ l; 10 μ M) was injected into the left (n=15) or right (n=13) IMHV only, or bilaterally (n=15), (5 μ l per hemisphere) in order to test if the lesion study results can be reproduced with a pharmacological blocking technique that leaves the neuronal tissue intact. Control chicks for the experiments were injected with 0.9% saline into either the left (n=13) or right (n=13) IMHV only, or bilaterally (n=17). Injections were performed 30 minutes before training; testing was 1 hour after training in all cases.

Results

As expected the chicks that were injected in both hemispheres showed amnesia for the task (Figure 7.5; $\chi^2=6.036$, $p=0.0140$). Those injected with CHA into the left IMHV only also did not show recall for the task ($\chi^2=4.092$, $p=0.0431$). However, when the chicks were injected at the same dose and volume into the right IMHV only, they did not display amnesia, and avoided the MeA-covered bead ($\chi^2=0$, $p=1$).

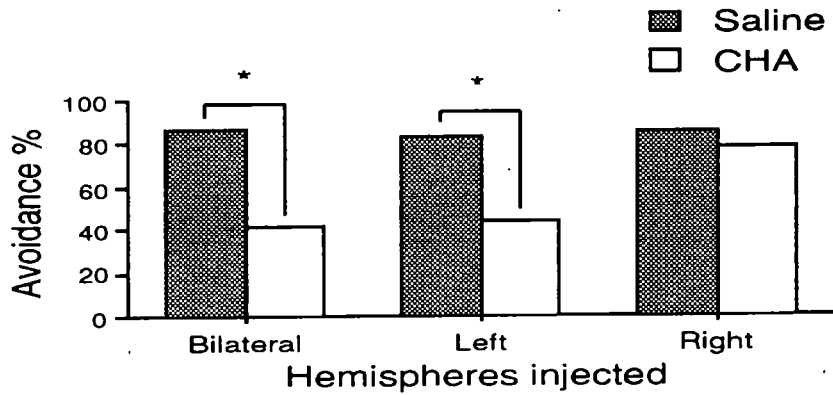


Figure 7.5. Lateralisation of the effect of cyclohexyladenosine (CHA) on the retention of the passive avoidance task: % avoidance of chicks injected (i.c.) with 10 μ M CHA, either bilaterally (5 μ l per hemisphere), left hemisphere only (5 μ l) or right hemisphere only (5 μ l) 30 minutes pre-training and tested 1 hour post-training. An asterisk * indicates significant differences between saline- and drug-injected chicks (χ^2 , $p < 0.05$).

Experiment 7.4. Is the effect of CHA due to the depression of locomotor activity?

Introduction

A potential problem encountered in *in vivo* adenosine work is the depressant effects on locomotor activity produced by adenosine and its agonists (Barraco et al., 1993) as well as respiratory and cardiovascular effects (Ribeiro, 1991).

To control for any potential locomotory effects of the drugs two tests were carried out, to determine the time it takes treated chicks to peck compared to their controls, and to find out whether chicks trained on the water bead were inhibited behaviourally so that they did not peck the bead.

Methods

Peck latency for CHA

Chicks were injected bilaterally (5 μ l per hemisphere), with either 10 μ M CHA (n=12) or 0.9% saline (n=11), 30 minutes before training. Training was carried out using a water bead in place of the MeA covered bead; testing was carried out 30 minutes later. The time from the presentation of the water bead to the time at which each chick pecked the bead was recorded at both training and testing. No chicks failed to peck the bead on both occasions.

Motor learning following i.c. CHA injections

Another test for determining whether the specific task has been learned, or whether any effects noted have been due to a locomotor disturbance, is to change the task so that chicks are trained on a water bead and to see how many do not peck subsequently at the bead during the second presentation. The numbers that avoid the bead should be as low as in the control chicks injected with saline if there are no locomotor effects of the

drug.

Chicks were injected bilaterally (5 μ l per hemisphere), with either 10 μ M CHA (n=15) or 0.9% saline (n=15), 30 minutes pre-training. Training was carried out using a water bead in place of the MeA covered bead; testing for recall of the task was carried out 1 hour later.

Results

The average peck latency for CHA-injected chicks was 2.6 ± 1.6 seconds (Figure 7.6A). The saline controls pecked on average 2 ± 1.62 sec after testing. The results were tested by a Mann Whitney U test and were not significantly different ($p > 0.05$).

Chicks injected with 10 μ M CHA showed 20% avoidance towards the water bead (Figure 7.6B). 26.67% of those injected with saline avoided the bead. The difference between the recall of the two groups was again non-significant ($p > 0.05$, χ^2).

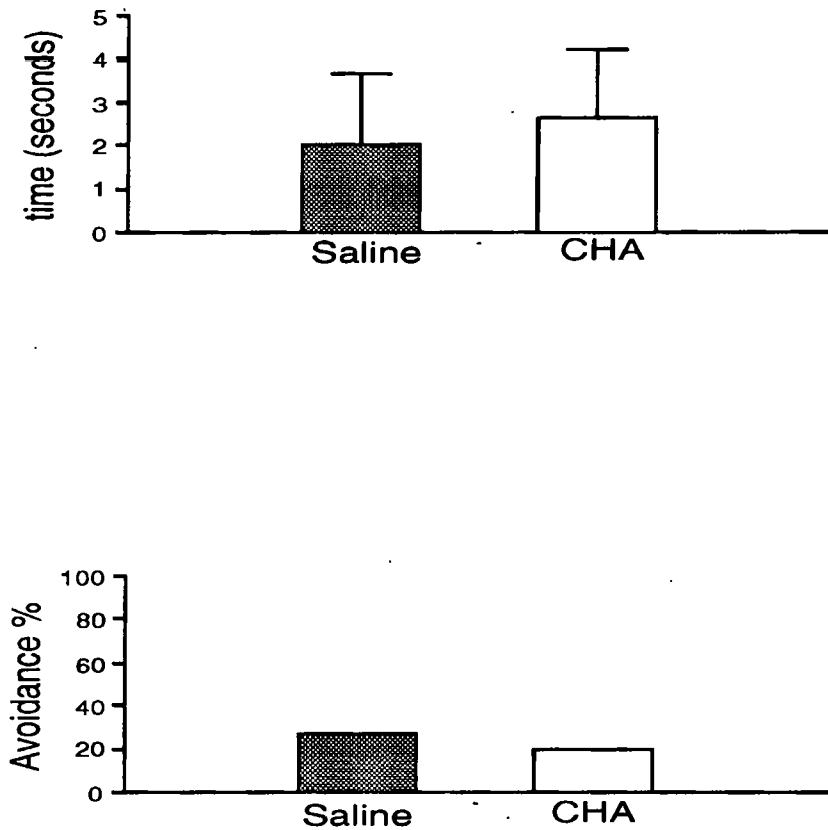


Figure 7.6. The effect of CHA on locomotor behaviour in the chick. **(A)** The peck latency of chicks injected bilaterally (5 μ l per hemisphere) with 10 μ M CHA compared to those injected with saline (\pm S.E.M.) and **(B)** the effect of bilateral injections of 10mM CHA on chicks pecking at the water bead compared to saline controls. Time of injection 30 minutes pre-training in all groups: trained on water bead and tested 1 hour after training.

Experiment 7.5. The effect of the adenosine A₁ receptor antagonist cyclopentyltheophylline (CPT) when injected together with CHA

Introduction

The adenosine A₁ receptor agonist CHA was found to inhibit the release of glutamate from the IMHV slice preparation (Chapter 6), and also to cause amnesia for the passive avoidance task without affecting the locomotor behaviour of the chick (Experiments 7.2 and 7.4). Cyclopentyltheophylline (CPT) had been shown to inhibit the CHA-induced decrease in glutamate release (Chapter 6). Hence, it was decided to examine whether CPT could arrest the CHA-induced amnesia shown in Experiment 7.2.

