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NITRIC OXIDE-ACTIVATED MECHANISMS UNDERLYING MEMORY FORMATION USING A PASSIVE AVOIDANCE TASK FOR THE DAY-OLD CHICK

(Volume one)

Submitted by

Thomas M. Edwards BSc(Hons)

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Department of Psychology

School of Psychology, Psychiatry & Psychological Medicine

Faculty of Medicine

Monash University

Clayton, Victoria, 3800

Australia

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Table of contents

Volume one

Volume two

List of figures

List of tables

List of Abbreviations

Biomolecules

Cation channels

BK_{Ca}	large conductance calcium-activated potassium channel
CNG	cyclic nucleotide-gated ion channel
IP_3	inositol 1,4,5-triphosphate
K_{ATP}	ATP-activated potassium channel
K_{Ca}	calcium-activated potassium channel
K_{LIG}	ligand-activated potassium channel
K_{STR}	stretch-activated potassium channel
K_v	voltage-dependent potassium channel
RyR	ryanodine receptor calcium release channel

Enzymes

CaMKII	calcium-calmodulin protein kinase-II
GC	guanylyl cyclase
PARP	*poly*(ADP-ribosyl) polymerase
PDE	phosphodiesterase
pGC	particulate isoform of guanylyl cyclase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
sGC	soluble isoform of guanylyl cyclase

Ions

Ca^{2+}	calcium
K^+	potassium
Mg^{2+}	magnesium
Na^+	sodium

Miscellaneous biochemical terms

ADP	adenosine diphosphate
ATP	adenosine triphosphate

cADPR	cyclic-ADPribose
CICR	calcium induced calcium release
$[H^3]$	tritium labelled
NADH	nicotinamide-adenine dinucleotide phosphate
STOP	stable tubule-only polypeptide

Neurotransmitters and hormones

AMPA	α-amino-3-hydroxy-5-methylisoxazolepropionate
GABA	γ-aminobutyric acid
NMDA	*N*-methyl-D-aspartate

Nitric oxide synthase-related terms

BH_4	tetrahydrobiopterin
eNOS	endothelial isoform of nitric oxide synthase
FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
iNOS	inducible isoform of nitric oxide synthase
NADPH	hydrogenated nicotinamide adenine dinucleotide phosphate
nNOS	neuronal isoform of nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen

Second messengers

cAMP	cyclic-adenosine monophosphate
cGMP	cyclic-guanosine monophosphate

Brain structures

HV	hyperstriatum ventrale
IMHV	intermediate medial hyperstriatum ventrale
LPO	lobus parolfactorius

Drugs and chemicals

Abbreviations of drugs and chemicals used in the current research programme

DMSO	dimethyl sulfoxide
IbTX	iberiotoxin

MeA	methyl anthranilate
MSB	menadione sodium bisulfite
NaCl	sodium chloride salt (saline)
L-NAME	L-N^G-nitroarginine methyl ester
NOVO	novobiocin
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one

Abbreviations of drugs referred to in cited literature

L-AArg	N^G-amino-L-arginine
AP5	2-amino-5-phosphonovalerate
ascorbic acid	ascorbate, Vitamin C,
8-Br-cGMP	8-Bromoguanisine-3',5'-cyclic monophosphate
ChTX	charybdotoxin
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
DEA-NO	diethylamine-NO
DGG	gamma-D-glutamylglycine
DNP	2,4-dinitrophenol
DNQX	6,7-dinitro-quinoxaline-2,3-dione
DPE	diphenylephedine
DPI	diphenyleneiodonium chloride
ebselen	PZ51
KCl	potassium chloride
L-MArg	N^G-methyl-L-arginine
MB	methylthione chloride (methylene blue)
MCPG	(RS)-alpha-methyl-4-carboxyphenyl glycine
MIBG	meta-iodobenzyl-guanidine
MSG	glutamic acid – monosodium salt
MSO	methionine sulfoximine
D-NAME	D-N^G-nitroarginine methyl ester
NDGA	nordihydroguaiaretic acid
L-NNA	L-N^G-nitroarginine. Also known as: L-NA, L-NOARG, NO-Arg, L-NArg and L-Arg
NPLA	*N*-propyl-L-arginine
7-NI	7-nitroindazole
L-NMMA	N^G-monomethyl-L-arginine
NOCys	*S*-nitroso-L-cysteine
pCPT-cGMP	Rp-isomer of 8-(4-chlorophenylthio)-cGMP
SIN-1	3-Morpholinosydnonimine
SNAP	S-Nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside (sodium ferricyanide)
TBT	tributyltin

TEA	tetraethylammonium
tocopherol	vitamin E

Drug administration terms

ic	intracranial
icv	intracerebroventricular administration
ip	intraperitoneal

Memory-related terms

Gibbs & Ng model of passive avoidance memory formation

DR	discrimination ratio
ITM	intermediate-term memory
ITM-A	intermediate-term memory phase A
ITM-B	intermediate-term memory phase B
LTM	long-term memory
mDR	mean discrimination ratio
STM	short-term memory

Synaptic processes

EPSP	excitory post-synaptic potential
FEPSP	field excitory post-synaptic potential
LTD	long-term depression
LTP	long-term potentiation
PPF	paired-pulse facilitation

Data analysis terms

Statistical terms

ANOVA	analysis of variance
SEM	standard error of the mean

Abstract

The role of nitric oxide (NO) in memory formation has been established in a variety of learning tasks and across a number of species. This includes passive avoidance learning in the day-old chick whereby a persistent retention loss is observed by 40 minutes post-training following inhibition of nitric oxide synthase (NOS). However, the mechanism(s) through which NO facilitates memory formation remain poorly understood. Only one NO-dependent pathway has been clearly identified in memory formation, that is, the activation of guanylyl cyclase (GC) and ultimately protein kinase G (PKG). It is the central aim of this thesis to investigate mechanism(s) through which NO may be involved in passive avoidance learning using a single trial task for the day-old chick. Surprisingly, the pharmaco-behavioural investigations reported in this thesis do not support a relationship between NO and GC or PKG activity in memory formation processes. Similarly, the up-regulation of *mono*ADP-ribosylation or the activation of cyclic nucleotide-gated ion channels or large conductance calcium-activated potassium channels, could not account for the role of NO. In contrast, peroxynitrite formation and ryanodine receptor calcium release (RyR) channel activation were found to be likely candidate mechanisms following NO activation in passive avoidance learning. Inhibition of peroxynitrite production using 300μM or 800μM Trolox, or blockade of the RyR channel with 5mM dantrolene, produced a persistent retention loss from 40 minutes post-training, consistent with the retention function following NOS inhibition. In addition, the effective times of administration for NOS, peroxynitrite and RyR channel inhibition were all similar. The conclusion that at least one role of NO in memory for this task is via peroxynitrite and RyR channels is supported by previous research demonstrating peroxynitrite production is NO-dependent and either NO or peroxynitrite are capable of directly activating RyR channels. In addition, RyR channels have been directly implicated in memory formation. While the function of this NO-dependent pathway in memory formation cannot be determined from this research, it is speculated that release of calcium from RyR channel-dependent intracellular stores, and thus the up-regulation of various calcium-dependent channels and enzymes, could be required for long-term potentiation-like processes, result in noradrenaline release or have vasodilatory effects.

Statement of authorship

The work contained within this thesis has not been used for the assessment of any other degree or diploma at Monash University or another institution.

All supporting work produced by other persons has been acknowledged in full within the text of this thesis.

The behavioural experiments reported in Chapters 3, 4 and 5 were completed with the limited technical assistance of Ms Elena Hartley, Dr Marie Gibbs, Dr Pauleen Bennett, Mr Rob Whitechurch and Ms Mara Silins. The behavioural studies reported in Chapters 3 and 4 have been published and copies have been included in Appendix D.

The experiments undertaken for this thesis were approved by the Monash University, Department of Psychology Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Thomas M. Edwards BSc(Hons)

19/7/2002

Date

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Mark Twain on memory...

"[memories are the] little threads that hold life's patches of meaning together."

CHAPTER 1 -

Introduction

SECTION 1: Introduction

SECTION 2: Nitric oxide production, localisation and role in memory formation

1.2.1 Nitric oxide synthase localisation, physiological roles and the production of nitric oxide

1.2.2 Nitric oxide affects memory formation in a wide variety of behavioural paradigms

SECTION 3: Biochemical mechanisms and cellular processes through which nitric oxide may facilitate memory formation

1.3.1 The biological chemistry of nitric oxide

1.3.2 Nitric oxide-stimulated mechanisms implicated in memory formation

1.3.3 Nitric oxide is implicated in cellular processes thought to underlie memory formation

1.3.4 Nitric oxide-stimulated mechanisms are implicated in cellular processes thought to underlie memory formation

1.3.5 Summary of the role of nitric oxide-stimulated mechanisms in cellular processes thought to underlie memory formation

SECTION 4: The Gibbs and Ng model of memory formation

1.4.1 Background

1.4.2 The role of nitric oxide in passive avoidance learning using the day-old chick

1.4.3 Cellular processes aligned to nitric oxide-stimulated mechanisms previously investigated using passive avoidance learning for the day-old chick

SECTION 5: Aims of the present study

CHAPTER 1 - INTRODUCTION

SECTION 1 : Introduction

Nitric oxide (NO) is a highly labile radical affecting a myriad of biological processes. Initially described as a mediator of vasodilation, NO has since been demonstrated to also initiate numerous immunological and neuronal functions. Such neuronal functions include long-term potentiation (LTP), long-term depression (LTD) and neurotransmitter release. These neuronal functions of NO, along with the indirect action of NO-controlled cerebral vasodilation, impact upon behaviour and in particular memory processing.

NO has been implicated in memory formation for a large number of species from invertebrates to primates and across a wide variety of learning tasks. However, many paradigms lack sufficient independent replication and those paradigms widely studied, such as spatial learning for rats, often demonstrate contradictory findings. One memory paradigm in which the role of NO has yielded consistent results between research groups is the single-trial passive avoidance task for day-old chicks. This paradigm has a number of significant advantages including the use of innate pecking behaviour as the basis of a naturalistic learning style. More importantly, the passive avoidance task for the day-old chick is a single trial task thereby allowing the time of learning to be clearly identified. In turn, a high degree of temporal specificity can be achieved with respect to the time of retention loss onset and to the persistence of the retention loss. In this way common times of retention loss may suggest relationships between cellular processes.

The studies presented within this thesis set out to examine the role of NO in passive avoidance memory formation using the day-old chick. Specifically, little is known of the biochemical mechanisms through which NO may facilitate the consolidation of memory formation. Therefore, it is the central aim of this thesis to identify NO-activated memory-specific mechanisms.

The literature reviewed within this chapter is divided into three sections. The first concerns the cellular localisation of nitric oxide synthase (NOS) and the production

of NO, but primarily focuses on the role of NO in memory formation. The second section discusses the currently limited understanding of the biochemical mechanisms through which NO might bring about memory formation. Through a discussion of behavioural studies, and studies detailing NO-activated mechanisms common to physiological processes thought to underlie memory formation, several candidate NO-activated mechanisms are put forward. The third and final section discusses the usefulness of a single trial passive avoidance discrimination learning task developed for the day-old chick as a means to study NO-activated pathways relevant to memory formation. Finally, the aims and direction of the current research are more fully outlined.

SECTION 2 : Nitric oxide production, localisation and role in memory formation

1.2.1 Nitric oxide synthase localisation, physiological roles and the production of nitric oxide

Excluding the production of NO by disproportionation, or through the reduction of nitrate in acidic or highly reducing environments found in disease states (Zweier, Samouilov & Kupp, 1999), NOS is primarily responsible for the production of NO. Specifically, NOS is responsible for the conversion of the amino acid L-arginine, with oxygen, into L-citrulline and NO in the presence of the hydrogenated form of nicotinamide-adenine dinucleotide phosphate (NADPH). The cofactors flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), calcium (Ca^{2+}) (for the neuronal and endothelial isoforms (Cho et al., 1992)), calmodulin, tetrahydrobiopterin (BH_4) and a heme moiety are also required for NOS activity (Abu-Soud & Stuehr, 1993; Chen, Tsai, Berka & Wu, 1997; Gachhui et al., 1996; Lowe et al., 1996; Masters et al., 1996; McMillan & Masters, 1995; Watanabe, Hu & Hidaka, 1997) (see Table 1.1).

There exist three known isoforms of NOS; namely, the neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) isoforms which are also referred to as NOS types I, III and II respectively (see Table 1.1). nNOS has been most clearly characterised in the neurons (Dawson & Dawson, 1996) and astrocytes (Murphy et al., 1993) of the central nervous system, including the spinal cord, and is not c ıocalised with a single neurotransmitter which suggests a broad role in brain function. nNOS is also present in the peripheral nervous system, including the myenteric plexus of the gut, the pelvic plexus leading to innovation of the penis, the autonomic nerves in the outer adventitial layers of the cerebral cortical and retinal blood vessels, and in discrete

ganglia and fibres of the adrenal medulla (Dawson & Dawson, 1996). nNOS has also been characterised in the macula densa of the kidney (Huan, Dawson, Bredt, Snyder & Fishman, 1993), and skeletal muscle (Kobzik, Reid, Bredt & Stamler, 1994).

Initially characterised in blood vessels, eNOS also has a wide distribution and is present in kidney tubular epithelial cells, in interstitial cells of the canine colon and in the neurons of the central nervous system (Dawson & Dawson, 1996).

The third isoform, iNOS, is predominantly found in macrophages, but is also present in other immunological cells such as neutrophils, some lymphocytes, eosinophils and splenic Kupffer cells, hepatocytes, alveolar macrophages, histiocytes and mast cells. Astrocytes, microglia, and neurons may also express iNOS under pathological conditions (Dawson & Dawson, 1996).

The physiological roles of the three isoforms are reflected in their respective locations. nNOS has been implicated in a number of neuronal processes which may have behavioural outcomes. Most notably nNOS may be involved in LTP, acting as a retrograde transmitter, as a messenger in LTD, or in directly modulating neurotransmitter levels all of which may underlie memory formation (see section 1.3.3). nNOS may also modulate behaviour, through the release of sex hormones such as luteinising hormone-releasing hormone (Rettori, Canteros, Renoso, Gimeno & McCann, 1997) and gonadotrophin releasing hormone (Herbison, Simonian, Norris & Emson, 1996).

Consistent with its endothelial location eNOS is thought to control vasodilation, both peripherally and within cerebral blood vessels. Cerebral vasodilation may also have behavioural consequences related to learning and memory which will be discussed at greater length in section 1.3.3.4. In addition to nNOS, the localisation of eNOS to the hippocampus, an area commonly associated with memory formation, is suggestive of a role for both isoforms in memory formation. Such a role for both nNOS and eNOS has been demonstrated in a number of behavioural studies using specific antagonists in conjunction with a passive avoidance learning in the day-old chick (Rickard & Gibbs, in preparation - b; Rickard, Gibbs & Ng, 1999).

The third NOS isoform, iNOS, predominates in the immune system and affects a number of physiological functions, including platelet adhesion and aggregation (Salvemini & Botting, 1993), immune complex disease (Mulligan, Hevel, Marletta & Ward, 1991), and macrophage function. iNOS is also active in processes as diverse as inflammation, anti-tumour responses and parasite clearance (Adams, Franzblau, Varvin,

Hibbs & Krahenbuhl, 1991; Aliberti, Machado, Gazzinelli, Teixeira & Silva, 1999; Green, Meltzer, Hibbs & Nacy, 1990; Rodrigues, Ribeirao & Boscardin, 2000; Shoda et al., 2000).

In keeping with the theme of this thesis NOS localisation in the rodent and avian brains are similar as described by Bredt and Snyder (1992), Brüning (1993), Brüning, Funk and Mayer (1994) and Vincent and Kimura (1992). Brüning (1993), utilising NADPH-diaphorase staining in adult chicks determined a broad localisation of NOS in both neurons and in the endothelia of cerebral blood vessels. Brüning et al. (1994), who utilised immunocytochemical techniques found an almost identical distribution of nNOS in the neurons of the maturing avian brain.

NOS has been identified in memory associated brain regions in both mammal and avian species. For example, nNOS is located in the γ-aminobutyric acid (GABA)-ergic interneurons of the mammalian hippocampus (Dinerman et al., 1994; Dawson & Dawson, 1996) amongst other regions while eNOS is selectively concentrated in the olfactory bulb and hippocampus. Specifically, eNOS is present in the granule cells of the dentate gyrus and in the pyramidal cells of the CA1 region of the hippocampus (Dinerman, Dawson, Schell, Snowman & Snyder, 1994). In chicks, Brüning (1993) observed NADPH-diaphorase staining in memory associated regions of the avian brain such as the hyperstriatum ventrale (HV), including the intermediate medial portion of the hyperstriatum ventrale (IMHV), and the lobus parolfactorius (LPO) (Horn & Johnson, 1989; Rose & Csillag, 1985; Rose & Stewart, 1999; Serrano, Ramus, Bennett & Rosenzweig, 1992). For example, Brüning (1993) described NOS distribution in the posterior LPO as "high" (p 193-195) and HV as "moderate" (p 195). In broad agreement with Brüning (1993), Brüning et al. (1994) confirmed only minimal amounts of nNOS in the HV of the maturing avian brain using primary antibodies raised to porcine nNOS. As NOS levels in the HV and IMHV were moderate at best, it was of interest to determine if training altered NOS levels in this region. Ambalavanar, McCabe and Horn (1994) compared NOS levels in imprinted and naive chicks and detected only minimal NOS in the HV for either condition using NADPH-diaphorase staining. By way of explanation, von Bartheld and Schrober (1997) suggested that the LPO was the likely site of action of NO with respect to memory formation processes. Administration of NOS inhibitors into the region of the IMHV being indirect, acting instead on the locus coeruleus which has projections to both the LPO and HV.

Table 1.1

Summary of the Properties of the Three NOS Isoforms

Isoform	*major source*	*subcellular localisation*	*denatured molecular mass*	*substrates*	*prosthetic groups*	*regulation*	*number of amino acids*
nNOS	*brain*	*cytosol*	*160 kDa*	*L-arginine, O_2, NADPH*	*equimolar FAD/FMN, BH_4, heme*	*calcium, calmodulin*	*1429 (rat), 1433 (human)*
eNOS	*endothelial cells*	*membrane associated*	*133kDa*	*L-arginine, O_2, NADPH*	*equimolar FAD/FMN, BH_4, heme*	*calcium, calmodulin*	*1205 (bovine), 1203 (human)*
iNOS	*macrophages*	*cytosol*	*130kDa*	*L-arginine, O_2, NADPH*	*equimolar FAD/FMN, BH_4, heme*	*cytokine inducible, exogenous calcium independent*	*1144 (mouse), 1153 (human)*

Adapted from Förstermann and Garth (1996) and Dawson and Dawson (1996)

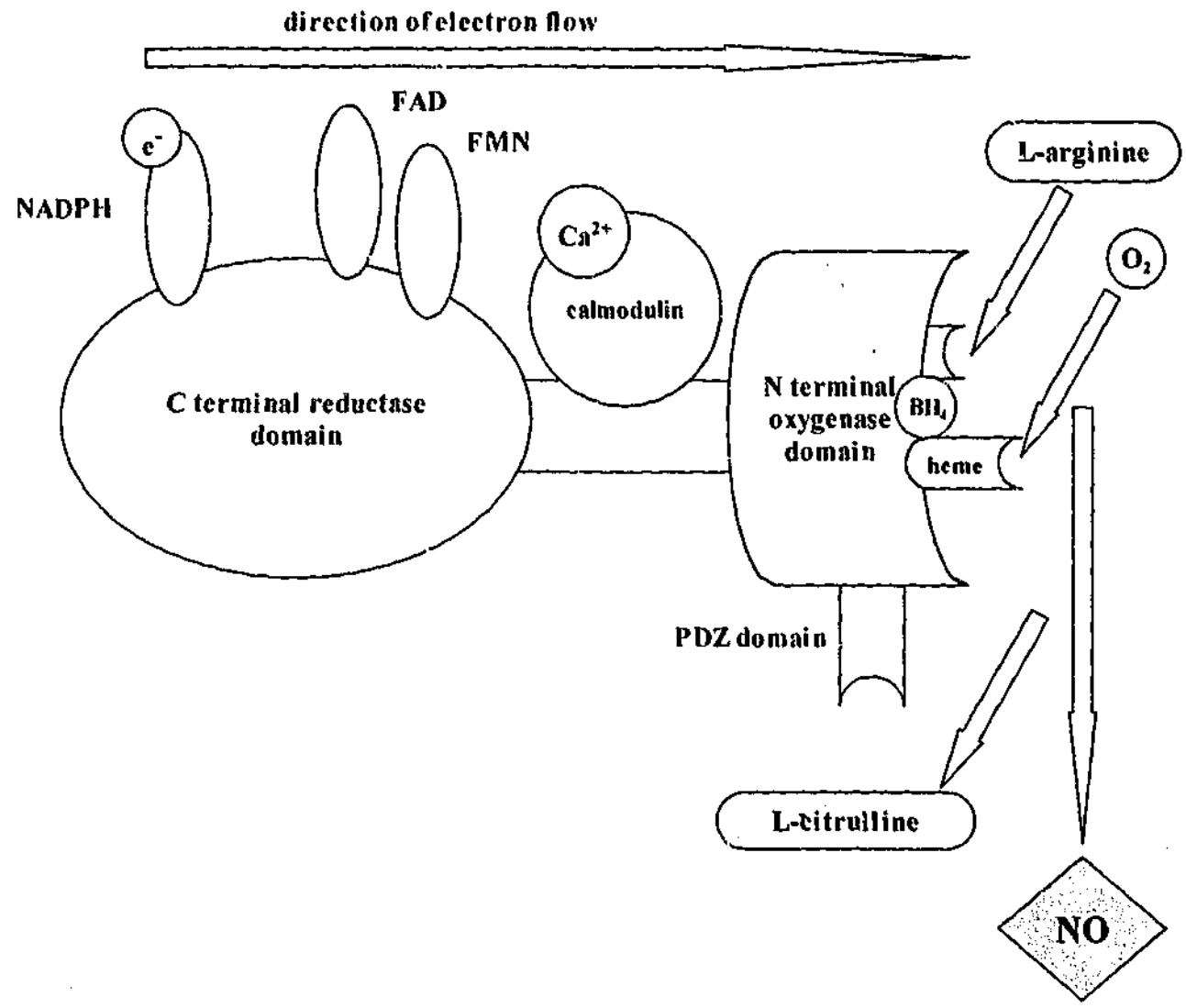


Figure 1.1 A representation of nNOS and the formation of NO.
nNOS domains are represented in mauve, co-factors in orange and Ca2+ in green. Electron transfer from NADPH to the catalytic site is indicated by the yellow arrow. The substrates L-arginine and oxygen are depicted in blue. Once bound to the catalytic site, containing the L-arginine binding site, BH4 and a heme group, L-arginine and oxygen are converted into L-citrulline (also depicted in blue) and NO (depicted in red).

1.2.2 Nitric oxide affects memory formation in a wide variety of behavioural paradigms

During the last decade the role of NO in memory formation has been investigated widely using pharmaco-behavioural studies. This field of research has established a broad requirement for NO in memory formation across both invertebrate species such as the honey bee (Hosler, Buxton & Smith, 2000; Muller, 1996), cricket (Jaffe and Blanco, 1994) and snail (Kemenes, Kemenes, Andrew, Benjamin & O'Shea, 2002; Teyke, 1996), and in a number of vertebrate species subjected to a range of

behavioural tasks. While acquisition for many, but certainly not all, tasks appears to be NO-dependent this may vary across species. Further, some drugs used to study NO-dependent learning may result in sensory-motor effects suggesting a role for NO when there may not be one.

Three broad categories of learning task often used in vertebrates are spatial, olfactory and avoidance learning tasks. For example, the role of NO in spatial learning has been widely studied but in rats remains controversial as some studies support a role for NO (Bohme et al., 1993; Chapman, Atkins, Allen, Haley & Steinmetz, 1992; Frisch et al., 2000; Prendergast, Buccafusco & Terry,1997a; Suzuki, Ikari, Hayashi & Iguchi, 1996; Zou, Yamata, Tanaka, Kameyama & Nabeshima, 1998) while others do not (Bannerman, Chapman, Kelly, Butcher & Morris, 1994; Blockland et al., 1999; Knepper & Kurylo, 1998; Tobin, Gorman, Baxter & Traystman, 1995).

Rodent models provide similarly complex findings for olfactory recognition tasks. While olfactory recognition appears to be NO-independent in the mouse (Brennan & Kishimoto, 1993; Okere, Kaba & Higuchi, 1996) the studies of Bohme et al. (1993) and Samama and Boehm (1999) found NO to be necessary for successful completion of an olfactory task using adult and neonate rats respectively. However, one of the most detailed studies of olfactory recognition was performed not with rodents but with ewes (Kendrick et al., 1997). Specifically, the authors found a requirement for NO in olfactory learning and demonstrated the action of NO to be most likely through the activation of guanylyl cyclase (GC).

Finally, avoidance learning has also been studied with rodents but investigations using chicks have also provided important insights. Rats provide contradictory findings with respect to the action of NO in avoidance learning tasks (Bernabeu, de Stein, Fin, Izquierdo & Medina, 1995; Bohme et al., 1993; Calixto, Vandressen, de Nucci, Moreno & Faria, 2001; Fin et al., 1995; Huang & Lee, 1995; Myslivecek, Hassmannova, Barcal, Safanda & Zalud, 1996; Telegdy & Kokavszky, 1997; Yilmaz, Kanit, Okur, London & Pogun, 2000). While, in murine models, the limited evidence available demonstrates that NO does not facilitate avoidance learning (Baratti & Boccia, 1999; Baratti & Kopf 1996). However, using variants of a single trial passive avoidance task for the day-old chick,

Hölscher and Rose (1992, 1993), Rickard, Ng and Gibbs (1998). Rickard et al. (1999) and Rickard and Gibbs (in preparation – a,b) have consistently demonstrated a loss of retention by 40 minutes post-training following administration of various NOS inhibitors.

Having provided a brief overview suggesting the role of NO in a variety of common tasks is not obvious, it is worth discussing some of these studies in more detail. In doing so, specific attention must be paid to both the task and species used as well as any findings suggesting sensory-motor effects.

1.2.2.1 Nitric oxide and spatial learning

One of the earliest studies on the role of NO in memory was performed by Chapman et al. (1992). Using the Morris water maze it was found that rats administered L-N^G-nitroarginine methyl ester (L-NAME) intracerebroventricularly (icv) prior to training displayed longer escape intervals, in a dose-dependent fashion, in comparison to rats administered the inactive stereo-isomer D-N^G-nitroarginine methyl ester (D-NAME). A third group received co-administration of L-arginine and L-NAME, the first drug as a challenge to the second, and again this control group located the platform significantly faster than did the group administered L-NAME alone. Having determined that NO was necessary for memory formation within this paradigm, Chapman et al. (1992) investigated whether NO was required for acquisition, memory storage or memory retrieval. To do this rats were well-trained prior to drug administration. One group of rats was administered L-NAME and another saline. Both groups showing similar performances within the Morris water maze suggesting NO is required for task acquisition.

Although Chapman et al. (1992) and a number of later studies (Bohme et al., 1993; Frisch et al., 2000; Suzuki et al., 1996; Zou et al., 1998) had apparently demonstrated that spatial learning in rats was NO-dependent, this has been challenged. One criticism of pharmaco-behavioural studies is whether the reported behaviour is due to inhibition of memory processes directly or due to sensory-motor effects. For example, L-NAME, a common inhibitor of NOS activity is known to impair spontaneous behaviour in rodents following chronic administration of 40mg/kg/day (Halcak et al., 2000). Importantly

however, Estall, Grant and Cicala (1993) found L-NAME administration impaired acquisition of a place navigation task noting that impairments were not the result of sensory-motor deficits or an effect upon motivation. Similarly, Ingram, Spangler, Kametani, Meyer and London (1998), Ingram, Spangler, Meyer and London (1998) and Meyer, Spangler, Patel, London and Ingram, (1998) concluded that both isoform-specific and isoform-non-specific inhibitors of NOS impaired task acquisition for a 14-unit T-maze without having an effect upon sensory-motor systems. Finally, Qiang, Chen, Wang, Wu and Qiao (1997) found that rats used in the Morris water maze showed a dose-dependent impairment in task acquisition following L-NAME administration. The capacity of rats to find the visible platform was not incumbered by concentrations of the drug greater than that found to impair memory formation (5μmol) therefore suggesting that the action of L-NAME was not due to sensory-motor effects.

Even so, other studies do suggest the presence of sensory-motor effects. Using the Morris water maze, Bannerman et al. (1994) initially concluded that 75 mg/kg L-NAME (icv) compromised spatial memory while not affecting retention of previously acquired spatial information. This was in accordance with the conclusions of Chapman et al. (1992). However, while Bannerman et al. (1994) excluded any gross sensory-motor disturbance as a possible alternative explanation for the observed behaviour they did suggest the possibility of a subtle sensory-motor effect. To test this, a visual discrimination variant of the water maze was used as visual discrimination learning is considered to be both hippocampal- and glutamate-independent and, thus, expected to be NO-independent. Therefore, if L-NAME affected NO-independent visual discrimination learning it could be concluded that any effects of the drug were related not to memory but to subtle sensory-motor disturbances. While both saline and L-NAME administered rats reached criterion at a similar rate there was a significant increased escape latency on the first day of training for the L-NAME treated group. It was suggested that this "first day" effect may be of relevance only in tasks which are acquired quickly, such as in the initial spatial memory task where training lasted only three days. Explanations given for the initial increase in escape latency included the possibility that the stress levels of the animal may have been increased when repeatedly placed back into the maze or if L-NAME produces a feeling of ill-being. Both of these alternatives could produce increased escape latencies without directly affecting memory processes. Finally, Bannerman et al. (1994) questioned the prior use of intraperitoneal (ip) administration of NOS inhibitors by Chapman et al. (1992), suggesting that

systemic effects of L-NAME, such as cardiovasculature responses, may also influence the subsequent behaviour without affecting memory.

Other issues associated with behavioural tasks are the determination of an effective concentration of drug and possible redundant systems which may overcome its effect. For example, Tobin et al. (1995) found no effect of L-NAME upon spatial learning using a "match to position task". 75mg/kg (ip) L-NAME which, according to *in vitro* assay, would inhibit 85% of NOS activity had no effect. However, a number of methodological issues may also explain this result. For example, it may be speculated that arousal brought about by the training regime may have overcome the effect of the inhibitor. Yet, it is more likely that the use of an *in vitro* assay to determine the optimum L-NAME concentration for an *in vivo* study led to doses that may have been erroneous when compared to effective doses determined by an *in vivo* dose response study. In addition, while 85% inhibition appears almost complete, the possibility of residual levels of NO production being enough to allow the completion of the spatial task, either alone or in parallel with other messengers, cannot be dismissed (Hölscher, Canevari & Richter-Levin, 1995). For example, Hölscher et al. (1995) reported that rats subjected to the Morris water maze produced only partial loss of spatial memory in a dose-dependent fashion when administered 10mg/kg L-N^G-nitroarginine (L-NNA). Similarly, only partial memory loss resulted when rats were chronically administered the arachidonic metabolism inhibitor nordihydroguaiaretic acid (NDGA) (10 or 20 mg/kg). Only when the two drugs were administered together was there a near complete inhibition of memory, suggesting both NO and arachidonic acid are necessary for spatial memory to occur.

Other studies have also questioned the role of NO in spatial learning. For example, Blokland et al. (1999) demonstrated, in adult rats, that icv administration of the NOS inhibition, L-NNA (30μg in 0.5μl saline), 45 minutes prior to a daily training session using the Morris water maze did not impair spatial learning. It was noted, however, that although L-NNA-treated rats were equal to the control groups with respect to escape latency and swim distance to platform they swam less close to the platform in the third and fourth days of training suggesting a different manner of searching not associated with spatial learning.

Curiously, another study using the Morris water maze found that eNOS knock-out mice demonstrated accelerated place learning (Frisch et al., 2000). Further, sensory-motor effects were discounted as the knock-out mice performed as well as wildtype

mice on a cued version of the task. It was also noted that there existed increased levels of a serotonin metabolite in the cerebellum, increased serotonin turnover in the frontal cortex, and an increased dopamine turnover in the ventral striatum.

In addition to the Morris water maze task there exist other spatial tasks and in such tasks NO may be necessary for memory formation to result. As mentioned above, Estall et al. (1993) determined a requirement for NO using a place navigation task excluding sensory-motor effects as the underlying cause of the altered behaviour. Similarly, Ingram et al. (1998a, b) and Meyer et al. (1998) utilised a 14-unit T-maze, finding NOS antagonists effective in blocking retention without the occurrence of sensory-motor effects. Alternatively, the radial arm maze has also shown to be effective (Yamada et al., 1995; Zou et al., 1998). For example, chronic infusion of the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA) (25mg/kg icv), to rats resulted in impaired performance in the radial arm maze task (Suzuki et al., 1996). Importantly, blood pressure was monitored and no change noted, thus excluding the possibility of systemic effects contributing to the observed behaviour.

Yet, even in alternate spatial tasks the role of NO in memory formation is controversial. One study which demonstrates this is by Knepper and Kurylo (1998) who studied both spatial and cued learning in rats using an operant conditioning chamber. Intra-peritoneal administration of L-NAME (75 mg/kg) had no significant effect on the acquisition or retention of either task, when compared with the respective saline control group.

NO involvement in spatial memory is therefore by no means conclusive. Its role may be species- or task-dependent, or be synergistic with other messenger molecules such as arachidonic acid. Inhibitors of NOS may in some cases result in altered systemic functions, such as blood pressure, or cause central nervous system effects not related to memory. It is therefore apparent that behavioural studies must test for these 'sensory-motor' effects so as to avoid potentially misleading results.

1.2.2.2 Nitric oxide and olfactory learning

Histochemical studies have reported that the encephalic structures which comprise the olfactory system have varying but significant levels of NOS. For example,

Brüning (1993) reported that the accessory olfactory bulb possessed very high levels of NOS while the olfactory bulb possessed only isolated NOS-positive neurons. Bredt et al. (1991) also concluded that the accessory olfactory bulb contained one of the highest levels of NOS in the mammalian central nervous system.

Consequently, Bohme et al. (1993) sought to determine if olfactory memory was NO-dependent using a social recognition test for rats. Olfactory recognition was impaired following the administration of the NOS inhibitor L-NNA (100mg/kg). Olfactory memory was also found to be NO-dependent in the studies of Samama and Boehm (1999) who used both a repetitive and a one trial olfactory associative learning task for neonate rats. Memory impairment was shown to occur for both tasks after the administration of L-NAME (50mg/kg ip) or 7-nitroindazole (7-NI) (30 mg/kg ip). For the one trial task, both inhibitors were utilised, but were only effective if administered before training, suggesting a role for NO in acquisition.

In contrast to the above studies, Brennan and Kishimoto (1993) made use of odour specific mating behaviour of mice. Attempts to block olfactory recognition of males by female mice using direct administration of either 1nmol or 10nmol L-NNA immediately anterior to the accessory olfactory bulb did not produce a significant decrease in mating behaviour. Another group of female mice were injected with both 10nmol L-NNA and 20nmol NDGA, a recognised inhibitor of arachidonic acid metabolism, but again there was no significant inhibition of mating. These results were confirmed for mice by Okere et al. (1996) who found that administration of NOS inhibitors failed to prevent mating by female mice to a stud male. However, they did show that sodium nitroprusside (SNP), a spontaneous NO-donor, facilitated olfactory memory during exposure to male pheromones in the absence of mating. This result was interpreted to suggest that NO may have a modulatory role in olfactory memory rather than a critical role.

Extending upon these studies, Kendrick et al. (1997) demonstrated a role for NO in olfactory recognition in *post partum* ewes and suggested the action of NO was through GC. Using *in vivo* microdialysis, glutamate (200μM) was infused into the accessory olfactory bulb and was found to increase NO and cyclic-guanosine monophosphate (cGMP) levels, presumably through the activation of N-methyl-D-aspartate (NMDA) and/or α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) receptors. Next, L-NNA (500μM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one

(ODQ) (200μM), inhibitors of NOS and GC respectively, were administered and both were found to prevent increased glutamate release. The NO-donor, S-Nitroso-N-acetylpenicillamine (SNAP) (1mM), dose-dependently increased both cGMP and glutamate concentrations. Taken together, these findings suggest a role for NO and GC in the accessory olfactory bulb associated with glutamate, a process consistent with a role for NO in LTP. To determine if olfactory memory was dependent upon NO-related processes Kendrick et al. (1997) bilaterally administered either 100μM L-NNA (icv), ODQ (200μM) or the ionotropic glutamate receptor antagonist gamma-D-glutamylglycine (DGG) (500μM) into *post partum* ewes. Olfactory recognition of their progeny was impaired, establishing a link between NO, cGMP and glutamate in olfactory memory. Finally, Kendrick et al. (1997) suggested that olfactory memory formation was NO-dependent as administration of an antagonist after memory formation failed to prevent recall.

1.2.2.3 Nitric oxide and avoidance learning

Passive avoidance learning, known also as inhibitory avoidance learning, uses such tasks as step-down or step-through inhibitory shock avoidance tasks with rodents or the passive avoidance learning task for chicks adapted from Cherkin (1971).

A number of studies have been conducted using either step-through or step-down inhibitory avoidance in rodents. Importantly, several of these have found that avoidance learning is NO-dependent and that the concentrations of drugs administered did not result in sensory-motor effects. For example, Baratti and Kopf (1996) used male mice in a single trial step-through inhibitory avoidance task and found that intraperitoneal injection of the NOS inhibitor L-NAME (3-100 mg/kg), but not the inactive stereo-isomer, D-NAME, impaired retention 48 hours post-training in a dose-dependent fashion. The performance of a control group which had not received foot shock during training was unaffected by the administration of L-NAME thus excluding systemic effects brought about by the route of drug administration or sensory-motor deficits generally. The effect of L-NAME (100mg/kg) was then challenged by co-administration of the NO precursor L-arginine (300mg/kg) or of the inactive stereo-isomer D-arginine. When challenged with L-arginine the resulting behaviour was equivalent to that of the saline control while the challenge with D-arginine resulted in

impaired memory. Finally, the action of NO on task acquisition was deduced by administration of L-NAME either immediately post-training or at 180 minutes post-training with only the first group displaying significant impairment. In a similar study, Myslivecek et al. (1996) was also able to exclude sensory-motor effects and systemic effects due to the route of administration for an inhibitory avoidance task applied to neonate rats. Comparing injection route to criterion attainment they found a dose-dependent inhibition of learning following administration of either 5mM or 10mM L-NNA (ip or icv) while measuring spontaneous motor behaviour, heart rate and brain and body temperature.

However, while Fin et al. (1995) also noted a role for NO in the inhibitory step-down shock avoidance task for rats using L-NNA (2.0μg/0.5μl) administered into the hippocampus 10 minutes prior to, or immediately post-training. Administration of the spontaneous NO-donor, SNAP, (5.0μg/0.5μl) improved retention when administered immediately post-training and at times far later than those found effective for L-NNA. SNAP was effective when administered 60 or 150 minutes post-training, but not at 300 minutes post-training. It may be speculated that the action of SNP was therefore upon NO-dependent pathways not normally associated with memory formation around the time of training.

In addition to the pharmaco-behavioural studies of Fin et al. (1995), Bernabeu et al. (1995) linked behaviour to neural biochemistry by utilising the step-down foot shock task for male rats to establish that increased NOS activity was observed immediately after training but not at 60 minutes post-training. In agreement with the biochemical data and with the studies of Fin et al. (1995), intrahippocampal administration of L-NNA (2.0μg/0.5μl) 10 minutes prior to training or immediately post-training resulted in impaired performance, while administration 60 minutes post-training had no effect.

In contrast to the previous studies, Bohme et al. (1993) found no evidence of NO-dependent memory for either a step-through or step-down task. Bohme et al. (1993) explained their negative result by suggesting that shock avoidance utilises different memory regions which are NO-independent. However, the severity of the shock imparted may have been sufficient to overcome any partial inhibition of memory by L-NNA. For example, Bohme et al. (1993) utilised a 0.7mA shock for 20 seconds during training whereas Fin et al. (1995) applied only a 0.4mA shock for 1 second.

A similar foot shock avoidance task was employed by Telegdy and Kokavszky (1997). Administration of 1.25μg/2μl to 20μg/2μl (icv) of the NO precursor L-arginine,

before or immediately after training, increased avoidance latency times. The inactive stereoisomer D-arginine had no effect, demonstrating improved memory as might be expected from past studies. If administered 6 hours post-training L-arginine was ineffective, which is also consistent with previous studies suggesting a role for NO in acquisition. 2.5μg/4μl and 5μg/4μl (icv) of L-NNA were both successful in preventing the action of L-arginine. However, when administered alone, L-NNA failed to inhibit passive avoidance memory. While appearing contradictory, these results may again be explained by arguing that administration of L-arginine results in aberrant NO production, similar to the administration of NO-donors, and augments those processes active in foot shock learning. Therefore L-NNA would attenuate the action of L-arginine but not be active when administered alone.

Other avoidance tasks have provided more consistent findings such as the single trial passive avoidance task developed for the day-old chick (Cherkin, 1971). In this task, chicks initially peck at a coloured bead which is subsequently coated in a non-toxic chemical taste aversant. When tested at a specific time post-learning, the chick passively avoids pecking a similar dry bead. The first to test if passive avoidance learning in the chick was NO-dependent were Hölscher and Rose (1992, 1993). They demonstrated memory retention loss by 30 minutes post-training after intraperitoneal administration of the NOS inhibitor, L-NNA (2mmol), one hour before training (Hölscher & Rose, 1992). Co-administration of L-arginine and L-NNA prevented memory retention loss (Hölscher & Rose, 1992). However, as in other studies mentioned above intraperitoneal administration of L-NNA may result in systemic effects which alter performance without affecting memory *per se*. Hölscher and Rose (1993) repeated the study using intracranial administration of L-NNA (2-8mM) and confirmed that memory was lost by 30 minutes post-training. The effective times of administration were further characterised by Hölscher and Rose (1993) who found administration of 4.5mM L-NNA resulted in retention loss when tested 120 minutes post-training if administered between 60 and 30 minutes before training (ip), or between 60 and 15 minutes before training (ic). Finally, they investigated lateralisation of this effect by administration of the antagonist to both cerebral hemispheres independently and found the effect to be bilateral.

Rickard et al. (1998) used a variant of the task employed by Hölscher and Rose (1992, 1993) which required discrimination between an aversive red bead and a non-aversive blue bead. Therefore chicks which demonstrated generalised avoidance, due

possibly to sensory-motor effects, could be excluded from the data analysis. Memory retention loss was observed by 40 minutes post-training when either L-NAME or L-NNA was administered intracranially, while co-administration of the NO donor, SNP, and the NOS inhibitor, L-NAME, produced no retention loss. One obvious difference between Hölscher and Rose (1993) and Rickard et al. (1998) was the effective times of administration for L-NNA. While Rickard et al. (1998) determined effective administration times for L-NNA between 30 minutes prior to training to 25 minutes post-training, Hölscher and Rose (1993) demonstrated memory loss did not occur when the drug was given earlier than 15 minutes before training. while administration times post-training were not tested. The reasons for these differences in administration times are not clear but may, to some extent, be due to a number of methodological differences, as outlined in Appendix B, and by Crowe and Hamalaine (2001). Finally, Rickard et al. (1994) also studied the role of NO in passive avoidance learning in the neonate chick using a weakly reinforced learning variant of the task. In this variant, the chemical aversant is diluted ten-fold in ethanol and retention persists for only 30 minutes post-training (Crowe, Gibbs & Ng, 1989). Rickard et al. (1994) showed that weakly reinforced chicks administered 150μM SNP (ic) demonstrated good retention until at least 180 minutes post-training. Taken together, the above studies clearly demonstrate a role for NO in memory for a passive avoidance task in day-old chicks.

1.2.2.4 Other learning paradigms implicating nitric oxide

In conclusion, the role of NO in memory formation has been studied most using spatial, olfactory and avoidance learning tasks. However, NO has also been implicated in other tasks and across a wide variety of species. While not investigated to the extent of those above it is apparent that tasks of object recognition using rats appear to be NO-dependent (Prickaerts, Steinbusch, Smits & de Vente, 1997; Samama & Boehm, 1999) as is conditioned place preference learning in the mouse (Martin & Itzhak, 2000) and condition taste avoidance (Prendergast et al., 1997a). In addition to rodents a number of other species have also been investigated. For example, eye-lid conditioning in the rabbit is NO-dependent (Allen & Steinmetz, 1996), as is delayed matching to sample learning in macaques (Prendergast, Terry, Jackson & Buccafusco, 1997b). Even in

goldfish the vestibulo-ocular reflex adaptation task appears to be NO-dependent (Smith & McElligott, 1995). Taken together, the breadth of species reliant on NO-dependent learning across a variety of tasks suggests that NO is an important mechanism underlying memory formation.

SECTION 3 : Biochemical mechanisms and cellular processes through which nitric oxide may facilitate memory formation

1.3.1 The biological chemistry of nitric oxide

While NO has been implicated in a number of memory paradigms the downstream biochemical mechanisms through which NO acts remain poorly understood. This is due, in part, to the large range of possible interactions NO may participate in. In brief, NO may activate, or inhibit, a large number of enzymes, modulate cellular ion balance by affecting intra- and extracellular membrane channels and form other reactive nitrogen species which are capable of interactions with proteins and lipids.

Before candidate NO-dependent downstream mechanisms can be assessed as to their role in memory formation it is worthwhile reviewing the variety of interactions NO may have with enzymes, ion channels and other radicals.

1.3.1.1 Nitric oxide interacts with enzymes

NO interacts with numerous enzymes but has been most clearly shown to bind enzymes with an iron, copper or iron-sulphur moiety within the catalytic site (Cooper, 1999) (refer Figure 1.2). Notable examples of heme-containing proteins binding NO include haemoglobin (Sharma, Traylor, Gardiner & Mizukami, 1987), myoglobin (Sharma, Isaacson, John, Waterman & Chevion, 1983), cytochrome c (Ascenzi, Coletta, Santucci, Polizio & Desideri 1994), catalase (Cooper, 1999) and GC (Stone & Marletta, 1994). It is through the activation of GC that NO is most commonly thought to act. In contrast, non-heme iron-containing proteins which interact with NO include

lipooxygenase (Nelson, 1987) and ferritin (Lee, Arosio, Cozzi & Chasteen, 1994). Cytochrome oxidase (Guiffre et al., 1996) is an example of an NO-reactive enzyme which contains both iron and copper. Finally, aconitase (Castro, Robalinho, Cayota, Meneghini & Radi, 1998) and NADH dehydrogenase (Clementi, Brown, Feelisch & Moncada, 1998) are representative of proteins with iron-sulphur centres and are also known to interact with NO. These few examples of common, and functionally important, enzymes illustrate the complex role of NO within the cell.

Not only can NO bind metal atoms in the catalytic site but may also alter the activity of enzymes at a distance from the catalytic site by nitrosylation of amino acid residues, such as those containing thiol groups (Castro et al., 1994; Clementi et al., 1998; Hausladen & Fridovich, 1994; Kenedy, Antholine & Beinert, 1997). Further, NO may alter enzyme activity by a number of indirect methods such as interaction with co-factors or catalytic products (Andersson, Leighton, Young, Blomstrand & Newsholme, 1998; Castro et al., 1998). Two examples of NO indirectly modulating enzyme function are the activation of the hexose monophosphate shunt by binding glutathione (Clancy, Levartovsky, Leszczynska-Piziak, Yegudin & Abramson, 1994) and the alteration of xanthine oxidase output by binding superoxide (Rubbo et al., 1994; Wink et al., 1993).

1.3.1.2 Nitric oxide commonly acts through guanylyl cyclase

As stated above, one of the energetically preferred reactions of NO is to bind the heme moiety of metallo-proteins. One such metallo-protein is GC and it is through the activation of the soluble form of this enzyme (referred to in this thesis simply as GC) that NO produces many of its known physiological actions. The basic premise of the GC-dependent pathway is that NO activates GC to synthesise the second messenger cGMP which, in turn, activates protein kinase G (PKG). PKG is then responsible for the phosphorylation of substrate proteins, including NOS and, by doing so, facilitates numerous physiological processes (see Figure 1.2) (Belsham, Westal & Mellon, 1996; Kanada et al., 1993; Range, Holland, Basten & Knox, 1997; Xu, Piper & Tseng, 1995).

The first direct evidence that GC was activated by NO came from the studies of Arnold, Mittal, Katsuki and Murad (1977) who bubbled NO gas through various tissue

preparations, including brain. They showed that, in all cases, cGMP concentrations were raised following exposure to NO gas and noted brain preparations were highly susceptible to NO activation of GC, indicating the potential importance of this pathway in brain functioning. Second, Arnold et al. (1977) found no increase in the level of another cyclic nucleotide second messenger, cyclic-adenosine monophosphate (cAMP). This underscored NO's sole preference for the cGMP second messenger pathway.

One obvious concern with many *in vitro* studies, including that of Arnold et al. (1977), is whether they sufficiently mimic *in vivo* conditions with respect to the concentration of NO administered. This question was specifically addressed by Marsault and Frelin (1992), who investigated whether physiological concentrations of NO elicited similar increases in cGMP levels as observed by Arnold et al. (1977). They placed NO producing cell types, including neuroblastoma and aortic endothelial cells, in close proximity to NO-sensitive cerebral capillaries and determined that cGMP levels were indeed raised in the target tissue. The validity of this experiment may be questioned as high numbers of NO-producing cells in close proximity to vascular tissue could conceivably produce super-biological levels of NO. However, it should be noted that the small diffusion radius of NO means that only a proportion of the NO-producing cells present would release NO that acted on the target vascular tissue.

While NO is a major activator of GC, there are a number of alternative agonists of GC which ultimately result in the activation of PKG (refer Figure 1.2). Therefore, any action of PKG cannot necessarily be attributed to NO. For example, there are two classes of GC: the soluble and the particulate class, as reviewed by Garbers (1993) and McDonald and Murad (1996). The isoforms of the particulate class are variously susceptible to activation by natriuretic peptides, prostaglandins and other agonists (Dizhoor & Hurley, 1999; Razandi, Pedram, Rubin & Levin, 1996; Schmidt, Lohmann & Walter,1993). Notably, the importance of natriuretic peptides in memory formation has been explored by Telegdy, Kokavszky and Nyerges (1999) who found C-type natriuretic peptide dose-dependently improved passive avoidance learning in rats.

There are also numerous isoforms of soluble GC (Schmidt et al, 1993) which may be suggestive of a number of agonists acting in place of, or in parallel with, NO to

increase GC activation (Monaco & Burke-Wolin, 1995). One such agonist is carbon monoxide (Kharitonov, Sharma, Pilz, Magde & Koesling, 1995; Sharma & Magde, 1999), which is chemically similar to NO and has been suggested to act in synaptic processes such as LTP (Hawkins, Zhuo & Arancio, 1994; Zhuo, Laitinen, Li & Hawkins, 1999) as well as being implicated, to a limited extent, in memory formation (Bernabeu et al., 1995b; Fin et al., 1994) (refer Figure 1.2). Not only have *in situ* hybridisation studies of brain slices suggested an overlapping distribution of GC and heme oxygenase, which synthesises carbon monoxide (Verma, Hirsch, Glatt, Ronnett & Snyder, 1993), but both NO and carbon monoxide have been suggested as candidate retrograde transmitters in LTP (Zhuo, Small, Kandel & Hawkins, 1993) and activators of vasodilation (Morita, Perrella, Lee & Kourembanas, 1995; Zakhary et al., 1996). However, carbon monoxide does appear to be an inferior activator of GC when compared to NO (Humbert, Niroomand, Fischer, Mayer & Koesling, 1990; Kharitonov et al., 1995; Stone & Marletta, 1994).

Activation of GC results in the production of the second messenger cGMP which is known to activate three classes of proteins: PKG isoforms, cyclic nucleotide-gated (CNG) ion channels, and phosphodiesterases (PDEs), as detailed in Figure 1.2 (McDonald & Murad, 1995). Generally, PKG-I and PKG-II are considered to be the main effector mechanisms of NO. While over 50 molecules are known to be phosphorylated by either PKG-I or PKG-II, very few of these have been identified, and even fewer have been studied in detail as to their functional role. One molecule which has been characterised is a novel member of the septin family and localised to the presynaptic nerve terminal (Xue et al., 2000). Another PKG substrate in nerve terminals is the stable tubule-only polypeptide (STOP), which may act by preventing the disassembly of tubules within the pre-synaptic bouton or axon (Wang, Mitchelhill, Kemp & Robinson, 1998). Other PKG specific proteins include G-substrate (Endo et al., 1999) and DARPP-32, which are also located in the presynaptic terminal (Wang & Robinson, 1997). In addition to PKG-specific substrates, there are many molecules which are jointly phosphorylated by PKG and other kinases. They include $GABA_A$ receptors, which are phosphorylated by PKG and calcium-calmodulin protein kinase-II (CaMKII) (McDonald & Moss, 1994), and the $\alpha 1$ subunit of the neuronal class C L-type Ca^{2+} channel, which is phosphorylated jointly by protein kinase C (PKC), CaMKII and PKG (Hell et al., 1993).

Other identified PKG substrates include the type I inositol 1,4,5-triphosphate receptor (IP_3 receptor), which is structurally and functionally similar to the IP_3 brain receptor (Komalavilas & Lincoln, 1996); the ryanodine receptor-related Ca^{2+} release (RyR) channel (Takasago, Imagawa, Furukawa, Ogurusu & Shigekawa, 1991) which is also directly activated by NO (Aghdasi et al., 1997; Stoyanovsky et al., 1997; Sun, Xin, Eu, Stamler and Meissner, 2001; Xu et al, 1998); vasodilator-activated phosphoprotein (Butt et al., 1994; Halbrugge, Friedrich, Eigenthaler, Schanzenbacher & Walter, 1990); $G_i\alpha$, leading to the inhibition of adenylate cyclase and thus blocking the production of cAMP (Pfeifer et al., 1995); the α-subunit of the Ca^{2+}-activated K^+ channel (K_{Ca}) (Alioua, Huggins & Rousseau, 1995); and phospholamban (Lincoln, Cornwall, Komalavilas, MacMillan-Crow & Boerth, 1996).

One cautionary note with regard to the activation of PKG isoforms is that they are not entirely specific to cGMP activation, being marginally activated by another cyclic nucleotide second messenger, cAMP (McDonald & Murad, 1995; Schmidt et al., 1993). However, *in vitro* studies suggest cAMP results in several orders of magnitude less activation than cGMP.

An alternate target for cGMP are the CNG channels which, together with cAMP-gated ion channels, provide a means to control ion influx into the cytosol via second messenger pathways. While classed as ligand-gated ion channels, CNG channels have many properties in common with voltage-gated ion channels. Initially characterised in both retinal cones and rods, as well as in olfactory epithelia they are found across a wide variety of species including bovine (Kaupp et al., 1989), human (Dhallan et al., 1992) and chick (Bonigk et al., 1993). Importantly for the current research, CNG channels have been widely reported in the central nervous system including memory related regions such as the hippocampus (Kingston, Zufall & Barnstable, 1996). Further research suggests a similar distribution in the cerebellum (Zufall, Shepherd & Banstable, 1997) another brain regions associated with learning. In addition to activation by cGMP, olfactory CNG channels have been shown to be directly activated by NO (Broillet, 2000; Broillet & Firestein, 1996, 1997, 1999).