Methods

To determine the specificity of the effect of CHA on learning the task, the specific A₁ antagonist CPT was injected along with CHA. CPT is a competitive antagonist at the A₁ site, although of lower affinity (11nm cf. 1.2nm for CHA). Therefore, the effect of injections 30 minutes pre-training with 10µM CHA only (n=17) was compared to injections of 10µM CHA with 100µM CPT (n=14) and with 0.9% saline (n=15). In addition, later injections of CPT only (100µM; n=14 for CPT; for saline n=15) were performed to find if CPT itself had any affect on acquisition. All chicks were tested 1 hour after training. The results were analysed using ANOVA (Statview).

Results

Figure 7.7 shows the effect of CPT competition with CHA at A₁ binding sites. CHA by itself produced significantly different results compared to saline (F=4.686, p<0.05) and CPT+CHA (F=4.328, p<0.05). No difference was found between CPT/CHA-chicks and those injected with saline (F=0.0018, p>0.05). CPT itself did not affect the acquisition of the task (data not presented graphically; χ^2 , p>0.05).

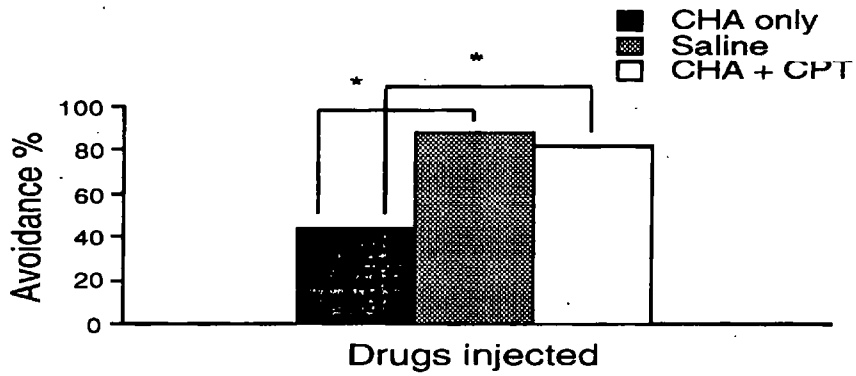


Figure 7.7. The effect of the A_1 antagonist CPT on the CHA-induced amnesia for the passive avoidance task. Chicks were injected bilaterally ($5\mu\text{l}$ per hemisphere) with either $10\mu\text{M}$ CHA-only, $10\mu\text{M}$ CHA and $100\mu\text{M}$ CPT, or 0.9% saline, 30 minutes pre-training and tested 1 hour post-training. Controls for each group consisted of chicks injected with 0.9% saline. An asterisk * indicates significant differences between groups ($p < 0.05$, ANOVA).

Discussion

The results described in this Chapter suggest the involvement, or at least the potential for involvement, of adenosine receptors in chicks following a one-trial passive avoidance task.

2-chloroadenosine (2-CADO) significantly impaired the recall of the passive avoidance task when injected at concentrations of 50mM and above. If it is assumed that the chick brain is of roughly 1ml in volume, the bilateral (5ml) injections would produce a concentration of 500nM if the drug dispersed throughout the entire brain. The highest concentration, 2mM, would represent 200mM if dispersed evenly through the whole brain. As 2-CADO is equipotent at A₁ and A₂ receptors, it could be expected that, certainly at the higher concentration, both receptor types are activated. As discussed in Chapter 6, it is thought that the actions of the A₂ receptor subtype is dominant to the A₁ receptor when higher concentrations of adenosine or adenosine agonists are applied to the tissue (Ameri and Juma, 1991). The results showing amnesia in chicks that had been given the higher dose of 2-CADO may be explained by either a direct action of A₂ receptor subtypes on memory formation, or by an effect of the receptor on locomotor activity (see below).

Cyclohexyladenosine (CHA) is a selective agonist of the adenosine A₁ receptor. Experiment 7.2 describes CHA-induced amnesia in the chick. Amnesia for the task was produced by a dose of 10mM or more (which would be equivalent to 100nM if dispersed throughout the whole brain). In a similar study in the mouse, Normile and Barraco (1991) described the effects of N⁶-cyclopentyladenosine (CPA), a selective A₁ agonist, on the retention of an inhibitory avoidance task. They showed that pre-training injections of CPA produced a dose-dependent impairment in memory for the task which could be blocked by a selective A₁ receptor antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine). From these results, they suggested that the activation of

A₁ receptors impairs the retention performance and information processing in the mouse. A later study (Normile et al., 1994) showed that injections of A₁ agonists into the nucleus accumbens impaired memory for the inhibitory avoidance task. The memory impairment was blocked by DPCPX. Therefore, the A₁ receptors in the nucleus accumbens were implicated in the processes underlying learning and memory, perhaps via modulation of the dopaminergic system that has been described in this area. The chick LPO has been suggested as being analogous to the nucleus accumbens (Reiner et al., 1983). This area is also rich in dopaminergic terminals. Therefore it is possible that CHA is acting to modulate dopamine release in this area, either directly or indirectly via other transmitters (amino acids or acetylcholine), to inhibit the acquisition of the task.

In a study by Zarrindast and Shafaghi (1994) the acquisition of a passive avoidance task in the mouse was inhibited by CHA and R-PIA (N⁶-phenylisopropyladenosine), both A₁ agonists. The administration of NECA (non-selective agonist) had no effect on the acquisition of the task. Low doses of A₁ antagonists blocked the CHA and R-PIA-mediated amnesia. These results are in agreement with the effects of CHA in the one-trial passive avoidance task paradigm used in this study on chicks, and again suggest the involvement of adenosine receptors in the acquisition of the task.

The results in Experiment 7.4 show that the locomotor activities of the chicks were not disturbed following injection with the specific adenosine A₁ receptor agonist, CHA. The latency to the first peck was found not to have been affected, and the ability of the chicks to learn a task did not differ from those injected with saline. This suggests that the results obtained with the chicks trained on MeA to avoid the bead when presented at test were not influenced by any potential motor dysfunction, and were due to the specific effect of the drug on the learning of the task. Barraco et al. (1993) demonstrated that CPA, also an A₁ agonist, had no effect on the locomotor

activity of mice when injected into the nucleus accumbens (also see Barraco et al., 1994). However, previously it has been shown that bilateral injections of the A₂ agonist CGS 21680 into the nucleus accumbens in the mouse impaired locomotor activity in a dose-dependent manner (Barraco et al., 1993). In addition, the non-selective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) also reduced the locomotor activity. Barraco et al. (1993) found that the depression in locomotor activity that accompanied the activation of the A₂ receptor when stimulated by these drugs was significantly antagonised by treatment with an adenosine A₂ receptor antagonist.

In addition, Suzuki et al. (1993) tested a variety of adenosine receptor antagonists on scopolamine and R-PIA-induced amnesias. They found that some of the selective A₁ antagonists attenuated these amnesias at doses that did not induce an increase in spontaneous locomotor activity.

The effect of CHA was lateralised, such that pre-training injections, into the right IMHV only, did not produce amnesia for the task, but those to the left IMHV and bilateral injections did (Experiment 7.3). These results are in agreement with the lesion data: chicks that received a sham left IMHV lesion whilst receiving a lesion to the right showed avoidance levels similar to those usually associated with saline-injected chicks (Patterson et al., 1990). In their study, chicks receiving true unilateral left IMHV lesions showed amnesia for the task. These results were later corroborated by Gilbert et al. (1991), who also showed that pre-training lesions of the right IMHV do not produce amnesia. Injections of CHA before training interfere with the successful acquisition of the task when they are applied to the left IMHV, but injections to the right IMHV do not, suggesting that the right IMHV is not necessary for successful acquisition, even though it is thought that the right IMHV is generally required for distributing information to the LPO (Gilbert et al., 1991). It can be postulated that the action of CHA is to inhibit the release

of transmitters via the A₁ receptor system or to produce post-synaptic hyperpolarisations, both of which disturb the biochemistry of the IMHV sufficiently to inhibit acquisition. No lateralisation was found in either adenosine or glutamate release at the 30 minute time point however, with increases in both transmitters following the training task (see Chapters 4 and 6).