The third class of cGMP activated proteins are PDEs, which are not only regulated by cyclic nucleotide second messengers but regulate intracellular levels of cyclic nucleotide second messengers inturn. There are, at least, seven families of PDEs presently identified. In brief, type I is a Ca^{2+}/calmodulin activated isoform which has a lower affinity for cAMP than cGMP. Type II hydrolyses both cAMP and cGMP and may be activated by cGMP. Types III and IV are cAMP specific but, importantly, type III activity is inhibited by the presence of cGMP while type IV is insensitive to both cGMP and Ca^{2+} / calmodulin. Types V and VI are cGMP-specific and both contain cGMP non-catalytic binding sites, suggesting a homeostatic mechanism to control intracellular cGMP concentration. Finally there has been another class of PDEs recently characterised and given the name type VII (Carvajal, Germain, Huidobro-Toro & Weiner, 2000).

In summary, NO is most often thought to act through activation of GC. In turn GC is known to activate three classes of proteins, PKG, CNG channels and PDEs. Therefore these three classes represent some of the effector mechanisms for NO.

1.3.1.3 *Nitric oxide activates* mono*ADP-ribosylation*

While NO has traditionally been considered to act primarily via the GC- and PKG-dependent pathway, there are numerous other enzymic pathways by which NO may affect physiological systems. One such pathway which has shown promise as being memory-related is that utilising *mono*(ADP-ribosyl) transferase (refer Figure 1.2). ADP-ribosylation is a common biochemical mechanism requiring the transfer of an ADP-ribose group from NAD^+ to a protein resulting in an alteration to its activity. ADP-ribosylation has been observed in DNA repair, cell differentiation and malignant transformation, as well as being an important post-translational modification for many proteins. However, the relationship between ADP-ribosylation and brain function is still tenuous.

Early work on the ADP-ribosyl transferase pathway was carried out by Brüne and Lapetina (1989) who administered the spontaneous NO donor SNP to a preparation of platelet cells. They observed ADP-ribosylation of a 39kDa protein. To determine whether ADP-ribosylation was a down-stream result of GC activation, cGMP analogues were administered to the platelet preparation in place of SNP and were found to have no

effect. Brüne and Lapetina (1989) also isolated the cytosolic fractions of a number of tissues, including brain, and again demonstrated ADP-ribosylation of the 39kDa protein.

Following the work of Brüne and Lapetina (1989), Williams, Li, Gu and Jope (1992) prepared homogenates from rat cerebellum, striatum, thalamus, cerebral cortex and hippocampus. ADP-ribosylation of three proteins, including a 39kDa protein, was observed in each sample after administration of SNP. Williams et al. (1992) then investigated the role of ADP-ribosylation by using different cell fractions and discovered that for a 47kDa and a 49kDa protein, a translocation to the cytosol occurred after ADP-ribosylation. Without knowing the identity of these proteins, the physiological relevance of the translocation cannot be ascertained; but it is important in that it suggests another NO-dependent mechanism which may impinge on neuronal function, leading to possible effects upon behaviour.

Later studies by Huang and Lee (1995) and Sullivan, Wong and Schuman (1997) identified several proteins within hippocampal synaptosomes which were subject to NO-dependent ADP-ribosylation. While the identities of these proteins also remain unknown, their molecular masses were determined to be 42, 48, 51, 54, 74kDa respectively. The 54 and 74kDa proteins were found to be brain specific, while the 42 and 51kDa proteins were more ubiquitous in their localisations.

1.3.1.4 Nitric oxide directly activates ion channels

Although NO is best characterised as having effects upon enzymes, another broad class of proteins which NO has been demonstrated to directly interact with are ion channels. NO has been shown to directly activate at least three types of ion channels; CNG channels, also activated by cGMP and cAMP, large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels and the RyR channel.

Both Ahmad et al. (1994) and Broillet and Firestein (1996) used electrophysiological techniques to observe the action of spontaneous NO donors on rod and olfactory-type CNG channels. Importantly, Broillet and Firestein (1996) found not only that the spontaneous NO donors resulted in the direct activation of CNG channels, but also that single CNG channels produced identical amplitudes, conductance levels and mean open/close times for both NO and the NO-independent activator cAMP. Further, NO appeared to be a more potent activator as the open probability of a CNG

channel increased 2-fold when NO donors were applied to patches compared to that resulting from saturated cAMP levels. However, Lynch (1998) has since disputed these findings, having found that NO appeared to be inhibitory to the activation of cAMP-dependent CNG channels in the rat olfactory receptor neuron. Even so, Broillet (2000) has since identified a cysteine residue necessary for the direct activation of olfactory-type CNG channels by NO.

The second class of ion channels thought to be directly activated by NO is the BK_{Ca} channel which is one class of Ca^{2+}-activated K^+ (K_{Ca}) channels. Although evidence exists to suggest NO opens K_{Ca} and BK_{Ca} channels through the up-regulation of GC (Ferrer, Marin, Encabo, Alonso & Balfagon, 1999; Jiang, Thoren, Caligiuri, Hansson & Pernow, 1999; Plane, Hurrell, Jeremy & Garland, 1996; Taguchi et al., 1996) electrophysiological studies have also established the role of NO as a direct activator of BK_{Ca} channels (R.J. Lang, Harvey, McPhee & Klemm, 2000; Mistry & Garland, 1998). For example, NO was first implicated in the direct activation of BK_{Ca} channels by Bolotina, Najibi, Palacino, Pagano and Cohen (1994). They found that endogenous or exogenous NO resulted in vasodilation through the action of BK_{Ca} channels, as determined by the use of the specific channel antagonist charybdotoxin (ChTX). Notably, the GC inhibitor methylene blue (MB) was ineffective in blocking NO-activated vasodilation. However, MB is suggested to be membrane impermeable casting doubt on these findings (Cohen et al., 2000; Kontos & Wei, 1993). Even so, cell-free membrane patches have also revealed that NO could directly activate single Ca^{2+}-activated K^+ channels. The investigation of Bolotina et al. (1994) were later supported by the investigations of Fukami et al. (1998) and Chen, Wu and Yen (1999) who found both MB and the cell permeable and GC specific antagonist, ODQ, only partially inhibited NO- and K_{Ca}-dependent vasodilation suggesting a GC-independent mechanism of action upon K_{Ca} channels generally.

At a broad level, there is evidence that NO modulates K_{Ca} channel activity in neurons. For example, Cetiner and Bennett (1993) found that NO modulates K_{Ca} channels in post-ganglionic neurons of avian cultured embryonic ciliary ganglia. While the effects of GC were not tested for the authors suggest that the action is independent of the effect of cGMP. Schmachtenberg and Bacigalupo (1999) used olfactory receptor neurons from *Caudiverbera caudiverbera* and *Xenopus laevis* and found that NO

activated BK_{Ca} channels directly. In a later study, Schmachtenberg and Bacigalupo (2000) used whole-cell patch-clamp techniques in conjunction with dissociated toad olfactory receptor neurons and again determined that NO can directly activate BK_{Ca} channels. Finally, Ahern, Hsu and Jackson (1999) demonstrated that NO directly activates BK_{Ca} channels in posterior pituitary nerve terminals and activation of BK_{Ca} channels, by NO, occurred in the presence of GC inhibitors. Interestingly, a NO-derived radical, peroxynitrite, may also activate BK_{Ca} channels. Tang et al. (2001) demonstrated increased activity for BK_{Ca} channels following oxidisation of methionine residues. As the NO-related molecule, peroxynitrite, is a known oxidant, along with NO, it may be speculated that either activates BK_{Ca} channels independently of GC and PKG.

The third type of ion channel known to be directly activated by NO is the RyR channel. Although once thought to be inhibited by NO (Meszaros, Minarovic & Zahradnikova, 1996), more recent studies by Stoyanovsky, Murphy, Anno, Kim and Salama (1997) demonstrate NO donors oxidise the RyR channel and increase the open probability of RyR channels in striatal and cardiac muscle, resulting in Ca^{2+} release into the cytosol. This work was confirmed by Aghdasi, Reid and Hamilton (1997) and Xu, Eu, Meissner and Stamler (1998). Aghdasi et al. (1997) suggested that the open probability of RyR channels was proportional to the level of NO production, with low levels of NO actually inhibiting channel opening thus reconciling the studies of Meszaros et al. (1996) and Stoyanovsky et al. (1997). More recently Hart and Dulhunty (2000) have confirmed that NO can both activate and inhibit RyR channel function by direct action. However, in opposition to the evidence above they suggest that at higher concentrations NO inhibits RyR channel opening. As to the mechanism of activation, both Hart and Dulhunty (2000) and Anzai, Ogawa, Ozawa and Yamamoto (2000) suggested that NO or nitrothiol can oxidise or nitrosylate exposed thiol groups on the RyR receptor and, through the resultant conformational change increase the channel's open probability. A more recent study by Sun et al. (2001) has suggested NO nitrosylates a specific cysteine residue (C3635) of the RyR1 isoform leading to channel opening. Further, such a mechanism also appears plausible for the actior of the NO-derived radical peroxynitrite acting directly upon such channels. Therefore, through both pharmacological and electrophysiological techniques, NO has been observed to directly activate CNG, BK_{Ca} and RyR channels.

1.3.1.5 Nitric oxide forms other reactive nitrogen species

NO may also affect physiological processes by the formation of reactive nitrogen oxide species such as peroxynitrite. In turn, the actions of such species on proteins can be various, including the nitrosylation of thiol and amine groups (Wink, Grisham, Mitchell & Ford, 1996) most notably acting on tryptophan and tyrosine residues. Reactive nitrogen oxide species can also complex with transition metals if present within the active site of enzymes (Wink et al., 1996). In this way reactive nitrogen species may act in a similar capacity to NO itself.

One important reactive nitrogen species is peroxynitrite which is formed from the reaction of NO with superoxide (Carreras, Pargament, Catz, Poderoso & Boveris, 1994) (refer Figure 1.2). Peroxynitrite reacts with proteins via sulfhydryl groups to form disulfides (Radi, Beckman, Bush & Freeman, 1991a) and by adding nitrate groups to tyrosine and tryptophan residues, resulting in alteration of protein function (Aspée & Lissi, 2000; Di Stasi, Mallozzi, Macchia, Petrucci & Minetti, 1999; Halliwell, 1997; van der Vilet, Eiserich, Halliwell & Cross, 1995a; van der Vilet, Eiserich, O'Neill, Halliwell & Cross, 1995b). In fact, Beckman et al. (1994) and Beckman and Koppenol (1996) suggested that most tyrosine nitration of proteins was the result of peroxynitrite *in vivo*. Peroxynitrite will also oxidise many biological substrates (van der Vilet et al., 1995a, b), including both protein and non-protein thiols (Radi et al., 1991a), protein sulfides (Moreno & Pryor, 1992; Pryor, Jin & Squadrito, 1994), and can even react with lipids, resulting in lipid peroxidation (Darley-Usmar, Hogg, O'Leary, Wilson & Moncada, 1992; Radi Beckman, Bush & Freeman, 1991b; Rubbo et al., 1994). Further, peroxynitrite has also been observed to react with other classes of biomolecules including low-density lipoprotein (Graham et al., 1993) and deoxyribose (Beckman, Beckman, Chen, Marshall & Freeman, 1990). In such ways peroxynitrite may act in place of NO to alter biological functions.

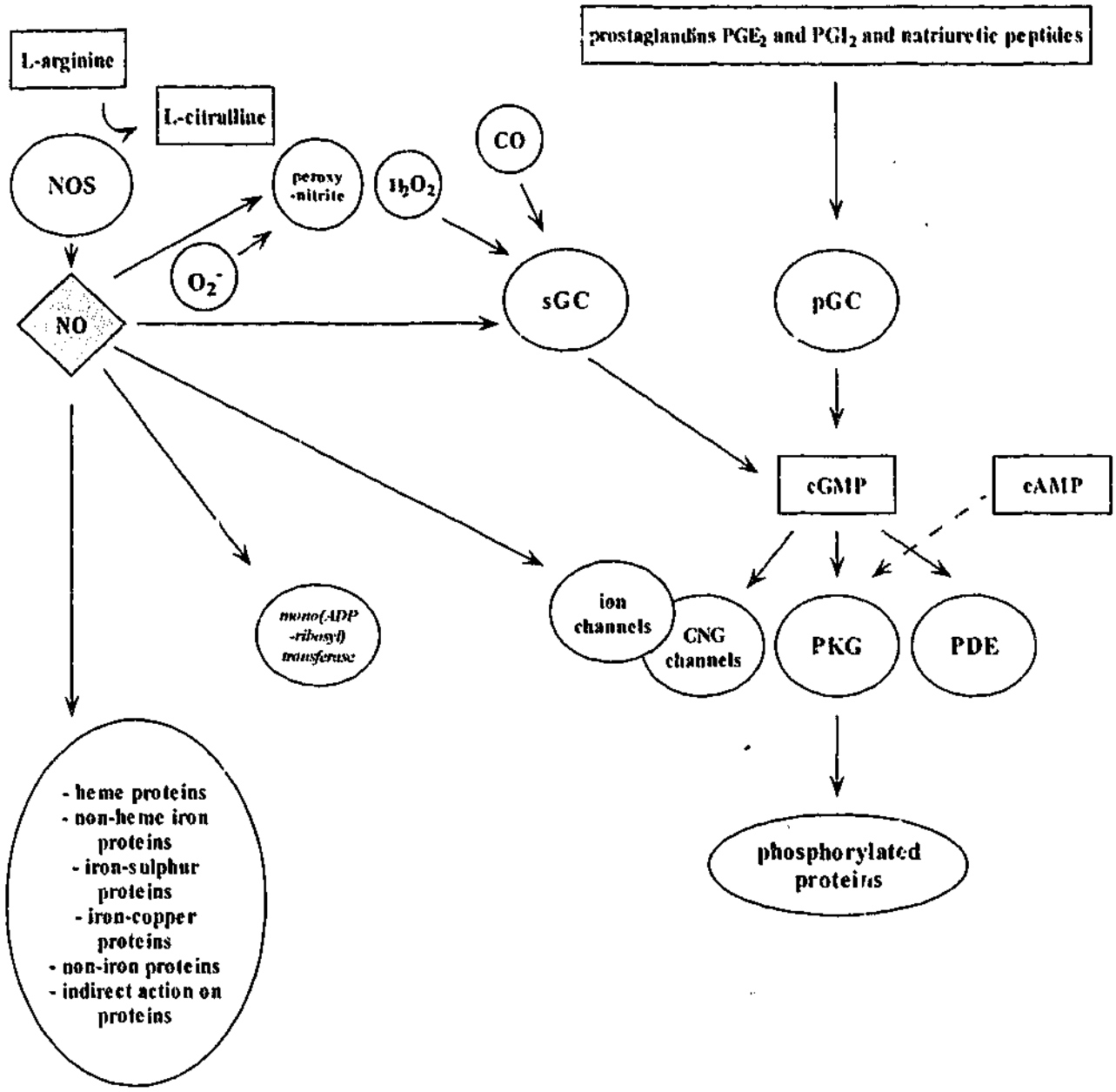


Figure 1.2 A summary of the complex biochemical interactions of NO.

NO is marked in red, enzymes in yellow, radicals in grey, substrates and activators of NOS and GC in pink, cyclic nucleotides in green, ion channels in aqua and proteins are marked in mauve. The dashed line between cAMP and PKG denotes a slight action by this second messenger on PKG while the overlap of CNG channels and ion channels denotes that CNG channels are a subset of ion channels. For clarity, interlacing of pathways has not been shown.

1.3.2 Nitric oxide-stimulated mechanisms implicated in memory formation

The breadth of NO's actions makes any discussion of potential downstream mechanisms in memory formation difficult. However, having categorised NO's broad ability to interact with enzymes, ion channels and radicals (see Figures 1.2 & 1.3) to affect cellular function it is possible to discuss behavioural evidence implicating a limited number of these interactions in memory formation. Specifically, the role of GC and some cation channels has been studied using pharmaco-behavioural techniques providing direct evidence for their role in memory formation. In contrast, ADP-ribosylation has only been studied with respect to its role in altering synaptic efficacy.

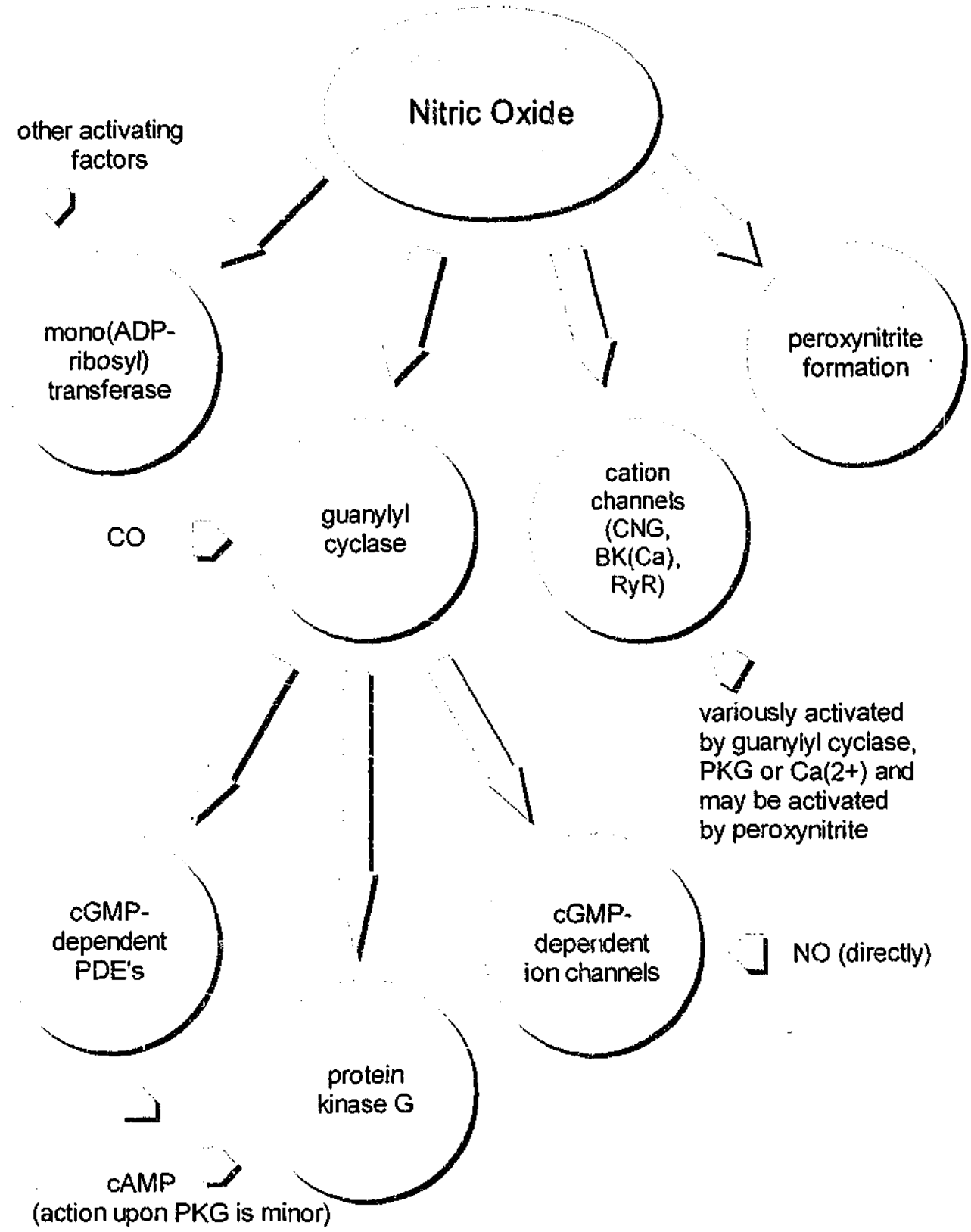


Figure 1.3 The major pathways through which NO may affect memory processes. Note that each effector mechanism has other activators (represented by blue arrows), that CNG channels are activated by cGMP and by NO directly and that peroxynitrite may also act in place of NO under some circumstances but has not been represented as doing so.

1.3.2.2 Guanylyl cyclase-dependent pathways implicated in memory formation

Consistent with the well characterised role of NO activating GC, behavioural studies have been used to implicate GC in memory formation. For example, recent studies by Kemenes et al. (2002) have implicated NO and GC in long-term memory using an appetitive single-trial associative conditioning task for the snail *Lymnaea stagnalis*. They noted the period of NO sensitivity appears to extend up to 5 hours post-training, testing 24 hours post-training. In addition it was determined that the specific inhibitor of GC, ODQ, was effective in blocking retention 24 hours post-training if administered 10 minutes post-training. However, no other times of administration were tested.

Even so, most studies demonstrating a role for NO and GC in memory formation have used mammals trained on a limited number of tasks. For example, Bernabeu et al. (1997) and Kendrick et al. (1997) have directly administered inhibitors of GC and observed memory loss in rodents subjected to an aversive task and in ewes subjected to an olfactory task respectively. Like Bernabeu et al. (1997), Izquierdo et al. (2000) utilised a one-trial step-down inhibitory avoidance task for rats finding cGMP necessary for both short-term and long-term memory in the CA1 region of the hippocampus. In addition to studies simply using an antagonist of GC activity, Yamada et al., (1996) challenged the action of the NOS inhibitor, L-NAME, with the cGMP analogue, 8-Bromoguanisine-3',5'-cyclic monophosphate (8-Br-cGMP), and found retention was restored. In this way suggesting that NO may activate GC to elicit memory formation.

Further, the activation of PKG has also been studied in a behavioural context. Bernabeu et al. (1997) and Izquierdo et al. (2000) have suggested, using a combination of biochemical analysis and behavioural testing, that PKG is necessary for memory formation in rats subjected to a single-trial step-down inhibitory avoidance task and that the action of PKG is consistent with the action of both NO and GC (Bernabeu, Schmitz, Faillace, Izquierdo & Medina, 1996; Bernabeu et al., 1997). However, Izquierdo et al. (2000) suggested a role for PKG activation in the CA1 region of the hippocampus only

during long-term memory stage and not in the entorhinal cortex. In agreement with this, Serrano et al. (1994) showed that PKG inhibition resulted in memory loss after 60 minutes for chicks trained on a single-trial passive avoidance task. This time of retention loss coincides with the onset of the protein synthesis-dependent long-term memory (LTM) stage according to the Gibbs and Ng model (Gibbs & Ng, 1979a).

A second class of cGMP activated proteins is the CNG cation channel. Although localised in regions such as the hippocampus (Kingston et al., 1996a), there is little direct evidence suggesting a role for these channels in memory formation. One behavioural study has compared the action of Ca^{2+} channel antagonists and have found verapamil, a drug which preferentially inhibits CNG channels, affected retention in rats for the elevated plus maze (Jankowska, Pucilowski & Kostowski, 1991). In a separate study, verapamil, unlike other Ca^{2+} channel antagonists which facilitated retention, was without effect when mice were tested using a step-through inhibitory avoidance task. However, in accordance with the action of the other Ca^{2+} channel antagonists tested, verapamil did facilitate retention for a linear maze task using a time of drug administration immediately after the final trial. However, verapamil was without effect if given 1 hour before training suggesting that it does not affect task acquisition (Quartermain, Garcia deSoria & Kwan, 2001). Finally, Lee and Lin (1991) found verapamil, amongst other drugs, was effective in blocking retention for rats trained on a one-way inhibitory avoidance task when tested 24 hours post-training. Although inferential, *Drosophila eag* mutants possesses a leg-shaking phenotype and display learning difficulties (Finn, Grunwald & Yau, 1996; Schmidt et al, 1993; Griffith, Wang, Zhong, Wu & Greenspan, 1994). Warmke and Ganetzky (1994) have determined a family of *eag* genes in a range of species including *Drosophila*, mouse and human. Although the gene products appear responsible primarily for altering K^+ flux there is considerable homology between *eag* and genes coding for CNG channels (Guy, Durell, Warmke, Drysdale & Ganetzky, 1991).

The third class of GC-activated proteins are the cGMP-dependent PDEs. Prickaerts et al. (1997) linked the inhibition of cGMP-dependent PDEs to an alteration in the behaviour of mammals. Specifically, rats were subjected to an object recognition test and given zaprinast (3 and 10 mg/kg ip), a phosphodiesterase class V inhibitor, immediately after exposure to two identical objects. After a one hour delay it was observed that rats within the drug group spent more time exploring a new object than

did the control group. Further, the highest dose of zaprinast (10mg/kg) reversed a memory deficit induced by the NOS inhibitor 7-NI using the same task. While a slight rise in arterial blood pressure was noted it was considered to be too small to affect behaviour. However, the facilitation of memory noted by Prickaerts et al. (1997) can be interpreted along with the studies of Baratti and Boccia (1999) as due to a rise in intracellular cGMP concentrations and not necessarily due to a direct role for PDEs in memory formation. Better evidence for the role of cGMP-dependent PDEs in memory formation comes from studies using *Drosophila* mutants. For example, the 'dunce' mutant is behaviourally characterised by memory and learning difficulties and codes for a mutant cAMP-specific cGMP-modulated phosphodiesterase (Schmidt, et al 1993).

Taken together, there exists a number of pharmaco-behavioural studies using mammal and avian species suggesting a role for PKG in memory formation. While evidence for the roles of CNG channels and PDEs in memory formation is not as convincing, there still remains evidence linking these cGMP-dependent proteins to memory processing.

1.3.2.3 Cation channels implicated in memory formation

There also exists a number of studies implicating cation channels in memory formation. Specifically, there exists strong evidence for the role of CNG, BK_{Ca} and RyR channels as directly activated by NO.

Evidence for the role of CNG channels in memory formation was discussed above. However, depending upon the class of CNG channel, they may be preferentially activated by either cGMP or cAMP while NO appears to modulate the activity of both classes as discussed in section 1.3.1.4. The evidence for CNG channels acting in memory formation is limited. At a general level CNG channels have been identified in the hippocampus (Bradley et al., 1997; Leinders-Zufall, Shepherd & Zufall, 1995; Kingston, Zufall & Barnstable, 1996 & 1999), but evidence relating CNG channels to memory processes *per se* relies on the pharmaco-behavioural studies discussed above (Janowska et al., 1991; Lee & Lin, 1991; Quartermain et al., 2001) and the inferential evidence linking the *Drosophila eag* mutant to CNG channel action in memory formation (Guy et al., 1991).

Schreurs, Gusev, Tomsic, Alkon and Shi (1998) conditioned rabbits and observed a strong relationship between the level of conditioning and membrane excitability in Purkinje cells which was present even after 1 month. Increases in membrane excitability related to conditioning could be mimicked in control rabbits by application of a variety of K^+ channel blockers including tetraethylammonium (TEA) and Iberiotoxin (IbTX). The latter of which is a specific BK_{Ca} antagonist. However this may not have affected memory processes *per se*. Of greater relevance was the study by Ghelardini, Galeotti and Bartolini (1998) who used a passive avoidance task for the mouse. Administration of a number of K^+ channel blockers 20 minutes before training, including ChTX which has a specificity for BK_{Ca}, prevented ATP-activated K^+ channel (K_{ATP}) agonist-induced amnesia. From both these investigations using specific inhibitors of BK_{Ca} channels, IbTX and ChTX, and inhibitors of K_{Ca} generally, it can be determined that BK_{Ca} channels most likely have a role in memory formation although it remains ill defined at present.

Unlike CNG and BK_{Ca} channels, the role of RyR channels in memory formation has been studied extensively in both clinical conditions associated with memory loss (Kelliher et al., 1990) and in various memory paradigms.

At a general level, molecular techniques have been employed by Zhao et al. (2000) who noted an increase in RyR2 mRNA and protein in the hippocampus of rats trained intensively using a water maze task. However, clearer evidence for a role for RyR channels in memory formation come from studies employing either a pharmacological antagonist or studies using gene knock-out techniques.

For example, using an invertebrate paradigm of classical conditioning, Blackwell and Alkon (1999) noted that dantrolene, a specific RyR channel blocker, disrupted training where a light stimulus is paired with a turbulence stimulus for the mollusc *Hermissenda crassicornis*. However, most studies have used mammals. Balschun et al. (1999) used RyR3 mutant mice. Initially they observed alterations in hippocampal synaptic plasticity noting no aberrant morphology, basal synaptic transmission or presynaptic function. Importantly, Balschun et al. (1999) then tested the RyR3 deficient mice in the Morris water maze only observing impairments in memory when a new platform location had to be learnt. Other 'knock-out' studies include those of Kouzu,

Moriya, Takeshima, Yoshioka and Shibata (2000) who, like Balschun et al. (1999), used mutant mice lacking a functional RyR3 channel and showed impairments of performance in contextual fear conditioning, passive avoidance and Y-maze learning. RyR3 channel deficient mice also showed reduced fear response in the elevated plus-maze test.

Using pharmacological agents in place of gene knock-out techniques, Ohnuki and Nomura (1996) blocked the action of the RyR channel in mice using dantrolene. When retention was tested using a step-through inhibitory avoidance task 6nmol dantrolene shortened the response latency. While 6nmol dantrolene did not appear to affect acquisition 10nmol appeared to have a deleterious effect on acquisition and, like 6nmol, shortened the response latency when retention was tested. When mice were trained and tested using a radial arm maze 20nmol dantrolene impaired maze-choice accuracy and increased error numbers. Similarly, Salinska, Bourne and Rose (2001) used a non-discrimination variant of the single trial passive avoidance task for neonate chicks and found that dantrolene impaired retention when administered at either 30 minutes before training or 30 minutes after training with significant retention loss at 3 hours post-training, but not at the earlier time of test 30 minutes post-training. Taken together there exists substantial evidence for the role of RyR channels in memory formation in a range of species and tasks including a variant of the passive avoidance task used in this thesis.

Another NO-dependent pathway which could be implicated in learning and memory results in the formation of peroxynitrite. While there is comparatively little evidence to support a role for peroxynitrite in memory formation Levin et al. (1998) genetically disrupted superoxide production and therefore peroxynitrite production. When the mutant mice were placed in the win-shift 8-arm radial maze to test spatial memory a severe impairment of spatial memory resulted. Interestingly, mice engineered to over express superoxide production also displayed noticeable impairments in performing the task thus suggesting specific concentrations of NO must be maintained for proper function.

1.3.3 Nitric oxide is implicated in cellular processes thought to underlie memory formation

Although NO is well characterised as necessary in memory formation the possible biochemical mechanisms through which it may facilitate memory are not. This is due in part to the wide range of biochemical interactions NO may undertake with enzymes, ion channels and other radicals. However, while many of NO's biochemical actions have not been adequately studied in a behavioural context, a number have been studied in detail within the context of being necessary for cellular processes implicated in memory formation. There are a number of cellular processes thought to underlie memory formation of which LTP, LTD, neurotransmitter release and cerebral vasodilation are perhaps the most recognised. Importantly each of these cellular mechanisms has been shown to be NO-dependent. By identifying NO-dependent mechanisms implicated in such cellular processes it may be possible to limit the number of NO-dependent mechanisms needed be studied behaviourally to ascertain those through which NO affects memory formation.

1.3.3.1 Nitric oxide-dependent long-term potentiation of synapses as a cellular process underlying memory formation

Nearly twenty five years after Hebb (1949) put forward his postulate relating neuronal processes to information storage, Bliss and Lomo (1973) discovered a neuronal process which possessed many attributes described by Hebb (1949). Known as LTP, this synaptic process results in a long-lasting enhancement of synaptic transmission brought about by repeated stimulation of afferent fibres and is observed electrophysiologically as a sustained increase in excitory post-synaptic potentials (EPSPs).

Evidence for LTP as a key cellular mechanism responsible for memory formation comes from a number of studies including those using anatomical (Green & Greenough, 1986; Greenough & Volkmar, 1973; Greenough, Volkmar & Juraska, 1973; Horn, Bradley & McCabe, 1985), pharmacological (Davis, Butcher & Morris, 1992;

Izquierdo, 1994; Morris, 1989; Kemenes et al., 2002; Morris, Anderson, Lynch & Baudry, 1986), genetic (Abeliovich et al., 1993; Aiba et al., 1994; Grant et al., 1992; Huerta, Sun, Wilson & Tonegawam, 2000; Kishimoto et al., 1997; Rondi-Reig, Libbey, Eichenbaum & Tonegawa, 2001; Silva, Stevens, Tonegawa & Wang, 1992; Silva et al., 1992; Tonegawa et al., 1996; Tsien, Huerta & Tonegawa, 1996) and behavioural approaches (Mitsuno, Sasa, Ishihara, Ishikawa, & Kikuchi, 1994) or a combination thereof.

The classical model of LTP induction (Collingridge & Bliss, 1987; Collingridge, Kehl & McLennan, 1983) begins with the depolarisation of a glutamatergic-positive post-synaptic neuron by the release of glutamate from the pre-synaptic bouton. This depolarisation is responsible for removing the voltage-dependent Mg^{2+} block upon the NMDA receptor-associated ion channels in the post-synaptic bouton (see Figure 1.3) (Mayer & Westbrook, 1987; Mayer, Westbrook & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984). Once the Mg^{2+} block has been overcome, there occurs a flux of Ca^{2+} ions into the post-synaptic bouton (Lynch, Larson, Kelso, Barrionuevo & Schottler, 1983; Malenka, Kauer, Zucker & Nicoll, 1988) resulting in the activation of Ca^{2+}-dependent enzymes including PKC (Hu et al., 1987; Linden, Sheu, Murakami & Rottenberg, 1987; Malinow, Madison & Tsien, 1988; Malinow, Schulman & Tsien, 1989), phospholipase A2 (Linden et al., 1987) and CaMK-II (Malinow et al., 1989; Silva et al., 1992a, b).

However, LTP maintenance ultimately results in increased amounts of transmitter to be released presynaptically from potentiated synapses (Bekkers & Stevens, 1990; Bliss, Douglas, Errington & Lynch, 1986; Feasey, Lynch & Bliss, 1986; Malgeroli & Tsien, 1992; Malinow et al., 1989). Therefore LTP is induced post-synaptically and maintained pre-synaptically (see Figure 1.4).

If induction is post-synaptic and maintenance pre-synaptic, then a retrograde transmitter must exist which links the induction and maintenance of LTP. It is in this capacity that NO is thought to act within LTP (Bohme, Bon, Stutzmann, Doble & Blanchard, 1991; Haley, Wilcox & Chapman, 1992; Nowicky & Bindman, 1993a, b; O'Dell et al., 1991; Schumann & Madison, 1991; Zhu & Luo, 1992) (see Figure 1.4). It must be noted, however, that other molecules such as arachidonic acid (O'Dell et al.,

1991), platelet activating factor (Kato, Clark, Bazan & Zorumski, 1994; Wieraszko, Li, Kornecki, Hogan & Ehrlich, 1993) and carbon monoxide (Zhuo et al., 1993) have also been proposed to act as retrograde messengers. Although there is evidence supporting arachidonic acid, platelet activating factor and carbon monoxide as retrograde messengers this particular role for each has been questioned (Alkadhi, Al-Hijailin, Malik & Hogan, 2001; Doucet & Bazan, 1992; Izumi, Clifford & Zorumski, 1992; Williams, Errington, Lynch & Bliss, 1989, Wieraszko et al., 1993). Even although NO has been suggested as a retrograde messenger in a number of studies there still remains some controversy as to the exact role of NO in LTP. For example, gene knock-out studies which have disrupted a single NOS isoform have shown no significant disruption of LTP (Doreulee et al., 2001; Haul, Godecke, Schrader, Haas & Luhmann, 1999; O'Dell et al., 1994). However, due to the overlapping localisations of nNOS and eNOS in regions such as the hippocampus these results may reflect the action of redundant mechanisms rather than the role of NO in LTP *per se*. Williams et al. (1993) and Murphy, Williams, Bettache and Bliss (1994) both questioned any role of NO in LTP at physiological temperatures. In addition to these studies are those of Bon and Garthwaite (2001), Chetkovich, Klann and Sweatt (1993), Gribkoff and Lum-Ragan (1992), Haley, Malen and Chapman (1993), Lum-Ragan and Gribkoff (1993) and Zhuo, Kandel, & Hawkins (1994) which suggested a more subtle modulatory role for NO rather than that of a simple retrograde messenger linking post-synaptic induction and presynaptic maintenance.

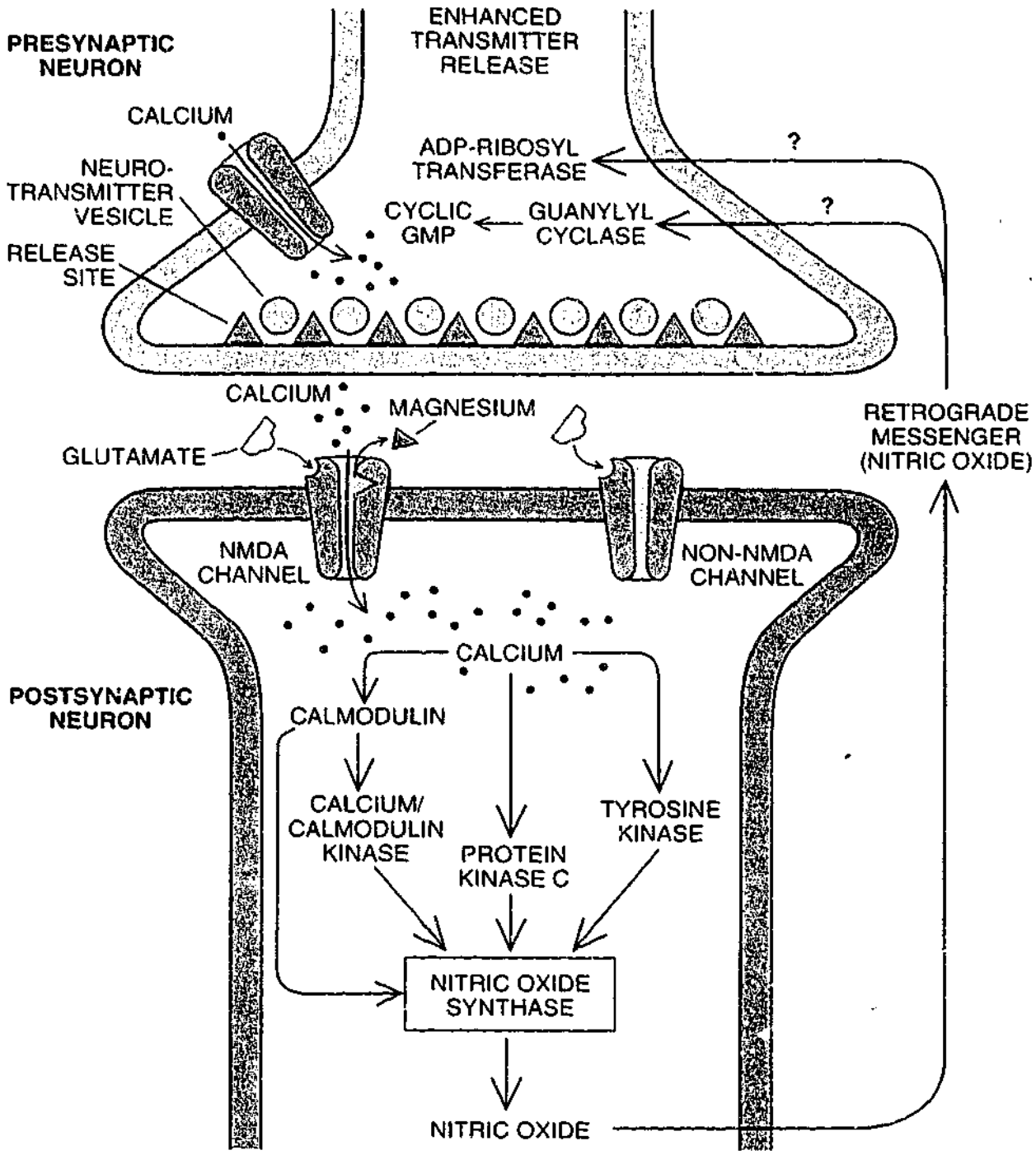


Figure 1.4 The role of NO as a retrograde messenger linking the post-synaptic induction of LTP to pre-synaptic maintenance (Kandel & Hawkins, 1992).

1.3.3.2 Nitric oxide-dependent long-term depression of synapses as a cellular process underlying memory formation

In addition to LTP, another mechanism by which synaptic efficacy is altered is LTD. Therefore LTD may also have ramifications upon information storage (Hebb, 1949). However, in this mechanism synaptic efficacy is reduced. Although not studied

to the extent of LTP, this process nevertheless appears capable of affecting memory formation at a cellular level (Manahan-Vaughan & Braunewell, 1999). LTD has also been localised to brain regions found to be active in learning and memory, including the hippocampus and cerebellum (Bear & Abraham, 1996) and importantly for this thesis, in avian hippocampal slices (Margrie, Rostas & Sah, 2000).

While LTP is considered to be NMDA receptor-dependent, several varieties of LTD have been identified in the hippocampus based upon receptor utilisation and the stimulus employed (Bashir, Jane, Sunter, Watkins & Collingridge, 1993; Kemp & Bashir, 1997; Kemp, McQueen, Faulkes & Bashir, 2000). These different forms of LTD may also be co-expressed in the hippocampus (Kemp et al., 2000). Of these, the most obvious is the NMDA receptor-dependent variety which has been observed in the CA1 region of juvenile and adult rats (Kemp et al., 2000; Kemp & Bashir, 1997; Nicoll, Oliet & Malenka, 1998; Oliet, Malenka & Nicoll, 1997). Other forms of LTD suggested include metabotropic glutamate receptor-dependent LTD (Nicoll et al., 1998; Oliet et al., 1997) and NMDA-independent metabotropic glutamate receptor-independent LTD (Domenici, Berretta & Cherubini, 1998) as well as GABA receptor-dependent LTD (Caillard, Ben-Ari & Gaiarsa, 1999).

Regarding the role of NO in hippocampal LTD, there is only limited evidence suggesting both NO-dependent and NO-independent forms of hippocampal LTD (Gage, Reyes & Stanton, 1997). Most clearly in support of NO-dependent LTD are the studies of Reyes-Harde, Potter, Galione and Stanton (1999), who used the NO donor SNAP to induce NMDA-dependent LTD at Schaffer-collateral-CA1 synapses *in vitro*.

Unlike NMDA-dependent hippocampal LTD, a model which has been extensively characterised and is considered NO-dependent is cerebellar LTD. Although not hippocampal, this model remains important with regard to learning and memory as the cerebellum has been implicated in motor learning for human subjects (Ghilardi et al., 2000), prism adaptation tasks in macaque monkeys (Baizer, Kralj-Hans & Glickstein, 1999), non-motor associative learning in humans (Drepper, Timmann, Kolb & Diener, 1999), and retention of spatial information in mice (Hilber, Jouen, Delhaye-Bouchaud, Mariani & Caston, 1998).

Briefly, cerebellar LTD can be brought about when a Purkinje cell is synapsed to both parallel and climbing fibres (Ito, 1989). The mechanism by which LTD results begins with the influx of Ca^{2+} ions into the parallel fibres, activating NO production

(Schuman & Madison, 1994). Within Purkinje cells, PKC phosphorylates α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) receptors to down-regulate their function. However, this depression would not be maintained if not for the diffusion of NO, as phosphatases restore the receptor's original conformation. It is thought that NO acts to down-regulate phosphatase activity through the action of PKG (Crepel, Hemart, Jaillard & Daniel, 1996; Crepel & Krupa, 1988; Linden & Connor, 1991). Therefore, in this synaptic process NO acts as an anterograde messenger in contrast to its role in LTP as a retrograde messenger (Daniel, Hemart, Jaillard & Crepel, 1993, Izumi & Zorumski, 1993; Lev-Ram, Nebyelul, Schumann, Huang & Tsien, 1997; Renyolds & Hartell, 2001; Shibuki & Okada, 1991).

1.3.3.3 Nitric oxide-dependent neurotransmitter release as a cellular process underlying memory formation

Neurotransmitters underlie all behaviour and have been extensively studied with regard to their role in memory formation. While Vizi and Kiss (1998) reviewed the evidence for the action of glutamate, GABA, acetylcholine, noradrenaline and serotonin in the hippocampus, Ellis and Nathan (2001) specifically related the action of a number of neurotransmitters to human working memory. Importantly for the current thesis however are the pharmaco-behavioural studies seeking to block the action of a number of neurotransmitters in the day-old chick and assessing the effect of such treatments on passive avoidance learning. Specifically, Rickard et al. (1994, 1995) blocked both NMDA and non-NMDA glutamate receptors and established a persistent loss of retention by 90 minutes post-training. Similarly, the role of noradrenaline has been studied using the passive avoidance task for the day-old chick. From the investigations of Crowe, Ng and Gibbs (1990, 1991a, 1991b), O'Dowd, Gibbs, Ng, Hertz and Hertz, (1994a), O'Dowd, Gibbs, Sedman and Ng (1994b) and O'Dowd, Barrington, Ng, Hertz and Hertz (1995) noradrenaline appears responsible for initiating glycogenolytic processes leading to the formation of the protein synthesis-dependent LTM stage of memory formation as defined by the Gibbs and Ng model (1979a).

Numerous studies have determined that NO is intimately involved in synaptic transmission by regulating transmitter production, secretion, uptake or receptor activity.

On a broad scale, NO modulates the function of tyrosine hydroxylase, which is the rate limiting enzyme for the production of all catecholamines (Gonzalez, Llorente & Abreu, 1998; Rodriguez-Pascual, Ferrero, Miras-Portugal & Torres, 1999). Notably, NO modulates the production and release of five common neurotransmitters, namely, GABA (Jones, Loiacono, Moller & Beart, 1994), glutamate (Kano, Shimizu-Sasamata, Huang, Moskowitz & Lo, 1998; Montague, Gancayco, Winn, Marchase & Friedlander, 1994), acetylcholine (Lonart, Wang & Johnson, 1992; Prast & Phillipu, 1992), dopamine (Kiss, Hennings, Zsilla & Vizi, 1999; Lonart, Cassels & Johnson, 1993;. Zhu & Luo, 1992) and noradrenaline (Chu & Etgen, 1996; Jones et al., 1994; Kiss, Sershen, Lajtha & Vizi, 1996; Kaye, Wiviott, Kobzik, Kelly & Smith, 1997; Lauth, Hertting & Jackisch, 1993; Lonart et al., 1992; Lauth, Hertting & Jackisch, 1993; Lonart & Johnson, 1995a, b; Montague et al., 1994; Satoh, Murayama & Nomura, 1996; Stout & Woodward, 1994; Stout & Woodward, 1995). In addition the $GABA_A$ (Robello et al., 1996) and NMDA (Lei et al., 1992) receptors have also been shown to have their activity modulated by NO.

1.3.3.4 Nitric oxide-dependent cerebral vasodilation as a cellular process underlying memory formation

NO was first recognised as a vasodilating agent (Furchgott, & Zawadzki, 1980; Grutter, Grutter, Lyon, Kadowitz, & Ignarro, 1981; Palmer, Ferrige & Moncada, 1987). Importantly for this thesis a number of studies, from disparate areas of investigation, suggest that vasodilation is important in memory formation. Although not directly affecting memory formation, in contrast to the action of synaptic processes, cerebral vasodilation appears to facilitate increased nutrient levels to those areas of the brain which are highly metabolically active during memory processing.

A number of neuroimaging studies have shown a correlation of blood flow with memory processing in the temporal lobe, hippocampus and various other brain regions (Grasby et al., 1994; Gur et al., 1993; Kopelman Stevens, Foli & Grasby, 1998; Salmon et al., 1996; White et al., 1999). In

particular de la Torre et al. (1992) correlated spatial dysfunction in elderly rats with decreased cerebral vasculature while Grady et al. (1995) demonstrated that elderly people who tested poorly on an encoding task also possessed poor vasculature to the hippocampus and cortex.

Animals subjected to various learning tasks have also provided much information relating cerebral vasodilation to memory formation. Using a weakly reinforced variant of the single trial passive avoidance task for the day-old chick it was found that the spontaneous NO-donor, SNP (Rickard et al., 1994), and the α_1-adrenergic receptor blocker, prazosin (Rickard, 1995), resulted in retention persisting into the LTM stage as defined by Gibbs and Ng (1979a). This was in contrast to chicks which did not receive either of these drugs. Both SNP and prazosin are known vasodilators but act independently of each other. Importantly, as the effects of the spontaneous NO-donor SNP mimic those of prazosin it can therefore be speculated that vasodilation leading to persistent retention is NO-dependent in the day-old chick. Further evidence in support of NO-dependent vasodilation underlying memory formation has been reported by Rickard et al. (1999). They showed that administration of diphenylpheḍiné (DPE), an eNOS antagonist, produced a memory retention deficit 40 minutes post-training using the strongly reinforced variant of the task. Although nNOS inhibition has also shown to result in retention loss at this time (Rickard & Gibbs, in preparation - b), eNOS is well characterised in the endothelia of blood vessels, amongst other tissues, and the effect of DPE is consistent with the time of effect for both SNP and prazosin. Therefore, there exists considerable evidence for the role of NO-dependent cerebral vasodilation in memory formation in the day-old chick.

The perceived role of vasodilation in memory formation is to increase the level of nutrients to brain regions which are highly metabolically active following training. Studies which support this role include those which have sought to determine the effects of glucose administration on memory formation in both humans (Hall, Gonder-Frederick, Chewning, Silveira & Gold, 1989; Manning, Parsons & Gold, 1992) and rats (Messier, 1997). Using an object recognition test, mice which had been administered glucose at the time of learning in comparison to those mice which received saline or a delayed injection of glucose spent greater time exploring a newly presented object which was interpreted as discrimination learning (Messier, 1997). Similarly, in a different paradigm Allweis, Gibbs, Ng and Hodge (1984) showed that both hypoxia and

anoxia induced 2.5 or 5 minutes post-training resulting in a temporary memory retention loss between 20 and 50 minutes post-training using a single trial passive avoidance task for the neonate chick.

1.3.4 Nitric oxide-stimulated mechanisms are implicated in cellular processes thought to underlie memory formation

Although NO can affect the function of a vast array of enzymes and a number of cation channels, NO appears to facilitate each cellular processes discussed through only a limited number of mechanisms. These mechanisms include the activation of GC and PKG, the activation of *mono*(ADP-ribosyl) transferase and the opening of CNG channels, BK_{Ca} channels and RyR channels. Finally, NO may act through the formation of peroxynitrite.

1.3.4.1 The role of guanylyl cyclase in cellular processes thought to underlie memory formation

The first recognised, and most well studied NO-dependent mechanism, is the activation of GC leading to extensive phosphorylation through the action of PKG. This mechanism is found necessary in all the cellular processes underlying memory formation mentioned previously.

There exists much evidence for the involvement of NO, GC and PKG in LTP. For example, East and Garthwaite (1991), Chetkovich, Klann and Sweatt (1993), Zhuo, Hu, Schultz, Kandel and Hawkins (1994) and Arancio, Kandel and Hawkins (1995) demonstrated early on that hippocampal LTP is NO, GC and PKG dependent. These findings were then confirmed by Boulton, Southam and Garthwaite (1995) who were the first to use the specific GC antagonist, ODQ, to more clearly verify the action of GC in LTP. More recent studies include those by Lu, Kandel and Hawkins (1999) who studied the 'late-phase' of LTP with respect to NO, GC and PKG for first time. While evidence existed for NO in the 'early phase', inhibitors of NOS, GC and PKG also

blocked the 'late phase' of LTP. These studies are also supported by Arancio et al. (2001) who even localised PKG-I to the presynaptic bouton which is consistent with the action of NO as a retrograde messenger in LTP. However, a number of studies have also found that NO may act independently of GC and PKG in LTP (Barcellos et al., 2000; Jacoby, Sims & Hartell, 2001; Kleppisch et al., 1999; Schuman et al., 1992, 1994; Selig et al., 1996) suggesting the existence of other mechanisms.

In addition to LTP, Zhuo, Kandel and Hawkins (1994) demonstrated a role for NO and GC in LTD in the hippocampus using low frequency stimulation. This was confirmed by Gage, Reyes and Stanton (1997) who found ODQ blocks low frequency stimulated hippocampal LTD while a cGMP analogue promoted LTD. cGMP production can result in the activation of a number of protein classes. Importantly, Reyes-Harde et al. (1999) found that hippocampal homosynaptic LTD ultimately required the activation of PKG. In comparison cerebellar LTD is also GC-dependent. For example, Daniel, Hemart, Jaillard and Crepel (1992) showed exogenous cGMP to be effective in initiating cerebellar LTD and these results have since been confirmed by a number of studies including Boxall and Garthwaite (1996), Hartell (1994, 1996) and Wu, Wang, Rowan and Anwyl (1998).

GC has also been implicated in neurotransmitter synthesis and release. For example, Rodriguez-Pascual et al. (1999) used chromaffin cells of the bovine adrenal medulla to suggest NO affected the function of tyrosine hydroxylase through activation of GC and PKG and in doing so modulated the production of all catecholamines.

A number of studies also implicate NO, GC and PKG in neurotransmitter release. Jones et al., (1994) found NOS and GC inhibitors increased [^{3}H]GABA release. Using pharmacological agents, Robello et al. (1996) suggested NO may also modulate the action of GABA by reduction of $GABA_A$ receptor function. This was brought about by the action of NO, independent of GC in the forebrain, but was GC-dependent in the cerebellum. In addition, Lonart et al. (1993) found the cGMP analogue, 8-Br-cGMP, to enhance noradrenaline release. Glutamate and aspartate release *in vivo* have also been investigated and been shown to be GC and PKG dependent (Sluka & Willis, 1998). However, one of the most comprehensive *in vivo* studies is that of Trabace and Kendrick (2000) who utilised *in vivo* microdialysis to see whether NO modulated striatal neurotransmitter release in the rat through cGMP and/or peroxynitrite production. 100μM or 200μM ODQ resulted in a dose-dependent

decrease in acetylcholine, serotonin, glutamate and GABA concentrations. Finally, Meffert, Premack and Schulman (1994) used a fluorescence method to study exocytosis of vesicles from hippocampal synaptosomes. This process was found to be NO-dependent but curiously GC-independent.

NO-dependent vasodilation also appears to act predominantly through GC and PKG. The most relevant studies to this thesis are those using cerebral arteries. GC and PKG were found to be fundamental to cerebral vasodilation both *in vivo* and *in vitro* (Dostmann et al., 2000; Faraci & Sobey, 1999; Kruuse et al., 2001; Sobey & Faraci, 1997).

1.3.4.2 The role of ADP-ribosylation in cellular processes thought to underlie memory formation

Although GC and PKG may account for much of the action of NO there are a number of other pathways through which NO may act. For example, Schuman, Merffet, Schulman and Madison (1992) found H-8, an inhibitor of PKG, failed to block LTP following strong tetanic stimuli. In contrast, three different inhibitors of ADP-ribosyl transferase inhibited LTP. These results were supported by Schuman, Merffet, Schulman and Madison (1994) who found that cGMP analogues and PKG inhibitors were without effect upon CA1-localised LTP, while inhibitors of ADP-ribosylation were effective and appeared to act presynaptically. This is consistent with the role of NO as a retrograde messenger in LTP (O'Dell et al., 1991) and suggests that ADP-ribosylation may act in place of GC under some circumstances. Further, Kleppisch et al. (1999) utilised PKG knock-out mice to impair LTP in the CA1 region of the hippocampus. However, no loss in LTP was observed. Further, inhibition of LTP by administration of the specific GC inhibitor, ODQ, was without result suggesting a GC and PKG-independent form of LTP. To determine if NO was active within this model inhibitors of both NOS and the NO-related NMDA receptor were administered with a reduction in LTP noted. Finally, Kleppisch et al. (1999) administered the ADP-ribosyl transferase inhibitor nicotinamide (niacinamide) and it effectively blocked LTP. More recently, Barcellos, Bradley, Burns and Webb (2000) determined that synaptic transmission in the IMHV of the chick, an

area intimately involved in memory formation (Rose & Csillag, 1985), was both NO-dependent and ADP-ribosyl transferase-dependent.

In contrast, ADP-ribosylation has not been found to play a role in either cerebellar or hippocampal LTD, noradrenaline release or vasodilation. For example, Blond, Daniel, Otani, Jaillard and Crepel (1997) found that the post-synaptic effect of NO in hippocampal LTD was not dependent upon ADP-ribosylation. This was further supported by Gage, Reyes and Stanton (1997) who found that ODQ blocked low frequency stimulated LTD in the hippocampus, but the ADP-ribosylation inhibitor nicotinamide did not. Similarly, inhibitors of ADP-ribosylation do not impair NO-induced noradrenaline release from rat hippocampal slices (Stout & Woodward, 1995). However, the limited investigations into ADP-ribosylation are mute as to any possible role in the release of other neurotransmitters or action in vasodilation.

1.3.4.3 The role of specific cation channels in cellular processes thought to underlie memory formation

Olfactory CNG channels have been shown to be directly activated by NO (Broillet, 2000; Broillet & Firestein, 1999) and expressed in the hippocampus and cerebellum (Kingston et al. 1996; Zufall et al., 1997).

Attenuation of LTP in the CA1 hippocampal region has been shown to result following the application of LY83583, a potent inhibitor of the olfactory CNG channels. However, LY83583 also blocks the action of GC (Zhuo, Hu, Schultz, Kandel & Hawkins, 1994). More convincingly, knock-out studies in mice lacking the olfactory type-I CNG channel α-subunit have demonstrated attenuation of LTP (Parent et al., 1997). Other knock-out studies include Parent et al. (1998) who used field potential recording to measure synaptic transmission in Schaffer-collateral-CA1 synapses in both wild-type and olfactory CNG channel α subunit-deficient mice. They found CNG channels contribute to the induction of LTP in the presence of weak stimuli. Measures of basal synaptic transmission were unaltered in the α subunit-deficient mice, however, using a theta-burst stimulation protocol (15 bursts of 4 pulses at 100Hz, 200ms burst interval, 5 sets at 0.1Hz) the mutant mice demonstrated a decreased initial amplitude of LTP and potentiation decayed faster.

CNG channels may also be involved in neurotransmitter release as is suggested by Savchenko, Barnes and Kramer (1997) who observed the relationship between GC and CNG channels in optic cones.

NO also appears able to directly activate BK_{Ca} channels which have also been found in memory-related regions such as the hippocampus (Shao, Halvorsrud, Borg-Graham & Storm, 1999). K_{Ca} channels, and BK_{Ca} channels specifically, have been implicated in synaptic transmission leading to neurotransmitter release (Augustine, Charlton & Horn, 1988) in both peripheral and pituitary neurons (Ahern, Hsu & Jackson, 1999; Cetiner & Bennett, 1993; Schmachtenberg & Bacigalupo, 1999; Schmachtenberg & Bacigalupo, 2000). One knock-out study implicating BK_{Ca} channels in neurotransmitter release is that of Wang, Saifee, Nonet and Salkoff (2001) who disrupted the SLO-1 gene. This gene codes for a BK_{Ca} channel subunit in *Caenorhabditis elegans*. They then measured evoked post-synaptic currents at the neuromuscular junction and found significantly increased quantal content in mutants primarily through increased duration of neurotransmitter release.

There also exists extensive evidence for the role of BK_{Ca} channels in vasodilation. However, the role of BK_{Ca} channels remains unclear, with a number of pharmacological studies suggesting activation of BK_{Ca} channels results from the direct action of NO or the action of NO on GC (Bolotina et al., 1994; Fukami et al., 1998; George, & Shibata, 1995; Sampson, Plane & Garland, 2001). While the patch clamp studies of Archer et al. (1994), Hampl, Huang, Weir and Archer (1995) and Li, Jin and Campbell (1998) suggest PKG is responsible for the opening of BK_{Ca} channels.

Besides CNG and BK_{Ca} channels, NO can also directly activate RyR channels and these channels more than any other have been implicated in the various cellular processes thought to underlie memory formation. In addition to direct activation by NO, RyR channels along with BK_{Ca} channels, appear also to be activated by PKG as suggested by Reyes-Harde et al. (1999) in a model of homosynaptic LTD.

RyR channels appear to have a complex role in LTP and LTD as noted by Katchman and Hershkowitz, (1993), Obenaus, Mody and Baimbridge (1989), Tekkök and Krnjević (199?) and Wang and Kelly (1997) all of whom have used pharmacological agents to inhibit RyR channels. In particular, O'Mara, Rowan and Anwyl (1995) used the common and specific RyR channel antagonist, dantrolene, to investigate LTD and LTP in dentate gyrus. Low frequency stimulation-induced LTD

was blocked by application of dantrolene while high frequency stimulation of LTP was enhanced by dantrolene. In contrast, Wang, Wu, Rowan and Anwyl (1996) found another antagonist, ryanodine, inhibited high frequency stimulation induced LTP in the dentate gyrus but facilitated low frequency stimulation induced LTP. However, yet another RyR channel antagonist, ruthenium red, blocked low frequency stimulation induced LTP. In addition to O'Mara et al. (1995), RyR channels have been implicated in cerebellar LTD by Kuroda, Inoue, and Mikoshiba (1995) using primary-culture Purkinje cells.

In contrast to pharmacological studies Futasugi et al. (1999) used RyR3 isoform knockouts. In the CA1 region of the hippocampus short tetanic bursts did result in LTP. However, this form of LTP was NMDA receptor-independent, being partially dependent upon metabotropic glutamate receptors. Further, RyR3 knock-outs were unable to induce LTD. In support of these findings Shimuta et al. (2001) found RyR3 knock-outs post-synaptically modulate AMPA receptor-related potentiation. Finally, Balschun et al. (1999) found RyR3 knock-outs showed impaired hippocampal plasticity by affecting post-synaptic events. This is interesting in light of NO's presumed action as a retrograde messenger (see Figure 1.4).

Two studies have so far implicated RyR channels in neurotransmitter release. Mothet et al. (1998) implicated RyR channels in acetylcholine release from cholinergic synapse in the buccal ganglion of *Aplysia* while He, Yang, Xie and Lu (2000) suggested neurotrophin-3 activates neurotransmitter release through the action of RyR channels in the *Xenopus* neuromuscular junction.

Finally, RyR channels have also been implicated in vasodilation (Porter, Rhodes, Reeve & Cornfield, 2001). Jaggar et al. (1998) have discussed the role of RyR channels in vasodilation, associating there action with K_{Ca} channels. This relationship between channels has more recently been explored by Furstenau et al. (2000).

1.3.4.4 The role of peroxynitrite in cellular processes thought to underlie memory formation

Peroxynitrite has been implicated in both neurotransmitter release and vasodilation. One of the most comprehensive *in vivo* studies on the effect of NO in neurotransmitter release is that of Trabace and Kendrick (2000) who utilised *in vivo* microdialysis to see whether NO modulates striatal neurotransmitter release in the rat through cGMP and/or peroxynitrite production. When a peroxynitrite scavenger was coperfused with NO-donors Glu and GABA concentrations increased. Perfusion of peroxynitrite decreased dopamine, dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid levels.

With respect to the role of peroxynitrite in vasodilation Wei, Kontos and Beckman (1996) used cerebral arterioles to implicate peroxynitrite in causing a dose-dependent dilation which was also blocked by an inhibitor of K_{ATP} channels. One of the constituents of peroxynitrite, superoxide, was also effective in causing vasodilation but appeared to act on K_{Ca} channels and not K_{ATP} channels. As the GC inhibitor LY83583 was ineffective in blocking the action of peroxynitrite or superoxide it can be suggested that their action was directly upon specific cation channels.

1.3.5 Summary of the role of nitric oxide-stimulated mechanisms in cellular processes thought to underlie memory formation

Section 3 of this chapter initially outlined the vast number of biochemical relationships NO shares with enzymes, ion channels and other radicals. All of which may affect cellular function. However, the aim of this thesis is to identify those mechanisms through which NO may facilitate memory formation. Notably, previous behavioural research has provided only a limited number of possible downstream mechanisms through which NO may act to facilitate memory formation. In addition,

cellular processes which may underlie memory formation have also provided important evidence identifying other NO-dependent pathways or consolidating the importance of pathways identified in behavioural studies.

In brief, behavioural studies have clearly demonstrated the role of GC-dependent pathways in memory formation with specific emphasis on the role of PKG as a prominent effector molecule. Similarly, BK_{Ca} and RyR channels and peroxynitrite have been implicated directly in memory formation to varying extents. In addition, a number of other mechanisms have been found to affect cellular processes implicated in memory formation. For example, ADP-ribosylation is a prominent mechanism underlying LTP. Similarly, CNG channels, BK_{Ca} channels, RyR channels and peroxynitrite have been found necessary in a variety of processes from LTP and LTD to neurotransmitter release and cerebral vasodilation.

SECTION 4 : The Gibbs & Ng model of memory formation

1.4.1 Background

Rodent-based tasks are useful to memory researchers as they provide a mammalian model of memory formation amenable to genetic manipulation. However, many common rodent-based tasks, including the Morris water maze for example, lack temporal specificity with respect to both the time of sensitivity to amnestic treatments and the time of retention loss onset. This is because these tasks are often multi-trial tasks where the time of learning cannot be exactly determined. Further, many rodent-based tasks are also limited by the time taken to train and test even small sample sizes, resulting in only a couple of times of test or the use of retesting (Izquierdo et al., 2000). Such retests can be misleading as retention levels are the result of a combination of memory traces from the original learning experience and the earlier testing.

Relationships between biochemical processes underlying memory formation may be suggested using behavioural tasks by observing common times of retention loss onset. Tasks which cannot clearly define the time of retention loss onset are therefore of limited value. To this end, a paradigm needs to be sought which is far more temporally defined. One such paradigm of memory formation is the single trial passive avoidance task performed on day-old chicks (adapted from Cherkin, 1971) which has given rise to the Gibbs & Ng model of memory formation (see Figure 1.5). It is recognised that this task uses immature chicks and that synaptic maturation continues for some weeks post-hatch (Rostas, Brent & Guldner, 1984; Rostas & Jeffrey, 1981), accompanied by alterations in kinase activity and phosphoprotein localisation (Wienberger & Rostas, 1988a, b). However, previous investigations by Hölscher and Rose (1992, 1993) and Rickard et al. (1998, 1999, in preparation – a,b) support the use of this task in pharmaco-behavioural studies investigating the role of NO in memory formation. The early expression of NOS in the chick, and therefore presumably NO-

activated mechanisms, suggests that this paradigm may also be generally comparable to other paradigms which use mature animals.

The major feature of the Gibbs and Ng model of memory formation using a single trial passive avoidance task in the day-old chick is the existence of three temporally specific and sequentially dependent stages of memory formation which can be observed both behaviourally and pharmacologically (refer Figure 1.5) (Gibbs & Ng, 1979a). According to the Gibbs & Ng model, each stage is demarcated by brief transient retention deficits which are thought to represent the cross-over between decay of the preceding phase and the expression of the new phase. Based on this division, short-term memory (STM) is formed by 5 minutes post-training and decays after 10 minutes post-training. Intermediate-term memory (ITM) is expressed between 20 and 50 minutes post-training, while long-term memory (LTM) forms by 60 minutes post-training and lasts at least 48 hours. It should be noted, however, that these three phases of memory are arbitrarily named and may not represent equivalent phases described by a cognitive approach in other species. Over time a more complex view has emerged, ITM has been divided into ITM phase A (ITM-A; 20-30 minutes post-training) and ITM phase B (ITM-B; 30-50 minutes post-training) and LTM may posses phases described as pre-90 minutes and post-90 minutes.

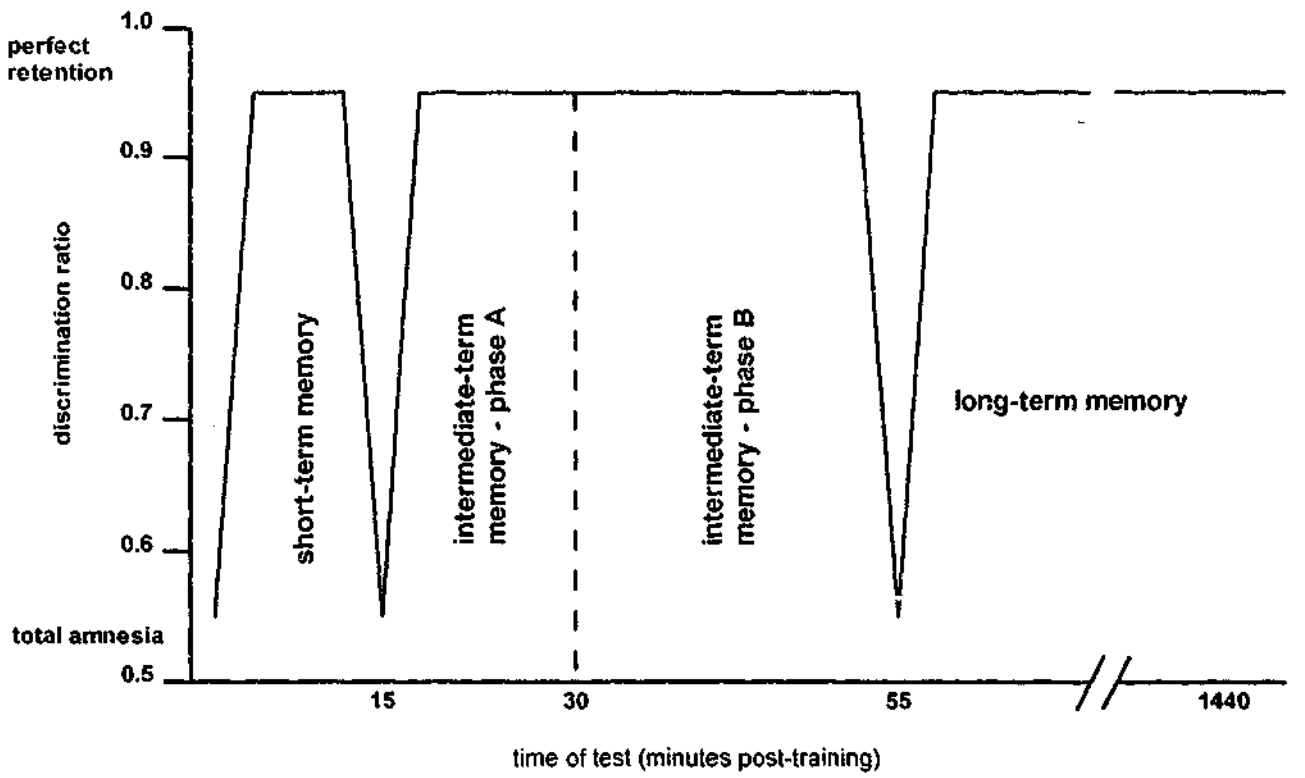


Figure 1.5 The three stages of the Gibbs and Ng model of memory formation using a single trial passive avoidance task for the day-old chick (Gibbs & Ng, 1979a).

Specifically, pharmacological intervention studies have revealed that STM is characterised by the action of agents, such as potassium chloride (KCl) and monosodium glutamate, which block memory retention by disrupting neuronal depolarisation (Gibbs & Ng, 1979b) and lanthanum chloride which non-specifically blocks Ca^{2+} channels (Gibbs, DeVaus & Ng, 1986; Gibbs, Gibbs & Ng, 1979a, 1979b).

The second stage of memory formation in this model is ITM. Beyond this simple demarcation, ITM can be divided into ITM-A and ITM-B due to the effect of the uncoupler of oxidative phosphorylation 2,4-dinitrophenol (DNP) which is effective in blocking retention during ITM-A (20 to 30 minutes post-training) if administered no later than 25 minutes post-training (Gibbs & Ng, 1984). In contrast, the action of cyclohexamide and iodoacetate marks the onset of ITM-B 30 minutes post-training if administered around the time of training (Gibbs & Ng, 1984; O'Dowd et al., 1994b). The division between ITM-A and ITM-B can also be observed behaviourally using a weakly reinforced variant of the task such that retention is progressively impaired from 30 minutes post-training (Crowe et al., 1989).

There are a number of processes underlying both ITM-A and ITM-B. Typically, ITM-A is characterised by a dependence upon oxidative metabolism (Gibbs & Ng, 1984), Na^+/K^+ pump activity (Gibbs & Barnett, 1976) and cholinergic activity (Zhao, Feng, Bennett & Ng, 1997). In contrast ITM-B is dependent upon a number of disparate processes including glycogenolysis (O'Dowd et al., 1994a, 1994b), glycoprotein synthesis (Crowe, Zhao, Sedman & Ng, 1994), PKC activity (Zhao, Sedman, Gibbs & Ng, 1994) and NOS activity (Rickard et al., 1998). Further, ITM-B consolidation appears dependent upon noradrenaline release (Crowe et al., 1989; Crowe et al. 1991a, b)

Finally, LTM is distinguished by its reliance on a number of protein related mechanisms including amino acid uptake (Robertson, Gibbs & Ng, 1978) and protein synthesis (Gibbs & Ng, 1984). Tyrosine kinase activity (Whitechurch, Ng & Sedman, 1997), protein phosphatase 2A activity (Bennet, Zhao & Ng, 2001) and glutamate receptor-dependent processes (Rickard & Ng, 1995; Rickard, Poot, Gibbs & Ng, 1994) are also features of the LTM stage.

It must be noted that variants of the single-trial passive avoidance task developed for the day-old chick have been used elsewhere in conjunction with pharmacological agents. Two examples of studies extending upon the work of the Ng and Gibbs research groups relate to investigations of Ca^{2+} channel subtypes and the role of various kinases

in passive avoidance memory. Initially Gibbs et al. (1979) and Gibbs et al. (1986) determined that the non-specific Ca^{2+} channel blocker, lanthanum chloride, impaired the STM stage. More recently, Clements, Rose and Tiunova (1995), Salinska et al. (2001) and Salinska, Chaudhury, Bourne and Rose (1999) have all studied the role of particular Ca^{2+} channel subtypes in passive avoidance memory using day-old chicks. While Salinska et al. (1999) found voltage- and ligand-gated Ca^{2+} channels to be active soon after training. Clements et al. (1995) found inhibition of N-type Ca^{2+} channels resulted in a loss of retention by 30 minutes post-training while inhibition of L-type Ca^{2+} channels did not impair retention when tested 3 hours post-training. Salinska et al. (2001) blocked another Ca^{2+} channel subtype, namely RyR channels and found that retention loss was not evident 30 minutes post-training but was observed at the later test time of 3 hours post-training.

Another variant of the passive avoidance task for day-old chicks has been used successfully by Serrano et al. (1994), Serrano, Rodriguez, Bennett and Rosenzweig (1995) and Serrano, Rodriguez, Pope, Bennett and Rosenzweig (1995) to study kinases. Following administration of PKC, protein kinase A (PKA) and PKG inhibitors, Serrano et al. (1994) demonstrated a loss of retention by 60 minutes post-training. Serrano et al. (1995) has since performed detailed studies administering various kinase antagonists into either the IMHV or LPO, bilaterally or unilaterally.

In conclusion, single-trial passive avoidance learning in the day-old chick has resulted in a temporally specific three stage model of memory formation and is well characterised with respect to the cellular processes underlying memory formation. The temporal specificity of retention loss onset afforded by the task allows the comparison of retention functions for cellular processes. In doing so, relationships between cellular processes may be suggested.

1.4.2 The role of nitric oxide in passive avoidance learning using the day-old chick

In addition to Hölscher and Rose (1992, 1993) who used a non-discrimination variant of the passive avoidance task Rickard et al. (1998) demonstrated that memory loss occurred by 40 minutes post-training following the administration of either L-NNA or L-NAME. Effective administration of L-NAME could occur between 5 minutes

before to 20 (Rickard, 1995) or 25 minutes post-training (Rickard et al., 1998) and L-NNA could be administered between 30 minutes before to 25 minutes after training using the discriminative variant of the single trial passive avoidance task. Rickard et al. (1998) also determined that co-administration of the spontaneous NO-donor, SNP, and L-NAME produced no deficit in memory retention for the strongly reinforced task.

The role of NO in passive avoidance learning was also studied using a weakly reinforced variant of the task. Rickard et al. (1994) administered 150μM SNP (ic) up to 25 minutes post-training, in accordance with the time of administration for L-NAME. In contrast to chicks administered saline, memory retention persisted until at least 180 minutes post-training. Taken together the above studies clearly demonstrate a role for NO in passive avoidance memory at the ITM-B stage.

More recently, Rickard et al. (1999), sought to determine which isoforms of NOS are necessary in passive avoidance learning in the day-old chick. Three nNOS inhibitors were found not to be effective at the concentrations tested. However, the specific eNOS inhibitor, diphenyleneiodonium chloride (DPI) (1μM, ic), resulted in a memory retention loss by 40 minutes post-training, a finding consistent with the previous studies of Rickard et al. (1994, 1998) and broadly consistent with Hölscher and Rose (1992, 1993). However, Rickard and Gibbs (in preparation - b) have shown that the nNOS-specific antagonist *N*-propyl-L-arginine (NPLA), administered at concentrations greater than those previously tested (50μM) effectively blocks retention from 40 minutes onwards. Interestingly, while DPI was known to have a range of effective administration times similar to those found using L-NAME and L-NNA (Rickard et al., 1998), NPLA was only effective if administered around the time of training.

Rickard and Gibbs (in preparation – a,b) have since characterised the effect of NPLA, DPI and L-NAME in hemispheric studies and in different brain regions, comparing the IMHV and LPO. In brief, L-NAME was not effective if administered bilaterally into the LPO. If injected into the left hemisphere IMHV, retention loss was observed only following administration times from 5 minutes before training to 5 minutes after training. In contrast the effective administration times if injected into the right hemisphere IMHV were from 15 minutes post-training to about 20 minutes post-training. Therefore L-NAME shows hemispheric differences in its effective times of administration.

1.4.3 Cellular processes aligned to nitric oxide-stimulated mechanisms previously investigated using passive avoidance learning for the day-old chick

A number of cellular processes have previously been found to underlie memory formation for the passive avoidance task. Some of these may be associated with the biochemical mechanisms activated by NO (see Section 2).

For example, NO is known to activate Ca^{2+} channel subtypes such as RyR channels. Generalised Ca^{2+} channel inactivation, using lanthanum chloride, results in retention loss during the earlier STM stage (Gibbs, Gibbs & Ng, 1979), preceding the retention loss observed using NOS antagonists. Importantly, Clements et al. (1995) found N-type Ca^{2+} channels active during the ITM-B stage, consistent with the time of action of NO, while retention loss onset following RyR channel inhibition occurred sometime between 30 minutes and 3 hours post-training (Salinska et al., 2001).

In addition, NO-activated CNG channels are not regarded as cation specific while BK_{Ca} channels are dependent upon Ca^{2+} to activate K^+ flux. Therefore the time at which various cations, other than Ca^{2+}, are required in memory processing is of relevance. Monosodium glutamate (4mM) and isotonic KCl (154mM) have been used in conjunction with the present task as neuronal depolarisers (Gibbs & Ng, 1979b) and inhibit STM formation. In contrast, oubain is known to block the Na^+/K^+ pump and has been found to result in retention loss during ITM-A (Gibbs & Ng, 1976).

NO has been determined most clearly to act through the up-regulation of GC leading to the activation of PKG. A non-discriminative variant of the present task has suggested PKG inhibition, using 16.7mM H-8, results in retention loss from 60 minute post-training (Serrano et al., 1994). However, H-8 is also known to inhibit PKA at high concentrations. This is supported by experiments using the discriminative variant of the task with a specific inhibitor of PKA which showed retention loss also from 60 minutes post-training (Zhao et al., 1995). Other kinases, such as tyrosine kinase have been implicated after the onset of the protein synthesis-dependent LTM stage (Whitechurch, Ng & Sedman, 1997) while phosphatases, necessary in a range of processes including cerebellar LTD, are required by 30 minutes post-training.

NO is also known to potentiate the release of noradrenaline in hippocampal tissue (Lonart et al., 1992; Lonart, Hertting & Jackisch, 1993; Stout & Woodward, 1994). As the action of NA becomes evident from the beginning of ITM-B (Crowe, Ng & Gibbs, 1989a; Crowe et al., 1990; Crowe et al., 1991b; Stephenson & Andrew, 1981) it is broadly consistent with the action of NO in ITM-B 10 minutes later. However, if NO is responsible for noradrenaline release in passive avoidance learning then it most probably does it through other mechanisms such as GC activation.

Finally, NO has often been implicated in LTP which is an NMDA receptor-dependent process. Although glutamate is believed necessary in a number of stages of the Gibbs and Ng model (Ng et al., 1997), inhibition of glutamate receptors, including NMDA receptors, resulted in a loss of retention after the onset of the LTM stage (Rickard et al., 1994b, 1995).

These studies prompt a number of further investigations into the possible mechanisms through which NO might bring about consolidation of LTM. For example, NO is known to lead to the activation of PKG. However, previous investigations into the role of PKG in passive avoidance learning have been problematic for a number of reasons (see Chapter 3). Alternatively, NO is known to activate RyR channels. Following administration of a specific inhibitor, retention loss onset occurred some time between 30 minutes and 3 hours post-training, using a non-discriminative variant of the passive avoidance task, and therefore may be found consistent with the retention function for NOS inhibition in future investigations.

Taken together the passive avoidance task for the day-old chick provides both temporal specificity and a previously established framework of cellular processes useful in determining through which mechanism(s) NO is likely to affect memory formation.

SECTION 5 : Aims of the present study

Extending upon the studies of Rickard et al. (1994, 1998, 1999, in preparation – a,b) the principal aim of this thesis is to use pharmaco-behavioural studies to suggest downstream mechanism(s) through which NO acts to consolidate the LTM stage according to the model of Gibbs and Ng (1979a).

NO most probably activates a number of memory processes as discussed in this chapter. However, as Rickard et al. (1998) identified a persistent loss of retention from 40 minutes post-training following the administration of L-NNA or L-NAME, only the candidate process which matches this retention function can be suggested to be NO-dependent within the current paradigm. Rickard et al. (1998) also identified a range of effective administration times for L-NNA and L-NAME, being from before the time of training up to 20 minutes post-training. Differences in pharmacokinetics between drugs makes comparisons of effective times of administration unsafe. However, if the mechanism identified to be NO-dependent through the finding of a common retention function with L-NNA and L-NAME also demonstrated a similar set of effective administration times this would add considerable weight to the finding.

Initial behavioural studies utilising the general NOS inhibitor L-NAME will seek to replicate the work of both Rickard et al. (1998) and Hölscher and Rose (1993) so as to establish continuity in technique between the current and past studies. In this way experimenter bias in the conduct of the behavioural studies can be tested.

Past research by Bernabeu et al. (1995, 1996, 1997) and Izquierdo et al. (2000), using single trial aversive learning in rats, has clearly established the role of PKG as the ultimate effector mechanism following NO activation of GC. Therefore, the role of PKG as an NO effector mechanism in single trial passive avoidance learning using the day-old chick will be explored in conjunction with specific pharmacological agents.

If PKG is not found to be an effector mechanism of NO then pharmacological agents will be used to test the action of other NO-dependent mechanisms implicated in memory formation. Potential mechanisms include NO activation of *mono*(ADP-ribosyl) transferase, CNG channels, BK_{Ca} channels, RyR channels or peroxynitrite production.

CHAPTER 2 -

GENERAL METHODOLOGIES

CHAPTER 2 - GENERAL METHODOLOGIES

SECTION 1 : Behavioural Methodologies

2.1.1 Introduction

The clearest evidence for NO-dependent learning comes from the study of spatial, olfactory and avoidance learning tasks. Among these, the passive avoidance learning task developed for the day-old chick has a number of advantages. For example, the task uses only a single learning trial and thus clearly establishes the time of learning. In single trial tasks, the time of retention loss onset relative to the time of learning can be accurately established. In doing so temporally precise comparisons between retention functions can be made and relationships between cellular processes suggested. One significant advantage of using day-old chicks is that drugs can be administered intracranially without surgery or anaesthesia. In addition, large sample sizes can be used and numerous data-points derived thereby removing the need for retesting as has occurred in some studies with rodents (Izquierdo et al., 2000). Although passive avoidance learning can be measured by the percentage avoidance to an aversive bead, measuring discrimination between an aversive and non-aversive bead allows detection of generalised avoidance or sensory-motor effects resulting from drug administration. In this way the discrimination variant of the task is designed to differentiate between the action of a drug upon memory processes or other possible confounding effects.

2.1.2 Animals and housing conditions

All behavioural studies were conducted in one of two laboratories. Each laboratory could hold a maximum of 240 chicks housed in pairs inside pens (20x25x20cm). These pens were constructed of wood, open at the top and bottom and designed such that 5 pens formed a single unit called a 'box'. In the front wall of each pen were drilled two rows of five holes (5mm diameter) at the chick's eye level (8 to 10 cm from the base). A series of shelves were constructed for holding the boxes in two horizontal rows. Further, between each box and the shelf was placed matt brown absorbent paper which was removed at the completion of each day's experiment and on which was scattered crushed poultry feed. Suspended 13cm above each pen was a 15W white light globe and all globes were connected in series (see Figure 2.1). Each laboratory had a reverse cycle heater which was used to pre-heat the pens to between 26-29°C if the radiant heat from the globes was insufficient.

Day-old white-Leghorn x black-Australorp chicks were obtained on the morning of each experimental day from a local hatchery and transported by van in large cardboard cartons (approx. 100 chicks per carton). Insulation against the cold was achieved in transport by placing shredded newspaper on the floor of the cardboard cartons.

Once in the pre-warmed laboratory, pairs of chicks were placed in individual pens. Chicks were housed in pairs to avoid isolation stress which has been demonstrated to interfere with the timing of the normal memory trace (de Vaus, Gibbs & Ng, 1980). One chick of each pair was marked on the head with an indelible black pen for the purposes of identification when recording data. Chickens were then grouped into 10 pairs for the purposes of data recording, as 20 chicks were used for each data-point. A 30 minute interval was allowed to elapse before the commencement of pre-training to allow the chicks to acclimatise to their surroundings. The chicks spent a large portion of this time pecking at the food. This reflects their innate tendency to peck at objects rather than a hunger response as they were still nourished from the yolk sac (Hogan, 1973).

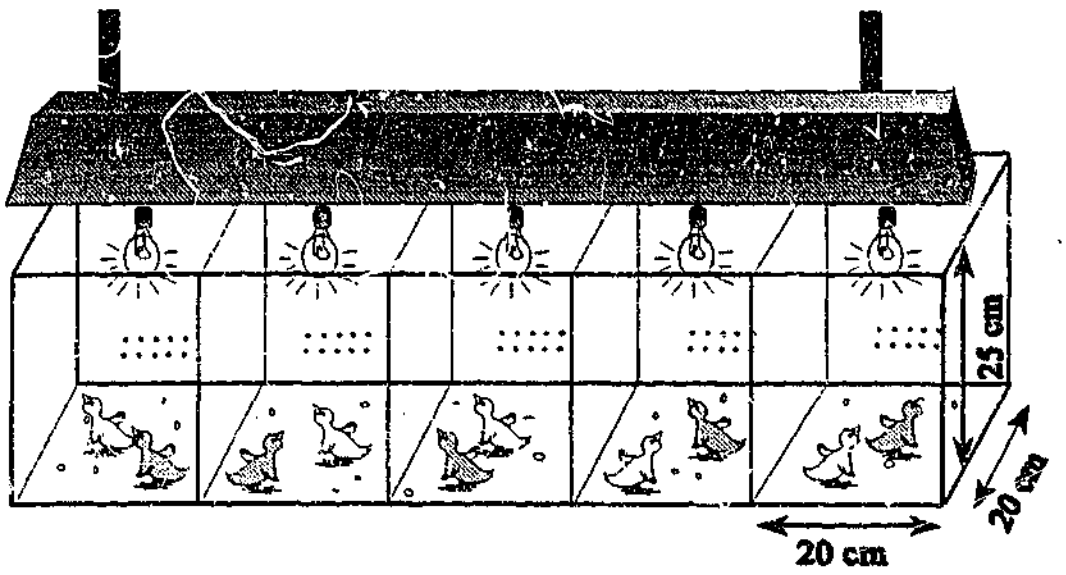


Figure 2.1 Housing for day-old chicks during the experimental procedure. (courtesy of Dr N. Rickard)

2.1.3 Task procedure

The behavioural task is a single trial discriminative passive avoidance task developed by Cherkin (1971) and adapted by Gibbs and Ng (1977). The task has three phases: pretraining, training and memory retention testing. Chicks were pretrained, trained and tested in pairs.

2.1.3.1 Pretraining

The purpose of pretraining was to familiarise the chicks with the presentation of a novel object and to introduce them to non-aversive red and blue beads. Pretraining commenced with the presentation of a small chrome bead (2.5mm diameter) attached to a 25 cm stiff wire and coated in tap water. Presentation for each bead is immediately preceded by a series of light taps to the front of the box at the level of the drilled holes to gain the attention of the chicks. The bead is then placed inside the box from above, approximately 2-3 centimetres from the chick at the level of their eyes. The duration of presentation for the initial chrome bead was usually less than 20 seconds but was defined as the length of time required to observe a short series of 'confident' pecks to

the bead. Confident pecks were described as pecking behaviour with the absence of fear responses such as cowering, active avoidance of the bead or long delays between pecks. Once presentation had occurred for all chicks, a second round of pretraining using the chrome bead was commenced with presentation lasting only a few seconds until another short series of 'confident' pecks to the chrome bead occurred.

Pretraining with the chrome bead was followed by pretraining with coloured beads identical to those used in the training and testing trials of the passive avoidance task. A red glass bead, dipped in tap water, was presented to each pair of chicks for 10 seconds, followed by presentation of a blue glass bead, also dipped in tap water, to each pair of chicks for the same duration (see Figure 2.2). These beads were attached to the end of a 25 cm length of stiff wire. The number of pecks each chick displayed to both red and blue beads was recorded using a hand held electronic recording device. A one hour interval ensued between pretraining and training during which time pharmacological agents were prepared.

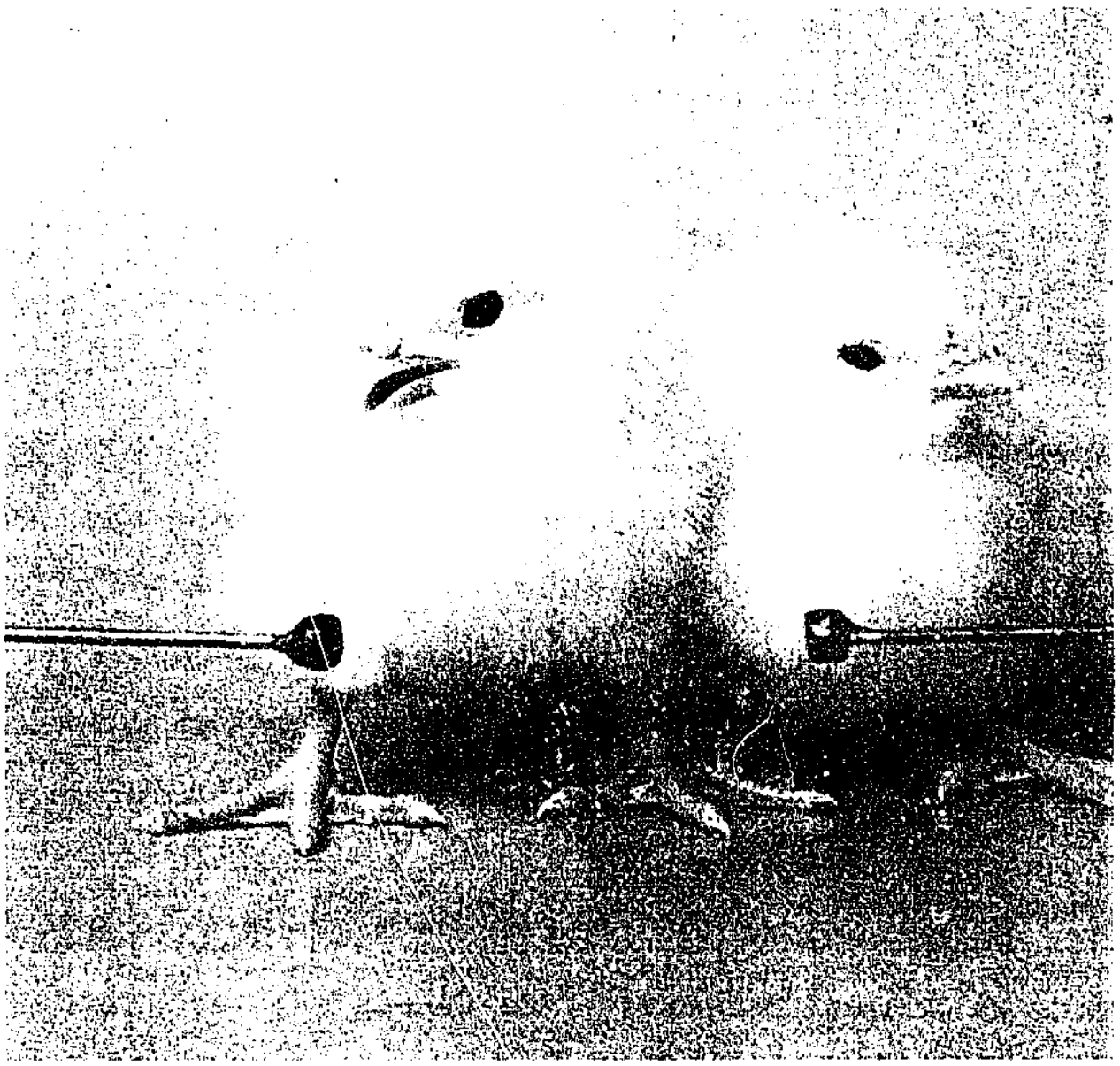


Figure 2.2 Day-old chicks with red and blue beads used in the passive avoidance task. (courtesy of D. Walsh - La Trobe University).

2.1.3.2 Training

A red bead identical to that used in pretraining was coated in a non-toxic chemical aversant, methyl anthranilate (MeA), and presented to each pair of chicks for 10 seconds. Most chicks pecking this bead display a typical disgust response which includes a pronounced shaking of the head and wiping of the beak on the floor of the pen. Few birds peck the aversive bead a second time during the training trial. Those birds which did not train, usually less than 10%, were excluded from the data analysis but for convenience underwent the testing trial. Again the number of pecks was recorded.

2.1.3.3 *Testing*

Retention testing occurred throughout the afternoon but no later than 4:30pm as the chicks are sensitive to light-mediated circadian rhythms (Aschoff & von Saint Paul, 1976). Relative to the time of training, retention testing occurred at various times post-training, between 10 minutes and 24 hours, depending on the purpose of the experiment. Testing was identical to the second stage of pre-training except a dry red and a dry blue bead were presented in succession. Without the administration of pharmacological agents, those chicks which trained typically refused the red bead but pecked the blue bead when presented. The number of pecks for both the red and blue bead were recorded.

2.1.4 Pharmacological intervention studies

Pharmacological agents were used to inhibit cellular processes of interest and were injected at various times relative to training depending on the purpose of the experiment. Two critical pieces of information can be gleaned from such studies: first, whether a specific cellular event is involved in memory processing; second, the time of retention loss onset represents the earliest time at which the cellular event is expressed in memory formation.

To deduce this information three studies must be undertaken in order:

1. a dose response study, which varies the concentration of the agent (typically an antagonist) while keeping time of administration and the time of retention test constant. Administration typically occurs immediately post-training, to avoid acquisition effects, while testing for retention loss occurs either 2 or 3 hours post-training. This study is used to determine if the biochemical mechanism in question is active in memory processing and then to determine what concentration of the antagonist used produces the optimum retention loss.
2. a time of administration study, which varies the time of drug administration while keeping concentration and retention test time constant. The concentration of the drug used is that determined in the dose response study while retention testing occurs at 2 or 3 hours post-training. This study determines the optimum time for

drug administration. Further, the range of effective administration times may provide an indication as to the time of activation of the biochemical mechanism being studied.

3. a time course study, also know as a retention function study, is undertaken only once the optimum concentration and time of administration have been determined. To demonstrate at what time after training retention loss first occurs the training-testing intervals extend from 10 minutes to 24 hours post-training. This type of study determines which stage of memory formation is dependent upon the biochemical mechanism by determining the time of retention loss onset.

The use of technical assistants, the large range of concentrations, times of administration and retention times meant that most experiments were performed blind; that is, a coding system could be used and referred to without revealing the experimental condition. Further, the large range of concentrations, administration times and retention times investigated meant that each experiment would typically occur over more than one experimental day.

2.1.4.1 Drug preparation

All drugs were prepared immediately prior to use from their solid form, reducing the risk of degradation through storage of aliquots. Those drugs which were not water soluble were first diluted in the recommended solvent, either ethanol or dimethyl sulfoxide (DMSO), and then serially diluted in saline (154mM NaCl). The final concentration of DMSO or ethanol was typically less than 1%. Those drugs which were water soluble were diluted immediately in 154mM NaCl (physiological saline). Control groups were administered the appropriate vehicle. A list of all drugs used in the present research programme and their respective solvents is given in Table 2.1.

Table 2.1

List of All Pharmacological Agents and Solvents Used in the Present Research Programme.

Action	*Drug*	*Manufacturer*	*Solvent*
NOS inhibitor	L-NAME (L-N^G-nitroarginine methyl ester)	Sigma (NSW, Australia)	saline
PKG inhibitor	H-8 (N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide)	ICN (California, United States of America)	saline
GC inhibitor	ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one)	Sigma (NSW, Australia)	DMSO
	LY83583 (6-Anilino-5,8-quinolinedione)	Cayman (Michigan, United States of America)	DMSO
mono(ADP-ribosyl) transferase inhibitor	menadione sodium bisulfite (2-Methyl-1,4-naphthoquinone sodium bisulfite)	Sigma (NSW, Australia)	saline
	novobiocin	Sigma (NSW, Australia)	saline
CNG channel inhibitor	verapamil	Sigma (NSW, Australia)	saline
BK_{Ca} channel inhibitor	iberiotoxin (IbTX)	Sigma (NSW, Australia)	saline
RyR channel inhibitor	dantrolene (1-[[[5-(4-Nitrophenyl)-2-furanyl]methylene]imino]-2,4-imidazolidinedione)	Sigma (NSW, Australia)	DMSO
peroxynitrite scavenger	Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Calbiochem (NSW, Australia)	ethanol

2.1.4.2 *Injection procedure*

All drugs were administered in 10µl volumes per hemisphere by free-hand injection with a Hamilton syringe (500µl capacity) fitted to a repeating dispenser. A 27.5 gauge needle was used, fitted inside a plastic sleeve which regulated the depth of injection to approximately 3.5mm. The duration of the entire injection procedure was less than 10 seconds per chick.

The target of each injection was the neostriatal/hyperstriatal complex within the chick forebrain (see Figures 2.3, 2.4), which has been demonstrated as a probable site of memory processing in the chick forebrain (Horn and Johnson, 1989; Rose & Csillag, 1985; Rose & Stewart, 1999; Sedman, O'Dowd, Rickard, Gibbs & Ng, 1991; Serrano et al., 1992). While this area generally contains only a low concentration of NOS-positive neurons (Brüning 1993; Brüning et al., 1994), NOS-dependent processes appear specific to this region. Administration of NOS inhibitors into another memory-related brain region, the LPO, did not produce a loss of retention on test (Rickard & Gibbs, in preparation - b).

Injections were performed free-hand by assistants who had undergone extensive practice on deceased chicks. Constant feedback with regard to the accuracy of injection site was provided by way of direct observation of the skull, such that once the skin had been removed the injection site was visible as a small deep red dot on the surface of the skull (see Figure 2.3). Further, examination of brain slices was undertaken by the research assistant to ensure administration into the neostriatal/hyperstriatal complex. Only when the assistant attained a >90% accuracy were they allowed to inject experimental chicks. Free-hand injections are possible as this site of injection can be identified topographically as being 3.5mm below the lateral boarders of the fontanel (see Figure 2.3). The accuracy of injection was again monitored, from time to time, by observation of the skull post-mortem using a random selection of 20 chicks from total of 120 used during the day.

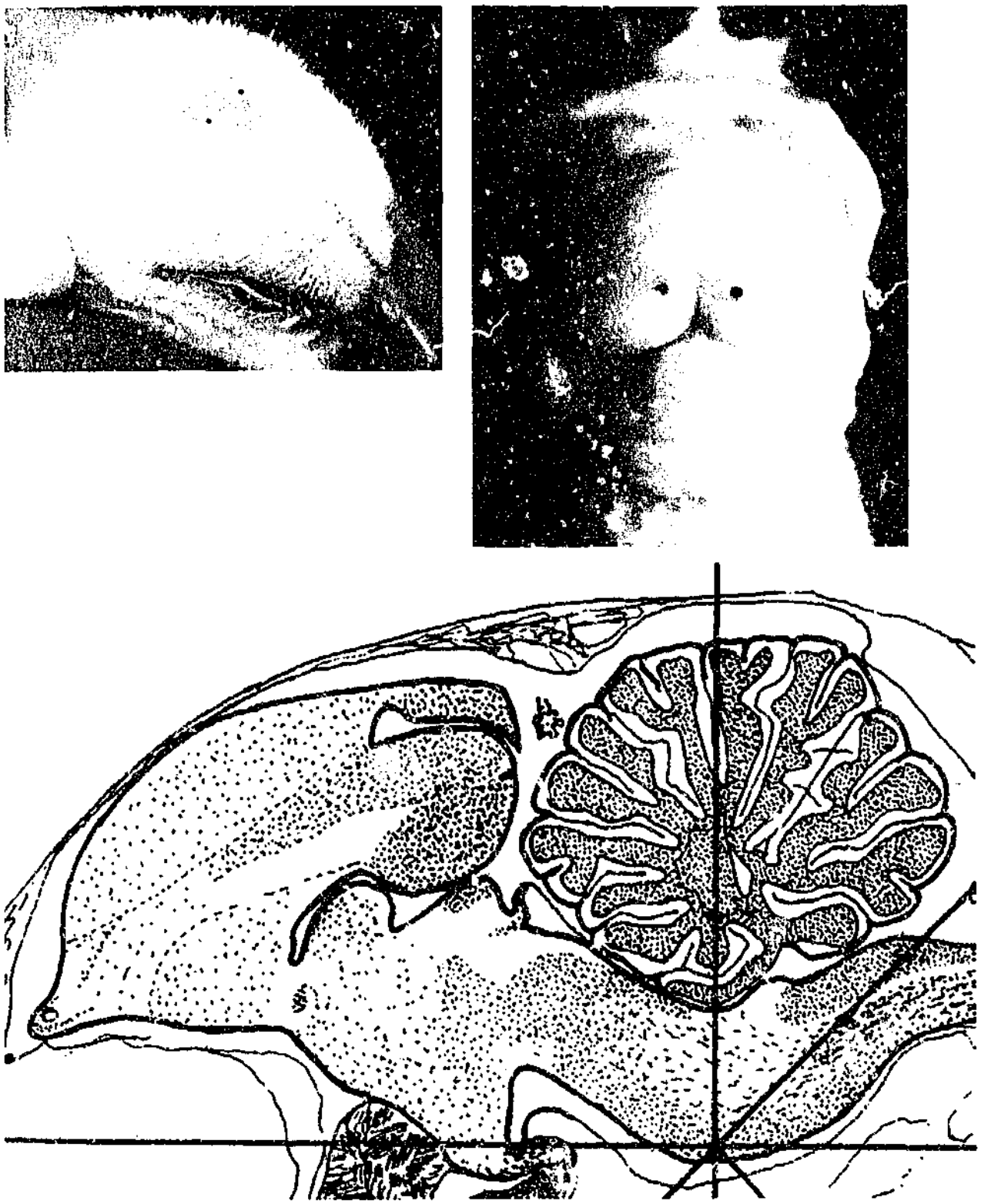


Figure 2.3 Visual representation of injection sites.

Top left - representation of both fontanelle and injection site location. Note that the injection site for each hemisphere is marked as a dark dot at the lateral borders of the triangular fontanelle. Top right - location of injection site into each cerebral hemisphere from above. Bottom - sagittal section of the chick brain noting the area of drug administration. Specifically the region of the neostriatal/hyperstriatal complex, containing the IMHV (marked in pink), is targeted (adapted from Kuenzel & Masson (1988) and courtesy of Ms E. Hartley, Monash University).

AId Archistriatum intermedium, pars dorsalis (Zeier and Karten)
AIv Archistriatum intermedium, pars ventralis (Zeier and Karten)
AL Area lentiformis
ALA Nucleus ansae lenticularis anterior [rostral]
Am Archistriatum mediale (Zeier and Karten)
APH Area parahippocampalis
CDL Area corticoidea dorsolateralis
CHCS Tractus cortico-habenularis et cortico-septalis
CIO Capsula interna occipitalis
CO Chiasma opticum
CPi Cortex piriformis
DA Tractus dorso-archistriaticus
DLAI Nucleus dorsolateralis anterior [rostralis] thalami, pars lateralis
DLAm Nucleus dorsolateralis anterior [rostralis] thalami, pars medialis
DMA Nucleus dorsomedialis anterior [rostralis] thalami
DSD Decussatio supraoptica dorsalis
DSV Decussatio supraoptica ventralis
FL Fasciculus prosencephali lateralis (Lateral forebrain bundle)
FPM Fasciculus prosencephali medialis (Medial forebrain bundle)
GLv Nucleus geniculatus lateralis, pars ventralis
HM Nucleus habenularis medialis
Hp Hippocampus
HV Hyperstriatum ventrale
ICT Nucleus intercalatus thalami
LAD Lamina archistriatalis dorsalis
LH Lamina hyperstriatica
LHy Regio lateralis hypothalami (Lateral hypothalamic area)
LMD Lamina medullaris dorsalis
N Neostriatum
nTSM Nucleus tractus septomesencephalicus (Nucleus superficialis parvocellularis)
OM Tractus occipitomesencephalicus
PA Paleostriatum augmentatum (Caudate putamen)
PCVL Plexus choroideus ventriculi lateralis (Choroid plexus within lateral ventricle)
PHN Nucleus periventricularis hypothalami
PVN Nucleus paraventricularis magnocellularis (Paraventricular nucleus)
QF Tractus quintofrontalis
ROT Nucleus rotundus
RSd Nucleus reticularis superior, pars dorsalis
SMe Stria medullaris
Tn Nucleus taeniae
TrO Tractus opticus
TSM Tractus septomesencephalicus
VL Ventriculus lateralis
VMN Nucleus ventromedialis hypothalami

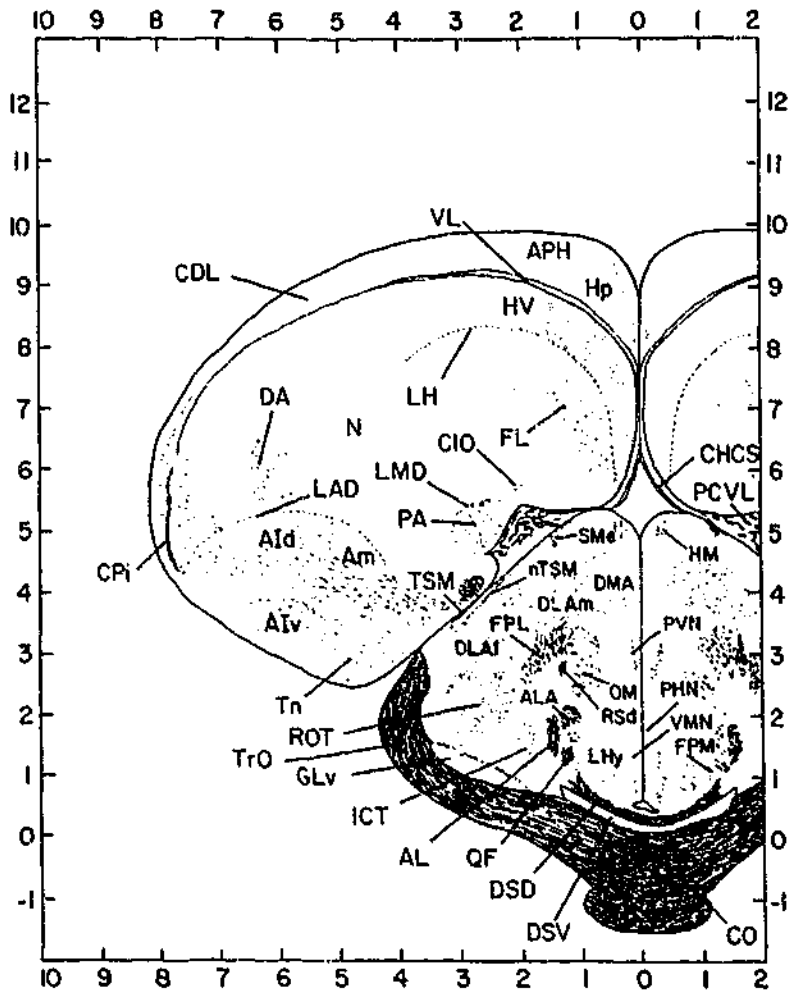


Figure 2.4 Coronal section of the chick brain showing the location of the IMHV.
While the IMHV (labelled in pink) is the target of drug administration the accuracy of free-hand injections are such that it is more conservative to state that the injection site is the neostriatal/hyperstriatal complex (marked as HV) . The depth of section along the rostral-cordal axis is equivalent to the area marked in pink for the sagittal view shown in Figure 2.3 (bottom). (Adapted from Kuenzel & Masson (1988) and courtesy of Ms E. Hartley).

2.1.5 Data collection

Data consisted of the number of pecks to a given bead, at a particular phase of the experiment, by an individual chick within a 10 second period. Data collection was by a hand-held recording device which down-loaded data at the completion of the experiment to a personal computer (IBM-compatible 90MHz Pentium) with the appropriate custom-made software (Dept. of Psychology – Monash University, 3800).

The recording device differentiated between chicks within each pair and between groups of chicks representing different conditions of the experiment. For all

experiments, pretraining, training and testing data were collected. For each chick, the data collected was the number of pecks to each bead during each phase of the experiment. Only pecking data derived from testing was used to determine the discrimination ratio (DR) for each chick as a measure of memory.