The results described in this chapter demonstrated that adenosine analogues interfered with memory formation, specifically at 30 minutes after training on the task. This would suggest that adenosine receptor activation occurs at this time. Adenosine receptor activation may modulate the release of amino acids, as demonstrated in Chapter 6, leading to either an enhanced efficacy or a loss in synaptic efficacy.

The results of Chapter 6 and 7 are discussed further, and in relation to the increases in transmitter release, in the General Discussion (Chapter 8).

Chapter 8: General Discussion and Future Directions

The initial aim of this thesis was to describe glutamate release from areas of the chick brain known to be biochemically active following a passive avoidance task (the lobus parolfactorius (LPO) and the intermediate medial hyperstriatum ventrale (IMHV), and to determine if glutamate release was changed at times that biochemical activity had been shown to be altered in previous studies. The extent to which these aims have been completed has been described in Chapters 4 and 5.

In addition to the release of glutamate, other putative amino acid neurotransmitters (aspartate, glycine and GABA) were also analysed to determine if increases in these occurred post-training. Also, a role for adenosine, a neuromodulator in mammalian brain, was investigated. The following briefly describes the main results from the work presented here, and the role of the three main neurotransmitters and adenosine, in memory formation, is explored in greater detail.

Chapter 3 described the background to amino acid analysis by high performance liquid chromatography (HPLC). The separation of the constituents of physiological media is dependent on their distribution between two phases or substrates. The detection of amino acids by UV absorbance requires PITC derivatisation. A methodology for HPLC amino acid analysis is described. The published method did not suffice to effect a complete separation of certain amino acids thought to be of importance as putative transmitters/modulators, notably the separation of glycine from glutamine. An outline of the factors that were altered to produce a more viable separation (k' , α and N) was given. The qualitative and quantitative aspects of amino

acid analysis by HPLC were examined briefly, showing that internal standards allow equivalent quantification between samples and that the amino acids can (generally) be identified according to their retention times and the addition of standards to "spike" the sample. In addition, slight modifications to the PITC derivatisation technique were described: the presence of amino acid contamination on glassware and the presence of salts present in the buffers used in the stimulated release of the amino acids produced many and diverse problems throughout the work. Also, the use of 4-aminopyridine, a very useful depolarisation agent, was not possible with the present HPLC methodology due to coelution of the molecule with glutamate and aspartate. Chapter 3 finished with a description of a refined HPLC methodology that could be used for amino acid analysis using a K^+ -stimulation paradigm.

In Chapter 4 slice incubations were used to investigate the release of amino acids from the intermediate medial hyperstriatum ventrale (IMHV) of the day-old chick following passive avoidance training. Release of amino acids was measured in media such that the Ca^{2+} -dependency of the release could be determined. No differences in the basal release of the amino acids between MeA-trained chicks and chicks that had pecked the water bead was found. However, an increase in the K^+ -stimulated Ca^{2+} -dependent release of amino acids was found at times after training in those chicks that had pecked at the MeA-covered bead and had subsequently remembered to avoid the bead. Glutamate release was found increased 30 minutes and 1 hour after training in the left IMHV, and 30 minutes, 3 and 6.5 hours post-training in the right IMHV: GABA and aspartate release were demonstrated in the left IMHV at 1 hour post-training and in the right at 3 hours (aspartate) and 6.5 hours (GABA) post-training. In addition, the Ca^{2+} -independent component of glycine release was found to be increased 30 minutes after training in the left IMHV in MeA chicks. The results were discussed in the light of other biochemical data that have been described in the IMHV following training.

Chapter 5 looked at the release of amino acids following passive

avoidance training in the lobus parolfactorus (LPO), an area of the chick brain thought to be equivalent to mammalian basal ganglia and involved in the integration of emotional behaviours (see Chapter 5). An additional time point of 24 hours was incorporated into the analysis, as changes in morphology are found at this time that may represent an increase in activity related to transmitter release (e.g. Stewart et al., 1992). Increased GABA and glutamate release were found at similar time points: at 30 minutes, 6.5 and 24 hours in the left hemisphere; and at 6.5 and 24 hours in the right LPO. Again, the results were discussed in relation to previous work that had shown this region to be involved in memory formation.

A potential neuromodulator in the chick brain is adenosine. The investigations carried out in Chapter 6 looked at the release of the purine from slices of IMHV tissue from both untrained chicks, in order to investigate some of the underlying pharmacological interactions between adenosine and the amino acids, and also from trained chicks in order to investigate any changes in release and to relate them to the changes described in Chapter 4 in the release of amino acids. The investigation was initiated as it had been found that the HPLC methodology employed to analyse amino acids was also applicable to analysis of some nucleotides, and that during the collection of data for the experiments performed in Chapter 4, it had been noted that there appeared to be a concomitant increase in adenosine at the same time as the glutamate. The experiments carried out as described in Chapter 6 substantiated these initial observations: there was a K^+ -stimulated release of adenosine from the left IMHV at 30 minutes and 1 hour post-training, and at 30 minutes, 3 and 6.5 hours post-training in the right IMHV of MeA-trained chicks as compared to water controls. The release was found to be both Ca^{2+} -dependent and Ca^{2+} -independent.

The effect of adenosine and its analogues on amino acid release was also investigated in Chapter 6. It was found that adenosine itself (at $100\mu M$ only) and the A_1 receptor-specific agonist cyclohexyladenosine (CHA) reduced the

release of glutamate from IMHV slices. The effect of CHA was eliminated by addition of an A₁ antagonist (CPT) to the media. No effects on the other amino acids analysed were observed. CGS 21680, a potent A₂ ('excitatory' adenosine receptor) agonist, increased glutamate release at a low dose (5µM). When the dose was increased in the media the effect on glutamate release was now abolished, but a decrease in GABA release was established by then.

NMDA and KCl both stimulated the release of adenosine. The effects of both were only noted in the collection periods after the stimulatory agent had been removed from the media, suggesting a delay in the processes involved in the release.

Behavioural pharmacological manipulations were carried out and described in Chapter 7. These investigations were in order to determine the effects, if any, of chemicals that act at the A₁ receptors in the chick. The drugs used did indeed produce amnesia, as predicted, in the day-old chicks for the task. The effect of the drug 2-CADO appeared to have set in by 30 minutes post-training, but not by 15 minutes. The amnesic effects were still apparent after 1 hour. A more specific A₁ agonist, CHA, was amnesic when injected at any time between 5 minutes and 1 hour before training. The effects of CHA were lateralised, such that no amnesia was observed when the compound was injected into the right only, and were not due to the inhibition of the natural pecking behaviour. When injected with the A₁ antagonist CPT the effect of CHA was no longer apparent, thus implicating the A₁ adenosine receptor in the formation of amnesia for the task.

The following sections deal with the release of the three amino acids (aspartate, glutamate and GABA), and of adenosine, from the chick forebrain after the passive avoidance task, and their relationships as regards one another and their role in memory for the task.

Bursting, GABA release and neuronal circuitry in the chick

GABA release was enhanced in MeA-trained chicks at various times post-training in a Ca^{2+} -dependent manner (see chapters 4 and 5, and section 1 of this Chapter). Evidence for a role of GABA in the chick brain comes from several sources. [^3H] muscimol binding was examined in 3-week-old birds by Stewart et al. (1988) who described high levels of binding in the IMHV and the LPO, although no lateralisation of binding was found in day-old chicks (Stewart and Bourne, 1987). Meza et al. (1985) found that GABA binding to membranes of the chick inner ear were at levels suggesting that receptors were present, and that GABA was a transmitter in this system. Clements and Bourne (1995) have recently carried out a behavioural pharmacological study of the effects of muscimol and bicuculline on the retention and acquisition of the passive avoidance task. Pre-training, bilateral injections of muscimol (GABA_A antagonist) produced amnesia for the task in chicks tested 10 and 30 minutes and 24 hours after training. These data are in agreement with those of Martijena and Arce (1994) who showed an increase in the binding (B-max: i.e. an increase in the number of receptors) of [^3H] flunitriazepam (a GABA receptor agonist) following training on the passive avoidance task as compared to chicks trained on the water bead. This increase was apparent at 30 minutes post-training, but not at 10 or 60 minutes, and the increase was also seen in water-trained chicks when comparing their 30 minute values with those at 10 and 60 minutes. This suggests that the GABA receptor increase is associated with early stages of memory formation. Both these lines of evidence are in agreement with the data found in the work presented in this thesis; that there is an increase in GABA-related activity shortly (in this study, at 1 hour) following training on the task. How can the increase in GABA be interpreted, and how does it relate to the increases seen in the release of glutamate?