The measurement of retention used was a DR, which is defined for a single chick as the ratio of the number of pecks of the blue bead compared to the total number of pecks of both the blue and red beads during the 10 second test trial. Any bird which did not train was excluded from the data analysis as was any bird which did not peck the blue, non aversive, bead during the test trial. Chicks which did not peck at the blue bead on testing were thought to display either generalised avoidance or non-specific avoidance due to factors such as somnolecence. Typically, no more than 15% of any group of 20 chicks were excluded. The mean DR for a group of chicks is a continuous variable which reflects the level of retention as a measure between 0.5 and 1.0, where 0.5 reflects no discrimination between the previously aversive red bead and non-aversive blue bead while 1.0 reflects perfect discrimination. Occasionally DR values less than 0.5 may result for a single chick. While such findings are difficult to explain as they reflect a preference for the previously aversive red bead it is of note that in such circumstances the overall total number of pecks tends to be quite small. Thus, while the relative number of pecks to the previously aversive red bead may appear large in comparison to the non-aversive blue bead, the absolute difference is often very small. Therefore, when DR's of less than 0.5 result it may reflect low levels of pecking during testing rather than specifically aberrant behaviour and, thus, a greater number of pecks to the previously aversive red bead may be considered chance rather than preference.

Another measure of memory used in passive avoidance learning for day-old chicks is percentage avoidance of the training bead which is simply a discrete measure of whether a chick pecks or avoids a previously aversive bead. The benefit of using a DR in preference to percentage avoidance is that percentage avoidance fails to detect birds which display non-specific pecking to both aversive and non-aversive beads; peck the aversive bead only once and then the non-aversive bead several times demonstrating poor memory; or birds which do not peck the non-aversive bead. In the second circumstance, this behaviour may have non-memorial motivational causes such as aggression, confusion or curiosity and may be misinterpreted as amnesia. Further, percentage avoidance fails to differentiate between chicks with mild retention losses and those with severe memory impairment. Percentage avoidance attributes all aversion to

discrimination ignoring the possible side-effects of pharmacological agents or aspects of the experimental design such as intracranial injections in producing aberrant behaviour.

2.1.6 Data analysis

Chicks which did not train or did not peck the non-aversive blue bead on test were excluded from the analysis. Further, the large number of treatment groups used in each experiment meant that most experiments occurred over more than one experimental day. In doing so it was necessary to replicate a limited number of treatment groups on each subsequent experimental day to exclude single-day effects. Therefore, exclusion of some chicks from the analysis and replication of some treatment groups resulted in different sample sizes amongst treatment groups.

Dose response studies were analysed first by one-way analysis of variance (ANOVA) and, if significant, were followed by post-hoc Dunnett's test where comparison of treatment means to a single control was of primary importance. For each of the time of administration and retention study a two-way ANOVA was conducted followed by simple main effects analysis if a significant interaction effect was observed. In all cases the Type I error rate was set at α=0.05. For all studies standard error of the mean was calculated for each condition as a measure of variance and displayed visually on all histograms. All analyses except the Dunnett's tests, which were performed manually, were performed on an IBM-compatible 90MHz Pentium computer using the Statistical Package for the Social Sciences (SPSS) for Windows version 10.

CHAPTER 3 -

THE ROLE OF GUANYLYL CYCLASE AND PROTEIN KINASE G ON PASSIVE AVOIDANCE LEARNING

SECTION1: Introduction

SECTION 2: Inhibition of nitric oxide synthase by L-NAME

3.2.1 L-NAME inhibition of nitric oxide synthase

SECTION 3: The effect of protein kinase G inhibition on passive avoidance learning

3.2.1 H-8 inhibition of protein kinase G

SECTION 4: The effect of guanylyl cyclase inhibition on passive avoidance learning

3.3.1 ODQ inhibition of guanylyl cyclase

3.3.2 LY83583 inhibition of guanylyl cyclase

SECTION 5: General discussion

CHAPTER 3 - The role of guanylyl cyclase and protein kinase G on passive avoidance learning

SECTION 1 : Introduction

There is evidence to suggest that nitric oxide (NO) is necessary for memory formation for a variety of behavioural tasks and species. The introductory chapter discussed a number of these in detail, including spatial, olfactory and inhibitory/passive avoidance learning. However, there exists comparatively little evidence as to the mechanism(s) through which NO might accomplish this. At a general level, NO may affect biological processes through activating haem-containing enzymes such as guanylyl cyclase (GC), activation of other enzymes such as *mono*(ADP-ribosyl) transferase, altering activity of ion channels, or through the formation of peroxynitrite. Of these mechanisms, the best characterised is the binding to, and activation of, GC. However, GC is not the effector mechanism *per se*, since it activates protein kinase G amongst other proteins which act to modify cellular processes (see Figure 3.1).

GC is known to activate three classes of proteins through the production of the cyclic nucleotide second messenger cGMP. The known targets of cGMP are PKG, PDEs and CNG channels (see Figure 3.1). The activation of each of these three protein classes has important biological ramifications. PKG is known to phosphorylate a range of protein substrates, many of which are found within the synapse and axon (Wang & Robinson, 1995). PDEs regulate second messenger levels and, thus, allow cross-talk between various transduction pathways. Finally, CNG channels, which have recently been found in hippocampal tissue (Kingston et al., 1996a), are transmembrane channels and are, in part, responsible for the overall ion balance of the cell. Of the three known classes of protein that cGMP binds, the action of PKG has been most clearly characterised with respect to memory formation. The aim of the experiments in this chapter was to determine the role of PKG and if GC in passive avoidance memory using the day-old chick, and in particular to determine if the action of PKG could account for the role of NO. Specific antagonists of NOS (L-NAME), PKG (H-8), and GC (ODQ &

LY83583) were administered as part of the pharmacological intervention studies undertaken and have since been reported by Edwards, Rickard and Ng (2002).

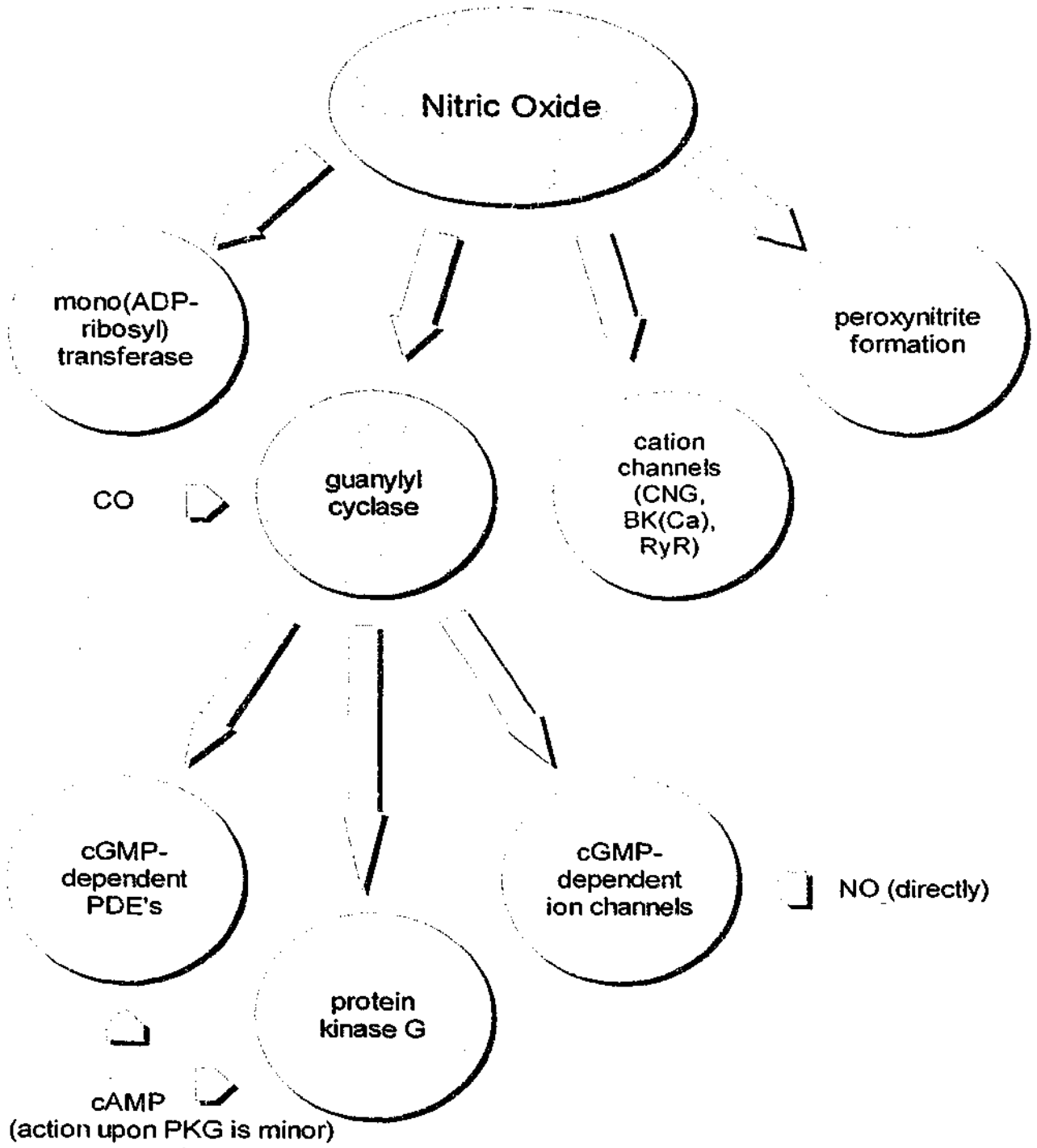


Figure 3.1 The major pathways through which NO may affect memory processes. Both guanylyl cyclase and PKG are marked in orange and the inhibition of these enzymes forms the basis of the studies reported in this chapter. Other activators of guanylyl cyclase or guanylyl cyclase-dependent molecules are noted by blue arrows. Further, some interlacing of pathways may occur but for clarity has not been represented herein.

SECTION 2 : Inhibition of nitric oxide synthase by L-NAME

Inhibition of NOS has been demonstrated to impair memory retention by 40 minutes post-training, using a single-trial passive avoidance task for the day-old chick (Hölscher & Rose, 1992, 1993; Rickard et al. 1998, 1999, in preparation - b). Initially, Hölscher and Rose (1992) administered 2mmol/0.5ml L-NNA intraperitoneally (ip), a dose which was previously found to be effective in blocking LTP in vitro (Böhme et al., 1991; Schuman et al., 1991). While this dose was effective in producing retention loss, the peripheral route of administration meant that the possibility of systemic NO-dependent cardiovascular effects could not be excluded. Hölscher and Rose (1993) later defended the use of ip administration by demonstrating a lack of state-dependency or sensory-motor effects. In this later study 8mM, but not 1mM, L-NArg administered intracranially resulted in a loss of retention when administered 1 hour prior to learning but not 5 minutes prior to training. In contrast, the more recent study by Rickard et al. (1998) found that 1mM L-NArg resulted in retention loss if administered intracranially when administered any time between 30 minutes prior to training and 25 minutes post-training. In spite of the differences in dose, time and route of administration, the time of retention loss observed by Hölscher and Rose (1992, 1993) and Rickard et al. (1998), was consistent occurring by 40 minutes post-training, thus affecting the ITM-B stage in the Gibbs and Ng model.

The likelihood that this effect was due to the inhibition of NOS is strong since retention loss also resulted by 40 minutes using another common NOS inhibitor L-NAME (0.5mM) (Rickard et al., 1998). L-NAME is a more appropriate non-selective isoform inhibitor of NOS, as L-NNA appears to preferentially inhibit nNOS (Hölscher & Rose, 1992). Intracranial administration of L-NAME displayed a broad range of effective doses between 0.5mM and 10mM, and a similar time of administration function as L-NArg, being effective when administered as late as 20 (Rickard, 1995) or 25 minutes post-training (Rickard et al., 1998).

The differences reported by Hölscher and Rose (1992, 1993) and Rickard et al. (1998) concerning effective doses, times of administration and, to a lesser extent, the time of retention loss onset may be due to variations in [illegible] between

researchers (Crowe & Hamalainen, 2001). For example, Rickard et al. (1998) used a discrimination index to test for retention in the passive avoidance task, as described in Chapter 2, while Hölscher & Rose (1992, 1993) used a discrete avoidance measure. This allowed Rickard et al. (1998) to detect sensory-motor effects produced by the drug, such as generalised avoidance or somnolecence, and remove such chicks from the analysis. Further Rickard et al. (1998) used only cockerels while Hölscher and Rose (1992, 1993) used chicks of both sexes and a different strain (black-Australorp x white-Leghorn cross compared with Ross Chunky).

3.2.1 L-NAME inhibition of nitric oxide synthase

3.2.1.1 Retention study for L-NAME

Using the methodology of Rickard et al. (1998), the purpose of the present experiment was to determine the reproducibility of their retention function. If the function is replicated all future behavioural experiments studying the mechanism of action of NO could be directly compared to the initial findings of Rickard et al. (1998). However, Rickard et al. (1998) did administer L-NAME 5 minutes prior to training. Handling of chicks and intracranial administration of a pharmacological agent prior to and so close to the learning event may confound later retention measurements through possible effects on acquisition. Therefore, the experiment was aimed at measuring retention using a similar time of administration but one which would leave training unencumbered by administration of L-NAME.

Method

Chicks were housed and trained as described in Chapter 2. Twenty chicks were used for each data-point. However, the final number of chicks for each data-point differed due to the exclusion of chicks from the analyses if they refused to train or to peck at the non-aversive bead during testing. Further, the experiment lasted for more than one experimental day, which necessitated the replication of some data-points as a

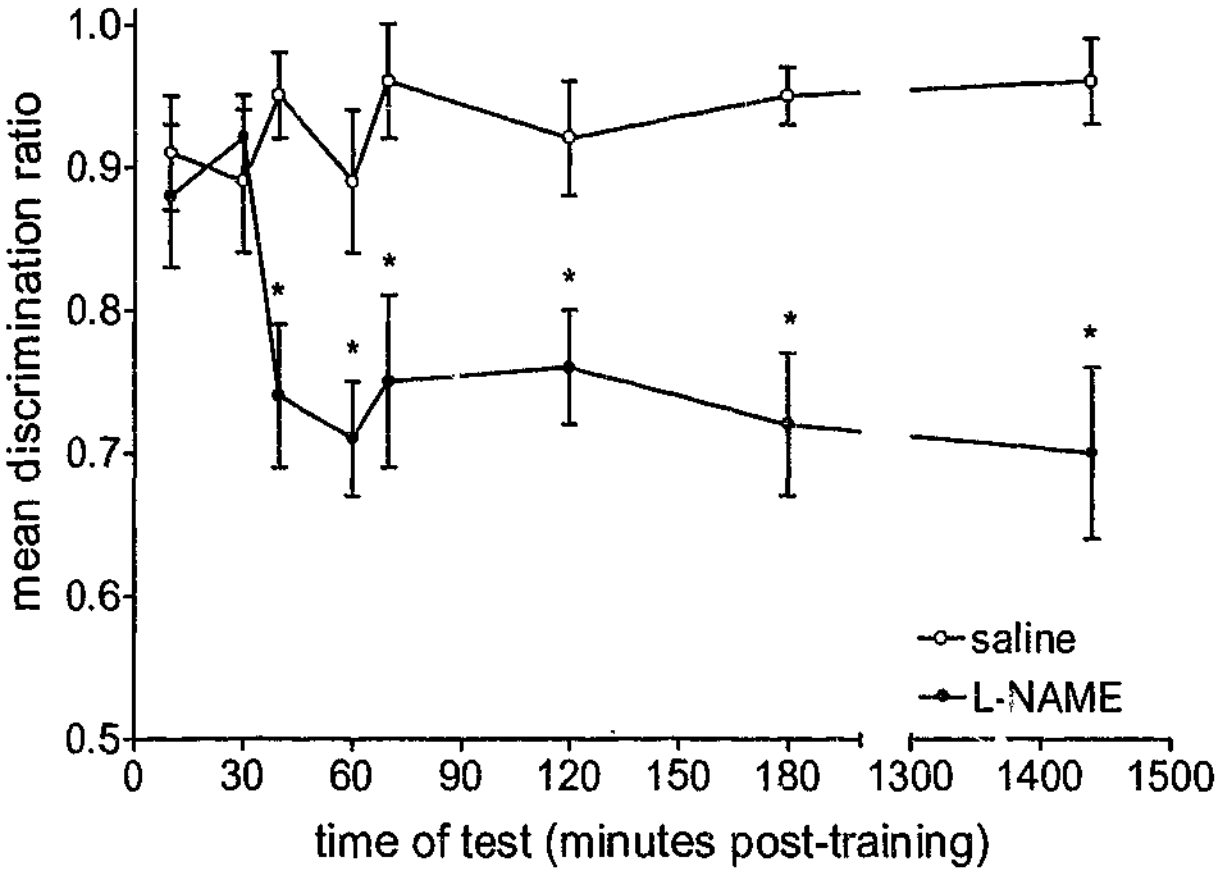
positive control. This further altered the number of chicks for a single data-point. In this retention study the number of chicks per data-point ranged from 12 to 29, with a mean of 18.

L-NAME (0.5mM) or saline was injected freehand into the region of the neostriatal/hyperstriatal complex in accordance with Rickard et al. (1998). The time of administration chosen was immediately post-training which is within the range of effective times of administration determined by Rickard et al. (1998).

Control groups were administered 154mM saline. Retention was tested at 10, 30, 40, 60, 70, 120 and 180 minutes post-training, as well as at 24 hours post-training.

Results

The retention functions for chicks administered either 0.5mM L-NAME or saline immediately post-training and tested at various times post-training are shown in Figure 3.2.



Saline n =	(16)	(20)(16)	(15)(17)	(15)	(18)	(17)
L-NAME n =	(12)	(18)(16)	(20)(17)	(17)	(29)	(23)

Figure 3.2 Retention function for the inhibition of NOS.
0.5mM L-NAME, compared with saline, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Chicks administered 0.5mM L-NAME demonstrated a persistent loss of retention from 40 minutes post-training to 24 hours post-training. A two-way ANOVA was performed, revealing a significant drug main effect ($F(1,291)=44.13$, $p<.001$) and a significant interaction effect ($F(7,291)=2.38$, $p<.05$), but not a significant time of test main effect $F(7,291)=0.99$, $p>.05$). Simple main effects post-hoc analysis confirmed a significant difference in retention between L-NAME and saline administered chicks at 40 minutes ($p<.005$), 60 minutes ($p<.005$), 70 minutes ($p<.005$), 120 minutes ($p<.005$), 180 minutes ($p<.001$) and 24 hours post-training ($p<.001$), but not at any other training-test intervals.

Discussion

The current findings that 0.5mM L-NAME produces retention loss by 40 minutes post-training when administered immediately post-training are consistent with the previous findings of Rickard et al. (1998), who used a time of administration 5 minutes before training. Further, only a small number of chicks were excluded from the data analysis due to not pecking at the non-aversive blue bead on testing thereby removing concerns raised by Bannerman et al. (1994) about the use of L-NAME in tasks acquired quickly.

In comparison with previous studies, minor variations in both magnitude and onset of the retention loss have been observed between research groups (Hölscher & Rose, 1992; Rickard et al., 1998) and may be due to differences in methodology or other environmental factors described above (Crowe & Hamalainen, 2001). While a temporal displacement of 10 minutes in the time of retention loss onset was noted, both Hölscher and Rose (1992) and Rickard et al. (1998) found NOS inhibition results in an impairment of retention during the ITM-B phase of memory formation as described by Ng & Gibbs, (1988) thereby providing behaviourally consistent results with respect to the stages of memory formation outlined by Gibbs and Ng (1979a). Importantly, the present study used the methodology of Rickard et al. (1998) with a post-training time of administration and demonstrated consistency with their findings. Thus, the loss of retention could not, in any measure, have been confounded by effects on acquisition.

SECTION 3 : The effect of protein kinase G inhibition on passive avoidance learning

A number of studies have demonstrated a role for PKG in memory formation using both rats and chicks subjected to passive avoidance learning. Studies by Bernabeu et al. (1997) and Izquierdo et al. (2000) clearly identified a role for cGMP activated PKG in memory formation using a step-down avoidance task for rats. Initially, Bernabeu et al. (1997) demonstrated that cGMP production temporally coincided with PKG activation immediately post-training. However, the effect of PKG activity upon retention was not established. To determine if increased PKG activity at the time of learning was simply the result of the application of the aversive stimulus or involved in memory formation *per se*, Izquierdo et al. (2000) utilised the same task but administered the specific PKG inhibitor, KT5823. Retention testing occurred 1.5 hours post-training, as a measure of short-term memory, and re-testing at 24 hours post-training, as a measure of long-term memory. It was noted that KT5823 had no effect on retention at test or retest when administered into the entorhinal cortex immediately post-training, although the infusion procedure took 30 seconds to complete. However, KT5823 was effective in blocking retention on re-test at 24 hours post-training when administered into the CA1 region of the hippocampus immediately post-training.

While such results would suggest an effect upon long-term memory formation, it cannot be deduced whether PKG is necessary for the consolidation or maintenance of long-term memory. This comes about as only a single time of test has been used to represent this latter stage of memory formation without knowing when the onset of long-term memory occurs. Further, this time of test, at 24 hours post-training, is actually a time of re-test. By measuring retention from animals already tested at 1.5 hours post-training the retention at 24 hours will be the product of the initial aversive learning experience and a second non-aversive learning experience associated with the original time of test. Therefore, the 24 hour time of re-test, although temporally distant from the 1.5 hour time of test, may not be a clear measure of long-term memory formation.

A major concern with the study of Izquierdo et al. (2000) is the choice of PKG inhibitor. While KT5823 is described as a specific inhibitor of PKG (Izquierdo et al., 2000) compared to H-8, which will also inhibit PKA at higher concentrations (Serrano et al., 1994), two studies have cast doubt on the reliability of KT5823. Wyatt, Pryzwansky and Lincoln (1991) found that KT5823 did not inhibit PKG in human neutrophils either *in vitro* or *in situ*. The addition of KT5823 to the neutrophil population did, in fact, result in dramatic shape changes, suggesting that this drug resulted in the overall activation of the cell population. A more recent study by Burkhardt et al. (2000) demonstrated that KT5823 failed to inhibit PKG using intact human platelet and rat mesangial cells, but was able to inhibit purified PKG. Nonetheless, the findings of Bernabeu et al. (1997) and Izquierdo et al. (2000) suggest the possibility that PKG activity in the hippocampus of the rat, is activated by cGMP immediately after training and is implicated in some aspects of memory processing.

Using a non-discrimination variant of the present single-trial passive avoidance task for juvenile chicks, Serrano et al. (1994) sought to investigate the role of PKG in memory formation. They administered 16.7mM H-8, intracranially 5 minutes prior to training and demonstrated a persistent loss of retention from 60 minutes post-training to the conclusion of the experiment at 120 minutes post-training. This corresponds to the LTM stage in the Gibbs and Ng model (Ng & Gibbs, 1989) and may be consistent with the findings of Izquierdo et al. (2000). However, Serrano et al. (1994) initially chose to examine only four concentrations of H-8, all of which were in the millimolar range then using only 16.7mM H-8 in subsequent retention studies. In using such high concentrations, Serrano et al. (1994) may have recorded non-specific drug effects which may not have been detected in the absence of a discrimination measure (refer to Chapter 2). More likely, the results of Serrano et al. (1994) may be attributed to the lack of specificity H-8 demonstrates towards PKG at higher concentrations. At high concentrations H-8 will inhibit a range of protein kinases including PKA (Serrano et al., 1994). This is further suggested by the findings of Zhao et al. (1995) who, using a single trial passive avoidance task for the day-old chick, determined retention loss following PKA inhibition to occur 60 minutes post-training. The same time as the retention loss observed by Serrano et al. (1994) using high concentrations of H-8. Furthermore, such concentrations were very high when compared with the micromolar concentrations of other kinase inhibitors used in previous passive avoidance studies

(Whitechurch et al., 1997; Zhao et al., 1995) or previous electrophysiological studies utilising H-8 (Matthies & Reymann, 1993).

3.3.1 H-8 inhibition of protein kinase G

Arguably, the best characterised pathway through which NO affects cellular processes is by activation of GC which in turn activates PKG. Further, PKG has been observed in previous behavioural studies to be necessary in memory formation (Serrano et al., 1994; Bernabeu et al., 1997; Izquierdo et al., 2000). Therefore it is reasonable that the action of PKG may underlie NO-activated memory formation processes in the current task. However, using NOS antagonists, Hölscher and Rose (1992, 1993) and Rickard et al. (1998) demonstrated the onset of retention loss by 40 minutes post-training, during ITM-B in the Gibbs and Ng model (Ng & Gibbs, 1989) which is in contrast to the findings of Serrano et al. (1994) who suggested the PKG antagonist, H-8, brought about retention loss from the onset of LTM. However, due to inherent limitations in their study a closer examination of the action of H-8 is necessitated. Most notable of these limitations being the restricted dose response range examined by Serrano et al. (1994), using millimolar concentrations of H-8, and the subsequent limited retention function using 16.7mM H-8. Not only are these concentrations considered very high and possibly result in the inhibition of kinases other than PKG, but the variant of the task employed by Serrano et al. (1994) could not assess sensory-motor effects as distinct from effects upon memory. In addition, a recent study by Crowe and Hamalainen (2001) has shown differences in a number of measured criteria between the variants of the passive avoidance task employed by different research groups using a single strain of chicks. They suggest, therefore, that comparisons between studies using different variants of the task may not be entirely appropriate without further investigation. Therefore it was deemed necessary to repeat the studies of Serrano et al. (1994) using a broader dose range, utilising a discrimination variant of the passive avoidance task and testing as late as 24 hours post-training.

3.3.1.1 Dose response study for H-8

Initially, a comprehensive dose response study was performed to determine if H-8 was amnesic at lower concentrations than those used by Serrano et al. (1994). If found, such a concentration would reduce the possibility of sensory-motor disturbances and inhibition of other protein kinases.

Method

Chicks were housed and trained as discussed in Chapter 2. Twenty chicks were used for each data-point, however, exclusions due to some chicks not meeting the training criterion and the refusal of others to peck the non aversive bead during testing resulted in data-points being based on groups of 15 to 17 chicks.

Chicks received either 154mM saline or one of various concentrations of H-8. The micromolar concentration range used was consistent with other studies within the Ng and Gibbs laboratory which have led to retention loss (Whitechurch et al., 1997; Zhao et al., 1995, 1996). The concentrations administered were 0.00μM (saline), 0.01μM, 1μM, 100μM, 150μM, 200μM, 250μM, 300μM or 500μM. The drug was injected freehand, using a Hamilton syringe (10μl per hemisphere), into the region of the neostriatum/hyperstriatum immediately after training. Post-training administration is preferred to avoid possible confounding effects of handling, the injection itself and the drug affecting the learning experience. Studies such as those of Bernabeu et al. (1996, 1997), Izquierdo et al. (2000) and Rickard et al. (1998) suggest that administration of H-8 immediately post-training would be most likely to result in a loss of retention.

Testing was conducted 120 minutes post-training, at a time when the Gibbs and Ng protein synthesis-dependent LTM stage is believed to have formed.

Results

Retention levels for chicks administered various concentrations of H-8 immediately post-training and tested 120 minutes post-training are shown in Figure 3.3.

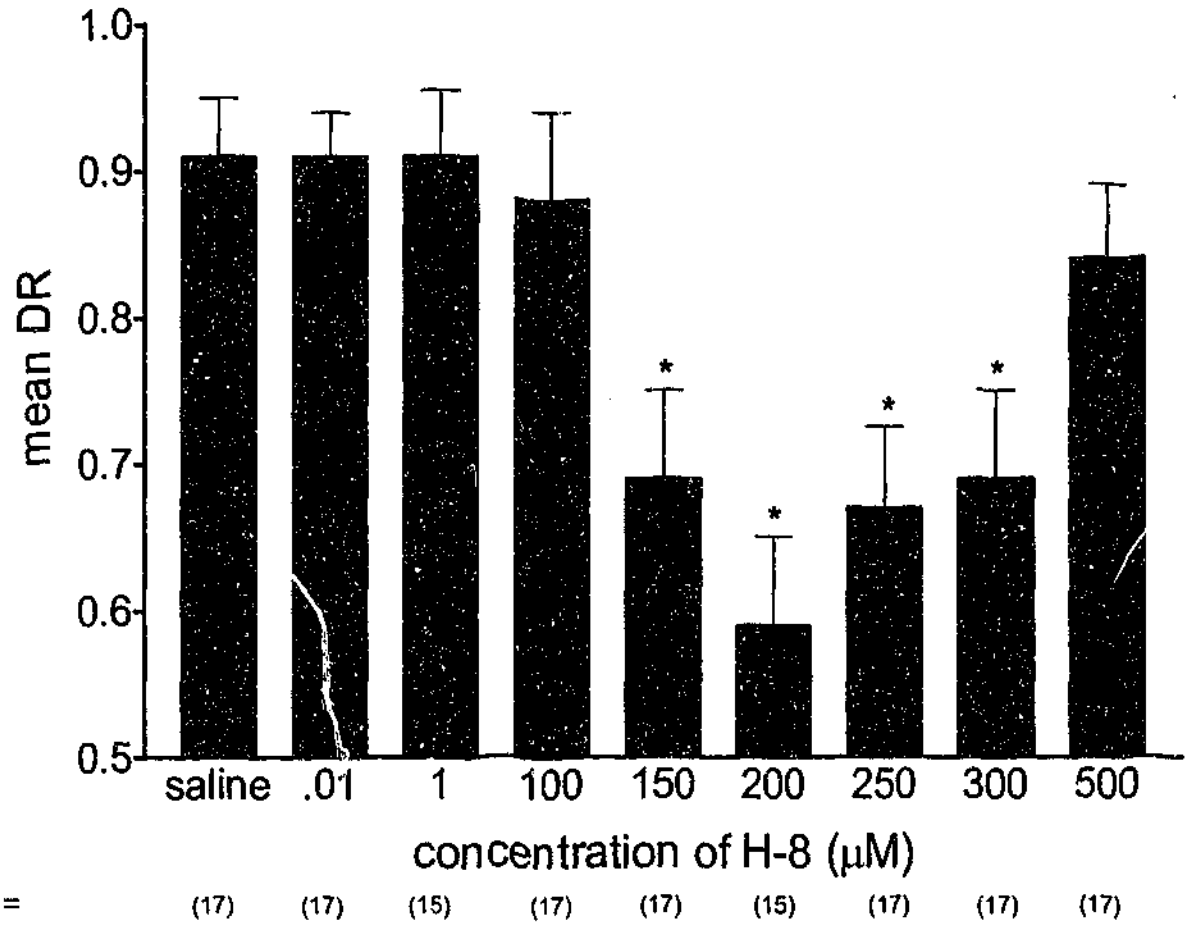


Figure 3.3 Dose response function for PKG inhibitor H-8.

Concentrations of H-8 were administered immediately post-training and retention was tested 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for individual groups of chicks, * $p<.05$.

Concentrations of 150μM, 200μM, 250μM and 300μM, but not lower or higher concentrations, appeared to result in a loss of retention. The maximum loss of retention was observed with 200μM H-8. A one-way ANOVA revealed a significant concentration effect ($F(8,140)=6.36$, $p<.001$). A Dunnett's post-hoc test revealed a significant loss of retention for chicks administered 150μM ($p<.005$), 200μM ($p<.001$), 250μM ($p<.001$) or 300μM ($p<.005$), but not other concentrations.

Discussion

The present dose response study demonstrated a range of concentrations of H-8 which were amnestic, with maximal retention loss observed using 200μM. The U-shaped time of administration function has been described by Calabrase and Baldwin (2001) as an example of hormesis. Such U-shaped curves are not uncommon in

toxicological research and demonstrate an attempt by the organism to achieve cellular homeostasis following the administration of a pharmacological antagonist.

In comparison, only four drug concentrations were used by Serrano et al. (1994) namely 3.0, 13.3, 16.6, and 19.9mM respectively and retention decreased from 78% to 23% with increasing dose. Excluding minor differences in methodology, Serrano et al.'s (1994) findings and those of the present study suggest that the dose response function for H-8 may be at least bimodal with effective dose ranges from 150μM to 300μM and >16.6mM. Such a dose response study may imply at least two effects of H-8. As H-8 has a greater specificity for PKG over other protein kinases it is preferable to use the lowest optimum concentration, being 200μM, as higher effective concentrations may be compromised by inhibition of other protein kinases or non-specific behavioural effects.

3.3.1.2 Time of administration study for H-8

A time of administration study was undertaken to determine if the effective times of H-8 administration matched those found with L-NAME (Rickard et al., 1998) and were consistent with those of Serrano et al. (1994) and Izquierdo et al. (2000). Like Rickard et al. (1998), Serrano et al. (1994) used an administration time of 5 minutes before training which is problematic as discussed previously (see section 3.2.1.1). However, unlike Rickard et al. (1998), Serrano et al. (1994) or Izquierdo et al. (2000) did not determine the range of effective administration times, thus being unable to determine if administration 5 minutes prior to training or immediately post-training was optimal. Although Izquierdo et al. (2000) used only a single time of administration, it was appropriate given Bernabeu et al.'s (1997) findings of increases in PKG activity immediately post-training using the same task.

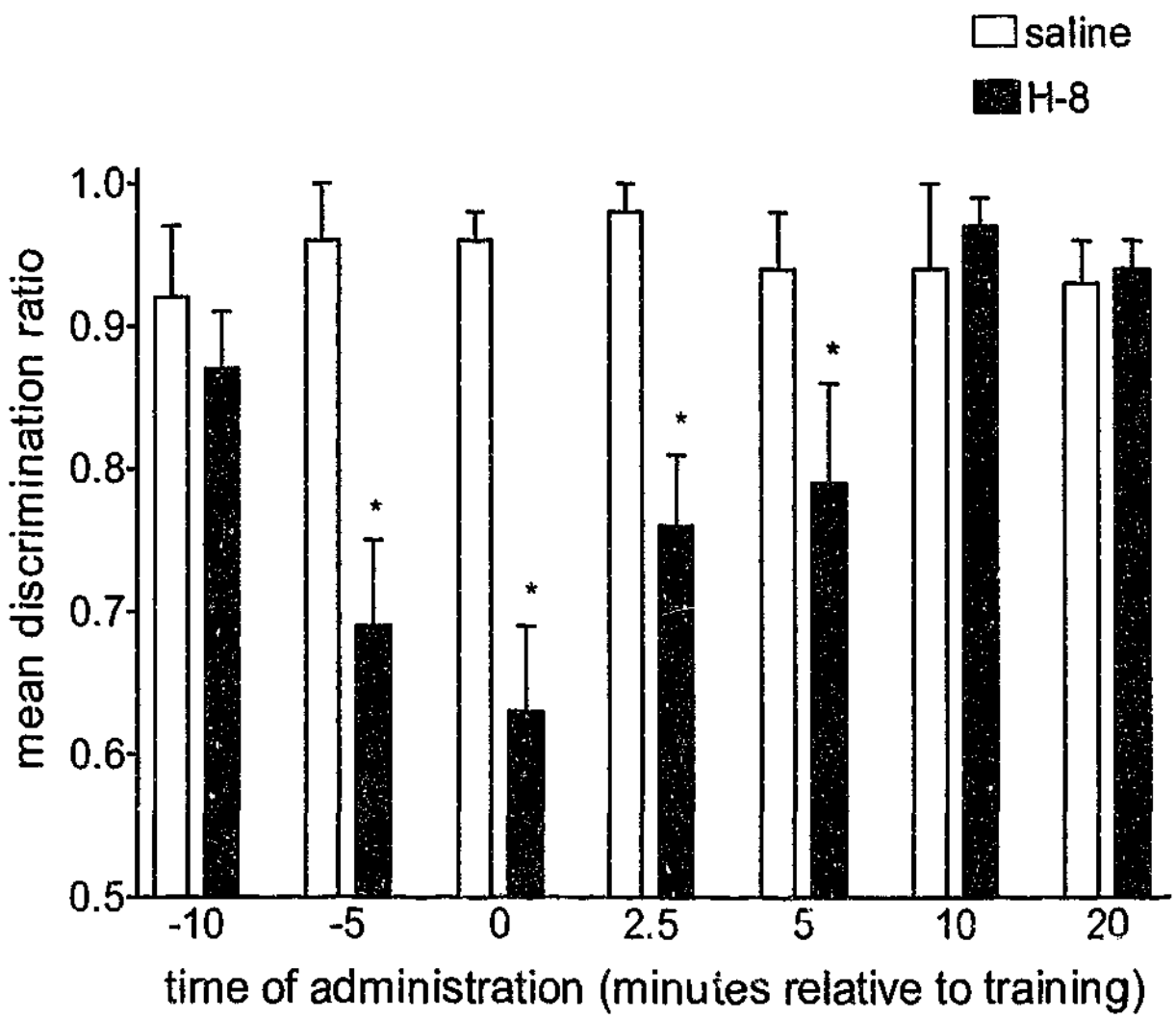
Method

Twenty chicks were used for each data-point although the final number of chicks per group ranged from 16 to 20 with a mean of 19. Chicks were housed and trained as previously described. Chicks were administered either 154mM saline or 200μM H-8 at different times relative to training. Administration occurred at –10 mins, -5 mins,

immediately post-training, 2.5 mins, 5 mins, 10 mins and 20 mins post-training as these times had been shown to be effective for the NOS inhibitor L-NAME (Rickard et al., 1998). The times of administration chosen included the times of administration previously used by Bernabeu (1996; 1997), Izquierdo et al. (2000) and Serrano et al. (1994). Chicks were tested for retention at 120 minutes post-training.

Results

Figure 3.4 describes the retention levels for chicks administered either H-8 or saline at various times relative to the time of training.



saline n =	(20)	(18)	(18)	(19)	(19)	(18)	(19)
H-8 n=	(20)	(18)	(19)	(17)	(18)	(16)	(20)

Figure 3.4 Time of administration function for the PKG inhibitor H-8.
200μM H-8, compared with saline, was administered at several times relative to training and tested at 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Figure 3.4 shows a considerable loss of retention for chicks administered H-8 between 5 minutes prior to and 5 minutes after training with no evidence of retention loss outside this range. A two-way ANOVA confirmed a significant drug effect ($F(1,245)=45.42$, $p<.001$), a significant time of administration effect ($F(6,245)=4.06$, $p<.001$), and a significant drug x time of administration interaction effect ($F(6,245)=6.38$, $p<.001$). Simple main effects post-hoc analyses demonstrated significantly lower retention levels for chicks administered 200μM H-8 either 5 minutes prior to training ($p<.001$), immediately after training ($p<.001$), 2.5 minutes post-training

($p<.001$) or 5 minutes after training ($p<.01$) than for chicks administered the vehicle at these times. No significant differences were found for the other times of administration.

Discussion

The above results demonstrate that H-8 is amnestic if administered between 5 minutes before training to 5 minutes after training. These results are consistent with the studies of Serrano et al. (1994) using a variant of the present task for the chick and the single trial aversive learning studies of Bernabeu et al. (1997) and Izquierdo et al. (2000) in the rat. Further, these results suggest PKG activity around the time of training.

The effective times of H-8 administration do not match those for L-NAME, being from 5 minutes before to 5 minutes after training in comparison to 5 minutes before training up to about 20 minutes post-training (Rickard 1995; Rickard et al., 1998), respectively. These findings would apparently imply that H-8 acts quickly *in vivo* as it is only effective if administered at times close to the learning event. Similarly, the brief period of effective administration times around the time of training suggest that it is activated close to the time of, and in response to, learning. This is in contrast to the broad range of effective administration times for L-NAME which extend up to 20 minutes post-training. Such a broad range most likely implies that the drug is not cleared from the cells quickly and that NOS is possibly activated at a distance to the learning event. Therefore it would appear that PKG activation may precede NOS activation. However, without specific knowledge of the pharmaco-kinetics of L-NAME and H-8 *in vivo* it is difficult to suggest this out-right. Therefore, although it would appear that PKG does not account for the action of NO associated with L-NAME inhibition of NOS a retention function study is necessary to clearly determine this. Finally, it must be noted that the nNOS inhibitor, NPLA, is effective only if administered from 5 minutes prior to training up to 2.5 minutes post-training (Rickard et al., in preparation – a,b) which is generally consistent with the effective times of administration of H-8. However, any association cannot be concluded without a comparison of retention functions.

3.3.1.3 Retention study for H-8

A retention function study was performed to determine at what time retention was first impaired by H-8. Serrano et al. (1994) had already performed a limited retention function study for H-8 using a non-discrimination version of the present avoidance task and found a loss of retention from 60 minutes post-training. However, such results were inconsistent with PKG as an NO-dependent mechanism as a difference of 20 minutes existed from the time of retention loss onset observed with L-NAME (Rickard et al., 1998). As stated earlier, methodological concerns with Serrano et al. (1994) may account for such differences. These include the use of a millimolar dose which was potentially problematic as H-8 can inhibit other kinases at high concentrations. Further, Serrano et al. (1994) could not account for sensory motor effects or other actions of the drug as only percentage avoidance was measured rather than the use of a discrimination ratio. Finally, the latest time tested by Serrano et al. (1994) was 120 minutes post-training. As later studies within this thesis demonstrate, retention may be restored for some drugs by 180 minutes post-training. Therefore an extensive retention function was investigated using 200μM H-8.

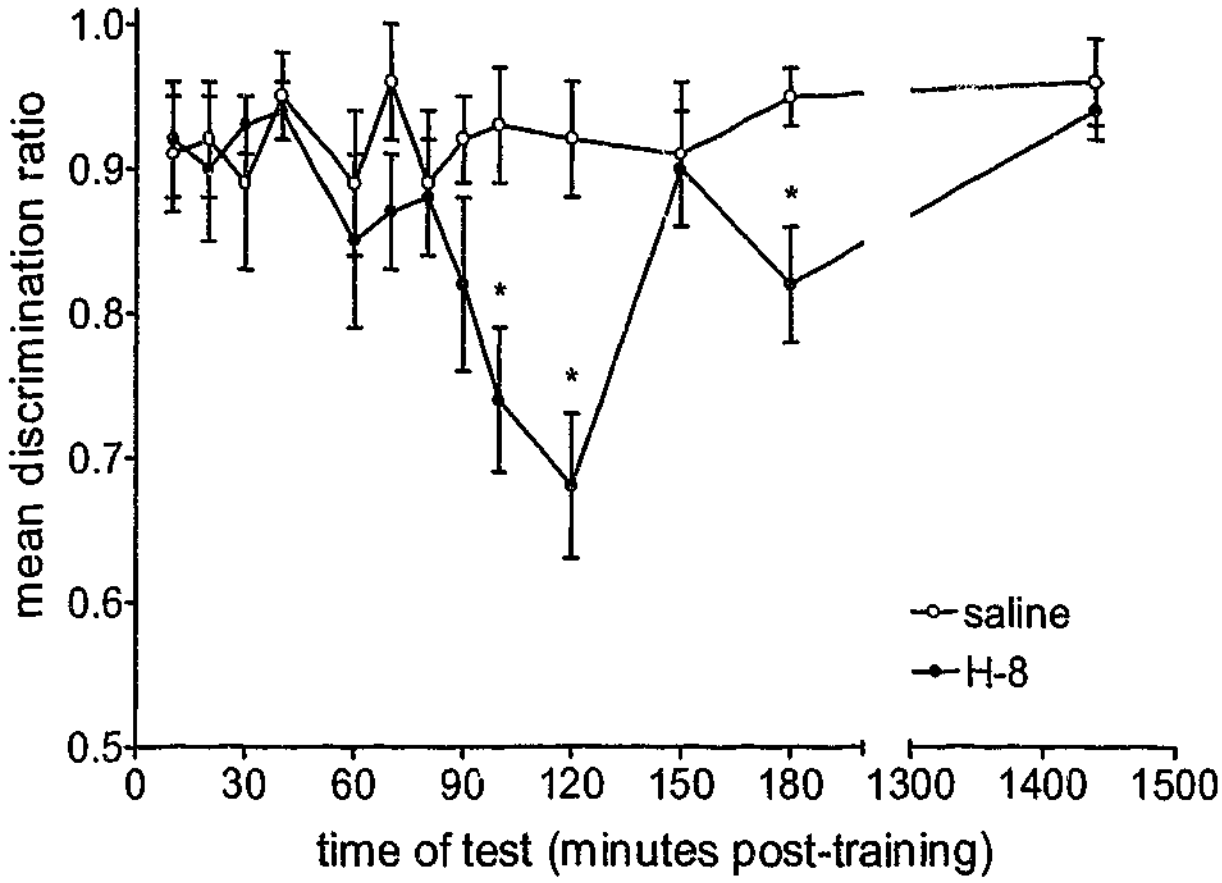
Method

Chicks were housed and trained as described previously in Chapter 2. Each data-point initially involved 20 chicks. However, the final number of chicks representing each data-point was between 14 and 38 with a mean of 18 due to the exclusion of chicks from the analyses and replication of some times of test across different experimental days.

Chicks were administered either 154mM saline or 200μM H-8 immediately post-training and tested at 10, 20 ,30, 40, 60, 70, 80, 90, 100, 120, 150 and 180 minutes and at 24 hours post-training.

Results

Figure 3.5 describes the retention functions for chicks administered 200μM H-8 or saline immediately post-training.



saline n =	(16)(14)(20)(16)	(15)(17)(15)(18)16)(15)	(15)	(18)		(17)
H-8 n =	(16)(17)(18)(38)	(15)(18)(19)(16)(27)(38)	(17)	(34)		(28)

Figure 3.5 Retention function for the inhibition of PKG.
200μM H-8 or saline was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

200μM H-8 resulted in a temporary retention loss centered at 120 minutes post-training. Test times at both 100 and 120 minutes showed a substantial loss of retention. Some retention loss also appeared to occur at 180 minutes post-training, with recovery by 24 hours post-training.

A two-way ANOVA yielded a significant drug main effect, ($F(1,490)=12.98$, $p<.001$), time of test main effect ($F(12,490)=1.99$, $p<.05$), and interaction effect ($F(12,490)=1.85$, $p<.05$) between drug and time of test. Simple main effects post-hoc analyses revealed a significantly lower level of retention in the H-8 treated chicks compared controls at 100 minutes ($p<.001$), 120 minutes ($p<.001$) and 180 minutes ($p<.01$) post-training.

Discussion

A transient retention loss beginning at 100 minutes post-training resulted following administration of 200μM H-8 immediately post-training with recovery by 150 minutes post-training and certainly by 24 hours post-training. While retention at 180 minutes post-training was significantly lower than that of saline-treated chicks it is possible that this difference was exaggerated by the unusually high retention level for the saline injected group and a slightly lower level of retention for the H-8 administered group. This may, therefore, be the result of sampling error.

This retention function is in stark contrast to that obtained by Serrano et al. (1994) who demonstrated permanent loss of retention from 60 minutes post-training with the differences in findings most likely due to the large differences in the concentrations of H-8 administered. Serrano et al. (1994) used 16.7mM concentration of H-8, however the dose response study for H-8 described in section 3.3.1.1 found that 200μM H-8 was sufficient to block retention. It is important to note that H-8, although most effective in blocking the activation of PKG (Ki=0.5μM), at higher concentrations also inhibits PKA (Ki=1.2μM) (Serrano et al., 1994). In this context it is of interest to note that Zhao et al. (1995) have shown that PKA inhibition results in a permanent loss of retention for day-old chicks trained on a single-trial passive avoidance task by 60 minutes post-training. It is, therefore, conceivable that Serrano et al. (1994) use of such a high H-8 dose inhibited PKA as well as PKG, and that the permanent retention loss at 60 minutes masked the temporary loss of retention after 100 minutes post-training for the inhibition of PKG as presently observed. Therefore, it is suggested that the present retention function is most likely due to inhibition of PKG while that derived by Serrano et al. (1994) is a consequence of PKA inhibition.

Transient retention losses are rarely observed in the current paradigm. To exclude drug effects, the time of retention loss must be related to the time of learning and not the time of H-8 administration. It can be deduced that the effect of H-8 is relative to the time of training by a comparison of the time of administration and retention functions found previously. In the time of administration study there is a 110 minute interval between the ineffective time of administration at 10 minutes post-training and the time of test at 120 minutes post-training. In the retention study the time of administration is immediately following training, therefore if the effect of H-8 was relative to the time of administration then the 110 minute time of test should also show

no retention loss. However, this was not observed suggesting the effect of H-8 is relative to the training event and not due to drug effects. This has since been confirmed in a retention study using a time of administration 24 hours post-training (see Appendix C).

With respect to the temporary nature of the retention loss observed in the present study Allweis et al. (1984) have suggested that such transient deficits indicate an effect upon memory retrieval rather than memory formation mechanisms. The reasoning behind this interpretation is that if memory formation is blocked then the trace is extinguished and no restoration of retention is possible. However, restoration of retention is possible if the underlying trace is not extinguished but simply that its ability to be expressed is temporarily blocked. This interpretation would suggest that there is a PKG-dependent stage of memory retrieval centred around 120 minutes post-training. This interpretation has recently been challenged by Bennett (1999) who suggested transient retention losses could in fact be due to effects upon memory formation processes as long as a multi-trace model of memory formation was assumed. However the interpretation of Bennett (1999) has yet to be tested using the current paradigm.

As discussed above, the range of effective administration times for H-8 occur around the time of training, consistent with those for the nNOS-specific inhibitor NPLA, but in stark contrast to the broad effective range of administration times for L-NAME which extend to about 20 minutes post-training. However, comparing the action of L-NAME, or NPLA, with H-8 upon retention shows obvious differences. While both L-NAME and NPLA (Rickard et al, 1998, in preparation - b) result in a persistent retention loss by 40 minutes post-training, H-8 results in a time of retention loss onset by 100 minutes post-training with restoration of retention by 150 minutes post-training. Therefore it cannot be concluded that H-8, inhibiting PKG, accounts for the actions of NO observed during the ITM-B stage of memory formation following administration of L-NAME.

SECTION 4 : The effect of guanylyl cyclase inhibition on passive avoidance learning

PKG is perhaps the best recognised effector mechanism for the action of NO, however, the studies presented so far suggest that PKG is unlikely to account for the action of NO in passive avoidance learning in the day-old chick. However, while it is unlikely that PKG is responsible for the actions of NO using the present task, the intermediary enzyme, GC, can also alter cellular biology through activation of either PDEs or CNG channels. Yet, these mechanisms are more obscure in their possible action upon memory processing. Thus, before directly testing the effect of either PDEs or CNG channels the action of GC will be evaluated. If GC inhibition does not share common characteristics with L-NAME with respect to effective times of retention loss onset then investigations into the role of GC-dependent mechanisms will not proceed.

3.4.1 ODQ inhibition of guanylyl cyclase

In pharmaco-behavioural investigations, it is important that the antagonist used is as potent and specific as possible. Ideally, the antagonist must not have secondary biochemical actions which may confound the results, nor be known to cause sensory-motor side-effects. Three of the best characterised GC antagonists are ODQ, LY83583 and MB. However, as MB is membrane impermeable (Kontos & Wei, 1993) and also inhibits NOS (Cohen et al., 2000) it is not appropriate for use in the present studies where the role of NO-activated GC, in contrast to other NO-dependent mechanisms, is being assessed.

In contrast, ODQ has been used frequently and in a variety of systems, to inhibit the soluble isoform of GC which is activated by NO. Boulton, Southam and Garthwaite (1995) found ODQ blocked hippocampal LTP production to the same degree as did administration of the NOS inhibitor, L-NArg. These results also suggest a relationship between NOS and sGC in the production of LTP. Garthwaite et al. (1995) extended this work by identifying ODQ as a potent,

selective and reversible inhibitor of sGC but not of the NO-insensitive particulate isoform of GC. Further, ODQ did not bind NOS, react with NO, or alter glutamate receptor function in the synapse. Finally, ODQ was found to have no effect on the production of other cyclic nucleotide second messengers, such as cAMP. Therefore, Garthwaite et al. (1995) concluded that ODQ was a highly efficient inhibitor of GC.

A recent study by Kemenes et al. (2002) has used a single trial appetitive task developed for the snail, *Lymnaea stagnalis*. Primarily seeking to identify a period of NO sensitivity leading to long-term memory formation they also briefly studied the role of GC within this paradigm. Administering ODQ (10μM) 10 minutes post-training resulted in a retention loss at test 24 hours post-training. As this time coincided with the extensive period of NO sensitivity previously identified, it was suggested NO activates GC leading to long-term memory formation.

Kendrick et al. (1997) studied olfactory memory formation in *post-partum* ewes. Using *in vivo* microdialysis of ODQ, they found that a 200μM concentration blocked cGMP production in the olfactory bulb. Even in the presence of a 1mM concentration of the spontaneous NO-donor SNAP, cGMP production did not increase, demonstrating the potency of ODQ. 200μM ODQ administered bilaterally to ewes from 6-24 hours before birth up to 8 hours *post-partum* impaired the ability to recognise progeny. This lack of recognition was attributed to an impairment of olfactory memory. Kendrick et al. (1997) also found that the effects of ODQ were reversible with recognition occurring by 16 hours *post-partum*.

Further confirmation of a role for GC in memory formation comes from Bernabeu et al. (1996) who found increased levels of cGMP up to 30 minutes post-training using a single trial step-down inhibitory avoidance task. In conjunction with these findings Bernabeu et al. (1997) and Izquierdo et al. (2000) found that administration of an alternate GC inhibitor, LY83583, immediately, but not at 30 minutes (Bernabeu et al., 1997), post-training resulted in retention loss. Taken together, these results suggest that GC may be activated around the times of training.

3.4.1.1 Dose response study for ODQ

The current experiment sought to determine if ODQ impaired retention using the discrimination passive avoidance task for the day-old chick. Previous *in vitro* studies

which have found ODQ to significantly attenuate either LTP or LTD have typically used doses in the micromolar range. For example, Boulton et al. (1995) found that concentrations of ODQ between 0.1μM and 10μM resulted in a dose-dependent decrease of NMDA receptor-activated cGMP production. In separate experiments they found 10μM ODQ reduced LTP production to a similar extent as 100μM N-Arg. Calabresi et al. (1999), Gage et al. (1997) and Wu, Wang, Rowan and Anwyl. (1998) found concentrations of 4μM or 10μM ODQ in a bath preparation effectively blocked LTD under various stimulation protocols although Wu et al. (1998) doubts a role for GC in LTP induction in the dentate gyrus. Finally, the *in vivo* studies of Kemenes et al. (2002) found 10μM ODQ effective in blocking retention for a single trial appetitive task using *Lymnaea stagnalis* while Kendrick et al. (1997) found 200μM ODQ effective in blocking olfactory recognition in ewes.

Method

Chicks were housed and trained as previously described in Chapter 2. Administration of ODQ was by freehand injection using a Hamilton syringe (10μl per hemisphere) into the region of the neostriatal/hyperstriatal region immediately after training. Twenty chicks were used for each data-point, although the final number for each data-point ranged between 16 to 20 chicks with a mean of 18.

A vehicle of physiological saline and <1% DMSO was used and concentrations of ODQ administered were 0.01μM, 0.1μM, 1μM, 10μM, 50μM, 100μM, 150μM, 200μM or 500μM. Retention was tested at 120 minutes post-training.

Results

Figure 3.6 describes the retention levels for chicks administered a range of concentrations of ODQ from 0.01μM to 500μM and tested 120 minutes post-training.

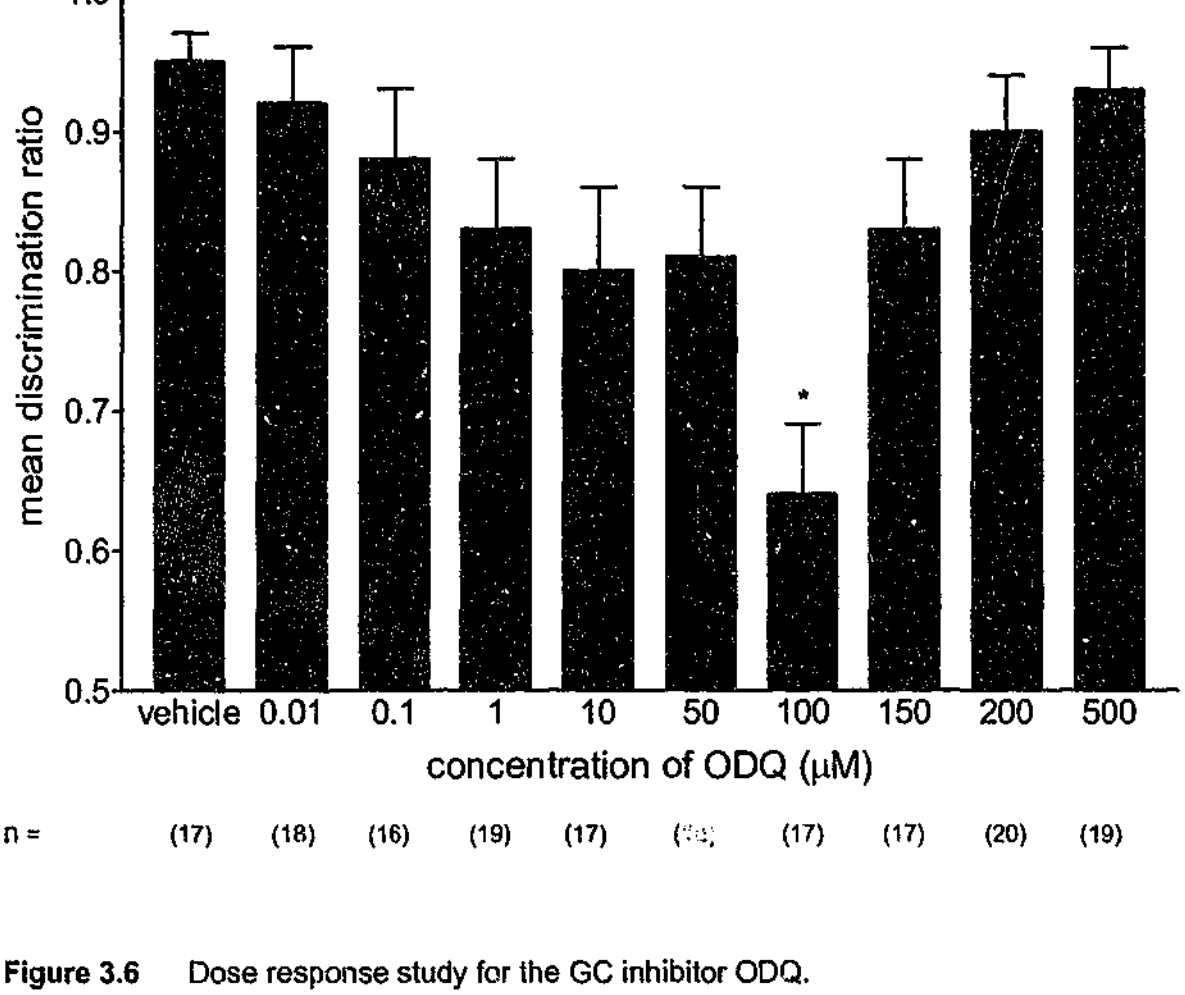


Figure 3.6 Dose response study for the GC inhibitor ODQ.

Various concentrations of ODQ were administered immediately post-training and retention was tested 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Only, 100μM ODQ resulted in a substantial loss of retention. A one-way ANOVA demonstrated a significant concentration effect ($F(9,166)=3.99$, $p<.001$) and a Dunnett's post-hoc analysis indicated a significant difference ($p<.001$) between the retention levels of chicks administered 100μM ODQ and chicks administered saline.

Discussion

Administration of various concentrations of ODQ immediately post-training resulted in a concentration-specific dose response curve with a significant retention loss occurring at 100μM. This concentration-specificity is markedly different from that observed in previous dose-response studies. For example, Rickard et al. (1998) found that concentrations between 0.5mM and 10mM of L-NAME were needed to block

memory formation. One explanation for the single low effective dose of ODQ presented here is that ODQ is a very specific and potent inhibitor of only the soluble form of GC, in comparison to the broad action of L-NAME upon all NOS isoforms reflected in a wide effective concentration range. However, a low effective concentration is consistent with the *in vivo* findings of Kendrick et al. (1997) but about 10 times higher than that used *in vivo* by Kemenes et al. (2002), or *in vitro* by Boulton et al. (1995), Gage et al. (1997), Wu et al. (1998) and Calabresi et al. (1999) to inhibit LTP and LTD. This difference to *in vitro* studies may be due to the diffusion characteristics of the drug in the chick brain as opposed to a slice preparation in a bath.

3.4.1.2 Time of administration study for ODQ

Rickard (1995) and Rickard et al. (1998) showed that L-NNA could be administered as early as 30 minutes before training and both L-NAME and L-NNA as late as 20-25 minutes post-training to produce retention loss. While such findings identify times of administration which will impair retention, at a more general level similarities in effective times of administration between drugs may also suggest underlying similarities in their method of action. When comparing the action of drugs on different enzymes, similar times of administration may tentatively be interpreted to mean temporally common times of enzyme activation. It must be noted, however, that between drugs, specific kinetic characteristics of each may result in some degree of variation in the effective times of administration. Similarly, drugs subject to degradation or clearance from the cell when injected at high concentrations may result in a broad range of effective administration times of which only the later times represent those when the enzyme would be active.

Previous investigations into the effect of GC inhibitors on memory formation have typically used, at most, a couple of times of administration, due to experimental limitations (Bernabeu et al., 1997; Izquierdo et al., 2000; Kemenes et al., 2002). One advantage of the single trial passive avoidance task for day-old chicks is that the time of learning can be clearly established. This allows the accurate determination of effective times of administration with respect to the time of learning. Therefore, a time of administration study was undertaken to examine a broad range of ODQ administration times.

Method

Chicks were housed and trained as per Chapter 2. The number of chicks representing each data-point ranged from 14 to 20, with a mean of 18, due to the exclusion of chicks which did not train or did not peck the non-aversive bead during training. Each chick received either 100μM ODQ or a vehicle of saline (154mM) containing <1% DMSO at either 10 and 5 minutes prior to training, immediately post-training and at 2.5, 5, 10, 20 and 25 minutes post-training. Each group of chicks was tested 120 minutes post-training.

Results

Figure 3.7 shows the loss of retention on testing at 120 minutes post-training for chicks administered either 100μ ODQ or a vehicle.

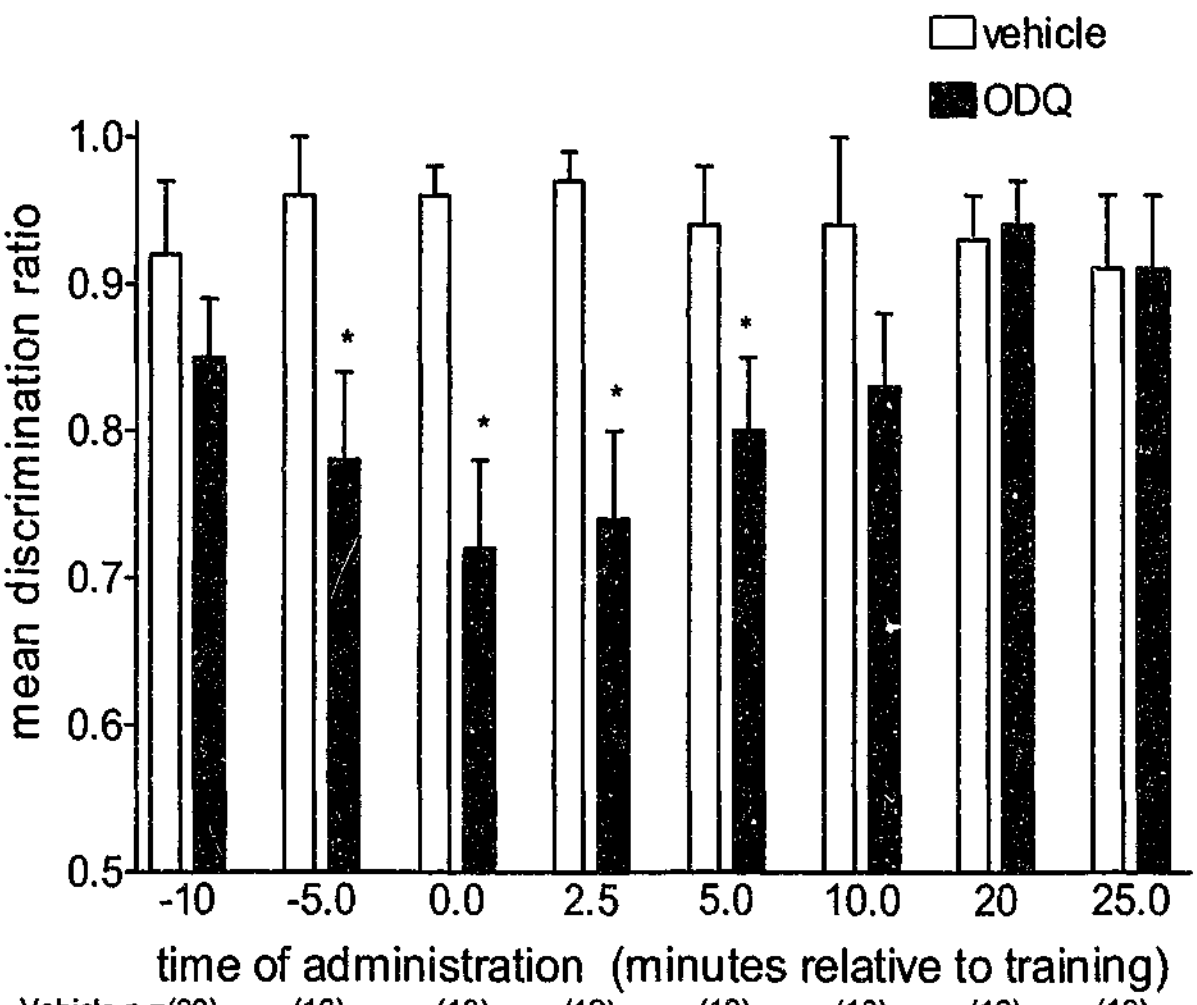


Figure 3.7 Time of administration function for the GC inhibitor ODQ.
100μM ODQ, compared with saline. ODQ or saline was administered at various times relative to training and tested at 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

A noticeable loss of retention is apparent at injection times around the time of training. The greatest retention loss appears to occur when ODQ was administered immediately post-training. A two-way ANOVA yielded a significant drug main effect ($F(1,264)=35.66$, $p<.001$) and a significant drug x time of administration interaction effect ($F(7,264)=3.05$, $p<.005$). The time of administration main effect ($F(7,264)=1.21$, $p>.05$) was not significant. Simple main effects analyses confirmed that chicks administered 100μM ODQ at 5 minutes prior to ($p<.001$), immediately after ($p<.001$), 2.5 minutes after training ($p<.001$) and 5 minutes after training ($p<.05$) demonstrated

significantly lower retention levels than chicks administered the vehicle at the same times.

Discussion

The current findings indicate a very restricted range of effective times of administration around the time of training, extending from 5 minutes before training to 5 minutes after training. This is consistent with the single trial aversive learning studies conducted by Bernabeu et al. (1996, 1997) and Izquierdo et al. (2000) on rats and generally consistent with the single trial appetitive task for *Lymnaea stagnalis* used by Kememes et al. (2002).

Kemenes et al. (2002) tested retention at 24 hours post-training for only one time of ODQ administration, 10 minutes post-training, which was found to be effective in blocking long-term memory. However, the most comprehensive studies detailing the action of GC in memory formation are those of Bernabeu et al (1996, 1997) and Izquierdo et al. (2000). Bernabeu et al. (1996), measured cGMP production at various times post-training, and found increased cGMP production immediately following the application of an inhibitory avoidance task. Even so, the increase persisted up to 30 minutes post-training. In comparison, Bernabeu et al. (1997) determined that the GC antagonist LY83583 was effective in a single trial avoidance task using rats when administered immediately post-training but not 30 minutes after training. The contrast in findings 30 minutes post-training between Bernabeu et al. (1996) and Bernabeu et al. (1997) may result if GC activity has ceased but cGMP has not been completely hydrolysed by PDEs. In subsequent pharmaco-behavioural studies Izquierdo et al. (2000) confirmed administration of LY83583 immediately post-training was effective in blocking retention at 1.5 hours and on retest 24 hours post-training.

In contrast to ODQ, L-NAME was found to inhibit retention when given as late as 20 minutes post-training. As with H-8, depending on the pharmacokinetic properties of L-NAME and ODQ, the difference in effective times of administration may suggest that GC is activated around the time of learning while NOS is activated after 20 minutes post-training and thus GC activation may precede the action of NO. This is supported to a limited extent by the studies of Gooderham, Soames, Rice, Boobis and Davies (1991) who determined that an alternate quinoxaline compound demonstrated rapid clearance from murine tissues. Therefore it is likely that ODQ acts on GC quickly and

thus GC is activated soon after learning while L-NAME acts on NOS sometime later. However, recent results by Rickard et al. (in preparation - b) show a similar set of effective administration times as those for ODQ when the nNOS-specific inhibitor, NPLA is administered. Taken together the pharmaco-kinetics of specific drugs render comparisons in times of effective administration times somewhat unreliable which is not the case with retention function studies. This comes about as if two enzymes are part of the same pathway leading to memory formation at a particular stage, as defined by Gibbs and Ng (1979a), then they must result in identical times of retention loss onset and show a persistence of retention loss.

3.4.1.3 Retention study for ODQ

In contrast with the three stage model of chick memory formation developed by Gibbs and Ng (1979a), stages of memory formation in the rat is described as either "short-term" or "long-term" (Izquierdo et al., 2000). In a broad sense, the chick protein synthesis-dependent stage may be equivalent to the long-term stage of rat memory formation as this stage is believed to represent the final laying-down of permanent memory. By analogy, both short- and intermediate-term memory stages in the chick may be considered to be equivalent to the short-term stage of rat memory formation. The behavioural studies of Bernabeu et al. (1997) and Izquierdo et al. (2000) have suggested a role for GC in the short-term stage of memory formation for rats trained on a single-trial step-down inhibitory avoidance task although Izquierdo et al. (2000) also found a loss of retention on retest 24 hours post-training. In this context, such findings would be consistent with the findings that NO activates GC since the passive avoidance studies of Hölscher and Rose (1992, 1993) and Rickard et al. (1998) demonstrated NOS inhibition to result in impairment of retention during the second phase of intermediate-term memory in the chick.

The possibility of NO activating GC in memory processing is reinforced by Bernabeu et al. (1995, 1996) who showed not only that NOS activity in the hippocampus increased immediately after training and returned to normal by 60 minutes post-training, but also that L-NNA could only be administered at either 10 minutes before or immediately after learning if memory was to be impaired. Consistent with this are the findings that cGMP formation was maximised immediately post-training

and while remaining elevated 30 minutes later, had returned to baseline levels by 60 minutes post-training. Therefore, there exists both behavioural and biochemical evidence for NO-induced cGMP production within memory processing.

The aim of the next experiment was to determine the time of retention loss onset following GC inhibition by ODQ. If the retention functions following NOS and GC inhibition are similar, it would be reasonable to conclude that NO is working through GC during memory formation for the passive avoidance task.

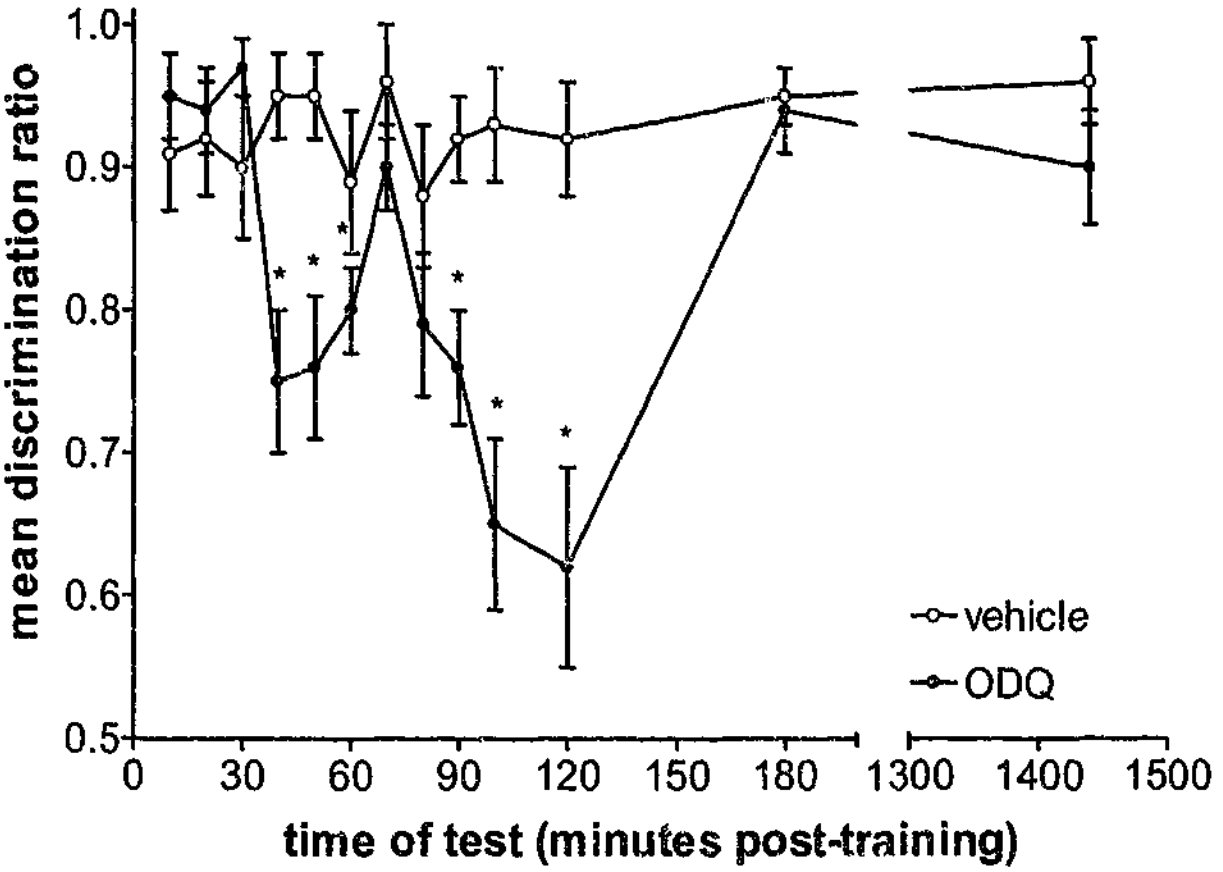
Method

Chicks were housed and trained as described in Chapter 2. The number of chicks per data-point ranged from 14 to 36 with a mean of 20, depending on the number of chicks excluded from the analysis and the replication of some data-points as stated earlier.

Chicks received either 100μM ODQ or a vehicle of 154mM saline, not containing more than 1% DMSO, immediately post training. Testing occurred at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120 and 180 minutes and at 24 hours post-training.

Results

The retention functions (mean DR and standard error of the mean at each training-test interval) for chicks administered 100μM ODQ or vehicle immediately post-training are shown in Figure 3.8.



Vehicle n =	(16)(14)(20)(16)(14)(15)(17)(15)(18)(16)(15)	(18)	(17)
ODQ n=	(17)(16)(18)(32)(31)(35)(35)(18)(36)(18)(13)	(18)	(26)

Figure 3.8 Retention functions for the inhibition of GC.
Chicks were given 100μM ODQ, or a <1%DMSO in saline vehicle immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Figure 3.9 shows two periods of retention loss for chicks treated with 100μM ODQ. Retention loss onset occurred 40 minutes post-training, with recovery by 70 minutes and a further period of retention loss beginning 90 minutes post-training with recovery by 180 minutes post-training at the latest. Normal retention levels then persisted at least until 24 hours post-training.

A two-way ANOVA revealed a significant drug main effect ($F(1,498)=31.88$, $p<.001$), a significant time of test main effect ($F(12,498)=3.28$, $p<.001$) and a significant interaction between drug and time of test ($F(12,498)=3.12$, $p<.001$). Simple main effects analyses demonstrated significant impairment of retention for ODQ-treated chicks, compared with controls, at 40mins ($p<.001$), 50mins ($p<.001$), 60mins ($p<.031$), 90mins ($p<.001$), 100mins ($p<.001$) and 120mins ($p<.001$).

Discussion

Administration of 100μM ODQ resulted in a biphasic retention function with the transient retention losses centered at 40 and 120 minutes respectively. Two features make this retention function unique within the current paradigm. First, a biphasic retention function has not been reported previously, following passive avoidance training. Second, the later temporary retention loss occurred after the consolidation of the protein synthesis-dependent LTM stage of the Gibbs and Ng model (Gibbs & Ng 1979a). While the retention function is obviously different from those for NOS and PKG inhibition, the onset of temporary retention loss at 40 minutes in the present study coincides with the onset of permanent retention loss for NOS inhibition. Furthermore, the second phase of temporary retention loss mimics that found for PKG.

While the pattern of retention loss is highly unusual, it is not inconsistent with the findings of Izquierdo et al. (2000), Kendrick et al. (1997) nor those of Kemenes et al. (2002) who found that ODQ impaired long-term memory formation in *Lymnaea stagnalis* at the single time tested, 24 hours post-training. Izquierdo et al. (2000) used an alternative inhibitor of GC, namely LY83583, and found that retention was inhibited in rats at 1.5 hours post-training and then on retest at 24 hours using a inhibitory shock avoidance task As discussed previously, stages of memory formation in the rat are described as "short-term" and "long-term". The short-term stage may be considered to incorporate the short-term and the intermediate-term memory stages in the chick while the long-term stage in both chicks and rats may be equivalent. Therefore, the loss of retention in the rat observed by Izquierdo et al. (2000) during both stages of memory formation could reflect the biphasic losses observed in the chick. However, Izquierdo et al. (2000) did not test retention at times between 1.5 and 24 hours or later than 24 hours. It cannot therefore, be determined whether the effects they observed were transient or not. Another concern with the study of Izquierdo et al. (2000) mentioned earlier was the retesting of animals at 24 hours. The 24 hour test may have been affected by the earlier retention test. Therefore, while it can be concluded that GC is necessary in the early stages of memory formation for rats, the permanency of this retention loss cannot be confidently inferred. More directly relevant, perhaps, was the observations by Kendrick et al. (1997) of a restoration of retention within 16 hours following cessation of ODQ infusion. This restoration is broadly consistent with the current findings

suggesting, in both cases, an effect on retrieval mechanisms rather than on memory formation pathways.

Before the present retention study can be fully interpreted, non-specific drug effects must be excluded. If drug effects were the underlying cause of the present results then it would be expected that the initial dose response study would demonstrate a progressive loss of retention as the concentration of ODQ increased. This was not observed, as the concentration of ODQ exceeded 100μM retention was progressively restored. Similarly, if the retention loss was the result of sensory-motor disturbances then a broad set of effective times of administration may be expected as the sensory-motor effect is a generalised toxic effect, not related necessarily to training or to the action of GC. Yet the effective times of administration occurred specifically around the time of learning suggesting a direct effect upon memory processes. Finally, it would be expected if sensory-motor disturbances were prominent, then large numbers of chicks would be expected to avoid both the aversive and the non-aversive bead on testing in all studies using ODQ. This also was not the case.

Nevertheless, the unique retention function generated by the administration of ODQ may still be the result of either subtle sensory-motor effects, or the concentration of ODQ used. To test these possibilities a number of experiments can be undertaken. First, to exclude subtle sensory-motor effects, it must be determined whether the retention loss is temporally related to the time of drug administration or the time of training. As with H-8, comparisons between the time of administration function and retention function are of value. As administration of ODQ 10 minutes post-training does not result in retention loss when tested 120 minutes post-training it may be surmised that there should be no loss of retention 110 minutes post-administration following administration immediately post-training if retention loss was due to a drug-effect. However, when administered immediately post-training, ODQ resulted in a loss of retention from between 90 and 120 minutes post-training. Therefore it is likely that the action of ODQ is related to the training event and not the time of administration. This was confirmed by a study administering ODQ 24 hours post-training and testing retention at times between 40 and 120 minutes post-administration with no retention loss observed (see Appendix C). Second, to detect dose-specific effects of ODQ in contrast to generalised effects of the drug, dose response studies can be conducted with different times of test. The initial dose response study using ODQ determined the latter transient retention deficit to be dose-specific. However, the earlier transient retention

deficit and the intervening restoration of retention may not be. Finally to verify the action of ODQ on GC the effect of another inhibitor of GC, LY83583, was determined. ODQ and LY83583 do not share common secondary actions upon other enzymes nor impair cGMP production in the same way. Therefore any common effect must be due to inhibition of GC. Only once these criteria have been forfilled can the bimodal retention function produced by ODQ administration be considered an accurate representation of GC inhibition within the current task.

3.4.1.4 Test of dose dependency for ODQ

The initial dose response study for ODQ found 100μM impaired retention when tested 120 minutes post-training. This time of test coincides with the later temporary retention losses observed in the initial retention study using ODQ (see section 3.4.1.3). However, the dose response characteristics of ODQ for the earlier temporary retention loss and the time period between the two transient retention losses were unknown. This experiment was to determine if the earlier transient retention loss was concentration-specific and if any concentration of ODQ brought about retention loss between the two transient losses observed previously in the retention study. In doing so, generalised or alternate effects of ODQ may be identified.

Method

Chicks were housed and trained in accordance with the methodology set out in Chapter 2. Chicks were administered 0μM (vehicle), 1μM, 10μM, 100μM, 500μM or 1000μM ODQ immediately post-training. Retention was tested at 40 minutes and 70 minutes post-training in separate experiments. These times were chosen to reflect the first phase of retention deficit and the time of recovery, respectively.

Group numbers ranged from 14 to 18 with a mean of 17, tested at 40 minutes post-training, and from 15 to 20, with a mean of 18, tested at 70 minutes post-training. The differences in group numbers were brought about by the exclusion of chicks from the analyses which did not train or did not peck the non-aversive bead during testing.

Results

Figure 3.9(A) gives the mean DRs for the range of concentrations of ODQ used when retention was tested 40 minutes post-training. Figure 3.9(B) give the corresponding mean DRs for retention tests 70 minutes after training.

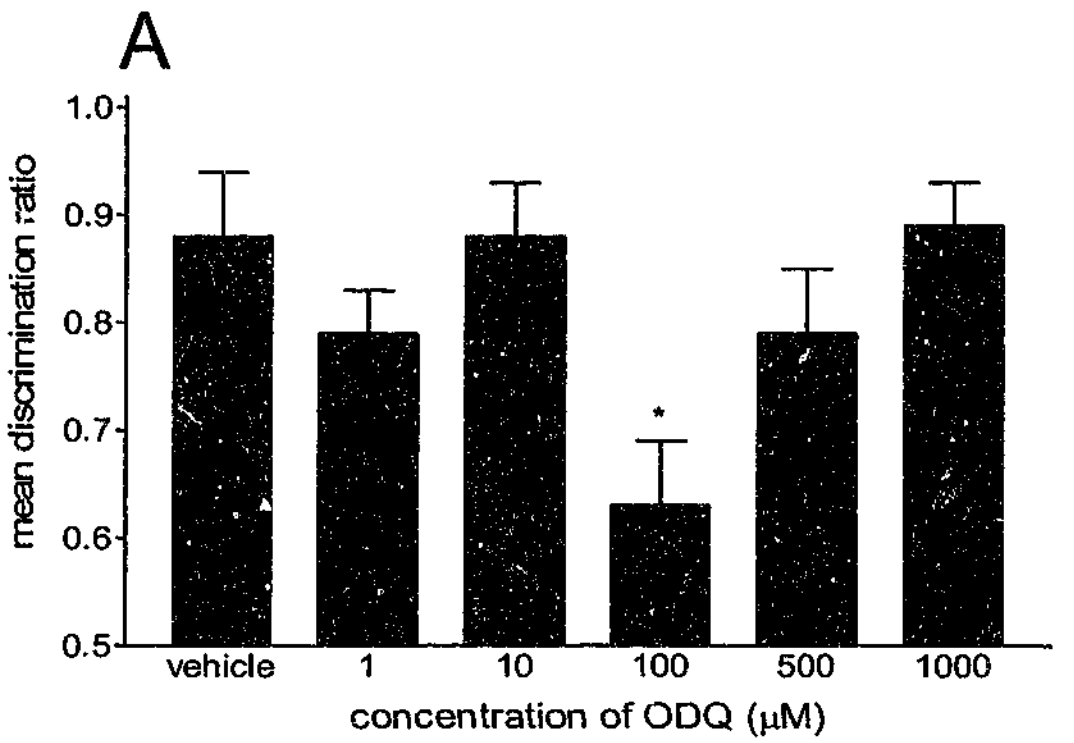


Figure 3.9 Dose response functions for ODQ tested 40 (A) or 70 (B) minutes post-training. The effect on retention of various concentrations of the GC inhibitor ODQ administered immediately post-training and tested at 40 minutes and 70 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for individual groups of chicks, * $p<.05$.

Figure 3.9(A) shows a retention loss at 40 minutes post-training only for chicks treated with 100μM ODQ. At 70 minutes post-training (Figure 3.9(B)) there was no obvious retention loss for any concentration of ODQ.

A one-way ANOVA was performed for each data set. For the 40 minutes post-training retention test, a significant treatment effect was found ($F(5,93)=3.27$, $p<.01$). Dunnett's post-hoc analysis found only the 100μM dose resulted in a significant difference in retention levels when compared with the vehicle ($p<.05$). The 70 minutes post-training test revealed no significant treatment effect when analysed using a one-way ANOVA ($F(5,100)=.77$, $p>.05$).

Discussion

The effect of ODQ tested at 40 minutes post-training is clearly dose-specific. 100μM ODQ resulted in a significant loss of retention, which was consistent with the initial dose response study tested at 120 minutes. Further, there was no retention loss observed using 1mM ODQ, a concentration higher than that used in the initial dose response study tested at 120 minutes post-training suggesting the dose response curve was not bi-phasic. The dose-specific nature of the retention loss suggests that the effect is directly upon memory processes, since if retention loss was a product of drug toxicity then retention loss may be proportional to drug concentration.

As retention tested at 70 minutes post-training was not impaired by any concentration of ODQ tested, it is suggested that there are two distinct temporary retention losses caused by the administration of ODQ. Taken together these results suggest that ODQ has a dose-specific effect upon memory recall at both 40 and 120 minutes post-training, with recovery at 70 and 180 minutes respectively.

3.4.2 LY83583 inhibition of guanylyl cyclase

To further clarify the nature of the unique retention function observed following ODQ administration it would be informative to replicate the studies using an inhibitor of GC whose mechanism of action upon GC is different to ODQ, which did not share any secondary effects with ODQ and has been used previously in behavioural studies.

If the second GC antagonist results in the same findings as ODQ, this would confirm the action of ODQ upon GC.

Two other GC inhibitors have been well characterised, namely MB and LY83583. However, as MB is membrane impermeable and acts directly on GC, like ODQ, it does not provide a useful alternative. In contrast, LY83583 is membrane permeable and acts on GC via different mechanisms to ODQ and does not share common secondary-effects with ODQ.

LY83583 is more complex than ODQ with respect to both its specificity and mode of action. There has been dispute as to whether LY83583 is sGC specific or will inhibit both sGC and pGC isoforms (Malta, 1989; Malta, MacDonald & Dusting, 1988). As for secondary actions upon other enzymes, LY83583 does not inhibit the production of cAMP (Schmidt, Sawyer, Truex, Marshall & Fleisch, 1985), but will inhibit glutathione reductase at high concentrations (Luond, McKie & Douglas, 1993).

In contrast to the direct inhibition of GC brought about by ODQ, LY83583 may inhibit cGMP production in up to three different ways. Mülsch, Busse, Liebau and Förstermann (1988) determined two mechanisms by which LY83583 may interfere with cGMP production. *In vitro* studies using rabbit endothelial cells found that LY83583 directly inhibited GC, but at higher concentrations than required for LY83583 to generate superoxide radicals. Therefore, LY83583 will scavenge NO by the production of superoxide radicals at all concentrations, but only directly inhibit GC at higher concentrations. Thus, it may be argued that production of superoxide radicals is the prime mechanism through which LY83583 acts. A third mechanism through which LY83583 may block cGMP production was suggested by Sundqvist and Axelsson (1993) who demonstrated that LY83583 consumes NADPH. As NADPH is required for NOS function, it may be surmised that LY83583 may impede the action of NOS as well as GC. The direct action of LY83583 upon NOS was further reinforced by more recent studies which have suggested that LY83583 is a potent inhibitor of rat cerebellar NOS (Luo, Das & Vincent, 1995). If so, this is problematic if LY83583 is being used to differentiate between the actions of NOS and GC, as in the present research. However, studies by Malta et al. (1988, 1989) and Mülsch, Luckhoff, Pohl, Busse and Bassenge, (1989) clearly demonstrate the action of LY83583 upon GC. In a series of experiments, LY83583 was shown to be capable of blocking NO-independent but GC-dependent functions such as atrial natriuretic peptide (ANP) induced vasodilation. Rightly or not,

the authors interpreted these results as indicating that the prime action of LY83583 was upon GC.

Further complexity in the action of LY83583 was demonstrated by Schmidt et al. (1985) who found that although LY83583 resulted in decreased cGMP formation in a number of guinea-pig tissues, including cerebellum, splenic tissue appeared to be resistant to LY83583, while administration of LY83583 increased cGMP activity in cell-free preparation from guinea-pig lung. However, as suggested by Mülsch et al. (1988) determination of sGC activation in crude extracts may be complicated by the presence of other, endogenous, GC inhibitors such as ATP, haem-containing proteins and flavins, which may also be inhibited by LY83583, therefore leading to an overall activation of sGC. Therefore, it is important to note that the action of LY83583 appears to be more complex than that of ODQ.

Bernabeu et al. (1997) and Izquierdo et al. (2000) found that LY83583 impaired retention for a single trial step-down inhibitory avoidance task for male Wistar rats. Bernabeu et al. (1997) bilaterally implanted cannulae 1mm above the CA1 region of the hippocampus. Each rat received bilateral 0.5µl infusions of LY83583 (2.5µg per hemisphere equivalent to 20mM in the present research) or saline administered either immediately post-training or 30 minutes after training. Retention was impaired at 24 hours only when administration was immediately post-training. Using the same protocol as Bernabeu et al. (1997), Izquierdo et al. (2000) tested retention at 1.5 hours post-training to determine the effect of various drugs upon short-term memory processes, and retested 24 hours post-training to test their effect upon long-term memory processes. They found that LY83583 infused bilaterally into the CA1 region of the hippocampus over a 30 second period per hemisphere resulted in an impairment of retention at both test and retest, indicating an effect upon both short-term and long-term memory.

While previous research strongly suggests a role for GC in memory processing, the possible secondary effects of LY83583 on NOS may have confounded interpretation of results. This is suggested to some extent by the overlapping effective times of administration for NOS and GC inhibition in rats (Bernabeu et al., 1995; Bernabeu et al., 1997). Therefore, the present experiments sought only to use LY83583 to confirm the effects of ODQ.

3.4.2.1 Dose response study for LY83583

The concentrations tested were based on those found to be effective for ODQ in both LTP and behavioural studies (Boulton et al., 1995; Calabresi et al., 1999; Gage et al., 1997; Garthwaite et al., 1995; Kendrick et al., 1997; Wu et al., 1998). Although Bernabeu et al. (1997) and Izquierdo et al. (2000) found the equivalent of a 20mM solution of LY83583 effective in blocking retention, such high concentrations are not typically tolerated in the passive avoidance task for the day-old chick. In addition to obvious species differences, the disparity in tolerance limits may be accounted for as Bernabeu et al. (1997) and Izquierdo et al. (2000) infused LY83583 over a period of many seconds into rats in contrast to the discrete injection given to day-old chicks.

Method

Chicks were housed and trained as discussed earlier in Chapter 2. Groups of 20 chicks were initially used for each data-point, however, following exclusions and repetition of data-points across experimental days group sizes ranged between 15 and 36 with a mean of 26. Each group of chicks was administered a different concentration of LY83583: 0μM (vehicle), 0.1μM, 1μM, 10μM, 40μM, 70μM, 100μM, 150μM, 200μM, 250μM or 400μM. The drug was given immediately post-training. This concentration range was based on the effective concentration for ODQ (see section 3.4.1.1) and the time of administration was chosen based on the prior studies of Bernabeu et al. (1996, 1997) and to a lesser extent Rickard et al. (1998). The control vehicle was 154mM saline containing <1% DMSO. Testing occurred 120 minutes post-training, which coincided with the second temporary retention loss found using ODQ.

Results

Mean DRs for chicks administered the various concentrations of LY83583 and tested 120 minutes post-training are shown in Figure 3.10.

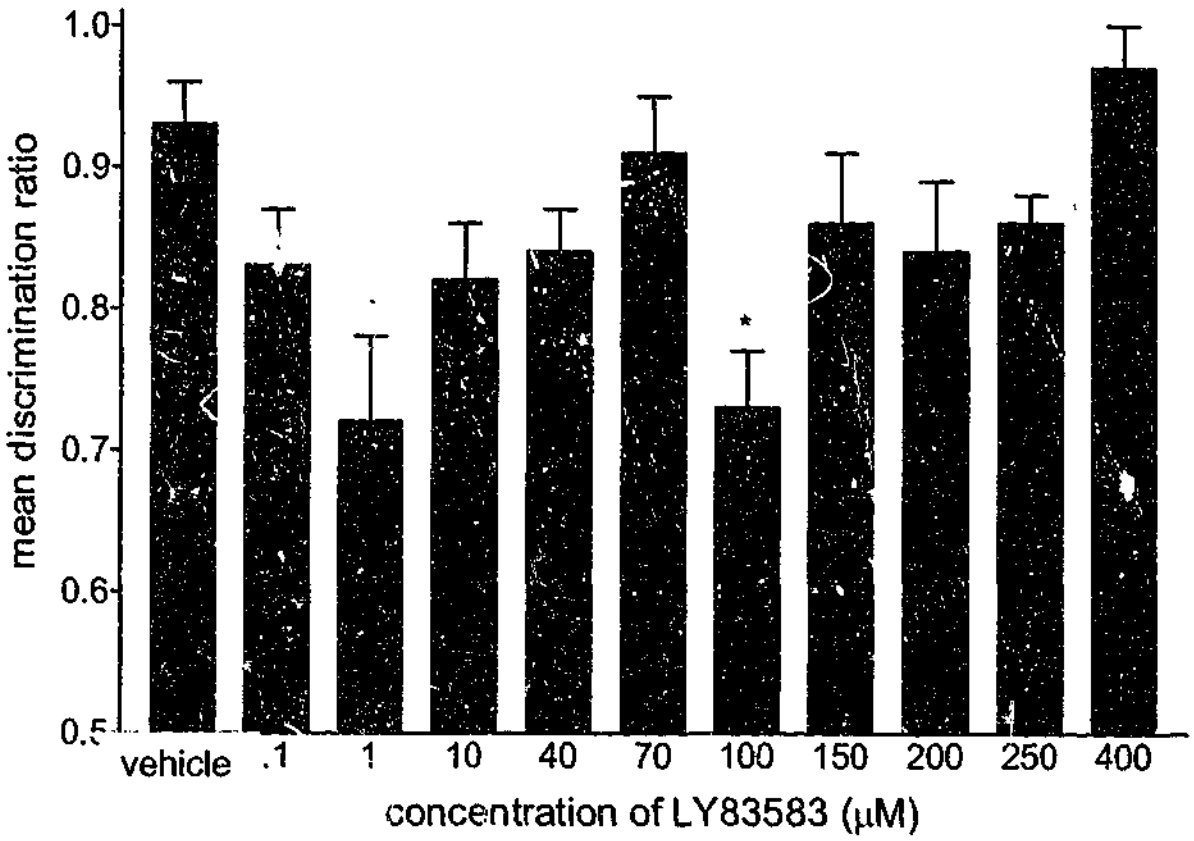


Figure 3.10 Dose response function for the GC inhibitor LY83583.
Various concentrations of LY83583 were administered immediately post-training and retention was tested 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Retention loss for the range of LY83583 concentrations from 0.1μM to 400μM resulted in a bimodal function. Substantial impairment of retention were observed with 1μM and 100μM LY83583. A one-way ANOVA yielded a significant concentration effect ($F(10,280)=3.445$, $p<.001$). Dunnett's post-hoc analysis showed significant impairment of retention, compared with controls, for chicks treated with 1μM ($p<.01$) and 100μM ($p<.001$) LY83583. No other concentration tested resulted in a significant retention loss.

Discussion

The result suggests at least a bimodal dose response function for LY83583. The effective concentrations of LY83583 in the present study were substantially different from those found effective for inhibiting avoidance learning in rats (Bernabeu et al., 1997; Izquierdo et al., 2000). While the present study found 1μM and 100μM

concentrations to be effective in inducing retention deficits, Bernabeu et al. (1997) and Izquierdo et al. (2000) used the equivalent of a 20mM solution. The large difference in doses between the present study and those of Bernabeu et al. (1997) and Izquierdo et al. (2000) may be due to both species and task differences. One anatomical difference between rats and chicks which may impact upon the concentration of drug needed is the greater intercellular space in the chick brain compared to the rat (Sykova, Jendelova, Svoboda, Sedman & Ng, 1990). It is reasonable to suggest that drug diffusion would be impeded in the rat. Therefore a higher dose may be required in the rat to produce a loss of retention.

3.4.2.2 Time of administration study for LY83583

The dose response study for LY83583 demonstrated that it was an effective amnestic agent when administered immediately post-training. The present experiment was aimed at establishing a time of administration function for LY83583.

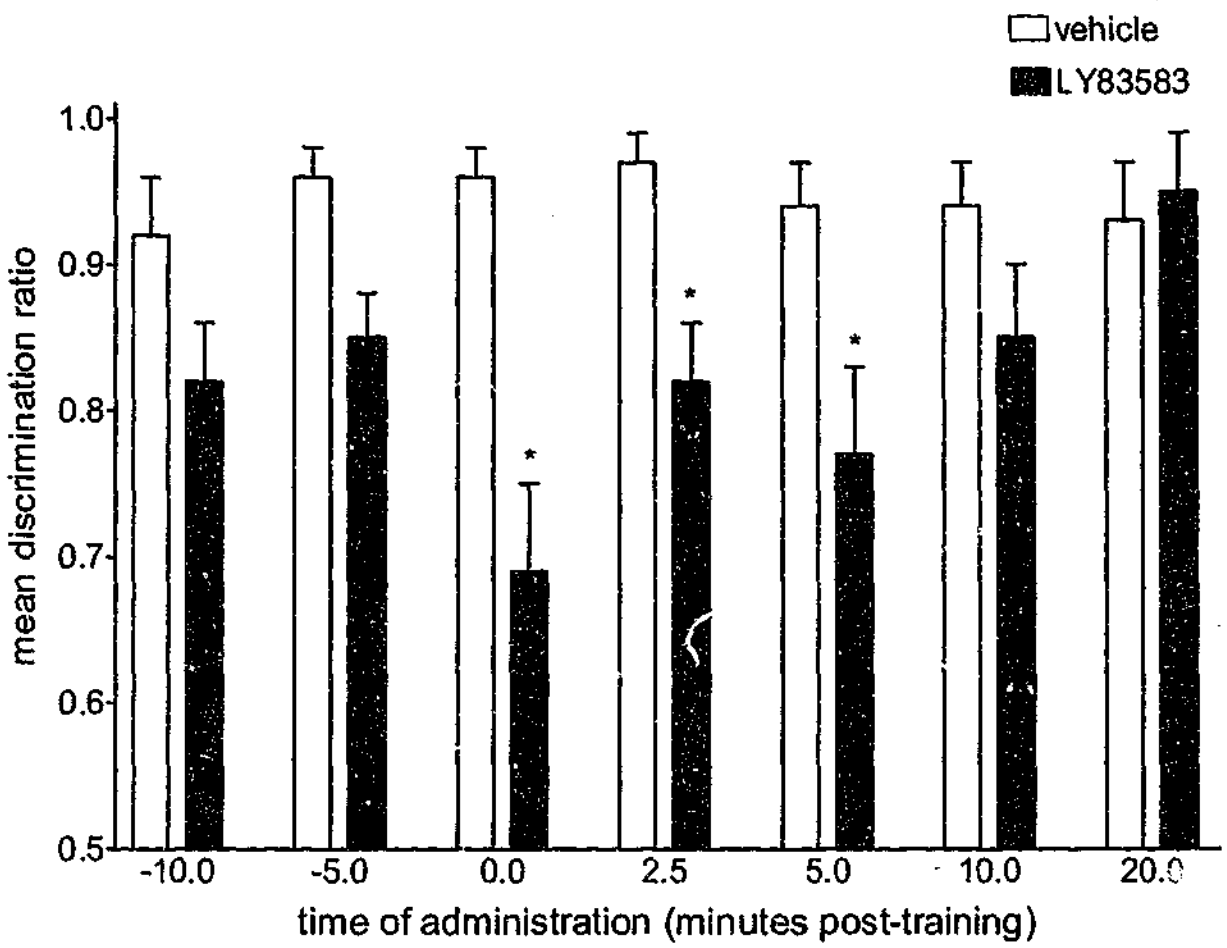
Method

Chicks were housed and trained as per Chapter 2. Each group of 20 chicks received either 100μM LY83583 or a vehicle (saline containing <1% DMSO). Although the dose response study for LY83583 resulted in a bimodal function with both 1μM and 100μM being effective, 100μM LY83583 was chosen for this experiment and the subsequent retention function experiment as 100μM ODQ had been previously found to impair retention. Bernabeu et al. (1997) and Izquierdo et al. (2000) had also shown millimolar doses of LY83583 to be effective in blocking retention in the rat, therefore suggesting 100μM and not 1μM to be the most likely effective concentration. Only if the effective times of administration and retention function did not match those for ODQ would 1μM LY83583 be tested. Times of administration for both LY83583 and the vehicle were 10 and 5 minutes prior to training, immediately post-training, and 2.5, 5, 10 and 20 minutes post-training.

The number of chicks for each data-point ranged from 14 to 20 with a mean of 17. Retention was tested at 120 minutes post-training.

Results

Mean DRs for chicks administered either 100μM LY83583 or a vehicle at a range of times relative to the time of training are given in Figure 3.11.



Vehicle	n =(20)	(18)	(18)	(19)	(19)	(18)	(19)
LY83583	n =(17)	(16)	(15)	(19)	(15)	(18)	(19)

Figure 3.11 Time of administration function for the GC inhibitor LY83583.
100μM LY83583, or a saline control vehicle, was administered at various times relative to training and tested at 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

A substantial loss of retention was observed with LY83583 given immediately after training. A two-way ANOVA revealed a significant drug main effect ($F(1,232)=29.57$, $p<.001$). However, neither the time of administration main effect ($F(6, 232)=1.43$, $p>.05$) nor the interaction effect ($F(6,232)=1.91$, $p>.05$) was significant. This suggests that LY83583 resulted in retention loss at all the times of administration used. However, the most substantial loss occurred immediately post-

training. A simple main effects analysis yielded a significant difference in mean DRs between drug and control groups when administration was given immediately post-training ($p<.001$), 2.5 minutes post-training ($p<.05$) and 5 minutes post-training ($p<.01$), but at no other time of injection. While post-hoc tests are strictly not justified in the absence of a significant interaction effect in a two-way ANOVA, they provide some informal indication that retention losses may be greater for some times of administration.

Discussion

It would appear from Figure 3.11 that administration of LY83583 must occur soon after the time of learning to cause a moderate retention loss. This is consistent with the effective times of administration for ODQ. The current research is also consistent with the studies of Bernabeu et al. (1997) and Izquierdo et al. (2000) who determined that administration of LY83583 immediately post-training resulted in amnesia.

Such a limited effective range of administration times would suggest the drug is effective in blocking the action of GC soon after learning and is removed from the cell shortly afterward. This is supported by limited evidence suggesting a similar isoquinonlinedione derivative is subject to fast removal from rat tissues except when in contact with gastric juices (Kim, Moon, Ryu & Lee, 2000).

3.4.2.3 Retention study for LY83583

Studies such as those by Bernabeu et al. (1995, 1996, 1997) and Izquierdo et al. (2000) suggest that NO affects retention for an inhibitory avoidance task by activating GC around the time of training. If preserved in the chick, this simple relationship would be expected to produce common retention functions following NOS and GC inhibition. While L-NAME resulted in persistent retention loss by 40 minutes post-training when administered from before training up to as late as 20 minutes post-training this did not occur following ODQ administration. ODQ produced a narrow range of effective administration times with administration of 100μM ODQ immediately post-training resulted in a biphasic retention function. Therefore it appeared that NO was unlikely to

activate GC in passive avoidance learning using the neonate chick. However, there exists other explanations for the biphasic retention function generated by ODQ which may clarify any possible relationship between NO and GC.

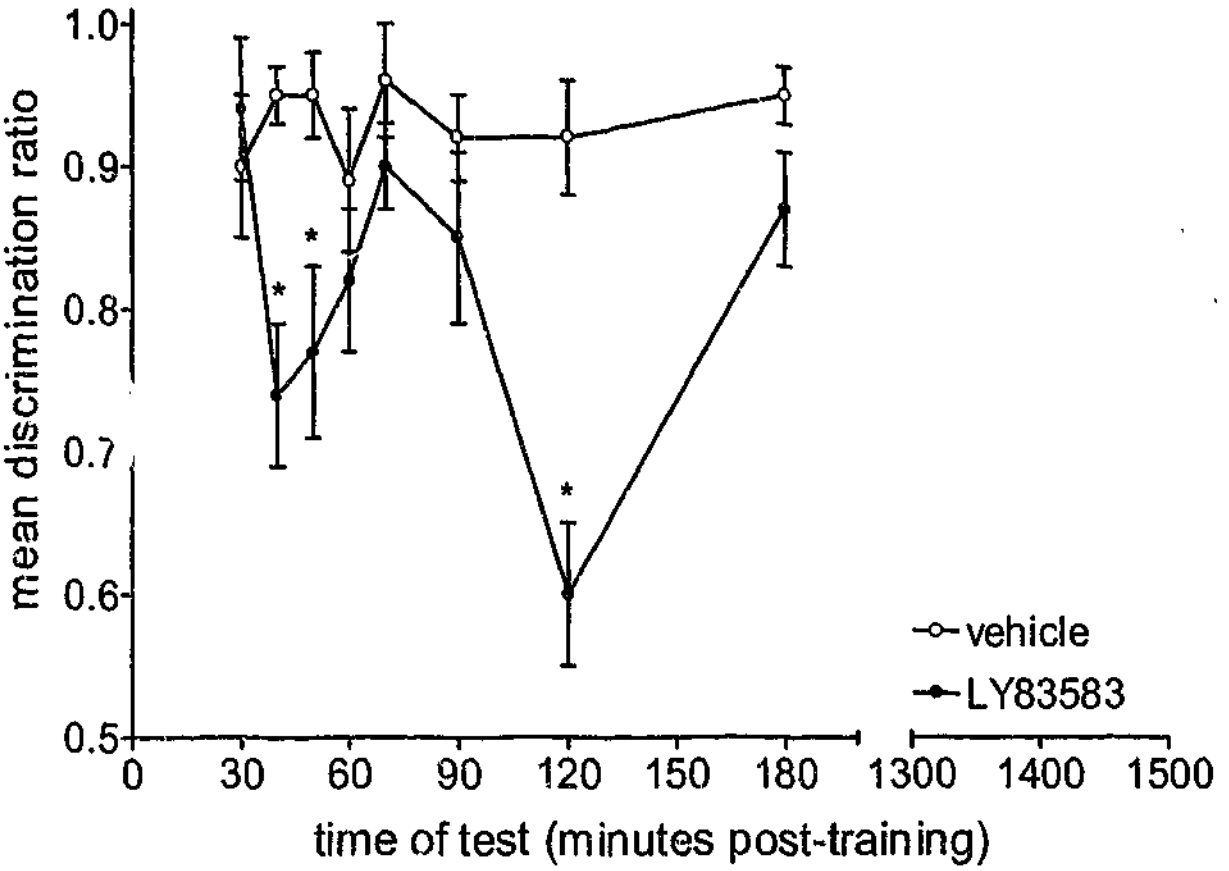
Gross sensory motor effects are considered unlikely to account for the transient retention losses as there was no obvious avoidance of the non-aversive blue bead in retention tests following administration of ODQ. Further, the retention losses were also related to the time of training and not the time of administration, and both phases of retention deficits were specific to the administration of 100μM ODQ. However, the pharmacological properties of ODQ may have resulted in subtle sensory-motor effects not easily distinguished from a genuine effect upon retention. The aim of the current experiment was to determine if an alternate GC inhibitor, LY83583, with different pharmacological properties to ODQ also produced a biphasic retention function.

Method

Chicks were housed and trained as described in Chapter 2. Groups of 20 chicks were administered either 100μM LY83583 or a vehicle (154mM saline with <1% DMSO) immediately post training and were tested at 30, 40, 50, 60, 70, 90, 120 or 180 minutes post-training. The final number of chicks in each group ranged from 14 to 38, with a mean of 19, as some chicks were excluded from the analyses while some data-points were replicated.

Results

Mean DRs for chicks administered either 100μM LY83583 or a vehicle immediately post-training and tested at various times post-training are given in Figure 3.12.



vehicle n = (20)(17)(14)(15)(17)(18) (15) (18)

LY83583 n = (17)(35)(17)(17)(18)(17) (38) (18)

Figure 3.12 Retention function for the inhibition of GC.
100μM LY83583, compared with a <1%DMSO in saline vehicle, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

100μM LY83583 given immediately post training resulted in a bimodal retention function with notable retention losses at 40, 50 and 120 minutes post-training. Recovery of recall occurred 70 and 180 minutes post-training for the two phases of deficits, respectively.

A two-way ANOVA yielded a significant drug main effect ($F(1,295)=24.25$, $p<.001$), a significant time of test main effect ($F(7,295)=2.82$, $p<.01$) and a significant interaction effect between drug and time of test ($F(7,295)= 3.12$, $p<.005$). Simple main effects analyses revealed significant differences in retention levels between chicks administered LY83583 and their matching controls at 40mins ($p<.001$), 50 mins ($p<.05$) and 120mins post-training ($p<.01$).

Discussion

These results are entirely consistent with those obtained using ODQ, with two phases of transient retention loss centred around 40 and 120 minutes post-training, respectively. The dose response and time of administration function for LY83583 are also broadly consistent with those derived using ODQ, suggesting a common action for both drugs, most likely upon GC soon after learning. Importantly, the retention study for LY83583 is also broadly consistent with the studies by Izquierdo et al. (2000) which, using LY83583, demonstrated a loss of retention both within the early stages of memory formation, tested 1.5 hours post-training, and during the late stage of memory, at 24 hours post-training for a passive avoidance task in rats.