A possible role for GABA in synaptic transmission efficacy in the formation of memory for the task may be in bursting activity in the IMHV and

LPO. [Bursting is defined here as “a high frequency train of action potentials superimposed on slow membrane depolarisations”. (Schneiderman and MacDonald, 1991)]. Following passive avoidance learning in the chick, Gigg et al. (1993 and 1994) and Mason and Rose (1987 and 1988) found that elevated bursting in the chick was associated with the memory formation. Bursting has several consequences for synaptic transmission: firstly, the likelihood of transmission of the impulse is enhanced, as single spikes may not generate an EPSP. Secondly, temporal summation will occur more readily as the interval between spikes may be shorter than the time course of a single EPSP, and thirdly, facilitation may occur reflecting an increased calcium concentration in the presynapse (Gigg, 1991).

It is thought that excitatory amino acid transmitters play an important role in the initiation and spread of synchronous bursting because EAAs can produce seizures (Neuman et al., 1989) and antagonists of EAAs act as anticonvulsants (Meldrum, 1984). The generation of bursting is dependent on the activation of the NMDA glutamate receptor by cellular depolarisation produced by excitatory input or by disinhibition. The depolarisation releases the Mg^{2+} voltage-dependent block of NMDA-activated calcium channels. Non-NMDA receptors are largely responsible for the bursts in the presence of Mg^{2+} and provide the initial depolarisation which triggers bursts by activation of NMDA and other voltage-dependent channels. NMDA receptors play a dominant role only when the extracellular Mg^{2+} is lowered sufficiently to relieve the calcium-channel blockade. The bursts generated are terminated by the activation of a calcium dependent outward K^+ current which sustains a slowly decaying after-hyperpolarisation.

Bradley et al. (1990) investigated the local circuitry common to the IMHV of the chick. Following a single stimulus, a biphasic response is seen. The second phase, of longer duration (15ms), is thought to be mediated by NMDA receptor responses. An important contribution to the duration of the response is thought to be the presence of positive feedback circuits in the

amino acid synapses, many of which are sensitive to NMDA." (Bradley et al., 1990; see Figure 8.1). This system may therefore provide the substrate for the synchronous bursting behaviour that is found in the IMHV accompanying the passive avoidance task. Indeed, it is thought by Schneiderman and MacDonald (1991) "that networks of neurons may produce bursts as a consequence of the architecture of their synaptic connectivity, particularly if the connections involve positive feedback."

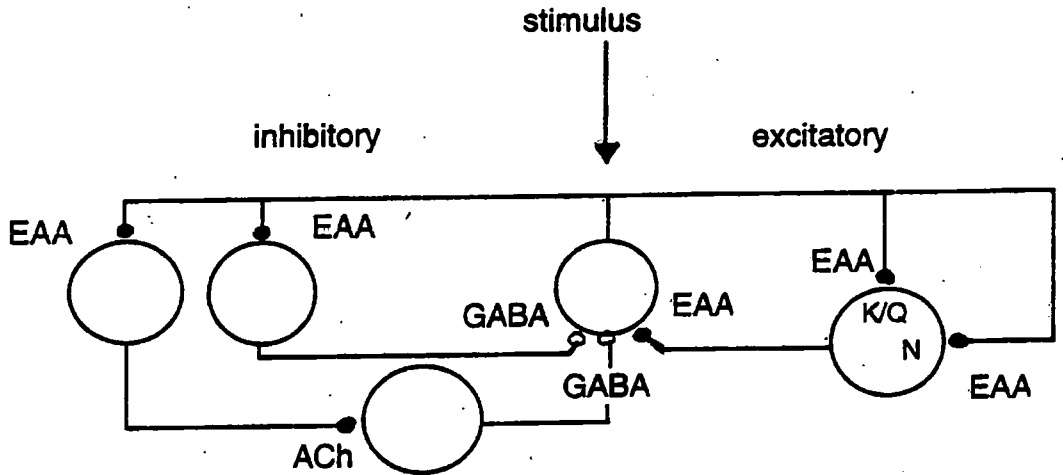


Figure 8.1. The diagram describes a neural circuit in the IMHV that satisfies the requirements of the results described by Bradley et al. (1990). See text for details. (●), excitatory synapses; (⊖), inhibitory synapses; EAA, excitatory amino acid; GABA, γ -aminobutyric acid; ACh, acetylcholine; N, NMDA receptor; K/Q, AMPA receptor. (taken from Bradley et al., 1990).

Bradley et al. (1990) demonstrated that the addition of bicuculline (GABA_A antagonist) made it apparent that the excitatory connections exposed are under powerful inhibitory influences.

As noted above, it is thought that inhibitory mechanisms limit excitation within networks of neurons (Schneiderman and MacDonald, 1991). Synchronous bursting may therefore require a decrease in the inhibitory tone produced by the release of GABA at usual physiological levels. In line with this, picrotoxin and bicuculline (both GABA_A antagonists) induce

produced by the release of GABA at usual physiological levels. In line with this, picrotoxin and bicuculline (both GABAergic antagonists) induce spontaneous bursting by blocking GABA_A-mediated IPSPs, thus allowing excitatory activity to spread through neural circuits (Schneiderman and MacDonald, 1991). Also, following repetitive stimulation of hippocampal slices (CA1) there is a decrease in the GABA-mediated inhibition (Kamphuis et al., 1990). The decrease in GABAergic inhibition may lead to an enhancement of NMDA responses: in the kindling model paired pulse depression is also reduced; the underlying mechanism may be GABA receptor down-regulation (Kamphuis et al., 1991). The desensitisation of GABA receptors may be enhanced by an increase in GABA release: the prolonged activation of the receptor-linked Cl⁻ channel by the endogenous agonist may lead to depression caused by intracellular chloride accumulation, following repetitive IPSPs, leading to a change in the chloride gradient and equilibrium potential (Tehrani and Barnes, 1988). In the hippocampus it has been found that there is a decrease in the paired pulse depression in the CA1 but there is also an increase in the exocytotic release of GABA (e.g. Kamphuis et al., 1990).

GABA_B-mediated events have also been implicated in bursting. GABA_B receptor-mediated events may produce significant suppression of inhibitory output to enhance signal transmission in the hippocampus (Mott et al., 1993). The GABA_B receptor-mediated depression of the IPSC occurs only during the synchronised activation of a network (Otis and Mody, 1992). Synchronised neural activity has been found to occur in the theta rhythm found in exploring rats (the theta rhythm, 3-12 Hz is thought to be important in certain learning tasks: Winson, 1978), and also in hippocampal sharp waves, which are thought to be involved in memory consolidation (Buzsaki, 1989); and the generation of this synchronised activity is thought to involve a suppression of inhibition (Buzsaki, 1989). The disinhibition of the GABA_B receptors is such that the transmission of the signal is enhanced only between 2.5 and 10 Hz (Mott et al., 1993). Thus, there is a filtering of signals allowing

selective enhancement during repetitive activation at frequencies in the range of the theta rhythm. LTP is induced by repetitive, synchronised activity (McNaughton et al., 1978), and as such the production of the theta rhythm or sharp waves may induce LTP (Buzsaki, 1989). Following on from this it has been found that the induction of LTP is dependent on GABA_B receptor-mediated disinhibition which relieves the Mg²⁺ block of the NMDA receptors and allows the expression of the EPSP_{NMDA} and LTP. The slow IPSC produced by GABA_B receptor activation would be able to counter the slow EPSCs produced by NMDA receptors, and thus potentially inhibit NMDA receptor-mediated events (Ling and Benardo, 1994). Davies et al. (1991) suggest that an autoreceptor system is in operation, so that the fatigue of the IPSPs required for EPSP summation is due to GABA feeding back and inhibiting its own release through an action on GABA_B receptors. Stimulation of GABA_B receptors inhibits the major excitatory and inhibitory afferent systems within the neostriatum, and is proposed as a local feedback mechanism with presynaptic receptors inhibiting either excitation or inhibition: a similar mechanism is proposed for the hippocampus (Nisenbaum et al., 1992). The slow IPSCs produced by GABA_B-receptor activation are decreased by blockade of ionotropic glutamate receptor antagonists and GABA_A receptors, suggesting that both glutamate and GABA_A-mediated events participate in the recruitment of GABA_B IPSCs. Moreover, GABA_A-mediated events have been shown to cause excitation of interneurons, which in turn trigger GABA_B IPSCs (Michelson and Wong, 1991). The depression of the fast GABA_A IPSCs will reduce the GABA_B IPSC.

As has already been alluded to above, the mechanisms responsible for GABA disinhibition might involve one of three processes: diffusion of transmitter out of the synaptic cleft, uptake of transmitter, and a change in transmitter release. The first is unlikely as it is a purely passive process. GABA uptake inhibitors have been found to prolong the IPSC decay (Thompson and Gahwiler, 1992). However, activity-dependent expression of

the slow IPSC was found to be associated with a decreased conductance, but the kinetics of the current were not changed, suggesting that there was no change in GABA uptake (Ling and Behardo, 1994). Ling and Benardo (1994) suggested that a decrease in GABA release may mediate the IPSC depression. It has also been found that the depression of GABA_A IPSCs may involve the presynaptic inhibition of GABA release because the repetitive stimulation does not affect conductances activated by GABA agonists. Interaction with GABA_A receptors may produce presynaptic inhibition and leads to a decrease in GABA release.

GABA release, therefore, plays an important role in maintaining the inhibitory tone of the neural environment. A reduction of the inhibitory tone can occur, which allows EPSP summation and a positive feedback mechanism to be employed. Increased release may act to produce a disinhibition of the pathway described by Bradley et al. (1990), via GABA_A-receptor desensitisation or via an increase in GABA_B slow IPSPs acting presynaptically on inhibitory interneurons. The increase in GABA_A receptors as described by Martijena and Arce (1994) appears to be contradictory to the model proposed by Bradley et al. (1990). However, it should be noted that the increases they found were for the whole forebrain, as specific areas were not examined individually. It is possible, therefore, that the increase in A-type receptors may be on the postsynaptic membranes of inhibitory interneurons. These receptors may be present, but hidden, during normal synaptic transmission, but following bursting activity or transient disinhibition are expressed subsequently following postsynaptic phosphorylation/kinase events.

Glutamate release

The increase in GABA release may underpin a decrease in inhibitory tone that allows cells to fire bursts of action potentials in response to a single synaptic input (Gigg, 1991). This bursting may allow LTP induction (and therefore memory formation in the chick?). In the chick IMHV, inhibitory inputs control excitation: an increase in GABA release may produce receptor desensitisation in the neural circuit. An increase in release of GABA following passive avoidance training was found in the chick IMHV and LPO (see Chapters 4 and 5). In addition to the increases in GABA release found in this work, there is also an increase in glutamate and aspartate following passive avoidance training. In order to produce changes that lead to memory formation, it appears that long term enhancement of excitatory transmission is required. Is there any evidence from LTP that suggests such a long-term enhancement, and what is the mechanism that may be involved in this enhancement?

Some of the quantal aspects of transmitter release in LTP have been referred to in Chapter 2 (e.g. Bekkers and Stevens, 1990). Evidence for an increase in presynaptic transmitter release accompanying LTP have mainly been determined by neurophysiological techniques investigating EPSCs or EPSPs evoked by paired pulses of electrical stimulation. Different statistical analyses are employed to determine whether a presynaptic component is responsible for LTP maintenance and whether the mechanism of the component is an increase in mean quantal content, m (the mean number of released quanta), or an increase in the response to each released quantum, q (mean quantal size).

Work by Xiang et al. (1994) evaluated three lines of evidence, based on different analyses (a graphical variance method, number of failures, and the relationship between paired-pulse facilitation and LTP). They concluded that there was indeed an underlying presynaptic component, in this case mossy-fibre, LTP. The mechanism appears to be an increase in m , the number

of quanta of transmitter released. The transmitter described is certainly glutamate, e.g. Weisskopf and Nicoll (1995) showed that antagonism of either NMDA or AMPA receptors would inhibit LTP maintenance. They also suggest that there is an increase in the probability of transmitter release during expression of LTP in the mossy-fibre pathway. These data are in agreement with Bekkers and Stevens (1990). In addition, Kamiya et al. (1991) also found an increase in transmitter release, and the increase persisted for up to 4 hours after tetanic stimulation.

Endogenous amino acid release has been looked at *in vitro* and *in vivo*. Bliss et al. (1986) and Lynch et al. (1985) showed that there is an increase in the release of glutamate between 1 and 2 hours after the induction of LTP. Direct monitoring of changes in glutamate levels using push-pull cannulae also suggest a causal relationship between LTP and the increase in neurotransmitter (Bliss et al., 1986). Ghisjen et al. (1992) investigated glutamate and GABA release from slices of CA1 in the hippocampus, 60 minutes post-induction of LTP. Using a similar release technique as has been applied to the work described in this thesis, they uncovered an increase in the K^+ -induced, Ca^{2+} -dependent release of both glutamate and GABA. These increases were found 60 minutes after the induction of LTP, again suggesting a long-lasting change in presynaptic activity producing a persistent increase in glutamate (and GABA) release.

From the above, it is evident that there is indeed an increase in presynaptic transmitter (glutamate/aspartate and GABA) release that accompanies LTP in the hippocampus of mammals, and that this release has been found to persist for at least 2 hours after the initiation of the stimulatory event. The results discussed in Chapters 4 and 5 can, therefore, be explained as a persistent enhancement of glutamate release required for the maintenance of a potentiation required for memory formation. A persistent increase in GABA release may also be required for continued disinhibition.

The NMDA receptor complex has been implicated in the study by

Burchuladze and Rose (1992). MK-801 (a non-competitive NMDA antagonist) was injected intraperitoneally at various times between 1 hour pre- and five minutes after training. Amnesia for the task occurred when the chicks were tested between 3 and 24 hours subsequently. However, if administered one or more hours after training there is no amnesia, implying that, as is the case in LTP, NMDA receptors are required only for initial phases of memory formation.

Following these initial investigations Steele (1995) carried out a series of experiments looking at the time course of glutamate receptor activation in the chick following the passive avoidance task. He found an increase in NMDA number in the left and right IMHVs, and the left LPO, 30 minutes, but not 3 or 6.5 hours, after training. AMPA receptors were found to be changed (in affinity) only at 6.5 hours after training.

These results suggest the involvement of NMDA receptors in the initial formation of memory for the task, whilst the AMPA receptors are crucial for the long term memory effects, since CNQX injections resulted in amnesia at 6.5 hours. This latter result is also analogous to LTP where increased AMPA receptor affinity is required for its long-term maintenance (Bliss and Collingridge, 1993).

In the chick it has previously been found that an LTP-like effect can be induced in the IMHV *in vitro* (Bradley et al., 1993). This effect, termed persistent potentiation, was eliminated by NMDA antagonists indicating that NMDA receptors mediate the potentiation. This suggests that NMDA receptor activation, required for memory formation in the chick, mediates potentiation and bursting in the chick and that enhanced glutamate release may initiate, or be produced by, these events.