Finally, the LY83583 retention function mimics the ODQ retention function rather than the L-NAME retention function. This is important for two reasons. First, it confirms that there is no clear relationship between NO and GC activation within the present paradigm because the retention functions following L-NAME and ODQ or LY83583 differ so dramatically. Second, Luo, Das and Vincent (1995) suggested that LY83583 may block cGMP production by inhibiting NOS. The above retention function is clearly different from that produced by inhibitors of NOS, such as L-NAME, and demonstrates that the concentration of 100μM LY83583, used here, is insufficient to inhibit the action NOS.

3.4.3 Interpretation of the effect of ODQ and LY83583 upon retention

Having confirmed that the biphasic retention function resulting from the administration of 100μM ODQ is an effect upon memory processes and not the consequence of subtle sensory-motor effects or an effect of drug concentration, it is now possible to interpret this retention function. The comparable action of ODQ and LY83583 would strongly suggest that GC is required for retention during the ITM-B stage and after the formation of the LTM stage. The Gibbs and Ng model (Ng & Gibbs, 1989) is predicated on sequentially dependent stages of memory. As these experiments show that retention is restored after each amnestic period, and thus the memory trace persists, it is reasonable to argue that GC is required for memory retrieval, and not

formation processes, during the ITM-B and LTM stages. However, the sequential dependence of this model has been questioned by Bennett (2000) who suggested that transient retention losses may imply multiple memory traces such that formation processes may still be affected by ODQ. To distinguish between memory formation and retrieval processes, the Ng & Gibbs laboratory use a variant of the one-trial passive avoidance task, known as the reminder task (Summers, Crowe & Ng, 1995, 1996, 1997). While it is beyond the scope of this thesis to conduct tests on the effects of GC inhibition with respect to the impairment of memory retrieval processes, it would be of value to consider using the reminder task in future studies to further explore the nature of the retention deficits produced by GC inhibition.

A hemispheric model of memory formation derived from monocular passive avoidance experiments with chickens is reviewed by Andrew (1991). Taking as its foundation the well characterised losses of retention at 15 and 55 minutes post-training (Gibbs & Ng, 1977), he suggests that both hemispheres have periods of high retention and low retention. These retention cycles are not in phase though, with the right hemisphere having a period of about 25 minutes and the left hemisphere a period of about 16 minutes. He suggests that highest overall retention occurs when the peaks of each hemisphere retention cycle temporally coincide. Therefore, within the present retention studies using ODQ or LY83583, one or both, temporary retention losses may be due to an innate trough in the cyclical retention of the day-old chick. Applying the work of Andrew (1991) to the earlier temporary retention loss following ODQ or LY83583 administration it is apparent that the peaks of the retention cycles for both hemispheres should coincide at 50 minutes post-training, a time at which retention is impaired following ODQ or Ly83583 administration. Thus we can suggest from this that the earlier temporary retention loss does not represent some underlying periodicy of retention inherent within the chick but suggests the action of ODQ and LY83583 upon memory processes.

Therefore, in conclusion it is possible to suggest that the biphasic retention function is due to two, temporally separate, actions of GC most likely affecting memory retrieval processes. Finally, there are aspects of the retention function for GC which are similar to the retention functions for L-NAME and PKG which may hint at a complex relationship even although NO does not appear to affect memory processing through GC.

SECTION 5 : General Conclusions

3.5.1 Possible NO-activated pathways in memory processing

There has been clear behavioural evidence demonstrating the necessity of NO in memory formation across a wide range of learning tasks and species (refer section 1.2.2). In particular, a role for NO in the ITM-B stage of passive avoidance learning using the day-old chick has been demonstrated (Hölscher & Rose, 1992, 1993; Rickard et al., 1994, 1998, 1999, in preparation- a,b). However, the mechanism through which NO affects memory processing for this task remained to be established.

Arguably, the best recognised mechanism through which NO acts to facilitate its diverse physiological roles is by activation of GC. This leads to the production of cGMP. cGMP, in turn, activates at least three classes of proteins, but is best known for its action upon PKG. PKG phosphorylates a range of proteins in various cell types, including neurons. Activation of NO, GC and PKG have been implicated in the biological mechanisms of memory in a number of pharmaco-behavioural studies (Bernabeu et al., 1995 & 1996 & 1997; Dittman, Quinn, Nevitt, Hacker & Storm, 1997; Izquierdo et al., 2000; Kemenes et al., 2002; Kendrick et al., 1997; L'Etoile & Bargmann, 2000; Serrano et al., 1994; Telegdy et al., 1999; Yamada et al., 1996).

If this pathway is necessary in memory processing then inhibition of either NO, GC or PKG should result in consistent times of retention loss onset. This has been most clearly suggested by the pharmaco-behavioural and biochemical studies of Bernabeu et al. (1995, 1996 & 1997) and Izquierdo et al (2000) who used a single trial avoidance task for rats and has also been supported by the studies of Kemenes et al. (2002). In addition, and excluding differences due to drug-specific pharmacokinetics, similar effective administration times for inhibitors of each enzyme would add further weight to any such findings. Thus, if NO affected memory processing through GC and PKG both ODQ and H-8 would be expected to result in a permanent loss of retention by 40 minutes post-training for the present single trial passive avoidance task in the chick and

possibly be effective if administered as late as 20 minutes post-training, since these results were observed with inhibition of NOS by L-NAME (Rickard et al., 1998).

3.5.2 The role of protein kinase G in memory processing

While cGMP may activate three classes of proteins, PKG was considered the most likely effector mechanism based upon the work of Bernabeu et al. (1997), Izquierdo et al. (2000) and Serrano et al. (1994). Both Izquierdo et al. (2000) and Serrano et al. (1994) demonstrated retention loss during 'long-term' memory formation in rats and chicks respectively. Yet, methodological issues in both studies suggested the need for further investigation. For example, Izquierdo et al. (2000) tested rats at 1.5 hours and then re-tested them 24 hours post-training as a measure of 'short-term' and 'long-term' memory. Retention levels observed during re-test may be affected by cellular events initiated by the retrieval event at 1.5 hours post-training. Furthermore, while KT5823 is presented as a specific PKG antagonist, culture studies by both Wyatt et al. (1991) and Burkhardt et al. (2000) demonstrated that KT5823 failed to inhibit PKG in intact human platelet and rat mesangial cells, although it inhibited purified PKG. Of greater relevance to the present research are the studies of Serrano et al. (1994) who used a variant of the present task to measure avoidance learning in neonate chicks. However, two methodological issues were of concern, Serrano et al. (1994) used a limited number of concentrations of the PKG inhibitor, H-8, within the millimolar range. Previous behavioural studies using a similar task demonstrated that nanomolar and micromolar concentrations of specific kinase inhibitors may have been effective in inducing amnesia (Zhao et al., 1995, 1996; Whitechurch et al., 1997). With such high concentrations, sensory-motor effects, not associated with memory, cannot be dismissed. However, while gross effects were accounted for, subtle effects may have occurred unnoticed as the variant of the task used by Serrano et al. (1994) measured percentage retention and not discrimination. H-8 is also not specific to PKG, but will inhibit other kinases at high concentrations. As Serrano et al. (1994) used 16.7mM H-8, the action of this drug upon other kinases must be considered.

Experiments reported in this Chapter investigated the effect of a range of micromolar concentrations of H-8 on memory for the single trial passive avoidance task

in neonate chicks. 200μM H-8 was found to be effective in inducing retention deficits. The effective times of administration for 200μM H-8 are immediately around the time of training and are consistent with the single time of administration used by Serrano et al. (1994). However, the onset of retention loss was at 90 minutes post-training and the loss was temporary. Retention levels returned to normal by possibly 150 minutes post-training. This transient loss may reflect interference of H-8 with retrieval mechanisms associated with memory retrieval after 90 minutes post-training. It is also significant to note in this regard that several treatments associated with the blockade of glutamate receptor subtypes (Rickard et al, 1994, 1995) show their effect at around 90 minutes post-training. As suggested earlier, there is a sense in which the Gibbs-Ng LTM stage may be said to involve at least two phases, changing over at around 90 minutes post-training.

What is important is that L-NAME yielded a persistent loss of memory from 40 minutes onwards, with no recovery evident by 24 hours post-training. This is consistent with the findings of Rickard et al. (1998) but starkly different from the effects of H-8 in the present study. It seems reasonable to suppose that the amnestic effect of L-NAME is unlikely to be due to an effect of NO inhibition that is mediated by blockade of PKG activity, although the latter may induce transient retention losses in a manner yet to be understood.

3.5.3 The role of guanylyl cyclase in memory processing

Although findings with H-8 exclude PKG as an effector molecule of the L-NAME associated NO activated pathway for memory processing, they say nothing of the possibility that NO may activate GC and, through this, another effector mechanism. Alternate GC-dependent effector mechanisms are PDEs or CNG ion channels which may be ultimately responsible for the action of NO. Rather than conducting dose response, time of administration and retention functions for both these potential effector mechanisms, for which there may not be specific pharmacological agents, a more parsimonious method was used. If the retention profile of GC inhibition matched those for NOS inhibition then it would be reasonable to argue that GC was activated by NO

during memory processing. If so, further studies could be carried out to differentiate the action of PDEs and CNG channels.

Of significance is the finding that the effective times of administration for L-NAME (Rickard et al., 1998) and those for the GC inhibitors, ODQ and LY83583, found in the present study were markedly different. While L-NAME administered from before training to 20 minutes post-training resulted in a significant retention loss (Rickard et al., 1998), ODQ and LY83583 had to be administered close to the time of training. However, possible differences in the pharmaco-kinetics of these drugs suggest that effective times of administration are likely to be a poor measure of common times of action. Most significant are the differences in retention functions between L-NAME and ODQ or LY83583. L-NAME resulted in a permanent loss of retention by 40 minutes post-training, while ODQ and LY83583 produced bimodal retention functions with temporary retention losses centered around 40 minutes and 120 minutes post-training. As with PKG inhibition, temporary impairment of retention is interpreted to be likely due to an effect upon memory retrieval mechanisms. These differences between the effects of L-NAME and ODQ or LY83583 suggest that the role of NO in passive avoidance learning is not likely to be mediated by GC activation.

3.5.4 An integrative approach

Although GC and PKG are unlikely to underlie the action of NO in passive avoidance learning associated with the action of L-NAME, it has been observed that both GC and PKG inhibitors must be administered around the time of learning. This has also been observed for the nNOS inhibitor, NPLA, (Rickard et al., 1999, in preparation- b). This may imply that nNOS, GC and PKG are activated in response to learning and is supported by the studies of Bernabeu et al. (1995, 1996 & 1997). However, the retention loss resulting from administration of NPLA is persistent. Therefore while retrieval mechanisms may be dependent upon GC during the ITM-B and LTM stages, little can yet be said about the role of NO in activating GC-dependent retrieval processes.

However, the transient retention losses at around 90 minutes post-training following ODQ, or LY83583, and H-8 administration do suggest a mechanistic relationship. It may be suggested that after the onset of the LTM stage memory

retrieval mechanisms are dependent upon GC and PKG. As will be discussed in Chapter 5 (section 5.2.1), another GC-dependent protein class, namely CNG channels, are believed necessary in retrieval processes after formation of the LTM stage. Therefore it may be hypothesised that the remaining class of GC-activated proteins, namely PDEs, are required down-stream of GC retrieval processes during the ITM-B stage.

CHAPTER 4 -

THE ROLE OF ADP-RIBOSYLATION ON PASSIVE AVOIDANCE LEARNING

CHAPTER 4 - The role of ADP-ribosylation on passive avoidance learning

SECTION 1 : Introduction

Nitric oxide (NO) is most often characterised as acting through GC and in turn PKG. However, there are a number of GC-independent mechanisms also facilitated by NO. One such mechanism is the activation of ADP-ribosylation (see Figure 4.1), which has been implicated in synaptic processes such as long-term potentiation (LTP). The pharmaco-behavioural studies discussed in this chapter have since been reported by Edwards and Rickard (2002).

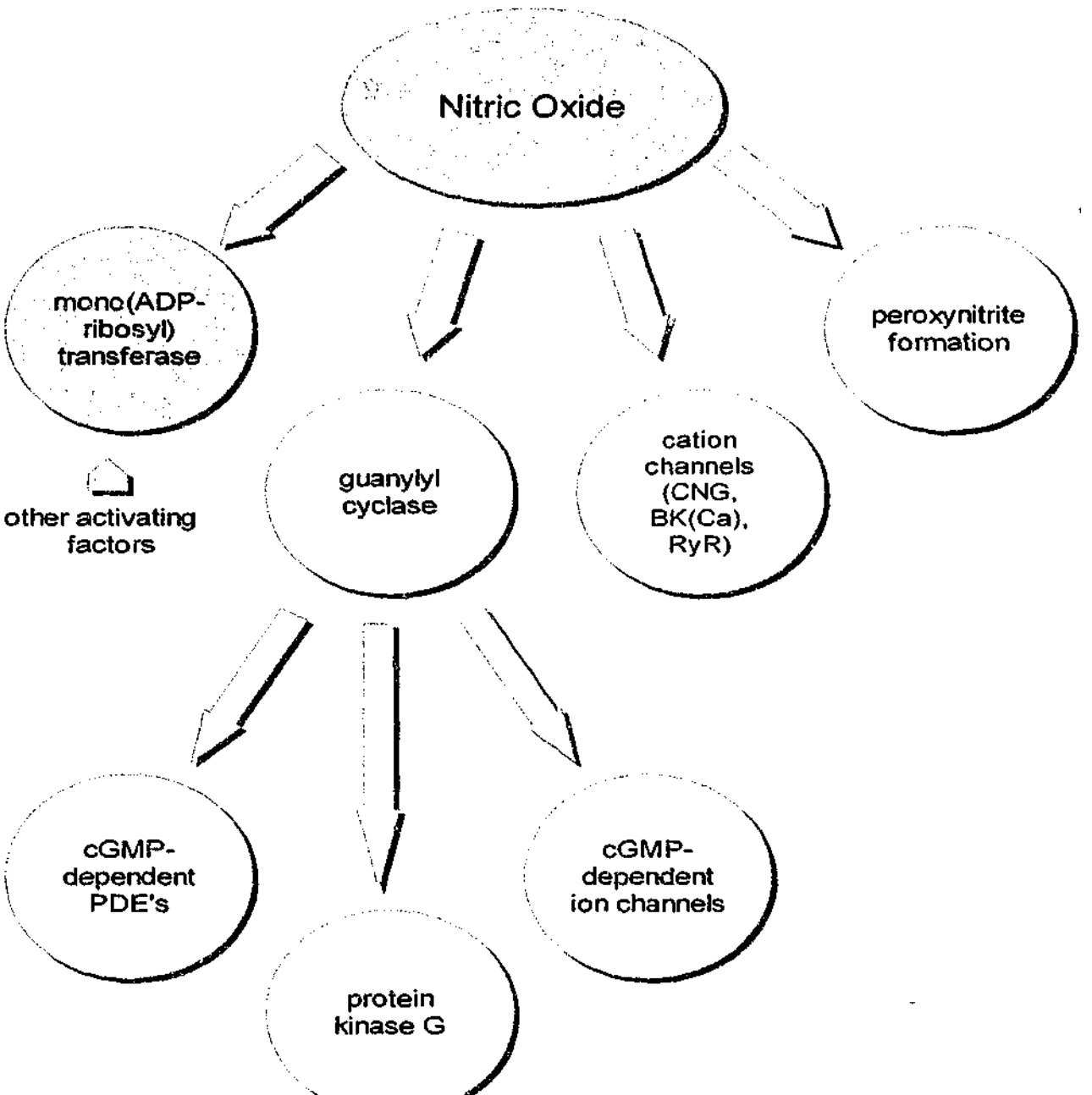


Figure 4.1 The major pathways through which NO may affect memory processes. Activation of *mono*(ADP-ribosyl) transferase is marked in orange. Note that some interlacing of pathways may occur but for clarity has not been represented.

ADP-ribosylation is a post-translational protein modification mechanism stimulated by ADP-ribosylation factors or by the presence of NO (Brüne & Lapetina, 1989; Dimler & Brüne, 1991). Conceptually, ADP-ribosylation is not unlike phosphorylation, however, rather than kinases attaching phosphate groups to a target protein, in this instance, (ADP-ribosyl) transferases attach ADP-ribose groups to the target protein. Originally sourced from NAD, ADP-ribose groups alter the activity of the target protein by changing its conformation. In opposition to ADP-ribosylation,

ADP-ribosylarginine hydrolases remove ADP-ribose groups from target proteins, fulfilling the same role as phosphatases in phosphorylation (Okazaki & Moss, 1996).

ADP-ribosylation can be of two types, *mono* or *poly*ADP-ribosylation. The *poly* form is most clearly characterised in nuclear processes such as DNA synthesis and repair, and is dependent upon *poly*(ADP-ribose) polymerase (PARP) to form branched ADP-ribose polymers (Okazaki & Moss, 1996). In contrast, *mono*ADP-ribosylation occurs in the cytosol and modifies a large number of proteins, including the G proteins G_s (Duman, Terwilliger & Nestler, 1991), G_i and G_o. G_s and G_i regulate adenylyl cyclase activity while G_o appears to regulate the function of ion channels or phospholipases (Hescheler, Rosenthal, Trautwein & Schultz, 1986; Moss & Vaughan, 1988; Tasi, Adamik, Kanaho, Halpern & Moss, 1987). Further, β/γ-actin (Matsuyama & Tsuyama, 1991), p33 (Mishima et al., 1991), p41 (Tao, Howlett & Klein, 1992), p39 (Brune & Lapetina, 1989), and integrin α7 (Zolkiewska & Moss, 1993) are all cytosolic proteins which are subject to *mono*ADP-ribosylation.

While a number of cytosolic proteins are known to be ADP-ribosylated, the functional consequences of this modification often remain unclear. Some known consequences of ADP-ribosylation include neutrophil and smooth muscle cell chemotaxis (Allport et al., 1996; Saxty, Yadollahi-Farsani, Kefalas, Paul & MacDermot, 1998), GC-independent vasodilation (Kanagy, Charpie, Dananberg & Webb, 1996) and prevention of apoptotic body formation through the ADP-ribosylation of actin filaments (Lodhi et al., 2001).

Importantly for the present research, a number of cytosolic proteins have been demonstrated to be ADP-ribosylated in the hippocampus, a brain region associated with memory formation. Further, hippocampal ADP-ribosylation has been shown to be in response to the presence of NO. For instance, Williams et al. (1992), Vezzani, Sparvoli, Rizzi, Zinetti and Fratelli (1994) and Hwang and Lee (1995) showed that a range of hippocampal cytosolic proteins were ADP-ribosylated in response to incubation of the dissected tissue with the spontaneous NO-donor SNP. Further, both studies confirmed that ADP-ribosylation was directly dependent upon NO and not mediated through the action of GC. One important consideration with respect to the present behavioural research using day-old chicks was that Williams et al. (1992) found that the ADP-ribosylation level of one protein was waning by 5 days *post-partum*, thus suggesting

some level of developmental regulation. As day-old chicks are used in the present research, developmentally regulated processes are of some interest. This work was furthered by Huang and Lee (1995) who not only detected increased ADP-ribosylation of specific proteins in rat hippocampal tissue following exposure to the spontaneous NO-donor, SNP but suggested the action of SNP in improving retention using an inhibitory avoidance task was the result of ADP-ribosylation. Although possible, this conclusion was almost speculative due to the range of actions NO may have in facilitating memory formation. In addition, super-physiological levels of SNP may also activate aberrant pathways leading to improved performance. In essence a study using an ADP-ribosylation antagonist would be required to determine such a role.

At a neuronal level, one notable function for ADP-ribosylation is in the production of LTP. This is of particular importance to the present research as LTP is a synaptic process thought to underlie memory formation for at least some tasks (Aiba et al., 1994; Davis et al., 1992; Grant et al, 1992; Izquierdo, 1994; Morris, 1989; Morris et al., 1986; Silva et al., 1992a; Tonegawa et al., 1996; Tsien et al., 1996). While a number of electrophysiological studies have determined a role for NO-dependent LTP (Bohme et al., 1991; Haley et al., 1992; Lonart et al., 1992; Nowicky & Bindman, 1993a, b; O'Dell et al., 1991; Schumann & Madison, 1991; Zhu & Luo, 1992), the mechanism through which NO acts remains controversial, with GC-dependent and independent forms of LTP being identified (Prabhakar, Short, Scholtz & Goy, 1997; Selig et al., 1996; Son et al., 1998). As to the mechanism through which NO facilitates GC-independent LTP several studies have suggested NO acts through *mono*(ADP-ribosyl) transferase. For example, Schuman et al. (1992) found ADP-ribosylation was necessary for long-term potentiation (LTP) in the memory-related CA1 region of the adult rat hippocampus. Pairing strong presynaptic tetanic stimulation with either AP5 or a membrane permeable analogue of cGMP resulted in a failure to induce LTP. Similarly, administration of the PKG inhibitor, H-8, prior to stimulation failed to block LTP. These results suggest that LTP is glutamate receptor mediated but is both GC- and PKG-independent. In contrast, inhibitors of ADP-ribosylation, namely nicotinamide (1mM), luminol (200μM) and vitamin K_1 (100μM) blocked LTP. Further, nicotinamide was suggested to act presynaptically, which is consistent with the retrograde messenger role attributed to NO by O'Dell et al. (1991). Finally, Schuman et al. (1992) sought to differentiate between the two forms of ADP-ribosylation. They

found no impairment of LTP following administration of a specific *poly*ADP-ribosylation inhibitor, benzamide (100μM), and thus concluded that LTP was mediated by *mono*ADP-ribosylation.

Schuman et al. (1994) consolidated this work by confirming that cGMP analogues and PKG inhibitors did not block the production of LTP in the CA1 hippocampal region of the adult rat using 1 second trains at 100Hz with one to four trains 15 to 30 seconds apart. However, both vitamin K_1 (100μM) and nicotinamide (10mM) blocked LTP, whereas the specific inhibitor of *poly*ADP-ribosylation, benzamide (100μM), did not. This evidence again suggests a role for *mono*ADP-ribosylation, rather than *poly*ADP-ribosylation, in the production of LTP. Further, the action of *mono*ADP-ribosylation was confirmed to be localised to the presynaptic terminal, as found by Schuman et al. (1992). Finally, in confirmation of the work of Williams et al. (1992), cell homogenates were found to significantly increase their ADP-ribosylation levels when spontaneous NO-donors were administered.

Two other studies which cast doubt on the importance of GC and PKG as the primary NO-activated pathway within LTP are those of Kleppisch et al. (1999) and Barcellos et al. (2000). Using mice which had both the PKG-I and PKG-II genes knocked-out, Kleppisch et al. (1999) found that LTP was unperturbed. Three stimulus paradigms were used: (1) strong tetanus 3x30 pulses, 100Hz, 5s pause between trains; (2) weak tetanus 50Hz for 0.5s; and (3) theta burst 10x4 pulses 100Hz, 200ms pause between pulses. Strong tetanic stimuli produced strong LTP while both the weak protocol and theta burst resulted in moderate LTP. The administration of the specific sGC antagonist ODQ was without effect after the administration of a theta burst, yet LTP was blocked by inhibitors of either NOS or NMDA receptors. This suggests that, at least within this methodology, LTP was both NO- and NMDA receptor-dependent, but GC- and PKG-independent. These findings mimicked the earlier results of Schuman et al. (1994). To test through which other NO pathway LTP was regulated, Kleppisch et al. (1999) used the ADP-ribosylation inhibitor nicotinamide (10mM) in conjunction with a theta burst and demonstrated a significant impairment in LTP.

Important for the present research was the work of Barcellos et al. (2000) who sought to determine the role of both GC and ADP-ribosylation in NO-stimulated LTP using the chick. They used a slice preparation of the chick intermediate medial hyperstriatum ventrale (IMHV), an area strongly associated with memory formation (Rose & Csillag, 1985). The spontaneous NO-donor diethylamine-NO (DEA-NO) was

added to the bath medium in conjunction with 300mA x 0.5ms delivered at 0.1Hz. The extracellular response was measured up to 80 minutes post-stimulus. They found a dose-dependent and reversible depression of the field response between 100 and 200μM DEA-NO. However, with a concentration of 400μM DEA-NO, wash-out of the drug failed to reverse the field response. Next, the specific sGC inhibitor ODQ was added to the bath prior to DEA-NO but failed to reverse the field depression. Interestingly, administration of the cGMP analogue 8-Br-cGMP resulted in a long-term, but not immediate, depression. In contrast to the lack of response shown by the GC inhibitor, the ADP-ribosylation blocker, nicotinamide (1mM), overcame the 400mg DEA-NO induced long-term depression. Taken together, these studies suggest that ADP-ribosylation, and in particular the *mono*-form, is responsible for mediating NO's role in LTP in species including the chick.

LTP is correlated to an increase in ADP-ribosylation (Schuman et al., 1994). This has been challenged by Duman, Terwilliger and Nestler (1991) who found that SNP-induced LTP was correlated with a reduction in the level of ADP-ribosylation. Therefore, while ADP-ribosylation affects synaptic processes, the overall balance of the ADP-ribosylation cycle may be more important than simply the action of *mono*ADP-ribosyl transferase as suggested by Duman et al. (1991).

In contrast to the view that ADP-ribosylation is implicated in synaptic processing, Blond, Daniel, Otani, Jaillard and Crepel (1997) showed that 10mM SNP induced cerebellar LTD, the functional opposite of LTP, but was not reversed by the application of 10μM nicotinamide. In comparison to the studies of Schuman et al. (1992; 1994), Kleppisch et al. (1999) and Barcellos et al. (2000), who used millimolar concentrations of nicotinamide, the absence of a response to the comparatively low concentration of 10μM nicotinamide is not surprising.

SECTION 2 : Comparisons of various antagonists of both mono and poly(ADP-ribosyl) transferase

All pharmaco-behavioural studies rely upon the specificity and potency of the antagonists chosen. A variety of *mono*(ADP-ribosyl) transferase inhibitors have been identified, although their potency and specificity are often poorly characterised. Even those agents whose specificity and potency have been characterised may lack verification of this *in vivo*. Finally, a number of *mono*(ADP-ribosyl) transferase inhibitors may be described as only relatively specific, inhibiting PARP as well. Therefore, the choice of inhibitors for the study of ADP-ribosylation as a NO-activated mechanism underlying memory formation is difficult.

Quantification of the potency and specificity of *mono*(ADP-ribosyl) transferase inhibitors was first undertaken by Banasik, Komura, Shimoyama and Ueda (1992) in an exhaustive survey using an *in vitro* assay system. Table 4.1 is adapted from the work of Banasik et al. (1992) and provides comparisons of specificity and potency for a number of common *mono*(ADP-ribosyl) transferase specific inhibitors, including menadione sodium bisulfite (MSB) and novobiocin (NOVO), which were used in the present studies. From these investigations Banasik et al. (1992) identified vitamin K_1 to be a potent and highly selective inhibitor of *mono*(ADP-ribosyl) transferase but also suggested NOVO and MSB to be useful in the study of *mono*(ADP-ribosyl) transferase.

Table 4.1

Comparison of Several Common mono*(ADP-ribosyl) transferase Inhibitors* in vitro

drug	*mono(ADP-ribosyl) transferase*			*poly(ADP-ribose) polymerase*		
	IC_{50} μM	% inhibition at 0.1mM	% inhibition at 1mM	IC_{50} μM	% inhibition at 0.1mM	% inhibition at 1mM
vitamin K_1	1.9	>86	>87	520	-	>72
vitamin K_3 (2-Methyl-1,4-naphtho-quinone)	120	42	95	-	-	
MSB (salt of vitamin K_3)	440	7	79	720	-	58
NOVO	280	11	100	2200	-3	16
MIBG (meta-iodobenzyl-guanidine)	-	-	-	-	-	-
nicotinamide	-	-	-	210	41	72
luminol	-	-	-	23	74	96
1,4-naphthoquinone	-	-	-	250	24	87

Adapted from Banasik, Komura, Shimoyama and Ueda (1992)

In vitro studies, however, only poorly replicate *in vivo* conditions. For instance, *in vitro* studies do not provide evidence on whether an inhibitor is membrane permeable or on how an inhibitor behaves within a complex biochemical environment where target proteins may be localised to specific regions of the cell or vary in their transcription levels. To overcome these problems, Saxty et al. (1998), extending upon the work of Allport et al. (1996), confirmed that a number of inhibitors of *mono*(ADP-ribosyl) transferase were membrane permeable and quantified their relative effects and toxicity using the biological measures of chemotaxis, chemokinesis, cell adhesion and viability within a culture system. Saxty et al. (1998) found vitamin K_3 demonstrated greater potency than vitamin K_1, novobiocin or nicotinamide, as shown in Table 4.2, which is in contrast to the *in vitro* studies of Banasik et al. (1992). Further, vitamin K_3 was the only drug to significantly inhibit *mono*(ADP-ribosyl) transferase mediated cell adhesion. Finally, while the reduction in cell viability was not significant for any inhibitor chosen, it must be noted that vitamin K_3 resulted in a slightly higher rate of cell loss, suggesting mild cytotoxic effects at higher concentrations. Therefore, the studies of both Banasik et al. (1992) and Saxty et al. (1998) strongly favour the use of four inhibitors in the investigation of *mono*ADP-ribosylation, namely vitamin K_1 or K_3, the sodium bisulfite salt of vitamin K_3 (MSB), and NOVO.

Table 4.2

Comparisons of Various mono(ADP-ribosyl) transferase Inhibitors Using a Culture System for Smooth Muscle Cells.

drug	*IC_{50} (μM) chemotaxis*	*IC_{50} (μM) chemokinesis*	*% cell adhesion at IC_{50} (μM) chemotaxis*	*% cell viability at IC_{50} (μM) chemotaxis*
vitamin K_3	22	20	55	83
vitamin K_1	95	>200	97	95
NOVO	165	98	100	89.5
nicotinamide	12000	23000	95	96

Adapted from Saxty et al. (1998)

In addition to these inhibitors a number of studies using biologically relevant systems have used alternate *mono*(ADP-ribosyl) transferase inhibitors. For example, Schuman et al. (1992; 1994) used luminol and nicotinamide to corroborate the action of vitamin K_1 (100μM) which effectively inhibited LTP. However, in their exhaustive survey of ADP-ribosylation inhibitors, Banasik et al. (1992) suggested luminol to be a relatively potent inhibitor of PARP with an IC_{50} of 23μM, equivalent to the well known inhibitor of PARP, benzamide. In addition, Schuman et al. (1992) used 200μM luminol to inhibit *mono*(ADP-ribosyl) transferase where as 100μM was found to result in 74% loss of activity for PARP *in vitro* (Banasik et al., 1992).

Nicotinamide was also used by Schuman et al. (1992; 1994), Kleppisch et al. (1999) and Barcellos et al. (2000) as a *mono*(ADP-ribosyl) transferase inhibitor but also appears to have significant action upon PARP. In addition, studies such as Schuman et al. (1994) and Wallis, Panizzon and Girard (1996) have described nicotinamide as an inhibitor of both *mono*(ADP-ribosyl) transferase and PARP. In particular, Schuman (1994) quoted the IC_{50} values for the inhibition of *mono*(ADP-ribosyl) transferase and PARP, as determined by Rankin, Jacobson, Benjamin, Moss and Jacobson (1989), to be 3400μM and 31μM respectively. Nicotinamide is, therefore, of doubtful utility in the study of *mono*ADP-ribosylation.

Metaiodobenzylguanidine (MIGB) has been widely used to inhibit *mono*(ADP-ribosyl) transferase and has been described as both "potent" (Lodhi et al., 2001) and "relatively specific" (Wallis et al., 1996). However, MIGB is inappropriate for use within the passive avoidance task for the day-old chick as MIBG is a functional analogue of noradrenaline (Smets, Loesberg, Janssen & van Rooij, 1990). Noradrenaline has been demonstrated to improve retention in a weakly reinforced variant of the present passive avoidance task (Crowe et al., 1990; 1991a; 1991b) during the NO-dependent memory phase (ITM-B). It may therefore be concluded that the use of MIBG would likely be a confounding variable in any interpretation of the data coming from the use of this task.

Therefore, there remains only four candidate *mono*(ADP-ribosyl) transferase inhibitors which appear to be both potent and specific; namely, vitamins K_1 and K_3, MSB (the sodium bisulfite salt of vitamin K_3) and NOVO.

However, data drawn from *in vitro* (Banasik et al., 1992) and culture studies (Saxty et al., 1998) with regard to the specificity and potency of an inhibitor may be of little relevance in behavioural studies. This being so, retention loss can be attributed

more clearly to *mono*(ADP-ribosyl) transferase inhibition if two inhibitors are used in separate experiments. If the inhibitors chosen share no secondary actions and inhibit *mono*ADP-ribosylation through different mechanisms but still result in matching retention functions then it can be concluded with a high degree of certainty that *mono*(ADP-ribosyl) transferase inhibition has resulted in the observed retention loss. NOVO differs in chemical structure from the other three alternate inhibitors which are vitamins. In the absence of studies detailing mode of action or secondary effects of NOVO its unique chemical structure may hint at differences to vitamins K_1, K_3 or MSB. NOVO is therefore deemed an appropriate choice as a useful antagonist in behavioural studies. Of the remaining inhibitors, Saxty et al. (1998) demonstrated a greater effect using vitamin K_3, in contrast to vitamin K_1, with respect to a number of biological measures. As Banasik et al. (1992) provided comparative data as to the relative specificity for *mono*(ADP-ribosyl) transferase and PARP for the sodium bisulfite salt of vitamin K_3, and not vitamin K_3 itself, only then was MSB, along with NOVO, deemed likely to provide the clearest evidence, if any, for the role of *mono*(ADP-ribosyl) transferase in passive avoidance learning. However, it must be noted that MSB may impair hydrogen peroxide and superoxide production (Allport et al., 1996; Sakagami, Satoh, Hakeda & Kumegawa, 2000). Since NO binds superoxide to form the bioactive radical peroxynitrite, MSB may compromise memory formation through either inhibition of *mono*(ADP-ribosyl) transferase or potentially by blocking superoxide formation.

SECTION 3 : The effect of mono(ADP-ribosyl) transferase inhibition on passive avoidance learning

4.3.1 Menadione bisulfite inhibition of mono(ADP-ribosyl) transferase

4.3.1.1 Dose response study for menadione sodium bisulfite

To de ermine if the specific *mono*(ADP-ribosyl) transferase inhibitor, MSB, impaired memory for the passive avoidance task in the chick a dose response study was undertaken. Further, if MSB induces amnesia, then an optimum concentration could be derived for use in subsequent time of administration and retention studies.

As outlined in the introduction to this chapter NO-dependent ADP-ribosylation is present in the hippocampus and has been identified as a mechanism through which LTP results. While this suggests that *mono*(ADP-ribosyl) transferase is a candidate mechanism in memory formation, no behavioural studies have been undertaken. Therefore, there is a lack of data to indicate what range of drug concentrations will be effective in blocking *mono*(ADP-ribosyl) transferase *in vivo*. Further, MSB has been used only to a limited extent to inhibit ADP-ribosylation and so, even in *in vitro* experimental systems, there is only limited evidence as to possible effective concentrations of MSB. However, using an *in vitro* assay, Banasik et al. (1992) determined that 0.1mM MSB reduced *mono*(ADP-ribosyl) transferase activity by 7%, while 1mM MSB reduced activity by 79%.

Further, as MSB is the sodium bisulfite salt of vitamin K_3, it is reasonable to discuss in a broad sense, prior studies using vitamin K_3. The most notable of these is that of Saxty et al. (1998) who showed that vitamin K_3, and thus presumably MSB, was capable of crossing the cell membrane, thus making it useful in *in vivo* studies. Vitamin K_3 then acted to alter biological measures such as chemotaxis and chemokinesis with an IC_{50} of no greater than 83μM. At an even more general level, vitamin K_1, which is a closely related structural analogue of vitamin K_3 (Chung et al., 1993), has been used to study biologically relevant processes such as LTP and synaptic transmission in hippocampal tissue. Specifically Schuman et al. (1992; 1994) found 100μM to be effective in blocking LTP. This is consistent with the studies of Banasik et al. (1992) who achieved greater than 86% reduction in activity of *mono*(ADP-ribosyl) transferase using 100μM vitamin K_1. Therefore, it may be expected that an effective concentration of MSB would occur between 100μM and 1mM.

Method

Chicks were housed and trained as previously described in Chapter 2. Administration of MSB was by freehand injection using a Hamilton syringe (10μl per hemisphere) into the region of the neostriatal/hyperstriatal cortex. Administration occurred immediately after training. Groups of 20 chicks received either the saline vehicle or 1μM, 10μM, 100μM, 200μM, 250μM, 300μM, 400μM or 500μM MSB. All groups were tested 120 minutes post-training as, at this time, temporary retention deficits were apparent following both ODQ and H-8 administration (refer Chapter 3) and long-term memory is thought to have been consolidated (Ng & Gibbs, 1988). While 20 chicks were used for each data-point, a failure to train or to peck the non-aversive bead on testing resulted in some chicks being excluded from later data analysis such that the effective sample sizes ranged from 14 to 19 with a mean of 17.

Results

Mean DRs for chicks administered a range of concentrations of MSB or saline immediately post-training and tested 120 minutes later is shown in Figure 4.2.

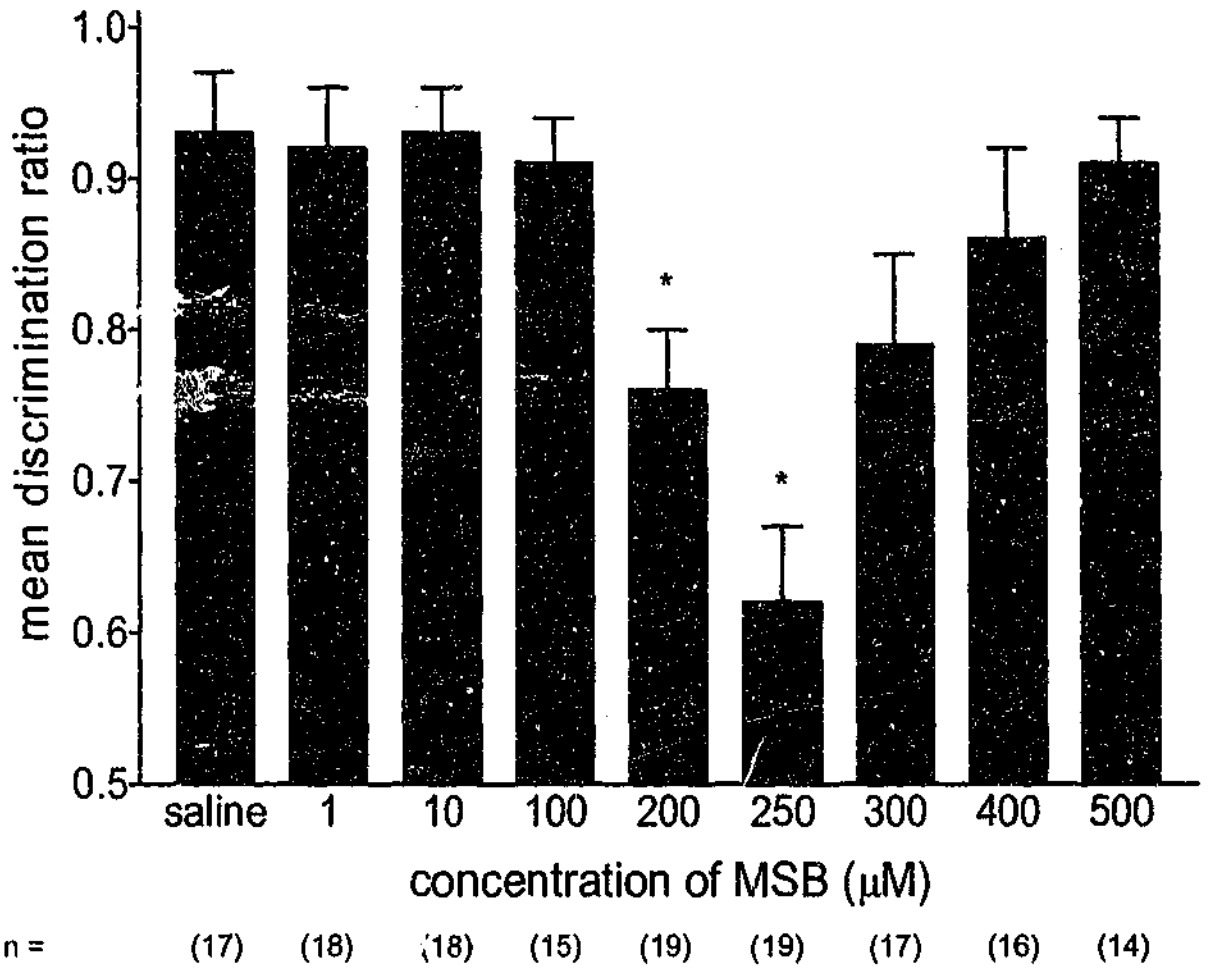


Figure 4.2 Dose response function for the mono(ADP-ribosyl) transferase inhibitor MSB. Various concentrations of MSB were administered immediately post-training and retention was tested 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * p<.05.

MSB at concentrations of 200μM and 250μM appear to have impaired retention when tested at 120 minutes post-training. Higher concentrations had less impact upon retention levels, resulting in a somewhat "U-shaped" dose response curve. A one-way ANOVA revealed a significant concentration effect ($F(8,144)=5.67$, $p<.001$) and Dunnett's post-hoc analysis indicated a significant difference between the retention levels of chicks administered saline and either 200μM ($p<.05$) or 250μM ($p<.001$), but not other concentrations of MSB.

Discussion

Administration of 200 or 250μM MSB resulted in a significant loss of retention when tested two hours post-training. This is the first demonstration that administration

of a *mono*(ADP-ribosyl) transferase inhibitor impairs retention for a learning task. "U-shaped" curves have been described by Calabrese and Baldwin (2001) as an example of hormesis and are not uncommon in toxicological studies.

The current observation that 250μM MSB is sufficient to induce memory loss is in contrast to the findings of Banasik et al. (1992) who showed that considerable inhibition of *mono*(ADP-ribosyl) transferase (79%) could only be achieved with 1mM MSB. This suggests that either a moderate inhibition of *mono*(ADP-ribosyl) transferase is required to compromise retention or, more likely, the difference between Banasik et al. (1992) and the present research illustrates the difficulties in comparing *in vitro* and *in vivo* studies.

4.3.1.2 Time of administration study for menadione sodium bisulfite

The aim in this experiment was to determine at what times of administration for MSB, relative to the time of training, would result in retention loss. Since MSB has not been investigated previously with respect to memory processing, the range of times at which memory is vulnerable to *mono*(ADP-ribosyl) transferase inhibition is not known. Nevertheless it is clear from the dose response study reported in section 4.3.1.1 that immediately post-training administration of MSB is amnestic. Therefore, it may be expected that times of administration surrounding training may also be effective.

Method

Chicks were housed and trained as described in Chapter 2. Each group of 20 chicks received either 250μM MSB or the saline vehicle at 10 or 5 minutes before training, immediately after training or at 2.5, 5, 10 or 20 minutes after training. These times of administration are consistent with the effective times of L-NAME administration (Rickard et al., 1998). Each group was tested 120 minutes post-training. While groups of 20 chicks were initially used at the different times of administration for MSB and saline, refusal to train or to peck at the non-aversive bead on testing resulted in the exclusion of chicks such that final sample sizes ranged from 14 to 20 with a mean of 18.

Results

Mean DRs for chicks administered 250μM MSB or saline at various times relative to training and tested 120 minutes post-training are shown in Figure 4.3.

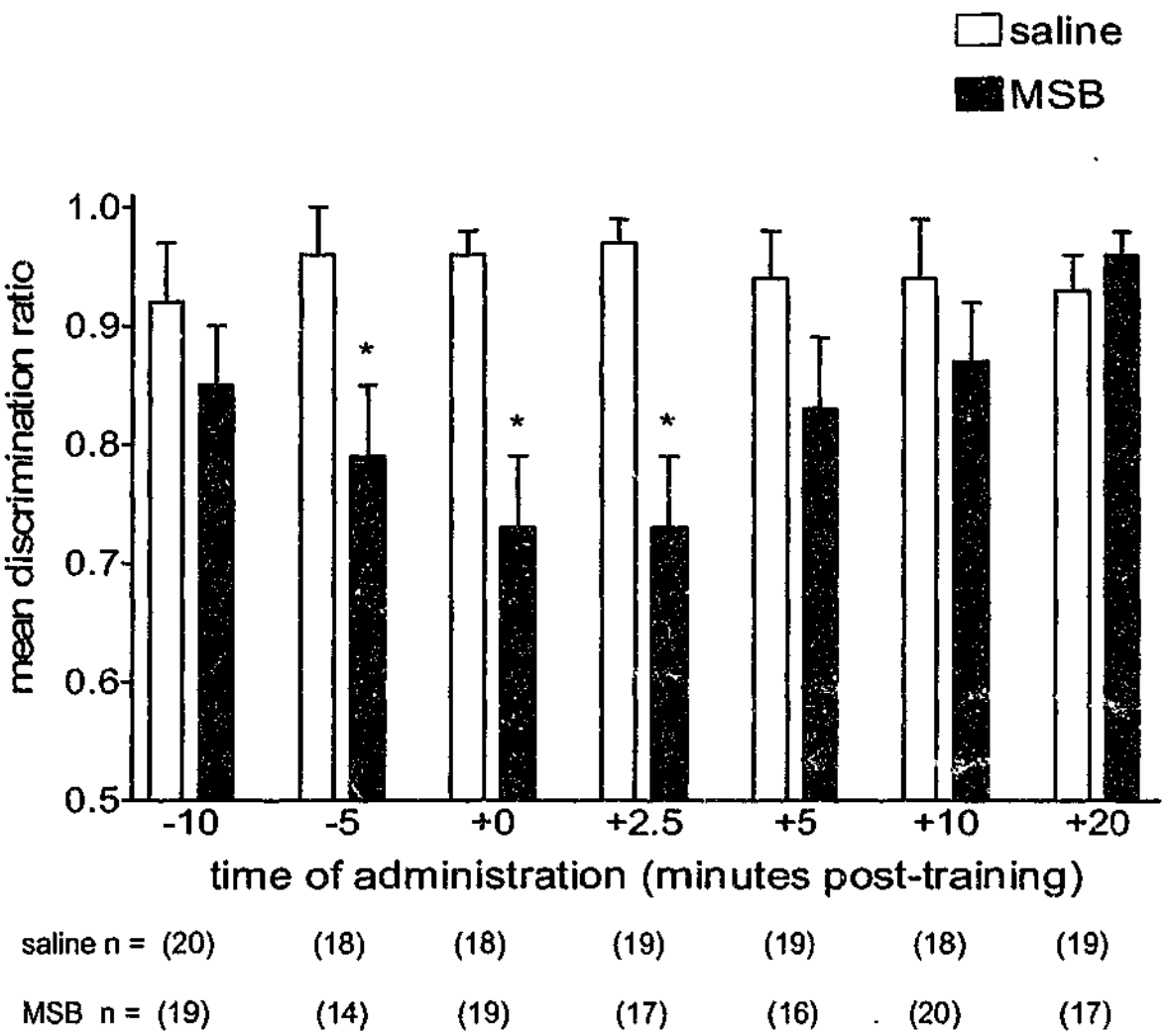


Figure 4.3 Time of administration function for the *mono*(ADP-ribosyl) transferase inhibitor MSB.

250μM MSB, compared with saline, was administered at several times relative to training and tested at 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

250μM MSB appeared to inhibit retention when given between five minutes before training and two and a half minutes after training (see Figure 4.3). A two-way ANOVA yielded a significant drug main effect ($F(1,239)=31.05$, $p<.001$) and a significant drug by time of administration interaction effect ($F(6,239)=2.75$, $p<.05$). However, the time of administration main effect was not significant ($F(6,239)=1.26$, $p>.05$). Simple main effects analysis found a significant reduction in mean DRs for chicks administered 250μM MSB at 5 minutes prior to ($p<.01$), immediately after

training (p<.001) and at 2.5 minutes after training (p<.001), compared with chicks given saline.

Discussion

It would appear that MSB must be administered around the time of learning to cause memory loss for this task. This time of administration function is quite different from that obtained from nitric oxide synthase (NOS) inhibition using the isoform non-specific inhibitor L-NAME, which could be administered up to 20 minutes post-training (Rickard et al., 1998). Such a brief effective period of drug administration is consistent with an action of MSB soon after the time of learning.

The studies of Hu, Wu, Chan, Wu and Whang-Peng (1996) demonstrated that vitamin K_3, at high concentrations, resulted in a fast clearance rate from plasma and erythrocytes in rabbits. They noted that the clearance half-life was possibly as high as about 20 minutes from both plasma and erythrocytes. Taking into account the slow clearance rates of other drugs being as long as hours, and the different experimental system being compared, the clearance half-life of MSB stated above is generally consistent with the brief range of effective administration times found in the current experiment.

It is however interesting to note that MSB shares similar effective times of administration with the nNOS-specific inhibitor NPLA (Rickard & Gibbs, in preparation - b), the GC inhibitors ODQ and LY83583, the PKG inhibitor H-8. However, these mechanisms would appear to be separate pathways in memory processing.

4.3.1.3 Time of retention loss study for menadione sodium bisulfite

Previous studies have focussed on the role of ADP-ribosylation within the context of LTP. As the present research is the first behavioural study undertaken using antagonists of *mono*(ADP-ribosyl) transferase, two possibilities as to the time of onset of retention loss were suggested. First, if ADP-ribosylation is an NO-initiated process associated with the action of L-NAME, retention loss may mimic the behavioural

studies for L-NAME and demonstrate persistent retention loss from 40 minutes post-training onwards. Alternatively, ADP-ribosylation, tyrosine kinases and glutamate receptors are thought to underlie LTP. Both tyrosine kinase and glutamate receptor inhibition result in the onset of persistent retention loss after the onset of the LTM stage using a passive avoidance task for the day-old chick (Whitechurch et al., 1997; Rickard et al., 1994, 1995). Excluding the possibility of ADP-ribosylation underlying cellular processes leading to memory at earlier memory stages, then ADP-ribosylation may also produce retention loss after the onset of the LTM stage in line with tyrosine kinase and glutamate receptor inhibitor.

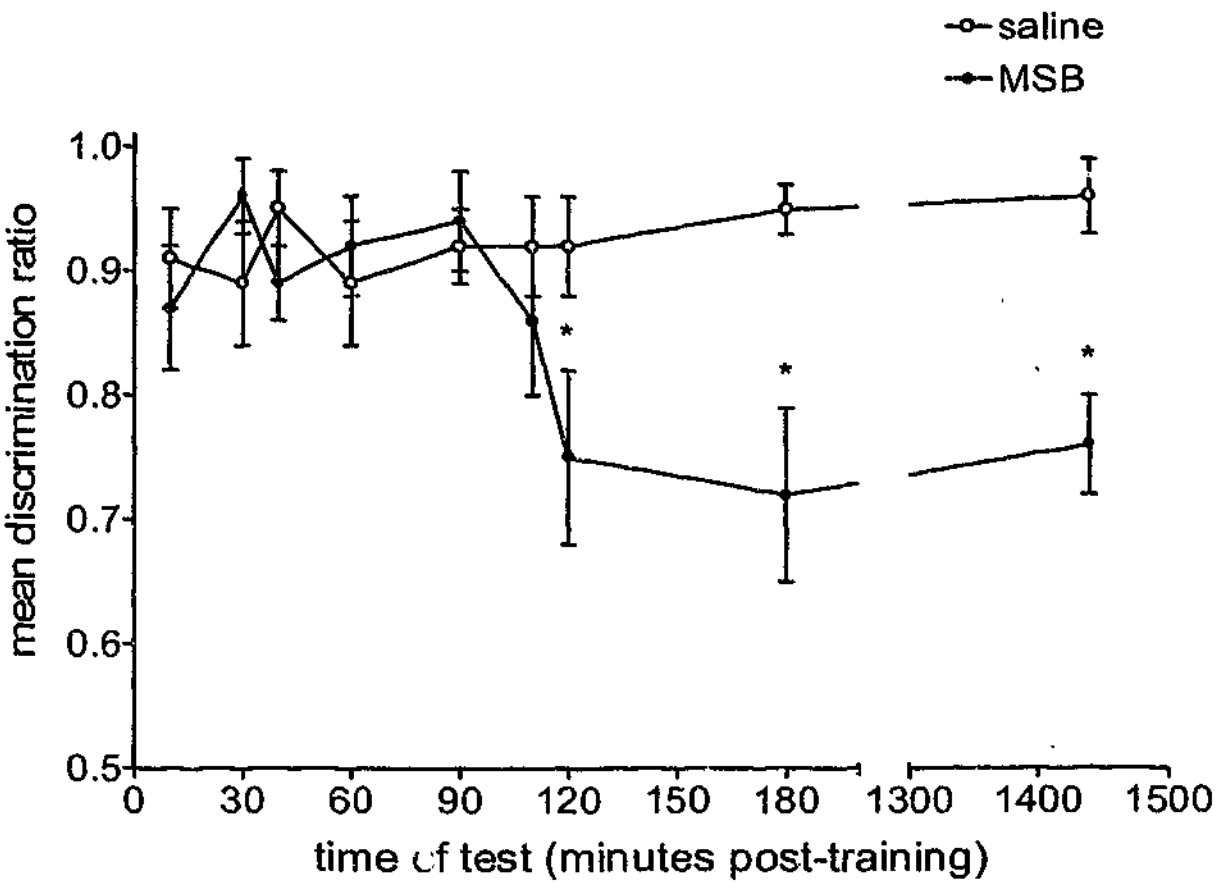
A retention study was therefore undertaken to determine at what time post-training memory retention was first impaired and whether the impairment was persistent or temporary.

Method

Chicks were housed and trained as discussed in Chapter 2. Groups of 20 chicks were given either 250μM MSB or the saline vehicle immediately post-training and were tested at 10, 30, 40, 60, 90, 110, 120 or 180 minutes post-training or 24 hours post-training. The number of chicks used per group ultimately ranged from 14 to 31 with a mean sample size of 18 due to exclusions from the data analysis and replication of data-points across experimental days.

Results

Retention levels for chicks administered 250μM MSB or saline immediately post-training and tested at various times up to 24 hours post-training are shown in Figure 4.4.



saline	n =	(16)	(20)	(16)	(17)	(18)	(16)	(15)	(18)	(17)
MSB	n =	(16)	(18)	(19)	(19)	(18)	(14)	(31)	(16)	(25)

Figure 4.4 Retention function for the inhibition of *mono*(ADP-ribosyl) transferase. 250μM MSB, compared with saline, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Administration of 250μM MSB immediately after training resulted in a progressive loss of retention after 90 minutes post-training with the loss persisting until at least 24 hours post-training. A two-way ANOVA revealed a significant drug main effect ($F(1,309)=8.77$, $p<.005$) and significant drug by time of test interaction main effect ($F(8,309)=2.68$, $p<.01$), but not a significant time of test main effect ($F(8,309)=1.43$, $p>.05$). Simple main effects analysis revealed significantly lower mean discrimination ratios in MSB-treated chicks compared to saline-treated controls at 120 mins ($p<.001$), 180mins ($p<.001$) and at 24 hours post-training ($p<.05$).

Discussion

Administration of 250μM MSB immediately post-training resulted in a significant loss of retention by 120 minutes post-training which extended to the completion of the experiment 24 hours post-training. Importantly, these results do not match the retention function for L-NAME inhibition of NOS. Therefore *mono*ADP-ribosylation does not account for the effects of NO in the current task associated with the action of L-NAME. However, the present retention function is consistent with the role of ADP-ribosylation as a post-translational mechanism for the modification of proteins as retention loss is first observed during LTM, a stage of memory formation dependent upon protein synthesis (Gibbs et al., 1977; Robertson et al., 1978; Gibbs & Ng, 1978).

Whitechurch et al. (1997) and Rickard et al. (1994) have reported a loss of retention by 90 minutes post-training following the inhibition of tyrosine kinase activity and blockade of glutamate receptors respectively. As indicated earlier, tyrosine kinases, glutamate receptors and ADP-ribosylation have all been implicated in LTP. Taken together, these findings are consistent with an LTP-like process underlying memory from about 90 minutes post-training.

It is also of interest that the onset of significant retention loss occurs by 120 minutes post-training which overlaps the second temporary retention loss caused by GC inhibition and the single temporary retention loss observed following PKG inhibition. However, little evidence exists for stage-dependent retrieval processes and what evidence there is has been derived from a 'reminder' variant of the present task. This variant is quite different to strongly-reinforced learning as it seeks to alter the memory trace associated with the original aversive trial by a second presentation of the aversive stimulus before testing of retention occurs (Summers et al, 1995, 1996, 1997). Therefore, any similarities in the time of onset of retention loss between transient and persistent traces is speculative at best.

While it is likely that MSB impairs retention by 120 minutes post-training through the inhibition of *mono*(ADP-ribosyl) transferase comparatively little is known of the action of this drug *in vivo*. Replication of the current findings with an alternate inhibitor of *mono*(ADP-ribosyl) transferase which does not share secondary effects with MSB, and acts upon *mono*(ADP-ribosyl) transferase through a different mechanism, would confirm the action of MSB on *mono*(ADP-ribosyl) transferase. To this end a

series of experiments using the alternate *mono*(ADP-ribosyl) transferase inhibitor NOVO was performed.

4.3.2 Novobiocin inhibition of mono(ADP-ribosyl) transferase

4.3.2.1 Dose response study for novobiocin

ADP-ribosylation inhibitors vary in their specificity and potency, and there is a lack of understanding of the behavioural effects of even common ADP-ribosylation inhibitors. Therefore, to confirm that the effects on memory of MSB was due solely to *mono*(ADP-ribosyl) transferase inhibition, another antagonist, NOVO, was examined. Importantly, NOVO does not appear share common secondary effects with MSB and is biochemically quite distinct, which suggests that it probably has a different mechanism of action upon *mono*(ADP-ribosyl) transferase from MSB. If both inhibitors result in identical retention functions, it can be suggested that retention loss is due to the inhibition of *mono*(ADP-ribosyl) transferase.

To determine if NOVO was amnestic, a dose response study was undertaken. While no behavioural studies had previously been conducted using NOVO, the culture studies of both Wallis et al. (1996) and Lodhi et al. (2001) suggest an effective dose between 500μM and 600μM respectively.

Method

Chicks were housed and trained as stated in Chapter 2. Administration of NOVO was by freehand injection using a Hamilton syringe (10μl per hemisphere) into the neostriatal/hyperstriatal region. Each group of 20 chicks received a different dose and due to a lack of prior behavioural studies with NOVO a wide range of concentrations were used: 0 (saline), 0.1, 1, 10, 50, 100, 250, 350, 500, 650, 800 and 1000μM. The drug or saline vehicle was administered immediately following training. Retention was tested 120 minutes post-training. While groups of 20 chicks initially

were used for each treatment condition, repetitions across experimental days and exclusions of chicks from the data analysis meant that ultimately group sizes ranged from 15 to 33 with a mean sample size of 20.

Results

Mean DRs of the chicks given the various concentrations of NOVO administered immediately post-training and tested 120 minutes post-training are given in Figure 4.5.

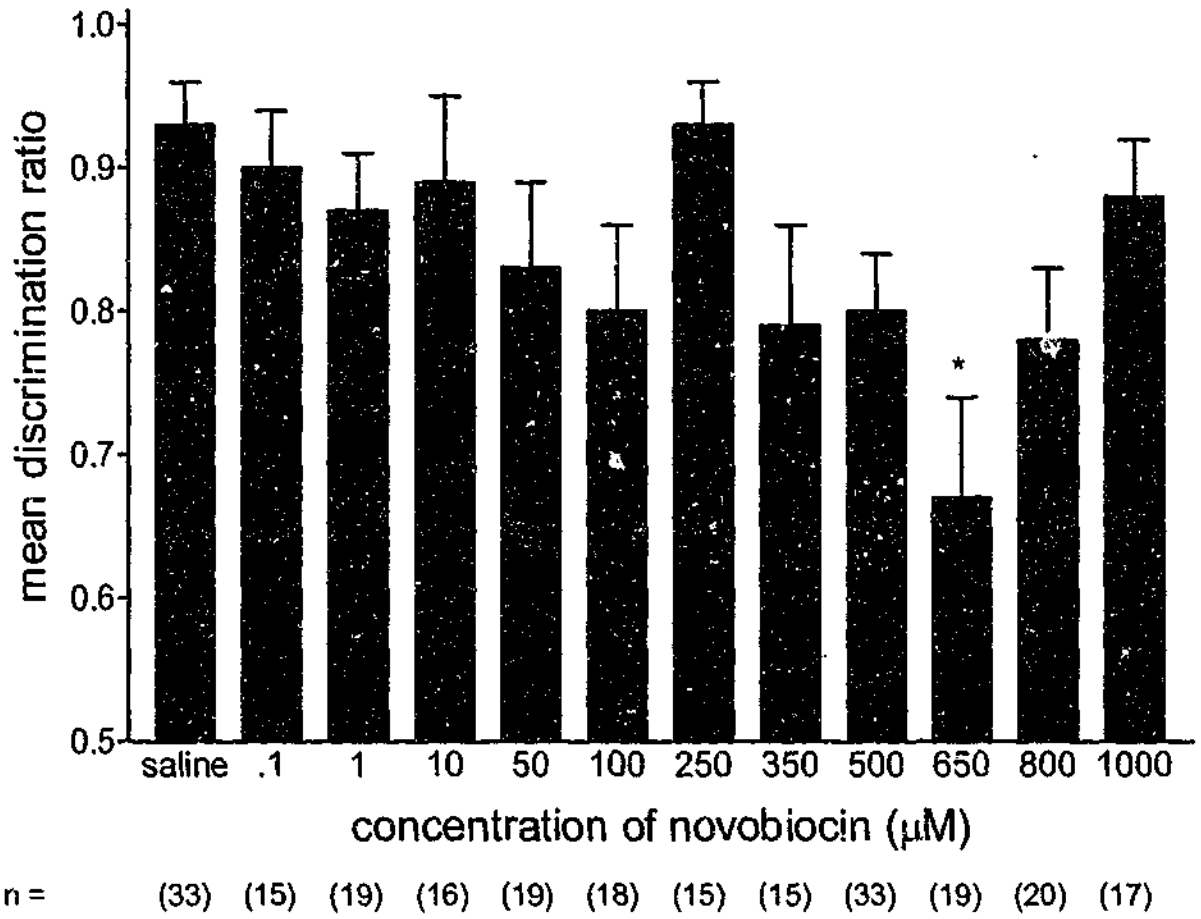


Figure 4.5 Dose response function for the *mono*(ADP-ribosyl) transferase inhibitor NOVO. Various concentrations of NOVO were administered immediately post-training and retention was tested 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

NOVO appeared to have some deleterious effect on retention across a wide range of concentrations. However, only at 650μM, was the retention loss pronounced. A one-way ANOVA demonstrated a significant concentration effect ($F(11,227)=2.41$, $p<.01$). Dunnett's post-hoc analysis indicated that the 650μM concentration was the only one

that yielded a significantly lower mean discrimination ratio than did the saline control ($p<.001$).

Discussion

Inhibition of mono(ADP-ribosyl) transferase using NOVO resulted in a very narrow dose response function with only 650μM NOVO, in the concentrations tested, significantly impairing retention. This effective dose for NOVO is consistent with both the culture studies of Wallis et al. (1996) and Lodhi et al. (2001) who respectively found 500μM and 600μM NOVO effective in culture.

This dose-specific retention loss may reflect the general specificity and potency of NOVO for mono(ADP-ribosyl) transferase, compared to MSB. However, if NOVO (IC_{50} = 280μM; Banasik et al., 1992) is more potent than MSB (IC_{50} = 448μM; Banasik et al., 1992), the optimum concentration should be lower than that for MSB. Yet, the optimum concentration for MSB was 250μM. This reversal in potency from *in vitro* to *in vivo* studies would appear to be inexplicable. However, this discrepancy may have come about due to the inherent nature of pharmaco-behavioural studies. For example, pharmaco-behavioural studies require a drug to pass from the extracellular fluid, through the cell membrane and then to act upon an enzyme rather than acting directly upon purified enzyme used in *in vitro* studies.

4.3.2.2 Time of administration study for novobiocin

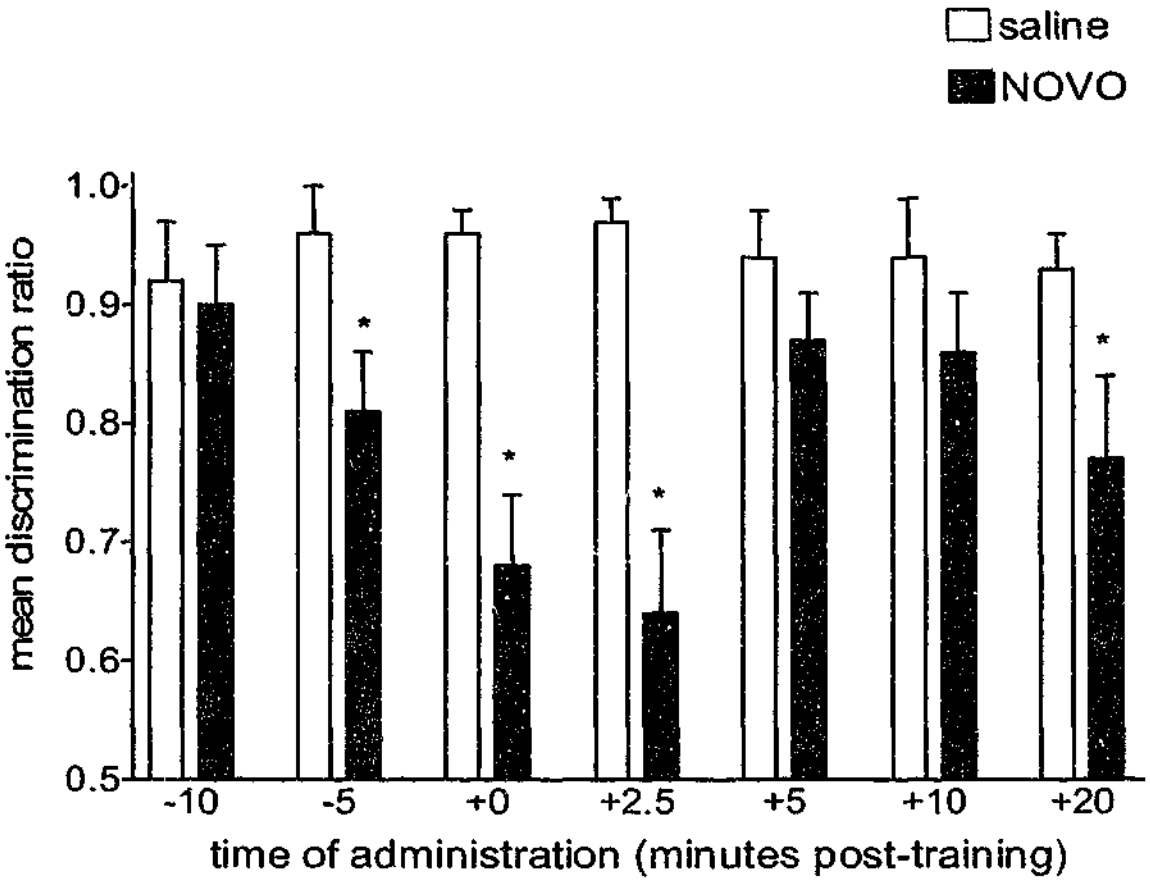
NOVO is amnestic in the current task with the greatest retention loss observed following administration of 650μM concentration. 650μM NOVO has been observed to be effective in compromising retention when administered immediately post-training which is consistent with the effective administration times of MSB. However, only if NOVO shares similar pharmacokinetic properties with MSB would a similar range of effective administration times be anticipated. The aim of the present experiment was to determine the range of effective administration times for 650μM NOVO.

Method

Chicks were housed and trained as discussed in Chapter 2. A different group of, initially, 20 chicks was used for each data-point but following exclusions from the data analysis group sizes ranged from 15 to 20, with a sample size mean of 17. Groups of chicks received either 650μM NOVO or saline at 10 or 5 minutes prior to training, immediately after training or 2.5, 5, 10 or 20 minutes after training. Retention was tested 120 minutes post-training.

Results

Mean DRs for chicks given 650μM NOVO or saline at various times before and after training, and tested 120 minutes after training, are shown in Figure 4.6.



saline n =	(20)	(18)	(18)	(19)	(19)	(18)	(19)
NOVO n =	(18)	(18)	(17)	(16)	(17)	(15)	(16)

Figure 4.6 Time of administration function for the *mono*(ADP-ribosyl) transferase inhibitor NOVO.

650μM NOVO, compared with saline, was administered at several times relative to training and tested at 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Retention loss was observed with 650μM NOVO given between 5 minutes before training and 2.5 minutes after training and at 20 minutes post-training. A two-way ANOVA revealed a significant drug main effect ($F(1,234)=47.33$, $p<.001$) and significant drug by time of administration interaction main effect ($F(6,234)=3.71$, $p<.005$). However, the time of administration main effect was not significant ($F(6,234)=1.80$, $p>.05$). Simple main effects post-hoc analysis revealed that chicks administered 650μM NOVO at 5 minutes prior to ($p<.05$), immediately after training ($p<.001$), 2.5 minutes after training ($p<.001$) and 20 minutes after training ($p<.05$) demonstrated significantly lower mean discrimination ratios than did chicks administered saline at the same times.

Discussion

NOVO effectively impaired retention when administered between 5 minutes before and 2.5 minutes after training, as well as at 20 minutes after training. The effective times of administration around the time of learning were similar to those found for MSB.

However, the significant retention loss observed for administration 20 minutes post-training was unexpected and suggests two possibilities. The first being sampling error, and the second being that the time of administration function for NOVO may be bimodal, with two periods of effective administration times. This second possibility may suggest two roles for NOVO in memory processing, one of which is possibly the inhibition of *mono*(ADP-ribosyl) transferase.

4.3.2.3 Time of retention loss study for novobiocin

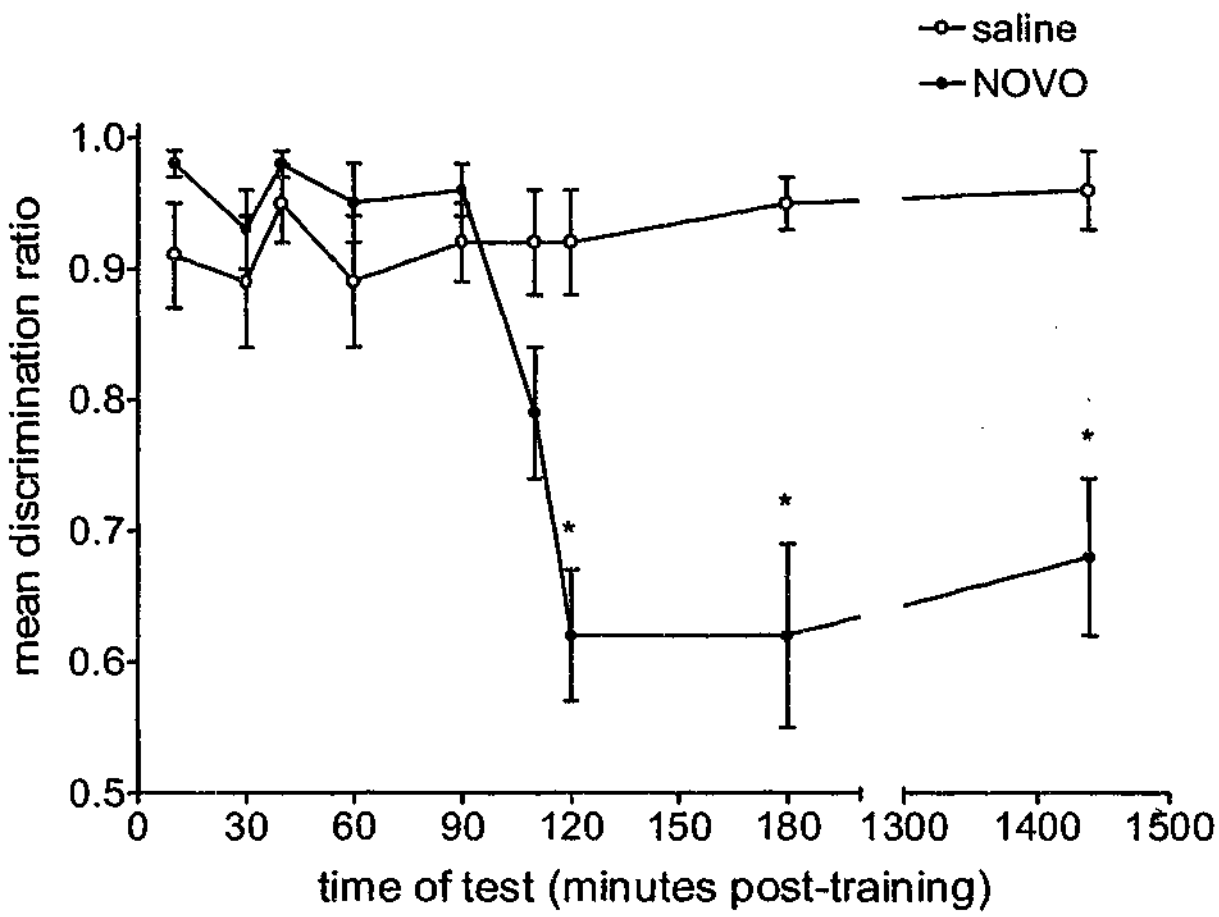
It was necessary to confirm the action of MSB upon memory retention by the use of another *mono*(ADP-ribosyl) transferase inhibitor, namely NOVO. To suggest that MSB and NOVO were acting on the same cellular mechanisms, NOVO would have to result in a loss of retention by 120 minutes post-training and this retention loss be persistent until at least 24 hours post-training.

Method

Chicks were housed and trained as stated in Chapter 2. Groups of 20 chicks received either 650μM NOVO or saline immediately post-training and were tested at 10, 30, 40, 60, 90, 110, 120 or 180 minutes post-training or 24 hours post-training. While groups of 20 chicks were used initially for each data-point group sizes ultimately ranging from 15 to 32 with a mean sample size of 19.

Results

Mean DRs for chicks administered 650μM NOVO immediately post-training and tested from 10 minutes to 24 hours post-training are shown in Figure 4.7.



saline n = (16)(20)(16) (17) (18) (16)(15) (18) (17)

NOVO n = (19)(17)(17) (19) (20) (17)(32) (17) (29)

Figure 4.7 Retention function for the inhibition of *mono*(ADP-ribosyl) transferase. 650μM NOVO, compared with saline, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Substantial retention loss in NOVO-treated chicks occurred by 120 minutes post-training and persisted until at least 24 hours post-training. A two-way ANOVA revealed significant drug ($F(1,320)=18.76$, $p<.001$) and time of test ($F(8,320)=5.83$, 001) main effects, and a significant drug by time of test interaction effect ($F(8,320)=8.10$, $p<.001$). Simple main effects analysis revealed a significant reduction in the mean discrimination ratio of NOVO-treated chicks compared with controls when tested at 120 mins ($p<.001$), 180mins ($p<.001$) and at 24 hours post-training ($p<.001$).

Discussion

The retention function for 650μM NOVO given immediately post-training resulted in essentially the same retention function as observed with MSB. This excludes the possibility that the effects of MSB may be due to inhibition superoxide or hydrogen peroxide production. The results confirm that *mono*ADP-ribosylation, probably occurring early after training, is necessary for the expression of memory after around 90 minutes post-training which appears to coincide with a later phase of the LTM stage.

SECTION 4 : General discussion

ADP-ribosylation is a common mechanism for post-translational modification of proteins by the transfer of an ADP-ribose group from NAD to the target protein through the action of ADP-ribosyl transferase. There are two forms of this reaction, the *poly* and *mono* forms, of which *poly*ADP-ribosylation occurs in the cell nucleus while *mono*ADP-ribosylation occurs in the cytoplasm.

While early studies had demonstrated the ability of spontaneous NO-donors to initiate the ADP-ribosylation of cytosolic proteins in the hippocampus (Williams et al., 1992; Vezzani et al., 1994) it was Schuman et al. (1992, 1994) who first demonstrated a role for *mono*ADP-ribosylation in the memory-associated process, LTP. Subsequent studies confirmed this (Kleppisch et al., 1999; Barcellos et al., 2000). However, the role of *mono*ADP-ribosylation in memory processing had not been assessed. The current research sought to determine if *mono*ADP-ribosylation has such a role, using two specific inhibitors to *mono*(ADP-ribosyl) transferase that do not share any common secondary action.

The results show that both MSB and NOVO are amnestic for the present passive avoidance task. MSB most notably impairs retention at a concentration of 250μM and NOVO 650μM. While the effective times of administration for MSB and NOVO were from 5 minutes before training to 2.5 minutes post-training, similar to that for the nNOS inhibitor NPLA, retention loss was not observed before 120 minutes post-training. In further contrast, L-NAME is amnestic when administered from before training to 20 minutes post-training and NPLA, DPI and L-NAME all result in persistent retention losses from 40 minutes onwards (Rickard, 1995; Rickard et al., 1998, 1999, in preparation - b). Therefore, it appears that if *mono*ADP-ribosylation is necessary for memory expression sometime after formation of the Gibbs and Ng LTM stage, it is *not* ADP-ribosylation brought about by NO associated with the effects of L-NAME.

It is, however, interesting to note that the limited range of effective administration times for both MSB and NOVO suggest a role for *mono*ADP-ribosylation soon after training, as was earlier suggested for both GC and PKG. Such a time of activation for each of these three enzymes is consistent with the activation of NPLA which similarly has a restricted effective period of administration around the

time of training. However, as mentioned above, the loss of retention due to NPLA is persistent occurring from 40 minutes post-training, and therefore either too early to account for the action of either GC, PKG or *mono*ADP-ribosylation or is affecting memory formation and not retrieval processes.

At a more theoretical level, it may be speculated that the similar effective times of administration for *mono*(ADP-ribosyl) transferase inhibitors, GC and PKG around the time of training may suggest the existence of a common activator. Further this common activator may be responsible for the initiation of both memory retrieval and memory formation processes. However, any interpretation of transient retention losses being equated to an effect upon retrieval processes is dependent upon a model of memory which has sequentially dependent stages. Recently, the sequentially dependent three stage model of memory formation proposed by Gibbs & Ng (1977) has been recently challenged by Bennett (1999) who suggested the possibility of overlapping memory traces being present and expressed differentially.

Taken together, these studies using MSB and NOVO do *not* provide clear evidence for ADP-ribosylation being down-stream of NO associated with the effects of L-NAME as a process underlying memory formation for the current task. However, this is the first demonstration that *mono*ADP-ribosylation is necessary for the maintenance of LTM.

CHAPTER 5 -

THE ROLE OF CATION CHANNELS ON PASSIVE AVOIDANCE LEARNING

SECTION 1: Introduction

SECTION 2: The effect of olfactory-type cyclic nucleotide-gated ion channel inhibition on passive avoidance learning

5.2.1 Verapamil inhibition of olfactory-type cyclic nucleotide-gated ion channels

SECTION 3: The effect of large conductance calcium-activated potassium channel inhibition on passive avoidance learning

5.3.1 Iberiotoxin inhibition of large conductance calcium-activated potassium channels

SECTION 4: The effect of ryanodine receptor calcium release channel inhibition on passive avoidance learning

5.4.1 Dantrolene inhibition of ryanodine receptor calcium release channels

SECTION 5: The effect of impaired peroxynitrite production on passive avoidance learning

5.5.1 Trolox inhibition of peroxynitrite production

SECTION 6: Summary of findings

Chapter 5 - The role of cation channels on passive avoidance learning

SECTION 1 : Introduction

Inhibition of NOS results in a loss of retention using a variety of memory paradigms as discussed in Chapter 1. These include passive avoidance learning in the day-old chick, which, following administration of a NOS inhibitor such as L-NAME, demonstrates retention loss from the ITM-B stage (Hölscher & Rose, 1992, 1993; Rickard, 1998) as defined by the Gibbs and Ng model (Gibbs & Ng, 1979a). While NO is known to activate enzymes such as GC and *mono*(ADP-ribosyl) transferase, these do not appear to be responsible for the actions of NO in passive avoidance learning for the neonate chick associated with the effects of L-NAME. This comes about as neither enzyme, when inhibited, resulted in a retention function, similar to L-NAME.

One large class of NO activated proteins which have been implicated in memory formation and in mechanisms that may underlie memory are the cation channels (see Figure 5.1). That is, ion channels which allow the passage of positive ions such as K^+, Na^+, Ca^{2+} and Mg^{2+}. Importantly, prior studies using variants of the single-trial passive avoidance tasks for the day-old chicks have implicated a variety of cation channels in memory formation (Clements et al., 1995; Gibbs et al., 1979; Gibbs et al., 1978; Gibbs & Barnett, 1976; Salinska et al., 2001).

Evidence also exists for the direct activation of a number of cation channels by NO. These include olfactory-type cyclic nucleotide-gated (CNG) cation channels (Ahmad et al., 1994; Broillet & Firestein, 1996; Broillet, 2000), large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) (Abdullah & Docherty, 1999; Atucha et al., 1998; Bolotina et al., 1994; Bradley, Buxton, Barber, McGaw & Bradley, 1998; Plane et al., 1996; Zhao, Wang, Rubin & Yuan, 1997; Zhou, Schlossmann, Hofmann, Ruth & Korth, 1998), and the ryanodine receptor Ca^{2+} release channel (RyR) (Aghdasi et al., 1997; Anzai, Ogawa, Ozawa & Yamamoto, 2000; Stoyanovsky et al., 1997; Xu et al., 1998). There is also some evidence that NO directly activates other channels including the

ATP-sensitive K^+ (K_{ATP}) channel (Armstead, 1996; Champion & Kadowitz, 1997; Csont et al., 1999; Hein & Kuo, 1999; Miyoshi, Nakaya & Moritoki, 1994; Murphy & Brayden, 1995; von der Weid, 1998; Wang, Bryan & Pelligrino, 1998; Wu, Chen & Yen, 1999), the small conductance K^+ (SK) channels (Lu, & Wang, 1996) and voltage-activated K^+ channels (Lu & Wang, 1996; Lovren & Triggle, 1998; Mule, D'Angelo & Serio, 1999) but any direct activation by NO remains equivocal especially for K_{ATP} channels (Kontos & Wei, 1996). The strongest evidence for a role for NO in memory formation via its action on cation channels is in the activation of CNG, BK_{Ca} and RyR channels.

The evidence for CNG and BK_{Ca} channels in memory processing is limited (Ghelardini et al., 1998; Jankowska et al, 1991; Lee & Lin, 1991; Quartermain et al., 2001). However, CNG channels have been implicated in hippocampal LTP (Parent et al., 1998) while NO-activated BK_{Ca} channels are intimately involved in vasodilation (Abdullah & Docherty, 1999; Atucha et al., 1998; Bolotina, et al., 1994 Plane et al., 1996). The latter possibly facilitating memory indirectly through increasing the supply of nutrients to metabolically active brain regions following learning. In contrast, the role of intracellular calcium stores generally (Blackwell, 2002), and RyR channel-sensitive stores specifically, have been implicated in memory formation directly (Balschun et al., 1999; Blackwell & Alkon, 1999; Kouzu, Moriya, Takeshima, Yoshioka & Shibata, 2000; Ohnuki & Nomura, 1996; Salinska et al., 2001; Schantz, Seo, Wong, & Pessah, 1997; Zhao et al, 2000). Further RyR channels have also been implicated in a variety of hippocampal cellular processes thought to underlie memory formation. These include LTP (Balschun et al., 1999; Obenaus et al., 1989; Shimuta et al., 2001; Tekkök & Krnjević, 1996; Wang, Wu, Rowan & Anwyl, 1996), paired pulse facilitation (Wang & Kelly, 1997) and LTD (O'Mara, et al., 1995; Reyes-Harde, Potter, Galione & Stanton, 1999). RyR channels have also been implicated in both GABA (Savić & Sciancalepore, 1998) and noradrenalin (Huang & Zhou, 1996) release in the hippocampus. The latter of which has been found vital to passive avoidance learning being necessary for the transfer of memory from labile to permanent storage (Crowe et al., 1990, 1991a, 1991b).

Finally, NO is known to form peroxynitrite of which both molecules share a number of biochemical characteristics including nitrosylation of tyrosine and tryptophan residues (Alvarez et al., 1996; Beckman, Estevez & Teng,

2000; Eiserich, Butler, van der Vliet, Cross & Halliwell, 1995; Hanafy, Krumenacker & Murad, 2001). Therefore NO may act through peroxynitrite to activate particular cation channels and thus affect memory formation.

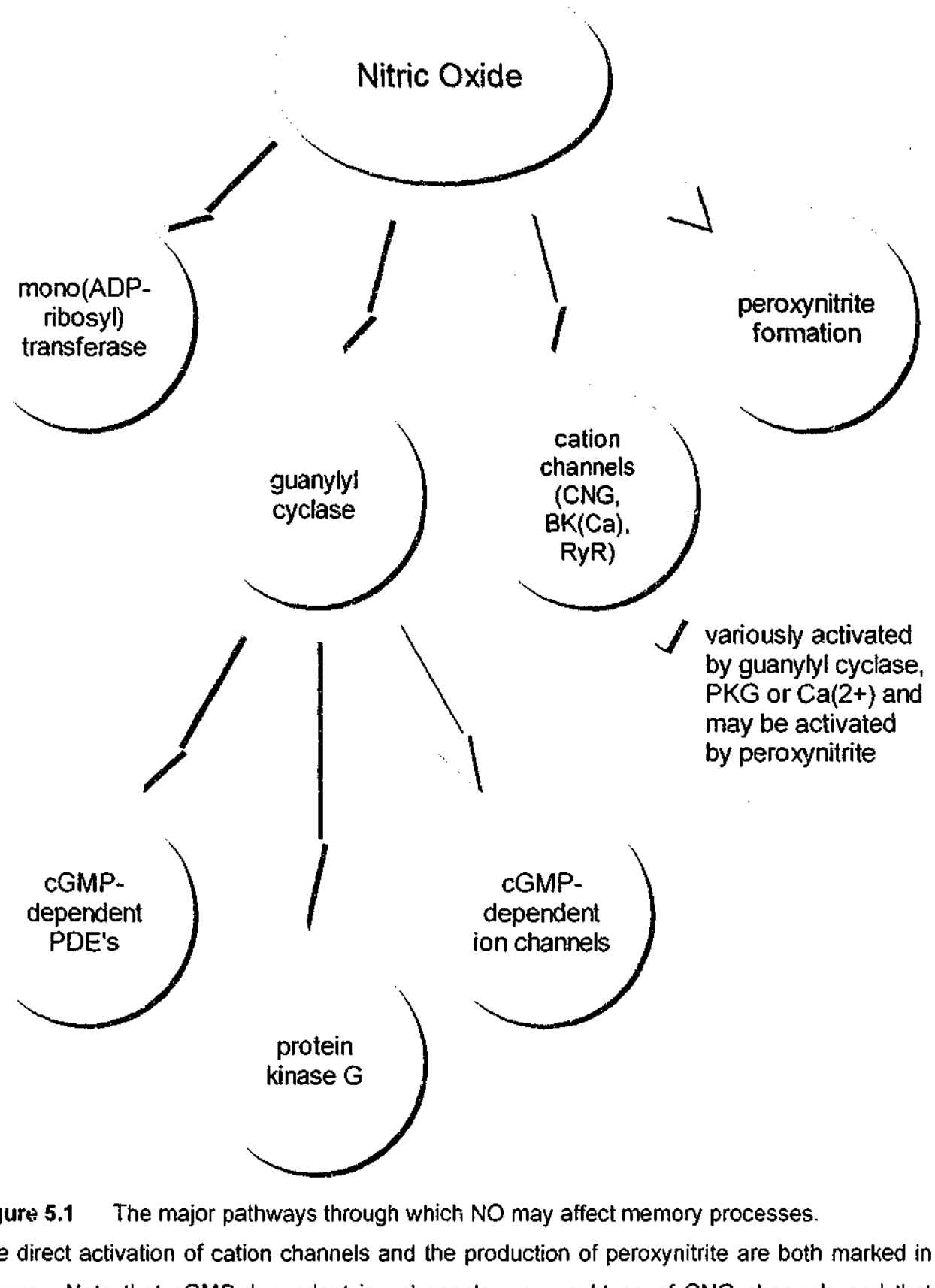


Figure 5.1 The major pathways through which NO may affect memory processes.
The direct activation of cation channels and the production of peroxynitrite are both marked in orange. Note that cGMP-dependent ion channels are a subtype of CNG channels and that peroxynitrite may interact with enzymes or channels represented but has not been shown in this figure for clarity.

SECTION 2 : The effect of olfactory-type cyclic nucleotide-gated ion channel inhibition on passive avoidance learning

CNG channels can be either olfactory- or rod-type. While both forms are considered to be relatively non-specific with respect to the passage of different cations such as K^+, Na^+, Ca^{2+}, Mg^{2+} (Kingston et al., 1996; Yao et al., 1995), it has been suggested that they preferentially move Ca^{2+} cations (Biel, Sautter, Ludwig, Hofmann & Zong, 1998; Zufall et al., 1997). Traditionally, CNG channels have been activated by the presence of cyclic nucleotides such as cAMP and cGMP, the latter produced through the activation of GC by NO. While olfactory CNG channels have similar specificity for both cAMP and cGMP cyclic nucleotides, rod CNG channels are preferentially activated by cGMP (Broillet & Firestein, 1996, 1997; Nakamura & Gold, 1987; Zufall, Firestein, & Shepherd, 1994).

In addition to being activated by cyclic nucleotides, CNG channels appear to be directly activated by NO (Ahmad et al, 1994; Broillet & Firestein, 1996 & 1997; Broillet, 2000). Using electrophysiological techniques, Ahmad et al. (1994) observed activation of CNG channels in the presence of NO donors in retinal ganglion cells, while Broillet and Firestein (1996) used single channel recordings to show that spontaneous NO-donors (NOCys and SIN-1) could directly activate olfactory neuron-type CNG channels. Their data also strongly suggested that NO not only directly activated olfactory-type CNG channels but was more effective than cAMP. However, these results were disputed by Lynch (1998) who suggested that NO appears to inhibit the activation of cAMP-dependent CNG channels in the rat olfactory receptor neuron. Even so, Broillet (2000) has identified the mechanism through which NO directly activates olfactory-type CNG channels. Specifically, NO interacts with a single cysteine residue on a number of channel subunits resulting in channel opening.

Inferential evidence supporting a role for CNG channels in memory formation comes from both histological and molecular investigations. Both rod- and olfactory-type CNG channels are localised to various brain regions including the memory-related

region of the hippocampus (Bradley et al., 1997; Leinders-Zufall et al., 1995; Kingston et al., 1996 & 1999). Molecular disruption of the α-subunit of the olfactory–type CNG channel has been shown to impair the development of LTP and result in quickened decay following theta-burst stimulation, compared to wildtypes, but not if high frequency stimulation was used (Precht et al., 1998). Further, the *Drosophila eag* mutant displays learning difficulties amongst other phenotypic characteristics (Finn, Grunwald & Yau, 1996; Schmidt et al, 1993; Griffith, Wang, Zhong, Wu & Greenspan, 1994). Although the *eag* gene products appear responsible primarily for altering K^+ flux there is considerable homology between *eag* and genes coding for CNG channels (Guy, Durell, Warmke, Drysdale & Ganetzky, 1991; Warmke & Ganetzky, 1994).

Evidence for the role of CNG channels in memory comes primarily from pharmaco-behavioural studies. However, although a number of CNG channel antagonists exist, many preferentially inhibit enzymes or other cation channels. For example, the protein synthesis inhibitor, neomycin (Ma & Michael, 1998), the tyrosine kinase inhibitor genistein (Molokanova, Savchenko & Kramer, 1999, 2000), the PKG inhibitor H-8 (Wei, Cohen & Barnstable, 1997), the dopamine antagonist pimozide (Dickinson, Smith & Mirenowicz, 2000; R. Lang et al., 2000) and the RyR channel antagonist ruthenium red (Lukyanenko et al., 2000; Ma & Michael, 1998) have all been reported as inhibitors of CNG channel function. Other CNG channel inhibitors include phosphatidylinositol 4, 5-bisphosphate (PIP(2)) (Womack et al., 2000), 3 types of polyamines (Lu & Ding, 1999) and pseudochetoxin (Brown, Haley, West & Crabb, 1999) which are also unsuitable due to secondary effects or poor characterisation. Finally, verapamil is an inhibitor of voltage-dependent Ca^{2+} channels, specifically L-type Ca^{2+} channels (Diaz & Dickenson, 1997; Gould & Hill, 1994), but has been also recognised as a potent inhibitor of CNG channels (R. Lang, 2000). Importantly, Clements et al. (1995), using a variant of the passive avoidance task for day-old chicks, could not find evidence of a role for L-type Ca^{2+} channels in memory formation when retention was tested at either 30 or 180 minutes post-training. This suggests that the most-notable secondary action of verapamil appears not to be implicated in memory formation at times associated with NO-dependence.

Calcium channel blockers, including verapamil have been used in a number of pharmaco-behavioural studies. Initially, chronic administration of verapamil, and not

diltiazem, had been found effective in overcoming the deleterious effects of social isolation upon performance in an elevated plus maze (Jankowska et al., 1991). In more recent studies, verapamil was found to facilitate retention for a linear maze task, consistent with the action of other calcium channel antagonists. However, verapamil did not change spontaneous alteration behaviour of mice tested in a T-maze nor did it affect retention using a step-through passive avoidance task similarly using young adult mice (Quartermain et al., 2001). Conversely, Lee and Lin (1991) found intrahippocampal administered verapamil, along with nifedipine, inhibited retention in rats trained using a one-way inhibitory avoidance task when tested 24 hours later. The differences in findings between Quartermain et al. (2001) and Lee and Lin (1991) may, in part, be attributable to species differences, comparing mice to rats, and also a result of subcutaneous versus intrahippocampal administration.

While it may appear that verapamil either has no effect upon memory formation, or acts in a fashion consistent with calcium channel antagonists it must be remembered that the above studies were conducted using rodent-based tasks which lacked temporal specificity due either to multiple training trials or limited times of test. Therefore if a more temporally precise task was employed, verapamil may be shown to clearly affect memory formation. Further, it is also possible that a greater number of test times will differentiate the effect of verapamil on retention from other Ca^{2+} channel antagonists. Finally, only if the action of verapamil is consistent with L-NAME would a second antagonist, also with a primary action upon CNG channels, be used to confirm the action of verapamil. This is brought about as the experiments in Chapters 3 and 4 have established a number of different retention functions for NO-dependent processes different to that determined by inhibiting NOS and therefore not associated with the effects of L-NAME.

5.2.1 Verapamil inhibition of olfactory-type cyclic nucleotide-gated ion channels

5.2.1.1 Dose response study for verapamil

Quartermain, Garcia deSoria and Kwan (2001) failed to note any effect of verapamil on passive avoidance learning for juvenile mice using a maximum dose equivalent to a concentration of 0.4M. In contrast, Lee and Lin (1991) found that intrahippocampal administration of the equivalent of 2M verapamil was effective in disrupting avoidance learning at 24 hours for rats. The aim of the present study was to determine if verapamil would result in a loss of retention using the passive avoidance discrimination task for the day-old chick when tested 120 minutes post-training.

As verapamil has not been used previously in passive avoidance learning for the day-old chick a broad range of concentrations were required for testing. Even although Lee and Lin (1991) had found molar concentrations of verapamil effective micromolar concentrations were tested as a wide variety of drugs had previously been found to be effective in this range for the current task. More importantly, if verapamil preferentially inhibits CNG channels then, by definition, the lowest effective concentration would be that which blocked CNG channel action. Effective concentrations in the micromolar range would also be less likely to induce sensory-motor effects.

Method

Chicks were housed and trained as described in detail in Chapter 2. Administration of verapamil occurred immediately post-training which is consistent with the effective times of administration for the NOS inhibitor L-NAME (Rickard et al, 1998). Groups of 20 chicks received either the saline vehicle or one concentration of verapamil. As verapamil had not been used previously in behavioural studies a wide range of concentrations was tested: 0.01μM, 0.1μM, 1μM, 10μM, 100μM, 300μM, 500μM, 600μM, 700μM, 800μM, 900μM, 1mM, 1.2mM or 1.5mM. All groups were tested 120 minutes post-training a time when the protein synthesis-dependent LTM

stage is believed to be consolidated (Ng & Gibbs, 1988). The number of chicks used for each concentration ranged from 17 to 38 as chicks were excluded if they did not train or did not peck the non-aversive blue bead at test. Replication of some concentrations also occurred over the two experimental days. The mean group size was 24.

Results

Retention levels for chicks administered a range of concentrations of verapamil, or saline, immediately post-training and tested 120 minutes post-training are shown in Figure 5.2.

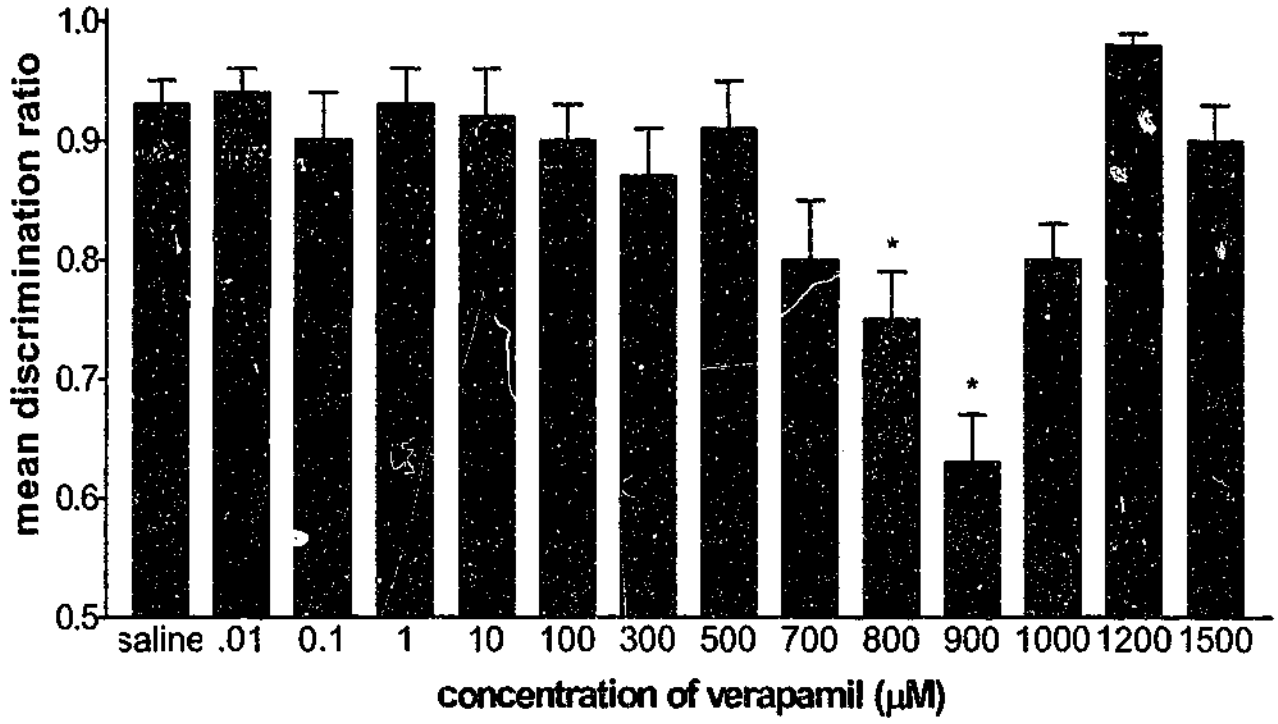


Figure 5.2 Dose response study for the CNG channel inhibitor verapamil. Various concentrations of verapamil were administered immediately post-training and retention was tested 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Concentrations of verapamil between 700μM and 1000μM appear to have impaired retention when administered immediately post-training and tested 120 minutes post-training. A one-way ANOVA revealed a significant concentration effect ($F(13, 320)=8.30$, $p<.001$) and Dunnett's post-hoc analysis showed that only the 800μM ($p<.001$) and 900μM ($p<.001$) verapamil-treated chicks showed significant depression in retention levels compared to saline administered chicks.

Discussion

Administration of a range of concentrations of verapamil immediately post-training resulted in an impairment of retention at both 800 and 900μM when tested 120 minutes post-training. The observed "U-shaped" dose response function may be the result of hormesis (Calabrese & Baldwin, 2001), such that at concentrations higher than 900μM, cellular clearance mechanisms are activated to remove the drug. Similar effects have been observed by Lee and Lin (1991), who also found a "U-shaped" dose response

function for verapamil when rats were tested following a one-way inhibitory avoidance task.

The optimum concentration of verapamil used by Lee and Lin (1991) was equivalent to 2M or 1μg infused in 1μl over 1 minute into the dentate gyrus of each hemisphere. However, as pharmacological agents may cause sensory-motor effects at high concentration it was deemed appropriate to test concentrations no higher than 1.5mM. In doing so it was found that 900μM was most effective in blocking retention which is considerably less than that found effective by Lee and Lin (1991). This difference in effective concentration may, at least in part, be attributed to both task and species differences.

5.2.1.2 *Time of administration study for verapamil*

Previous studies by Lee and Lin (1991) suggest that in rats subjected to a one-way inhibitory avoidance task verapamil impaired retention when administered either 30 minutes before training or immediately following training. While Quartermain et al. (2001) found inconsistent effects for verapamil when compared across several tasks, administration of verapamil immediately after the fifth and final training trial for a linear maze task was effective in facilitating retention while administration 1 hour prior to training was without effect. Although differing in the behavioural effect of verapamil, these findings are consistent with the above dose response study showing verapamil to be effective in blocking retention if administered immediately post-training.

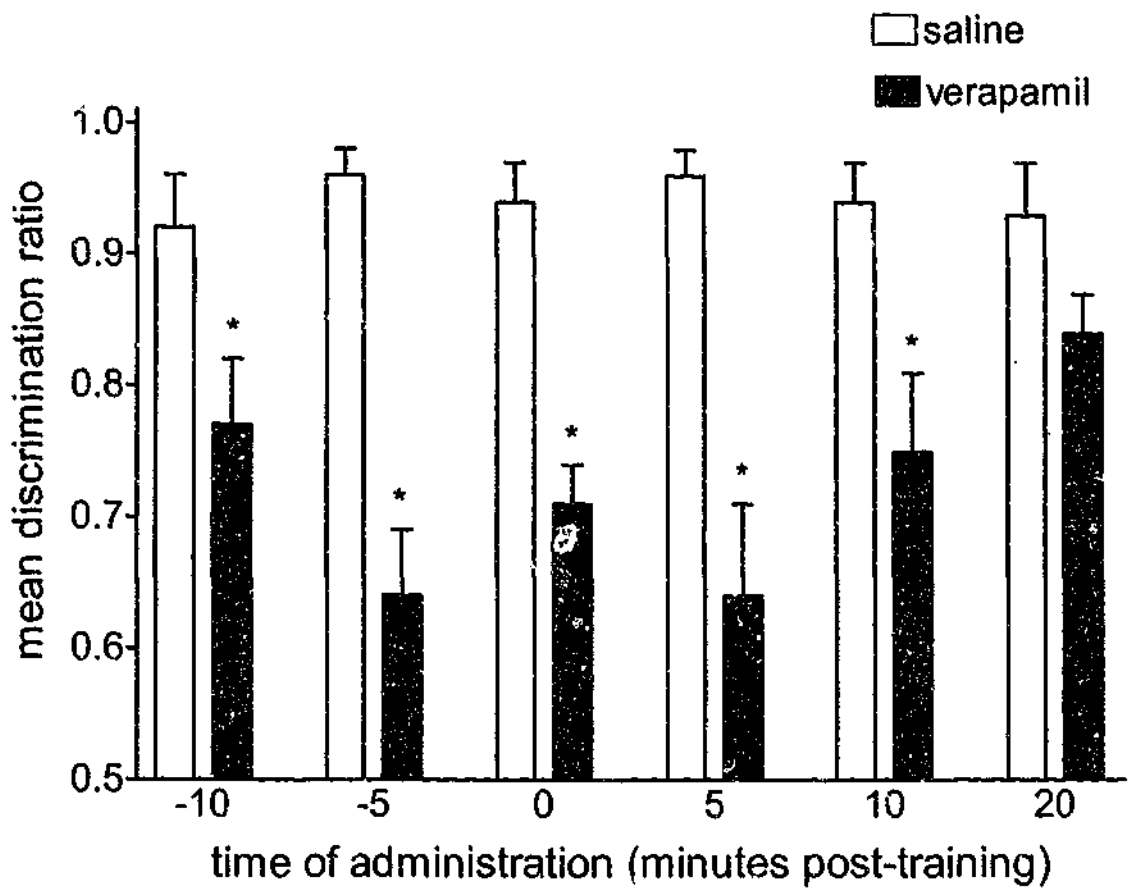
The current study was performed to determine the effective administration times for verapamil in the passive avoidance task developed for the day-old chick. Although effective administration times similar to those found for L-NAME would be suggestive of NO acting upon CNG channels, possible differences in pharmacokinetics makes this interpretation possibly unsafe. Ultimately a retention function study is required to suggest a relationship between NO and a downstream mechanism.

Method

Chicks were housed and trained as described in Chapter 2. Each group of 20 chicks received either 900μM verapamil or the saline vehicle at 10 or 5 minutes before training, immediately after training or at 5, 10 or 20 minutes after training. The times of administration chosen are consistent with the effective times of L-NAME administration (Rickard et al., 1998). Each group was tested 120 minutes post-training in accordance with the time of test used in the dose response study for verapamil. Final groups sizes ranged from 14 to 20 due to the exclusion of chicks which did not train or did not peck at the non-aversive blue bead on test. The mean of group sizes was 18.

Results

Retention levels for chicks administered 900μM verapamil or saline at various times relative to training and tested 120 minutes post-training are shown in Figure 5.3.



saline	n =	(20)	(18)	(18)	(19)	(18)	(19)
verapamil	n =	(19)	(14)	(19)	(16)	(20)	(20)

Figure 5.3 Time of administration function for the CNG channel inhibitor verapamil. 900μM verapamil, compared with saline, was administered at several times relative to training and tested at 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Retention levels for chicks administered 900μM verapamil between 10 minutes before and 10 minutes after training showed a marked decrease in retention relative to chicks administered saline. A two-way ANOVA was performed and revealed a significant drugs main effect ($F(1,208)=89.33$, $p<.001$) and drugs by time of administration interaction effect ($F(5,208)=2.80$, $p<.05$), but not a significant time of administration main effect ($F(5,208)=1.43$, $p>.05$). Simple main effects post-hoc analyses showed a significant difference in retention between chicks administered 900μM verapamil and saline at 10 ($p<.01$) and 5 minutes ($p<.001$) before training, immediately after training ($p<.001$), and at 5 ($p<.001$) and 10 ($p<.005$) minutes post-training.

Discussion

When tested 120 minutes post-training, retention was significantly impaired if verapamil was administered between 10 minutes before training up to 10 minutes after training. This range of times is consistent with effective administration times used by both Lee and Lin (1991) and Quartermain et al. (2001).

This relatively broad range of effective administration times appears to extend further post-training than the effective administration times for NO-activated mechanisms such as GC, PKG and *mono*(ADP-ribosyl) transferase inhibition, but not quite as far as L-NAME inhibition of NOS which is effective at 20 minutes post-training. Pharmaco-kinetic studies of radio-labelled verapamil show half-lives of between 23 and 73 minutes in various human tissues (Hendrickse, de Vries, Franssen, Vaalburg & van der Graaf, 2001). In general terms this rate of clearance appears slower than some other drugs previously discussed in this thesis and is therefore consistent with a broader range of effective administration times. However, the above findings do not discriminate between a period of CNG channel activation extending from the time of learning or a period of CNG channel activation occurring sometime after learning, of which the slow clearance rate for verapamil allows effective administration in advance of CNG channel activation. Therefore the period of CNG channel activation may overlap GC, PKG and *mono*(ADP-ribosyl) transferase activation around the time of training or CNG channel activation may occur after GC, PKG and *mono*(ADP-ribosyl) transferase activation but before NOS activation. One possible method to identify the period of CNG channel activation would be to use successively lower concentrations of verapamil noting any decrease in the breadth of effective administration times. The time(s) which correspond to the lowest concentration able to result in retention loss may suggest the time of CNG channel activation. In this way the relationship of CNG channels to GC and/or NO may be clarified.