Aspartate release

Changes in the Ca²⁺-dependent release of aspartate were recorded. No recent studies have shown aspartate to be involved in memory acquisition

or LTP (e.g. Ghijsen et al. (1992). Most authors no longer propose a transmitter-type role for aspartate in the mammalian CNS, and those that do may be recording a lack of specific uptake as opposed to a transmitter-like release (see Chapter 1). So why is there an increase in aspartate release found in the present study? A possible reason may be that aspartate is indeed a transmitter in the avian brain. More likely, I believe, is that the increase in aspartate release detected by the HPLC is actually due to the release of a chemical that co-elutes with aspartate, or that is initially released as a compound that subsequently degrades to aspartate. The potential identity of this compound may be N-acetylaspartylglutamate (NAAG). NAAG is a dipeptide that has been identified in synaptic vesicles and is released from neurons. Potassium-stimulated Ca^{2+} -dependent release of NAAG from a subset of putatively glutamatergic neurons, followed by degradation to glutamate (which will interact with EAA receptors), provides a possible mechanism/role for the peptide (Zollinger et al., 1994). The dipeptide exhibits a high affinity and specificity for NMDA receptors, and when injected into rat hippocampus it produced seizures (Zaczek et al., 1983). NAAG, and another possible candidate for the apparent increase in the release on aspartate, N-acetylaspartate (NAA), are found in the rat forebrain at sites that suggest that they may act to modulate the release of GABA by presynaptic autoreceptors (NMDA or mGluR-type receptors are suggested) (Moffett and Namboodiri, 1995). It is interesting to note that NAAG also has excitatory effects on (cultured) chick cerebellar neurons (Mori-Okamoto et al., 1987).

During work for this thesis, NAAG release from the chick brain was investigated (data not shown). NAAG was found, but in very small amounts (<10 pmol / 100 ml/ IMHV), and using a different HPLC method that also required UV absorbance at 210nm (the preparation for amino acid analysis appeared to break the dipeptide down into its constituent amino acids, such that even the standard NAAG prepared was detected as two peaks that coeluted with aspartate and glutamate, as suggested above). A major factor for

the small amounts analysed, assuming that the peptide is present in sufficient amounts originally, may be the membrane-bound peptidase activity that converts NAAG to glutamate and N-acetylaspartate.

From the above, it seems quite possible therefore that the aspartate found to be released following training was derived from a peptide, which also suggests that the glutamate found to be released at these times may also be derived from the peptide. No specific receptors appear to exist, (or rather, have not been described) for the products of the dipeptide, suggesting that if NAAG is released it is in the form of a "pretransmitter" (Zollinger et al., 1994) from which glutamate is cleaved. It could be postulated that, because aspartate, or the compound that coelutes with aspartate in the HPLC separation, is not increased in release until the later times of glutamate activation (at 1 hour in the left IMHV cf. the enhanced release of glutamate from the same region at this time but also earlier, at 30 minutes post-training), NAAG is released as well as glutamate, and the mechanisms of NAAG release or of its peptidase's activity is not expressed until activated (by the increased glutamate release?). Further work in this area is suggested at the end of this chapter.

Retrograde messengers

The mechanisms underlying the long-term enhancement of glutamate and GABA transmitter release are not clear. Arachidonic acid and nitric oxide have both been proposed as possible retrograde messengers that may enhance pre-synaptic release after its induction by receptor-mediated Ca^{2+} elevation. Arachidonic acid has been found to increase endogenous glutamate release in hippocampal nerve terminals (Lynch and Voss, 1990). As arachidonic acid enhances glutamate release, it may contribute to the maintenance of LTP by stimulating neurotransmitter release from presynaptic terminals.

A role for arachidonic acid in memory formation in the chick has been suggested by Hölscher and Rose (1994). Phospholipase A₂ (PLA₂) inhibitors produce amnesia for the passive avoidance task. Arachidonic acid is synthesised via PLA₂. In this way, it can be assumed that arachidonic acid is necessary for memory formation of the passive avoidance task. The time course of the experiments suggest that disruption of arachidonic acid production does not occur until 1.25 hours after training, and from this it can be implied that arachidonic acid may be involved as a retrograde messenger during the later stages of memory formation. Enhanced transmitter release (glutamate, aspartate and GABA) is seen in both IMHVs and LPOs 1 hour, and at subsequent times, post-training (see Chapters 4 and 5). This persistent increase may therefore be mediated by arachidonic acid production release from the postsynaptic membrane as a retrograde messenger.

Arachidonic acid stimulated PKC-dependent phosphorylation of GAP-43 was apparent after 5 minutes following the induction of LTP. From these data Luo and Vallano (1995) concluded that the proposed stimulated release of neurotransmitter that accompanies the phosphorylation of GAP-43 occurs during the early maintenance phase of LTP. This conclusion is in contrast to that of Hölscher and Rose (1994): an explanation may be a difference in the retrograde messenger activation, and source, following LTP

as compared to passive avoidance learning.

The substrate that is thought to regulate arachidonic acid activity is protein kinase C (PKC). Evidence for arachidonic acid-mediated PKC involvement include, that arachidonic acid enhances the phosphorylation of the PKC substrate GAP-43: GAP-43 phosphorylation has been correlated with an increase in transmitter release in hippocampal slices (Dekker et al., 1989). Also, activation of PKC and phosphorylation of GAP-43 have been associated with the persistence of LTP in the dentate gyrus and area CA1 of the hippocampus (from Luo and Vallano, 1995). Thus, arachidonic acid contributes to the maintenance of LTP by mediating increased neurotransmitter release via activation of presynaptic PKC and subsequent phosphorylation of GAP-43 (Luo and Vallano, 1995; also see Chapter 1)

Phorbol esters, activators of PKC, potentiate the sustained release of glutamate. Addition of phorbol esters before stimulation with KCl produces an increase in Ca²⁺-dependent glutamate release (Terrian and Ways, 1995). This increase in release was only apparent following at least 10 minutes exposure to the phorbol ester (Terrian, 1995). Again this suggests that PKC activation is apparent after stimulation, but that this activation, and the enhanced glutamate release that may accompany this activation, is delayed. Terrian and Ways (1995) suggested that CaM kinase is not involved in the evoked release of glutamate. The fact that PKC activity does not require CaM kinase activation could suggest that no CaM kinase activity is required for memory formation. However, Zhao et al. (1995) suggested that CaM kinase may be necessary for the initiation processes of LTP (and memory formation?) whilst PKC is required for the maintenance of the generated LTP.

PKC has been found to be necessary for memory formation in the chick (Burchuladze et al., 1990). Inhibitors of PKC produced amnesia for the task 3 hours post-training, and an increase in synaptic-membrane bound PKC at 30 minutes in the left IMHV. There is also a change in the level of phosphorylation of B-50 (GAP-43) in the left IMHV of MeA-trained chicks 30

minutes post-training (Bullock et al., 1990; also noted in Zhao et al., 1995). These results are consistent with the release of neurotransmitter seen at 30 minutes in the left IMHV in the present study, but does not explain the release seen in the right IMHV of MeA-trained chicks compared to water controls: though the levels of PKC appear to be roughly equivalent in the left and right IMHV of MeA-trained chicks at 30 minutes post-training, suggesting a similar level of transmitter release (Burchuladze et al., 1990). Also, Zhao et al. (1994) demonstrated that melittin (a PKC inhibitor) affected the second stage (ITM) of the proposed three-stage sequence in memory formation in the chick following passive avoidance learning (Ng and Gibbs, 1991). In addition, Bradley et al. (1992) showed that a persistent potentiation in the IMHV is enhanced following addition of phorbol esters; a protein kinase C inhibitor, H-7, prevented the induction of the potentiation.

In addition to PKC, cAMP-dependent protein kinase (PKA) may be involved in memory formation in the chick. PKA is involved in the activation of non-NMDA glutamate receptors which have been found to be associated with the maintenance of LTP (Wang et al. 1991). Cyclic AMP is increased under conditions that produce LTP (Stanton and Starvey, 1985). Also, a significant increase in whole forebrain levels of cAMP has been observed between 30 and 60 minutes following passive avoidance training in the chick (Brown, 1984). The use of PKA inhibitors showed that PKA was involved in the formation of long-term memory in the chick: the actions of the inhibitors did not occur until ca. 60 minutes post-training, and were apparent until 3 hours post-training, suggesting involvement of PKA in the establishment of the protein synthesis-dependent stage of memory formation, and a post-synaptic role for PKA (Zhao et al., 1995). A role for AMPA receptors in the consolidation of the passive avoidance task has been identified, because AMPA receptors increase in affinity at 6.5 hours post-training (Steele, 1995). At this time point there is an increase in glutamate release from the right IMHV of chicks, not seen in the left, suggesting an

increase in transmitter activity related to a second wave of protein synthesis.

In our laboratories Holscher and Rose (1993) demonstrated that nitric oxide (NO) production is necessary for the formation of memory in the passive avoidance task. N-nitro-L-arginine (an inhibitor of NO synthase) injected into the IMHV, left or right, before the training task produced amnesia from 30 minutes to 24 hours later. This suggested that NO is of importance in the first few minutes after the initiation of memory formation rather than in the later stages. Indeed it was thought that NO is but the first wave carrier of information and forms a first wave of retrograde signalling, reaching a peak of synthesis by 30-60 minutes after which NO production starts to decrease and arachidonic acid release, the presumed second wave, is increased.

NO was demonstrated to be of importance in the initiation of memory formation rather than in the later stages of memory consolidation in this study, which related well to the theory, as NO is rapidly produced after neuronal activation and destroyed after a few seconds (Bredt and Snyder, 1992). A form of long-lasting potentiation in the IMHV can be blocked by the NO scavenger, haemoglobin, which again suggests a role for NO in the IMHV (Bradley et al., 1992).

It has previously been shown that a correlation exists between the release of glutamate and NO after NMDA stimulation in rats (Rowley et al., 1993), and in slice work, glutamate and aspartate (Dickie et al., 1992) have been found to be released in a NO-dependent manner. As has previously been shown in this thesis, glutamate release was found to be elevated 30 minutes following the training task. NO production is elicited by joint stimulation of AMPA and mGluRs (Ito and Karachot, 1990) whilst NO release and cGMP production is NMDA dependent (Garthwaite, 1991). All these results suggest that presynaptic glutamate release and the postsynaptic activation of NMDA receptors are required for the increase in NO synthesis and the subsequent "induction" and maintenance of synaptic strengthening. NO, as has already

been outlined, is thought to be a retrograde messenger in LTP. Inhibition of NO synthesis can prevent LTP in hippocampal slices and dentate gyrus and in cortical slices (Nowicky and Bindman, 1993). NO diffuses through the cytosol and is absorbed by the haem group of a NO sensitive guanylate cyclase. Increase in cGMP activity then evokes an increase in glutamate release via an unknown mechanism. Glutamate uptake is also increased via an astrocytic based NO sensitive guanylate cyclase activated uptake mechanism: this may account for the increased glutamate uptake described in Chapter 4.

Modulation of amino acid release by adenosine

A role for adenosine in memory formation for the task was proposed in Chapters 6 and 7. Adenosine release was found to be elevated at the same times and in the same hemispheres where enhanced glutamate release was found.

The release of glutamate was affected by adenosine interactions. A₁ agonists inhibited release, whilst activation of A₂ receptors produced an enhanced release of excitatory neurotransmitter (this is in agreement with other studies in the mammalian system, e.g. O'Regan et al., 1992).

Adenosine has been implicated in long term potentiation (LTP). Dolphin (1983) found that a high intensity, high frequency train of electrical stimulation, when applied to the perforant path of the hippocampus, did not produce LTP when applied in the presence of the adenosine agonist, 2-chloroadenosine (2-CADO). The EPSPs produced by the stimulation were reduced by between 30 and 50% during 2-CADO perfusion. Extracellular application of adenosine will prevent LTP if applied after 1 minute, but not 5 minutes, after high frequency stimulation (Arai et al., 1990). The post-stimulation application of adenosine, suggests that changes in intracellular control systems linked to adenosine receptors can, during a brief period, interrupt the biochemical processes leading to the expression of LTP. Further to this, DeMendonca and Ribeiro (1990) found that the activation of A₁

receptors attenuated LTP.

At mossy-fibre-CA3 synapses, NMDA receptor activation is not required for LTP induction (see Chapter 2). The locus of both induction and maintenance is thought to be presynaptic. Alzheimer et al. (1991) described adenosine depression of the induction of LTP. This suggested that adenosine's presynaptic action is sufficient to interfere with synaptic transmission. Subsequent work (Asztely et al. 1994), has demonstrated that the inhibitory effect of adenosine was indeed indirect, via a decreased transmitter release at individual excitatory synapses, but that this action may not be potent enough, or may be present at only a relatively few synapses, such that LTP induction was not blocked. However, DeMendonca and Ribeiro (1994) described the actions of adenosine analogues on LTP in the hippocampus, and found, in agreement with previous data (e.g. Dolphin, 1983; Arai et al. 1983), that A₁ activation attenuated LTP, but that A₂ receptor agonists enhanced LTP induction. The discrepancies in these results might be due to GABA activation: in the studies that did not show a blocking effect of adenosine on LTP, GABA antagonists were used to reduce the inhibitory effect in the hippocampal synapses. This would suggest that adenosine acts in unison with GABA to produce the effect of LTP reduction seen with the application of adenosine and A₁ agonists. This is further substantiated by data that shows that adenosine potentiates the ability of muscimol to inhibit evoked potentials, and that these effects were mediated by a change in chloride conductance (Akhondzadeh and Stone, 1994). This interaction would provide a potent mechanism of inhibition if both were in action at the same time or/and in the same neurons/synapses.

Adenosine released during low levels of NMDA-receptor activity could inhibit the further release of glutamate and/or diminish postsynaptic responses (Burke and Nadler, 1988). Adenosine may provide a further inhibitory threshold for NMDA-receptor activation. Indeed, White and Hoehn (1991) suggest that released adenosine may serve to maintain the

selectivity of NMDA-mediated processes such as memory, learning and synaptic plasticity. Burke and Nadler (1988) suggested that, from their findings (80% reduction in the release of aspartate/glutamate following A₁ receptor activation), there may be not a decrease at each synapse by 80% but perhaps a suppression of release from 80% of terminals leaving the other 20% unaffected. This would lead to a selective deactivation of synapses and may play a role in the stabilisation of synapses required for the acquisition and retention of the memories.

As regards the formation of postsynaptic potentials, adenosine may play a vital role. At low (1 μ M) concentrations of adenosine, EPSCs are facilitated in the rat hippocampus whilst higher concentrations inhibited the EPSC (Garaschuk et al., 1992). Both NMDA and non-NMDA receptor-components of the EPSC were facilitated simultaneously. Also the application of this low concentration of adenosine was found to produce an increase in the amplitude of the EPSP, and this was due to an increase in glutamate release (Okada et al., 1992). A presynaptic action for adenosine was found by Lupica et al. (1992). Quantal analysis showed that application of CHA (A₁ agonist) reduced both the EPSP and *m*, the quantal content. Similar results were also described by Yamamoto et al. (1993) who described a suppression of the EPSP in the CA3 region. Such a suppression may be mediated by adenosine actions at P-type Ca²⁺-channels (Mogul et al., 1993). Thus, in this way adenosine may contribute to the extent of EPSP summation. Activation of excitatory, A₂, adenosine receptors is required in order to enhance excitatory amino acid release directly. Alternatively, A₁ (inhibitory) receptor activation may serve to inhibit the release of GABA from inhibitory interneurons: the actions are very dependent on the location of the receptors, and the receptor types: e.g. it has been demonstrated by Cunha et al. (1994) that ACh release in the hippocampus is differentially regulated, such that in the CA1 area only A₁ receptors modulate the release, in CA3 both A₁ and A_{2a} receptors modulate the ACh release, but in the dentate gyrus both receptor types are present but are

not activated by endogenous adenosine

Adenosine receptor agonists (both of the A₁ and A_{2a} type) have been found to inhibit the release of GABA in the rat cortex (Oregan et al., 1992). These findings were substantiated by Kirk and Richardson (1994) who described A_{2a} receptors present on GABAergic striatal nerve terminals that acted to inhibit the release of GABA. In addition, it has been suggested by Kirk and Richardson (1995) that the inhibition of GABA may be mediated by A_{2a} inhibition of PKC activity.

Adenosine was found to depress both EPSPs and IPSPs in the CA3 region of the hippocampus (Hasuo et al., 1992). However, the glutamate-induced hyperpolarising potential mediated by GABA released from interneurons was not affected: this suggests that adenosine did not directly inhibit the release of GABA from these cells.

Adenosine receptor-mediated events are also apparent in the second messenger systems. Adenosine interacts with mGluRs via an interaction with cAMP. DeLapp and Eckols (1992) found that forskolin-stimulated cAMP accumulation in rat cortex slices was in synergism with endogenous adenosine. The effect of caffeine and 8-phenyltheophylline, adenosine receptor blockers, and adenosine deaminase was to inhibit this forskolin-stimulated cAMP accumulation. Stimulation of cAMP in cortex slices by adenosine occurs through the low-affinity adenosine A_{2b} receptor (Mante and Minneman, 1990b). Hence, blockade of the adenosine A_{2b} receptor by adenosine receptor antagonists is implicated in the inhibition of cAMP accumulation (DeLapp and Eckols, 1992). The A_{2b} receptor-stimulated cAMP response can be potentiated in the presence of 1S,3R-ACPD, most notably when there is also an adenosine receptor antagonist present (Cartmell et al., 1992b), and this response is mediated through the same ACPD receptor that is coupled to phosphoinositide turnover (Alexander et al., 1992). In addition, 1S,3-R-ACPD also inhibited forskolin-stimulated cAMP accumulation (Cartmell et al., 1993). This inhibition is thought to be mediated via the

presynaptic mGluR2 receptor, although the effects have been found to be the opposite when applied to cAMP activation by adenosine A₂ receptors (Winder and Conn, 1993). The mGluR2 agonist DCG-IV reduced the release of adenosine induced by forskolin and cAMP formation (Casabona, 1994).

There appear, therefore, to be two opposing actions of metabotropic glutamate receptors with regard to the forskolin-stimulated accumulation of cAMP. These actions are probably associated with the interaction of glutamate with mGluR subtypes associated with inhibition (mGluRs2, 3, 4, 6 and/or 7; Casabona et al., 1994) and augmentation (mGluR1a) of cAMP accumulation (Cartmell et al., 1993).

Differential effects of adenosine A₁ receptor activation have been found to occur according to whether mGluR activation is present. Thus, A₁ receptor activation may enhance IP₃ formation induced by mGluR activation and also enhance Ca²⁺ mobilisation from internal stores (Ogata et al., 1994). However, in the absence of mGluR activation A₁ receptor activation may lead to hyperpolarisation, depressing the depolarisation-induced increases in Ca²⁺. It has been demonstrated in the rat hippocampus that there is a synergistic interaction between arachadonic acid and mGluRs, such that PKC activation and inositol phospholipid metabolism were enhanced when both agents were present (McGahon and Lynch, 1994). This suggests possible mechanisms for adenosine interactions with mGluRs, as both presynaptic receptors, mGluR subtype and A_{2b} receptor, can enhance inositol phospholipid metabolism, adenylate cyclase activity and PKC activity. This would suggest a potential excitatory or inhibitory feedback, as A_{2b} receptor activation has been demonstrated to enhance glutamate release (Simpson et al., 1992), which could interact with presynaptic mGluRs to enhance or diminish further glutamate release.

From these lines of evidence, it could be suggested that the increases in adenosine release described in the present work are consistent with the hypothesis that mGluRs are involved in memory formation for the task.

Increases in glutamate have been described at time points that show no increases in affinity or in number for NMDA or AMPA receptors in the IMHV. This suggests that mGluRs may be being expressed at these times, and that these mGluRs, acting synergistically with adenosine, act to enhance glutamate release via a system involving cAMP and adenylate cyclase activity. PKA may thus be activated (as has been discussed above), postsynaptically, perhaps to phosphorylate AMPA receptors and thus enhance their affinity at a later time (6.5 hours, Steele, 1995).

The effect of the increases of adenosine release on GABA are not clear. Evidence from other studies (e.g. Kirk and Richardson, 1994 and 1995) would suggest that adenosine might act to inhibit GABA release. However, evidence from the present work does not indicate that this is the case: enhanced release of GABA is found at times that are subsequent and simultaneous to increased adenosine release. Obviously the milieu of other transmitter interactions, the different messenger systems employed by the same transmitters at different neurons and the receptor systems involved will lead to a complex set of interactions that can only be guessed at using this release paradigm.

In conclusion, the data described in the present work suggest that glutamate and aspartate (or NAAG) and GABA show an enhanced and persistent release from the IMHV and LPO, following training on the task. Their release and interactions with pre- and post-synaptic receptors is vital for memory formation for the task. Adenosine also interacts with these amino acid transmitters and might act to modulate their actions in such a way as to be a filter that may determine the extent to which the internal representations are expressed.

Future Directions

1) Amino acid transmitter release has been measured following the task. In addition to the changes involving glutamate and GABA, and the amino acid system described by Bradley et al. (1990), other transmitter species may be involved in the formation of memory for passive avoidance training. The cholinergic system may be involved (e.g. the release of ACh is implicated in the Bradley et al. (1990) model). For example, 2 hours after training increases in the MHV in both anti-p65 and anti-SV2 are found, most notably in the right MHV (Bullock et al., 1987). These changes may be related to the changes found in neuronal bursting at this time point and it has been suggested that there is a cholinergic basis for this (Mason and Rose, 1987). Raised levels of acetylcholine have been found following training (Bullock et al., 1987), and the model described by Bradley et al. (1990) also describes a possible cholinergic input in the IMHV. In addition the adrenergic system may also be involved. Therefore, it is suggested that release of other, putative, neurotransmitters from the chick IMHV and LPO could be carried out to investigate a role for them in the task.

2) The neuropeptide N-acetylaspartylglutamate (NAAG) may be released from regions of the chick brain following the task. As has been discussed above, NAAG has been found to be released following depolarisation and is degraded to glutamate which will act at excitatory amino acid receptors (Zollinger et al., 1994). Therefore, it is suggested that initial immunohistological/cytochemical identification of NAAG in the chick forebrain is attempted. If the dipeptide is found, it is suggested that release of NAAG should be examined following passive avoidance training.

3) A very important set of experiments that could be carried out, is the *in vivo* microdialysis of transmitters (including adenosine) during passive avoidance training. This would allow real-time measurements to be taken, and with sampling carried out from a relatively small area (as compared to the gross studies using the brain slices) the timing of release can be directly compared:

does the release of one amino acid/purine precede another?

4) The experiments carried out for this thesis involved brain slices. Brain slices provide good experimental material because they contain mostly intact sets of neurons and glial cells, and thus should allow changes in transmitter release etc. that may be apparent in the intact animal. However, a potential drawback is that one cannot distinguish the relative contributions of glia and neurons to the biochemical changes. Thus, if possible, one could separate the glial material from the neuronal (neurosomes/synaptosomes) and measure changes in *both*. Discarding non-neuronal material may well end up in the discarding of important data (as regards receptor binding, uptake, and even transmitter release).

5) The work described in this thesis has shown the action of adenosine on memory formation in the chick following a passive avoidance task.

a) "Endogenous adenosine as well as uptake sites and A₁ receptors display localisations consistent with a neuromodulatory role in the avian retina" (Moffett et al., 1992): it may be possible to examine the distribution of adenosine receptors in the chick brain either via autoradiography or immunocytochemistry and the effect of the training paradigm on their distribution .

b) A₂ antagonists/agonists could be looked at for their effect on memory formation in the chick (e.g. using the weak aversive 10% MeA and an A₂ agonist might "improve" memory; A₂ antagonists should cause amnesia).

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