5.2.1.3 Time of retention loss study for verapamil

Behavioural studies such as those by Jankowska et al. (1991), Lee and Lin (1991) and Quartermain et al. (2001) have suggested a role for verapamil in memory formation using both avoidance and spatial learning tasks in rodents. In addition to the

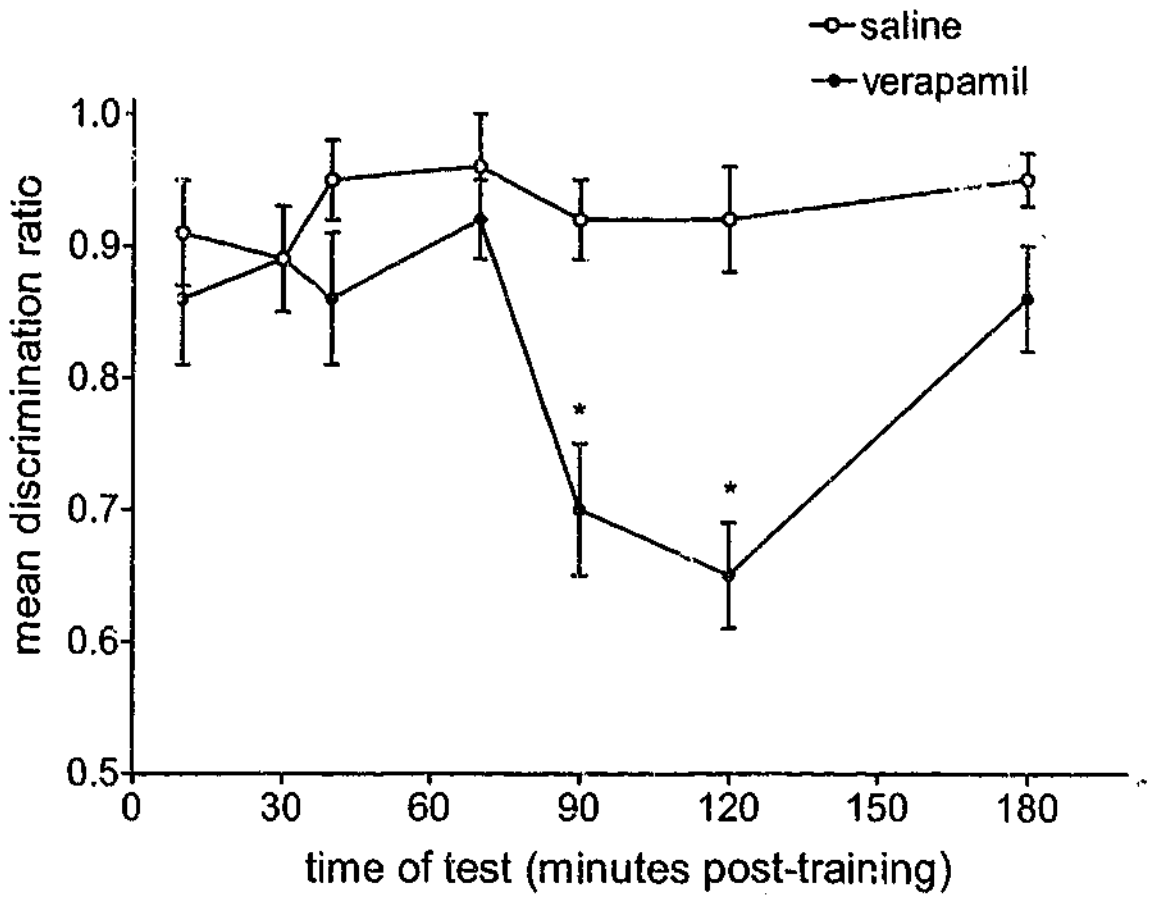
inconsistent effects of verapamil upon retention, these studies tested retention at only one time. Therefore, the time at which memory was first lost could not be inferred. The aim of the present study was to determine the time of onset of memory loss following verapamil administration using the passive avoidance task developed for the day-old chick. As NOS antagonists blocked retention from 40 minutes post-training up until at least 24 hours post-training (Rickard et al., 1998), it would be expected that if NO directly activated CNG channels, associated with the effects of L-NAME, retention loss following the administration of verapamil would also occur at 40 minutes post-training. However, CNG channels are also activated by cyclic nucleotides including cGMP. It is therefore also possible that the retention function following verapamil administration could be similar to that following GC inhibition, with temporary losses in retention around 40 and 120 minutes post-training (see Chapter 3).

Method

Chicks were housed and trained as discussed in Chapter 2. Groups of 20 chicks received either 900μM verapamil or the saline vehicle immediately post-training and were tested at 10, 30, 40, 70, 90, 120, 180 minutes post-training. The number of chicks used per group ranged from 15 to 38 with a mean group size of 21.

Results

Retention levels for chicks administered 900μM verapamil immediately post-training and tested at various times post-training are represented in Figure 5.4.



saline	n = (16)	(20)(16)	(17)	(18)	(15)	(18)
verapamil	n = (19)	(19)(20)	(18)	(20)	(38)	(34)

Figure 5.4 Retention function for the CNG channel blocker verapamil.
900μM verapamil, compared with saline, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Administration of 900μM verapamil resulted in a transient loss of retention after 60 minutes with recovery by 180 minutes post-training. A two-way ANOVA for 900μM verapamil revealed a significant drug main effect ($F(1,274)=24.80$, $p<.001$), a significant time of test main effect ($F(6,274)=3.61$, $p<.005$) and a significant drug by time of test interaction effect ($F(6,274)=2.96$, $p<.01$). Simple main effects post-hoc analysis revealed significantly lower mean discrimination ratios at 90 mins ($p<.001$) and 120 mins ($p<.001$) in chicks treated with 900μM verapamil compared to matching controls.

Discussion

Administration of 900μM verapamil immediately post-training resulted in a transient loss of retention after 60 minutes post-training with recovery by 120 minutes post-training. In contrast, L-NAME administration results in persistent retention loss from 40 minutes post-training. Therefore CNG channels cannot account for the action of NO associated with retention loss from 40 minutes onwards.

Verapamil is recognised to have a number of secondary actions, as do all CNG channel inhibitors. To precisely ascertain the effect of CNG channel inhibition on retention the effects of two inhibitors can be compared so long as they do not share common secondary effects. This was undertaken in Chapter 3, using ODQ and LY83583, to determine the role of GC and in Chapter 4, using MSB and NOVO, to determine the role of *mono*(ADP-ribosyl) transferase in memory formation. Specifically, comparative studies for GC inhibitors were undertaken as previous investigations had found GC to be the most likely downstream mechanism for NO in memory formation (Bernabeu et al., 1995, 1996, 1997; Izquierdo et al., 2000) while those studies reported in Chapter 3 had found a unique bimodal retention loss not previously observed and not consistent with the action of L-NAME. Comparative studies were undertaken in Chapter 4 as there did not exist a single inhibitor of *mono*(ADP-ribosyl) transferase thought specific enough to accurately represent the actions of *mono*(ADP-ribosyl) transferase in memory formation. From these studies it was also clear that NO-activated processes need not conform to the retention function determined following the administration of L-NAME. That retention loss at 40 minutes post-training may represent only the first of many NO-dependent retention periods. Therefore, although verapamil is not a very specific inhibitor of CNG channels, retention loss following its administration occurs much later than that observed using L-NAME excluding it as the possible mechanism through which NO acts during the ITM-B stage. For this reason comparative studies with an alternate inhibitor of CNG channels was not undertaken.

cGMP is known to activate three classes of protein: PKG, CNG channels and PDEs. Inhibition of cGMP production results in a bimodal retention function as discussed in Chapter 3 (Edwards et al., 2002), with transient losses of retention onset being at 40 and 90 minutes. Having found that PKG and CNG channel inhibition corresponds to the later transient retention loss for GC, it may be speculated that

phosphodiesterase activation lies downstream of GC at 40 minutes post-training. However, pharmacological inhibition of PDEs would be needed to confirm this possibility.

Given that the action of verapamil is likely to be upon CNG channels, the transient nature of the retention loss from 90 to 120 minutes post-training following verapamil administration is intriguing. As previously discussed, Allweis et al. (1984) suggested that transient retention deficits found in the present passive avoidance task were most likely the result of an effect upon memory retrieval and not formation mechanisms. As with other transient losses observed in Chapter 3, they appear to occur at the same time as do permanent retention losses induced by other agents, or exist for the duration of an identified memory stage. This is also true for the effects of verapamil, as both tyrosine kinase (Whitechurch et al., 1997) and glutamate receptor inhibition (Rickard et al., 1994, 1995) result in retention loss by around 90 minutes post-training. It appears that memory retrieval may consist of stages which share similar temporal parameters as the stages of memory formation although this remains speculative.

SECTION 3 : The effect of large conductance calcium-activated potassium channel inhibition on passive avoidance learning

There exists a wide variety of K^+ channels, including voltage-gated (K_V), calcium-activated (K_{Ca}), ligand-gated (K_{LIG}), ATP-activated (K_{ATP}) and stretch-activated (K_{STR}). Of these channels, K_{Ca} channels, especially the large conductance K_{Ca} (BK_{Ca}) channels, are recognised as intimately involved in various neuronal processes and in vasodilation. Many studies have suggested that NO may directly activate K_{Ca} and BK_{Ca} channels in particular.

BK_{Ca} channels have been identified in hippocampal CA1 pyramidal neurons (Shao et al., 1999), as well as in hippocampal neurons from cultures derived from a neonate source (Soh, Jung, Uhm & Chung, 2001), which is of particular relevance to the task used in the current studies.

NO has been shown to activate BK_{Ca} channels either through the activation of GC and PKG (Archer et al., 1994; Hampl et al., 1995; Li et al., 1998) or directly. Direct activation has been observed using patch-clamp techniques. For example, Schmachtenberg and Bacigalupo (1999, 2000) used olfactory receptor neurons from a range of species and found that NO activated BK_{Ca} channels directly. Further, Ahern et al. (1999) demonstrated that NO directly activates BK_{Ca} channels in posterior pituitary nerve terminals in rats. Importantly, the activation of BK_{Ca} channels by NO was shown to occur in the presence of GC inhibitors.

BK_{Ca} channel activation has also been implicated following learning. For example, Schreurs et al. (1998) measured the effects of classical conditioning on changes in Purkinje cell dendritic membrane excitability in a specific region of the cerebellum of the rabbit. Interestingly, increases in membrane excitability related to conditioning could be mimicked in control rabbits by application of a variety of K^+ channel blockers including IbTX which is a specific blocker of BK_{Ca} channels. However, only 4-aminopyridine, an inhibitor of both delayed rectifier K^+ channels and K_v channels (Kerr et al.,

2001; Thorneloe et al., 2001), reduced the observed transient hyperpolarisation. Therefore, while BK_{Ca} channels appear to be activated following learning as shown by the action of IbTX, they do not appear to be sufficient by themselves to induce memory formation.

Direct evidence of an association between BK_{Ca} channels and memory formation was provided by Ghelardini et al. (1998) who used a passive avoidance task for the mouse and found administration of a number of K^+ channel blockers, including the BK_{Ca} channel-specific blocker ChTX, 20 minutes before training, prevented amnesia brought about by the prior administration of K_{ATP} channel agonists. Taken together, these findings suggest that BK_{Ca} channels do have a role in memory formation although it remains ill defined at present.

As discussed in Chapter 1, one of the processes believed to modulate memory formation is cerebral vasodilation which increases nutrient levels to metabolically active brain regions following learning. While the few studies on the role of K_{Ca} channels, including BK_{Ca} channels, in the cerebral vasculature support cGMP-dependent K_{Ca} channel activation (Onoue & Katusic, 1997; Schuh-Hofer et al., 2001; Wang, et al., 1998), more common smooth muscle experimental systems also suggest a NO-dependent but GC-independent pathway leading to K_{Ca} channel activation. This has been suggested by both pharmacological and electrophysiological studies. Bolotina et al. (1994) demonstrated that the GC inhibitor MB was ineffective in blocking NO-activated, BK_{Ca} channel-dependent vasodilation. Interestingly, cell-free membrane patches revealed that NO could directly activate single Ca^{2+}-activated K^+ channels. Although MB is not an ideal antagonist of GC (Cohen et al., 2000; Kontos & Wei, 1993), these studies were supported by Fukami et al. (1998) and Chen et al. (1999), who found both MB and the cell permeable and sGC specific antagonist, ODQ, only partially inhibited NO-activated, K_{Ca}-dependent vasodilation, suggesting a GC-independent mechanism of action. Later studies by Mistry and Garland (1998) and R.J. Lang et al. (2000) confirmed that BK_{Ca} channels are directly activated by NO leading to vasodilation.

Studies using mesenteric smooth muscle also confirm these observations (Abdullah & Docherty, 1999; Plane et al. 1996). Coronary arteries have also been shown to have characteristics appropriate for GC-independent vasodilation, while being dependent upon NO and K_{Ca} channels (Taguchi et al., 1996; Ferrer et al., 1999; Jiang et

al., 1999). However, as with the studies above, there also appears to be a GC-dependent component to coronary artery relaxation.

However, several studies also dispute GC-independent vasodilation. Using mesenteric arteries, Adeagbo (1999) found that vasodilation could be blocked by the NOS inhibitor, L-NAME, by the GC antagonist ODQ, or by a BK_{Ca} channel antagonist. This finding was confirmed by patch-clamp experiments using microvessels from rat mesentery. Similarly, Carrier, Fuchs, Winecoff, Giulumian and White (1997) found that both a spontaneous NO donor, SNP, and a cGMP analogue induced vasodilation while vasodilation was attenuated by PKG inhibition. Similarly, evidence exists to suggest coronary arteries are not GC-independent in their production of vasodilation (Hernanz et al., 1999; Price & Hellermann, 1997). In other forms of smooth muscle, the role of GC-independent pathways mediating relaxation has also been disputed. Zhou et al. (1998) and Bradley et al. (1998) failed to find GC-independent vasodilation using human uterine smooth muscle.

In conclusion, NO has been demonstrated to directly activate BK_{Ca} channels in both neurons and smooth muscle cells. Further, there exists albeit indirect, behavioural evidence for a role for BK_{Ca} channels in memory formation. Finally, both synaptic transmission and vasodilation, considered to be physiological processes underlying memory formation, appear to involve BK_{Ca} channel activation. The aim of the current set of studies was to determine if BK_{Ca} channels were implicated in memory formation using the passive avoidance task for the day-old chick and, if so, whether this may be the pathway through which NO plays its role in memory formation.

5.3.1 Iberiotoxin inhibition of large conductance calcium-activated potassium channels

5.3.1.1 Dose response study for Iberiotoxin

Two common inhibitors of BK_{Ca} channels are IbTX and ChTX. While ChTX appears to be some what more potent (de-Allie, Bolsover, Nowicky & Strong, 1996; Tauc et al., 1993), IbTX appears to be more specific

in its ability to inhibit BK_{Ca} channels (Bang, Boesgaard, Nielsen-Kudsk, Vejlstrup & Aldershvile, 1999; Ohno-Shosaku, Kim, Sawada & Yamamoto, 1996). For these reasons IbTX has been selected for the current studies.

IbTX has been used widely in both neuronal and vascular preparations and in *in vivo* studies, thus providing an indication of an effective dose range for the current behavioural studies. For example, Martinez-Pinna, Davies and McLachlan (2000) found 20nM IbTX reduced prolonged after-polarisation of a subgroup of sympathet. neurons in the guinea pig celiac ganglia by 55%, while Li and Hay (2000) found 100nM IbTX effective in blocking the action of BK_{Ca} channels in postrema neurons. With respect to vascular preparations, Hohn, Pataricza, Toth, Balogh and Papp (1996) preincubated human saphenous vein with 90nM IbTX and found it blocked NO induced relaxation. Abdullah and Docherty (1999) used rat small mesenteric arterial rings and found both ChTX and 100nM IbTX effectively blocked GC-independent vasodilation. Similarly, Khan, Higdon and Meisheri (1998) found that 200nM IbTX or ChTX blocked nitroglycerine-induced relaxation in isolated dog coronary arteries. In another experimental model using bladder smooth muscle, Herrera, Heppner and Nelson (2000) found 100nM IbTX effectively increased contraction amplitude and duration. Importantly for the current behavioural studies, Bang et al. (1999) measured the hypotensive effect of nitroglycerine *in vivo* using conscious, chronically catheterised rats during an intravenous infusion of IbTX and found 100nM effective in preventing the action of NO-induced relaxation. Finally, Ghelardini et al. (1998) used 1µg/mouse of the alternate BK_{Ca} channel antagonist, ChTX administered icv, to block passive avoidance memory. At this concentration no sensory-motor effects were observed.

The aim in the current study was to determine if BK_{Ca} channels were involved in memory formation using the passive avoidance task for the day-old chick and if so at what concentration of IbTX would maximal loss of retention occur. *In vitro* and *in* vivo studies cited above suggest that a range of concentrations of IbTX between 20 and 200nM is likely to result in blockade of BK_{Ca} channels when administered *in vivo*. However, as the passive avoidance task for the chick differs significantly from previous studies from which this concentration range was determined, a wider range of concentrations was explored.

Method

Chicks were housed and trained as stated in Chapter 2. IbTX was administered immediately post-training, which is consistent with the effective times of administration for the NOS inhibitor L-NAME (Rickard et al., 1998). Each group of 20 chicks received either the saline vehicle or a different concentration of IbTX. The concentrations of IbTX administered were 1nM, 10nM, 50nM, 100nM, 250nM and 500nM and the time of test was 40 minutes post-training which is in contrast to previous dose response studies using a time of test 120 minutes post-training. Although this time of test is before the consolidation of the LTM stage the central aim of this thesis was to identify that NO-dependent mechanism which accounts for retention loss observed following L-NAME administration. As previous mechanisms studies in Chapters 3, 4 and 5 have described retention losses at various times post-training it is more efficient to have a time of test at 40 minutes post-training. Groups ranged in size from 16 to 20 with a mean of 18.

Results

Retention levels for chicks administered various concentrations of IbTX or saline immediately post-training and tested 40 minutes post-training are shown in Figure 5.5.

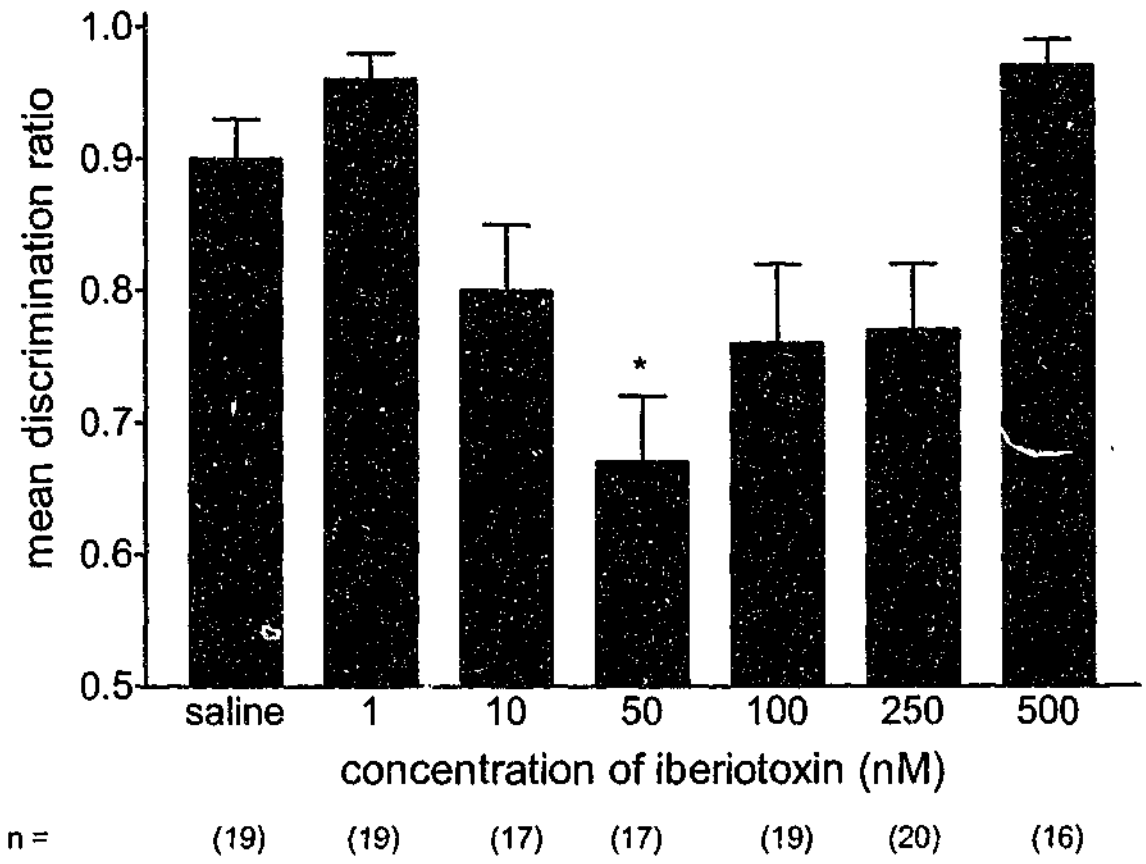


Figure 5.5 Dose response function for the BK_{Ca} channel inhibitor IbTX. Various concentrations of IbTX were administered immediately post-training and retention was tested 40 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Chicks administered 10-250nM IbTX appeared to demonstrate reduced mean DR's when compared with control chick administered saline. A one-way ANOVA revealed a significant concentration effect ($F(6, 120)=6.00$, $p<.001$) and Dunnett's post-hoc analysis showed a significant difference in retention between chicks administered saline and 50nM IbTX only ($p<.005$). No other concentration yielded a significant retention loss.

Discussion

Administration of a range of concentrations of IbTX between 1 and 500nM immediately post-training resulted in a "U-shaped" dose response curve when tested 40 minutes later, with maximum and significant retention loss observed using 50nM IbTX. It should be noted that verapamil, a CNG channel antagonist, also resulted in a "U-shaped" dose response function, both in the current research and in studies using an

avoidance task for rats (Lee & Lin, 1991), as suggested elsewhere this may reflect hormesis (Calabrese & Baldwin, 2001).

The current finding that 50nM IbTX optimally impaired memory is consistent with IbTX working through BK_{Ca} channels since previous investigators have found that BK_{Ca} channels were blocked with IbTX concentrations between 20nM and 200nM (Bang et al., 1999; Herrera et al., 2000; Martinez-Pinna et al., 2000; Li & Hay, 2000; Hohn, et al., 1996; Abdullah & Docherty, 1999; Khan et al., 1998). These results suggest that BK_{Ca} channels are necessary for some aspect of memory processing for the passive avoidance task in the chick and are broadly supported by the earlier studies of Ghelardini et al. (1998).

5.3.1.2 Time of administration study for Iberiotoxin

The central aim of this thesis is to identify a NO-dependent mechanism which, when pharmacologically inhibited, results in a retention function similar to that following L-NAME administration. Although likely differences in drug pharmacokinetics suggest that an inhibitor of a NO-dependent mechanism would not share identical effective times of administration with L-NAME such studies are necessary for two reasons. Effective administration times about the time of learning imply an action of the NO-dependent mechanism in response to the learning event. Alternatively, broad periods of effective drug administration, up to 20 minutes, would add considerable weight to retention study findings replicating the retention function for L-NAME. Therefore the aim of the current experiment is to determine the range of effective administration times for IbTX.

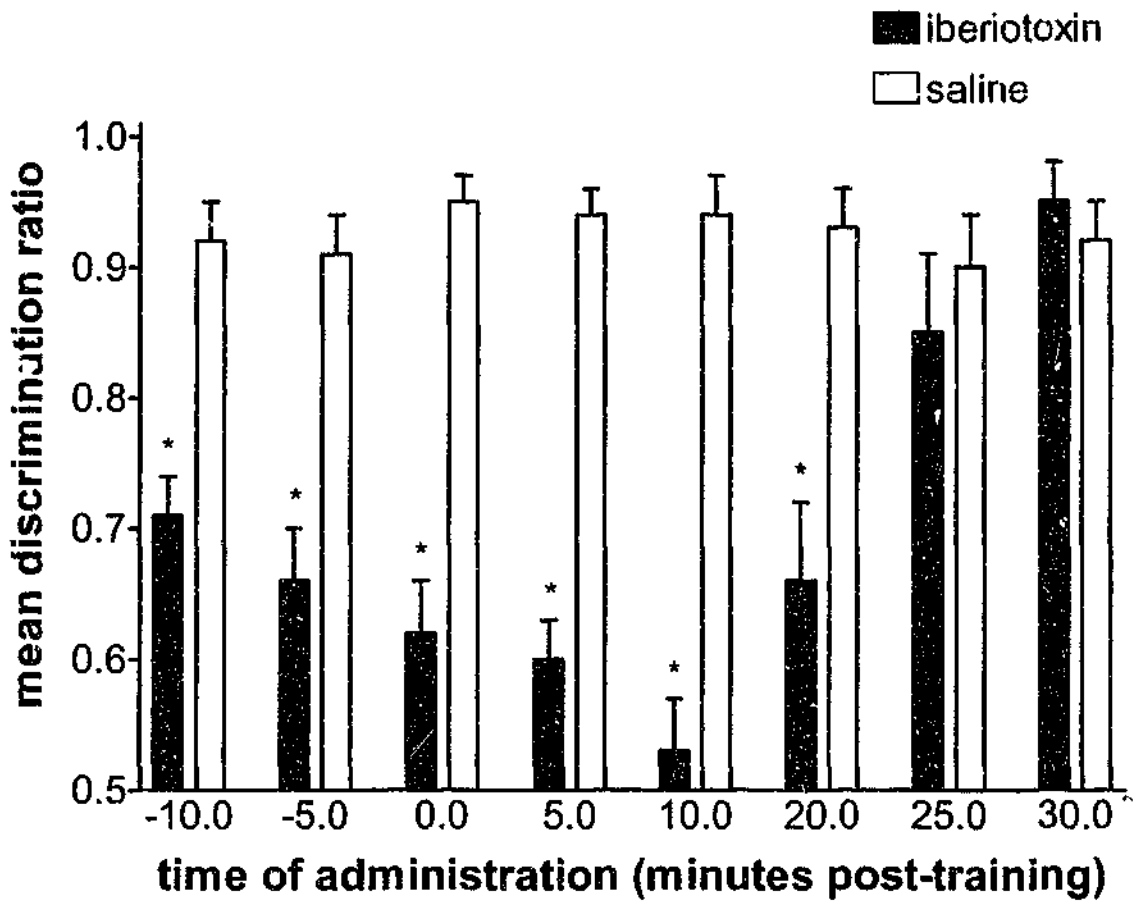
Method

Chicks were housed and trained as described in Chapter 2. Each group of 20 chicks contributed data to one time of administration data-point. Groups of chicks received either 50nM IbTX or saline at 10 or 5 minutes prior to training, immediately after training or 5, 10, 20, 25 or 30 minutes after training. Each group was then tested 40 minutes post-training which is both consistent with the time of test used in the dose response study for IbTX and the earliest time at which NOS inhibition by L-NAME

results in retention loss (Rickard et al., 1998). However, exclusion of chicks which did not train or did not peck at the non-aversive bead during testing and the replication of some time points across the two days of experimentation resulted in a range of group sizes from 14 to 38 with a mean of 21.

Results

Retention levels for chicks administered 50nM IbTX at various times relative to training and tested 40 minutes post-training are shown in Figure 5.6.



saline	n =	(20)	(18)	(18)	(19)	(18)	(19)	(19)	(14)
IbTX	n =	(20)	(19)	(19)	(19)	(18)	(38)	(38)	(19)

Figure 5.6 Time of administration function for the BK_{Ca} channel blocker IbTX. 50nM IbTX, compared with saline, was administered at several times relative to training and tested at 40 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

There appeared to be substantial retention losses for chicks administered 50nM IbTX between 10 minutes before training up to 20 minutes after training. A two-way ANOVA revealed a significant drug main effect ($F(1,319)=121.95$, $p<.001$), a significant time of administration main effect ($F(7,319)=3.84$, $p<.005$) and a significant drug by time of administration interaction effect ($F(7,319)=6.39$, $p<.001$). Simple main effects post-hoc analyses showed a significant difference in retention between chicks administered 50nM IbTX and saline at all administration times between 10 minutes before training and 20 minutes post-training (each $p<.005$).

Discussion

50nM IbTX was effective in blocking retention when administered between 10 minutes before training up to, and including, 20 minutes post-training. This time of administration function matched that obtained with L-NAME (Rickard, 1995; Rickard et al., 1998). This represents the first demonstration of a candidate NO-dependent mechanism within the present learning paradigm which has the same effective administration times as the NOS inhibitor L-NAME (Rickard, 1995; Rickard et al., 1998). Further, these characteristics are in marked contrast to the effective administration times of GC, PKG and *mono*(ADP-ribosyl) transferase.

5.3.1.3 Time of retention loss study for Iberiotoxin

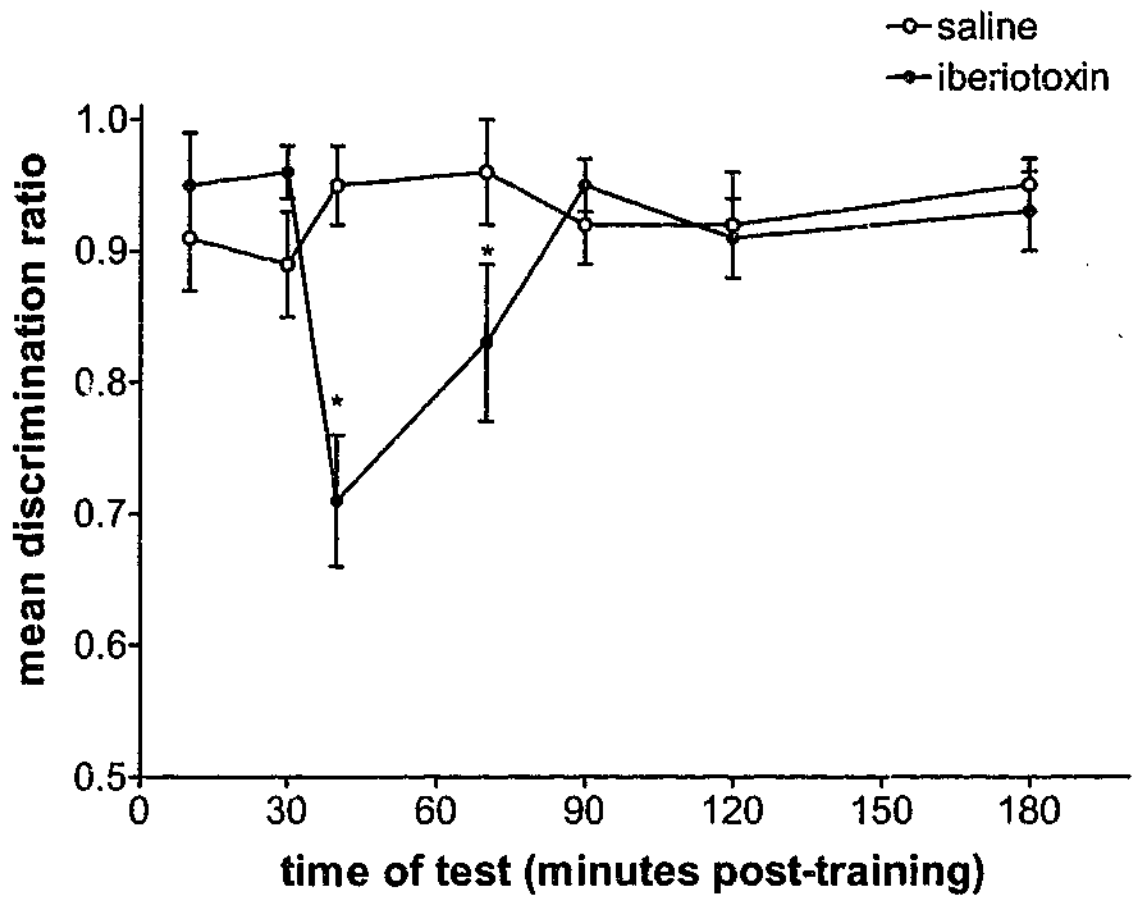
The aim of the current experiment was to determine whether inhibition of BK_{Ca} channels result in a similar retention function as observed following NOS inhibition. Inhibiting the production of NO is known to result in a persistent loss of retention by 40 minutes post-training (Hölscher & Rose 1992 & 1993; Rickard et al., 1998). If BK_{Ca} channel inhibition similarly results in a persistent retention loss by 40 minutes post-training then it may be concluded that BK_{Ca} channels account for the action of NO associated with the retention loss brought about by the administration of L-NAME.

Method

Chicks were housed and trained as described in Chapter 2. Groups of 20 chicks received either 50nM IbTX or saline immediately post-training and were tested at 10, 30, 40, 70, 90, 120 or 180 minutes post-training. Group sizes ranged from 15 to 20 with a mean of 17.

Results

Retention levels for chicks administered 50nM IbTX or saline immediately post-training and tested at various times post-training are shown in Figure 5.7.



saline n =	(16)	(20)(16)	(17)	(18)	(15)		(18)
IbTX n =	(15)	(16)(20)	(19)	(18)	(16)		(18)

Figure 5.7 Retention function for the inhibition of the BK_{Ca} channels.
50nM IbTX, compared with saline, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Administration of 50nM IbTX immediately post-training resulted in a transient impairment of retention beginning at about 40 minutes post-training and recovered by 90 minutes post-training. A two-way ANOVA revealed a significant time of test main effect ($F(6,228)=2.26$, $p<.05$) and a significant drug by time of test interaction effect ($F(6,228)=4.49$, $p<.001$) but not a significant drug main effect ($F(1,228)=3.65$, $p>.05$). A simple main effects post-hoc analysis showed a significant difference in retention levels between 50nM IbTX and saline treated chicks at 40 ($p<.001$) and 70 minutes ($p<.05$) post-training, but not before or after this training-test interval, respectively.

Discussion

Administration of 50nM IbTX immediately post-training resulted in a transient retention loss between 40 and 70 minutes post-training. NOS and BK_{Ca} channel inhibition share both a common set of effective administration times and time of retention loss onset. However, the transient nature of the retention loss induced by BK_{Ca} channel inhibition indicates that BK_{Ca} channels are unlikely to play a significant role in the action of NO in consolidating LTM formation in this paradigm. However, the identification of the action of BK_{Ca} channels in the ITM-B and early LTM stages is the first demonstration of a role for K^+ channels later than the ITM-A stage (Gibbs & Ng, 1979b).

While not mimicking the persistent retention loss found for NOS inhibition, it is of note that the temporary retention loss observed for BK_{Ca} inhibition was consistent with both the onset of retention loss for NO and the onset of retention loss observed for the earlier of two transient retention losses following GC inhibition (see Section 3.4.1.3). Specifically, GC inhibition resulted in two temporally distinct transient retention losses, one centred at 40 minutes post-training and the other at 120 minutes post-training. The similarities in the time of retention loss onset for NOS, GC and BK_{Ca} channel inhibition at 40 minutes post-training may initially suggest that in passive avoidance memory formation NO activates BK_{Ca} channels through GC. However, as GC acts through PKG and inhibition of PKG does not result in retention loss until 100 minutes post-training, it is unlikely that if NO does activate BK_{Ca} channels in passive avoidance learning that it would do so through GC. Therefore any action of NO upon BK_{Ca} channels in passive avoidance learning would appear to be through direct activation.

Transient retention losses have been interpreted as disruption of retrieval processes as inhibition of memory formation would result, by definition, in the termination of the memory trace, thus making re-establishment of retention impossible (Allweis et al., 1984). Therefore, the current results would also suggest that BK_{Ca} channels are necessary for memory retrieval and not formation. Interestingly, while the other transient retention losses reported coincide with known stages of memory formation, the temporary retention loss observed following BK_{Ca} channel inhibition extends from 40 to 70 minutes post-training. This includes the ITM-B stage and the onset of the LTM stage with restoration of retention occurring by 90 minutes post-

training. Interestingly, 90 minutes post-training is a time at which a number of processes appear necessary for passive avoidance memory formation (Rickard et al., 1994b, 1995; Whitechurch et al., 1997).

SECTION 4 : The effect of ryanodine receptor calcium release channel inhibition on passive avoidance learning

Another ion channel thought to be directly activated by NO is the RyR channel. Originally believed to be inhibited by NO (Meszaros et al., 1996), Stoyanovsky et al. (1997) demonstrated NO donors oxidise the RyR channel and increase the probability of opening of RyR channels in striatal and cardiac muscle resulting in Ca^{2+} release into the cytosol. Later studies by Xu et al. (1998) and Aghdasi et al. (1997) suggested that the probability of opening of RyR channels was related to the level of NO production, such that low levels of NO inhibited channel opening thereby reconciling the studies of Meszaros et al. (1996) and Stoyanovsky et al. (1997). Hart and Dulhunty (2000) have observed a similar effect but suggest NO directly activates RyR channels at low concentration and inhibits their opening at high concentrations. Specifically, Anzai et al. (2000) suggest that NO or nitrothiol can oxidise or nitrosylate exposed SH groups on the RyR receptor and through the resultant conformational change, increase the channel's opening. This has also been concluded by Hart and Dulhunty (2000). Specifically, Sun et al. (2001) has identified a particular cysteine residue (C3635) as being vital to the direct activation of the RyR1 isoform by NO. However, a review by Pessah and Feng (2000) suggest that hyperreactive thiols, of which only a few have been found, are more likely to act as redox sensors and in this way indirectly result in increased channel opening.

There are three isoforms of the RyR channel, denoted 1, 2 and 3, and it has been found that, along with direct activation by NO, RyR isoforms are activated by cyclicADP-ribose (cADPR). The role of ADP-ribosylation in passive avoidance memory formation was discussed in Chapter 4, but this process should not be confused with the production of cADPR. While *mono*ADP-ribosylation occurs through the action of *mono*(ADP-ribosyl) transferase, cADPR production results from the activation of ADP-ribosyl cyclase. Interestingly, ADP-ribosyl cyclase appears to be under the

indirect control of NO through the activation of GC (Clementi, Riccio, Sciorati, Nisticò & Meldolesi, 1996).

Evidence for the involvement of RyR channels in memory formation comes from a number of sources including histological studies which have identified RyR channels in memory related brain regions such as the hippocampus (Balschun et al., 1999; Futasugi et al., 1999; Sharp et al., 1993) across a wide number of species, including rabbits (Hakamata, Nakai, Takeshima & Imoto, 1992; Murayama & Ogawa, 1996), humans (Martin, Chapman, Seckl & Ashley, 1998) and chicks (Ouyang et al., 1993). RyR channel distribution also appears to be developmentally regulated (Mori, Fukaya, Abe, Wakabayashi & Watanabe, 2000) which is of importance in studies utilising the passive avoidance task developed for neonate chicks. Of further importance, Ouyang et al. (1993) used a mouse monoclonal antibody which recognised all three RyR channel isoforms and found high levels of staining in the cerebellum, moderate to low levels of staining in the parahippocampus and hippocampus, and low levels of staining in the hyperstriatum ventrale (HV) of day-old white Leghorn chicks. Since pharmacological agents are administered directly into the neostriatal/hyperstriatal complex containing the intermediate medial HV (IMHV), a region associated with memory formation (Rose & Csillag, 1985; Sedman et al., 1991), in the current studies, this finding is of particular interest. The low levels of staining found in the HV suggest that RyR channels are unlikely to be required for learning in this species. However, this finding may reflect methodological issues such as the use of a mouse antibody to detect RyR channels in the chick. One channel isoform may be of particular importance in memory formation but have a poor affinity to the antibody used. Further, it must be noted that NOS also demonstrated only moderate levels in the HV (Brüning, 1993) but that NOS antagonists are effective when administered to the region containing the IMHV but not another memory-associated region, namely the LPO (Rickard & Gibbs, in preparation - b). Therefore it may be that only a subpopulation of cells in the IMHV may possess NOS, and by implication, RyR channels, which may be important for learning and memory processing or that low levels of expression are sufficient to alter neuronal functions. Alternatively, the antagonists of NOS activation may act at a distance to the site of administration which may also be effective for RyR channel inhibition. However, this alternative appears unlikely as Greenberg, Hamada and Rysman (1997) found little diffusion of the NOS inhibitor L-NNA in the chick brain.

RyR channels are also necessary in cellular processes thought to underlie memory, including LTP and LTD, neurotransmitter release (He et al., 2000; Mothet et al., 1998) and vasodilation (Furstenau et al., 2000; Porter et al., 2001). For example, a large number of studies have supported the role of RyR channels in both LTP and LTD using either gene knock-outs for one of the RyR channel isoforms (Balschun et al., 1999; Futasugi et al., 1999) or a specific pharmacological antagonist such as dantrolene (Katchman & Hershkowitz, 1993; Obenaus et al., 1989; O'Mara et al., 1995; Reyes-Harde et al., 1999; Tekkök & Krnjević, 1996; Wang & Kelly, 1997), which appears to be effective in inhibiting RyR1 and 3 channels only (Zhao, Li, Chen, Louis & Fruen, 2001).

However, the clearest evidence for the role of RyR channels in memory formation comes from behavioural studies. Mice, chicks and the mollusc *Hermissenda crassicornis* have all been found to be dependent upon RyR channels for memory formation. Further, a number of different learning tasks have been utilised: including classical conditioning; contextual fear conditioning; spatial tasks, such as the Morris water maze, Y-maze, radial arms maze, and elevated plus maze; step-through passive avoidance task and a passive avoidance discrimination learning task.

While only limited evidence suggests impairment of RyR channels negatively affects memory formation using contextual fear conditioning in mice (Kouzu et al., 2000) or a classical conditioning task for *Hermissenda crassicornis* (Blackwell & Alkon, 1999). There is, however, considerably more evidence supporting the important role of RyR channels in memory formation for other tasks. For example, spatial learning has been shown to be RyR channel-dependent. Ohnuki and Nomura (1996) used 20nmol of dantrolene, equivalent to 2mM, to disrupt maze choice accuracy and increase the number of errors in the radial-arm maze task. Kouzu et al. (2000) used mutant mice lacking a functional RyR3 channel and compared their performance on various tasks to wild-types. In particular, RyR3 channel deficient mice showed impairments of performance in Y-maze learning and also reduced fear response in the elevated plus-maze test. RyR3 mutants have also been assessed for spatial learning in the Morris water maze. Balschun et al. (1999) observed an impairment in learning a new platform position but not the original position. This was shown by increased searching around the old location, indicating RyR3 disruption had no effect on retention of previously learned material. In contrast, Futasugi et al. (1999) also used RyR3 channel mutant mice and demonstrated increased spatial learning using Morris water

maze. During seven days of training no significant differences between wild-type and RyR3 channel mutants were apparent in the time taken to reach the hidden platform, but in the probe test, the RyR3 channel-deficient mice spent more time in the quadrant which had contained the platform than the wild-type mice. RyR3 channel-deficient mice also crossed the former platform site more often. Importantly, in a cued task, the performances of the mutant and wild-type mice were similar; thus, the improvements observed for the RyR3 channel-deficient mice were not due to swimming ability or motivation but to improved spatial learning. However, an allosteric place discrimination task based upon the Morris water maze was used to differentiate learning from non-learning behavioural effects (Kikusui, Tonohiro & Kaneko, 1999). They suggested that at high concentrations, dantrolene may have secondary actions upon retention not associated with learning and memory *per se.*

Although the role of RyR channels in spatial learning appears complex, the role of RyR channels in passive avoidance learning is far clearer. For exampie, Kouzu et al. (2000) used mutant mice lacking the RyR3 channel isoform and found impaired performance on a passive avoidance task. Ohnuki and Nomura (1996) used dantrolene in place of genetic disruption and subjected mice to a step-through passive avoidance task. 10nmol dantrolene increased the time taken to reach criterion in acquisition, and both 6 and 10nmol decreased the response latency in the retention test suggesting an impaired memory. No abnormal behaviour was observed, suggesting that, at these concentrations dantrolene was effective in impairing learning and/or memory processing without resulting in sensory-motor effects.

A variant of the single trial passive avoidance task used in this thesis was employed in a study of Ca^{2+} release *in vivo* and in synaptosomes accompanying AMPA or NMDA action (Salinska et al., 2001). They found dantrolene (equivalent to 5mM in the present thesis) inhibited memory when administered at 30 minutes before training and 30 minutes after training. Significant retention loss was observed at 3 hours post-training, but not at 30 minutes post-training and was not influenced by the use of an 8% DMSO vehicle. The limited times of test, however, do not allow for a clear description of the time of retention loss onset, occurring some time between 30 minutes and 3 hours post-training. In addition, differences observed in a number of measured criteria between variants of the passive avoidance task for neonate chicks, noted by Crowe and Hamalainen (2001), also necessitate the replication of Salinska et al's (2001) studies using the methodology outlined in Chapter 2.

In conclusion, NO has the ability to activate RyR channels directly, and isoforms of the RyR channel have been shown to be expressed in memory associated regions of the brain such as the hippocampus. The chick brain has also been shown to express all three isoforms of the RyR channel, although only sparsely in the memory associated region of the HV. RyR channels appear to be important in a number of processes associated with memory formation including synaptic plasticity, neurotransmitter release and vasodilation. Finally, a number of behavioural studies have directly shown the importance of RyR channels in various learning tasks. Therefore, direct activation of the RyR channel by NO is a possible candidate pathway to explain the role of NO in passive avoidance memory formation as distinct from memory retrieval processes. While previous studies have demonstrated the importance of RyR channels in memory formation there has not been any attempt to link the activation of RyR channels to NO production as a mechanism underlying memory processing.

5.4.1 Dantrolene inhibition of the ryanodine receptor calcium release channel

5.4.1.1 Dose response study for dantrolene

There are a limited number of antagonists to RyR channels, with dantrolene and ryanodine being the most widely used in both electrophysiological and behavioural studies. While protamine (Koulen & Ehrlich, 2000) and a class of compounds known as bastadins (Mack, Molinski, Buck & Pessah, 1994) are quoted as specific inhibitors of RyR channels, they are inappropriate for behavioural studies due to a lack of knowledge regarding their secondary actions and their limited experimental use. Neomycin is another common RyR channel inhibitor (Wang et al., 1996) and is commercially available; however, its specificity for RyR channels is poor as it also inhibits phospholipase C (Phillippe, 1994) and GTP-stimulated phospholipase D (Liscovitch, Chalifa, Danin & Eli, 1991). Whereas rapamycin, another RyR channel inhibitor, does not act on RyR directly instead binding the FKBP12 protein which complexes to RyR (Kaftan, Marks & Ehrlich, 1996). In this

capacity, Bennett (1999) has shown that rapamycin inhibits FKBP12 resulting in a transient loss of retention between 30 and 40 minutes post-training and a persistent loss of retention from 70 minutes post-training using a passive avoidance task for the day-old chick (Bennett, 1999). If RyR channels are found to be responsible for the action of NO in passive avoidance learning in the neonate chick associated with the action of L-NAME, then the findings of Bennett (1999) demonstrate the lack of specificity rapamycin has to RyR-dependent events. Further, Kaftan et al. (1996) found that in cardiac sarcoplasmic reticulum vesicle preparations, rapamycin significantly increased the probability of RyR channel opening, suggesting that rapamycin may also act as an agonist under some circumstances. Another common RyR channel inhibitor is ruthenium red, but electrophysiological studies indicated that this compound may inhibit both N- and P-type voltage-sensitive Ca^{2+} channel (Hamilton & Lundy, 1995).

Although rapamycin's effects upon retention have been studied briefly using a passive avoidance task for the day-old chick (Bennett, 1999) dantrolene remains the most specific, potent and commonly used inhibitor in both electrophysiological and behavioural studies. This specificity was studied both by Parness and Palnitkar (1995), who localised the effect of dantrolene to the endoplasmic reticulum, and Zhao et al. (2001) who found the RyR2 channel to be insensitive to dantrolene. Importantly, the *in vivo* studies of Wei and Perry (1996) observed that, in gerbils, 50mg/kg dantrolene did not alter brain or rectal temperature, suggesting no systemic effects at this dose.

As well as electrophysiological and behavioural studies, dantrolene has also been used *in vivo* and in culture to study neuroprotection. *In vivo* studies suggest that doses of between 10mg/kg (Hotchkiss & Karl, 1994) and 50mg/kg (Wei & Perry, 1996) are neuroprotective, as are concentrations between 0.4mM and 1.6mM (Zhang, Andou, Masuda, Mitani & Kataoka, 1993). This is in comparison to other culture studies that have used far more dilute concentrations of dantrolene (10μM) (Segal & Manor, 1992). With concentrations of dantrolene similar to those found to be effective in culture studies, electrophysiological studies have directly shown an impairment of either Ca^{2+} release from RyR-sensitive intracellular stores or an effect on synaptic efficacy. Concentrations of between 1μM and 100μM dantrolene have been found to be effective (Tekkök & Krnjević, 1996; Salinska, Ziembowicz, Stafiej, Zieminska & Lazarewicz, 1999) with most studies finding effective concentrations between 20μM (Katchman & Hershkowitz, 1993; Krnjević & Xu, 1989, 1996; Obenaus et al., 1989) and 50μM (O'Mara et al., 1995; Wang & Kelly, 1997).

More relevant to the current research are the concentrations of dantrolene found to be effective in behavioural studies. While 10mg/kg was found to result in sensory-motor effects when used in conjunction with the allosteric spatial learning task developed for rats by Kikusui et al. (1999), a number of other studies have found doses which appear to affect memory processing. Ohnuki and Nomura (1996) found both 6 and 10 nmol of dantrolene shortened the response latency in the step-through passive avoidance test demonstrating impairment of retention while 10nmol resulted in increased time taken to reach criterion. In contrast they noted that when using the radial-arm maze 20nmol dantrolene disrupted maze choice accuracy and increased error numbers again suggesting an impairment of retention. Similarly, Salinska et al. (2001), used a variant of the present passive avoidance task for the day-old chick and determined that the equivalent of 5mM dantrolene (corrected for the volume administered in the current studies) inhibited retention when tested at 3 hours post-training.

The aim in the present experiment was to determine whether the administration of dantrolene resulted in retention loss and to determine the optimum effective concentration of dantrolene following passive avoidance training.

Method

Chicks were housed and trained as detailed in Chapter 2. Administration of dantrolene occurred immediately post-training. Dantrolene was dissolved in DMSO followed by serial dilutions in physiological saline to obtain the working concentrations of between 0.001μM and 5mM. Notably, Salinska et al. (2001) found working concentrations of dantrolene containing 8% DMSO to be without effect upon retention. Further, dantrolene diluted in saline vehicles containing concentrations of DMSO as high as 20% have been used *in vivo* without DMSO having effects independent of dantrolene (Katchman & Hershkowitz, 1993; Ohnuki & Nomura, 1996). Working concentrations of dantrolene in the current studies did not contain greater than 8% DMSO in line with the methods of Salinska et al (2001).

Groups of 20 chicks received either the saline vehicle or one concentration of dantrolene. The concentrations of dantrolene used were 0.001μM, 0.01μM, 0.1μM, 1μM, 100μM, 300μM, 700μM, 1mM and 5mM. Note that 5mM was equivalent to that used by Salinska et al. (2001) after corrections for the different volumes administered.

Such a wide range of concentrations were tested as a detailed dose response study for dantrolene had not been previously attempted for the current task. Previous studies had also suggested that nanomolar concentrations of some channel antagonists are effective, such as IbTX while millimolar concentrations are more likely to be associated with sensory-motor effects which had not previously been tested for. Further, micromolar concentrations of various drugs had often been found effective in previous experiments. If a micromolar concentration of dantrolene could be found, this would reduce the possibility of sensory-motor effects that may be apparent with higher concentrations such as that used by Salinska et al. (2001). As with the administration of IbTX, all groups were tested 40 minutes post-training as this time of test represents the earliest time that retention loss results following administration of the NOS inhibitor L-NAME (Rickard et al., 1998). The final number of chicks used for each concentration ranged from 18 to 20.

Results

Retention levels for chicks administered various concentrations of dantrolene, tested 40 minutes post-training, are shown in Figure 5.8.

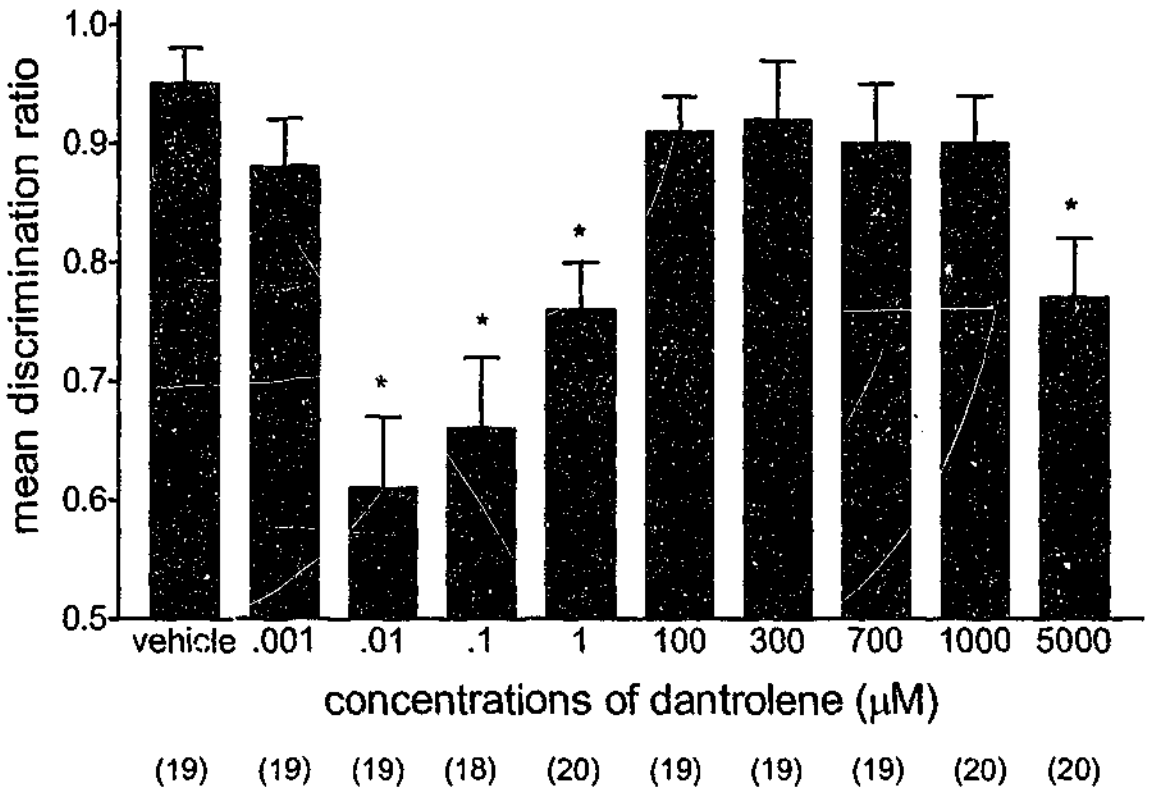


Figure 5.8 ɔse response function for the RyR channel inhibitor dantrolene. Various concentrations of dantrolene were administered immediately post-training and retention was tested 40 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Retention was impaired with concentrations of dantrolene between 0.01μM and 1μM. At higher concentrations retention levels were similar to those of controls, except at 5mM where retention once again appeared impaired. A one-way ANOVA revealed a significant concentration effect ($F(9, 183)=6.85$, $p<.001$) and Dunnett's post-hoc analysis indicated significant differences in retention between saline treated chick and chicks administered 0.01 ($p<.001$), 0.1 ($p<.001$), 1μM ($p<.05$) or 5mM ($p<.05$) dantrolene.

Discussion

Administration of dantrolene immediately post-training, at the range of concentrations used in the current study appeared to result in at least a bimodal dose response function when retention was tested 40 minutes post-training. Concentrations between 0.01μM and 1μM were effective in blocking retention, with maximal impairment for the 0.01μM concentration. In addition, however, 5mM dantrolene was

also effective in blocking retention in the present study, which is consistent with the concentration found by Salinska et al. (2001).

While it is not easy to interpret the underlying meaning of a bimodal dose response curve, the two distinct effective dose ranges may represent two separate actions of dantrolene. It may be initially thought that as dantrolene is specific to RyR channels then the lower effective concentration range would be that which acts directly on RyR channels. However, Salinska et al. (2001) showed 5mM dantrolene to act on RyR channels using a range of experimental techniques.

5.4.1.2 Time of administration study for dantrolene

Salinska et al. (2001) found that the equivalent of 5mM dantrolene was effective in blocking retention 180 minutes post-training if administered 30 minutes before or 30 minutes after passive avoidance training in chicks. Consistent with this, Ohnuki and Nomura (1996) found that administration of various concentrations of dantrolene five minutes prior to either aversive or spatial learning disrupted retention.

The aim in the present experiment was to determine the effective times of administration for 0.01μM and 5mM dantrolene. If, indeed, these two concentrations of dantrolene are acting via different memory processing mechanisms, differences in their effective times of administration may be evident.

Method

Chicks were housed and trained as described in Chapter 2. For each concentration of dantrolene, 20 different chicks were used for each data-point. Chicks received either the saline vehicle (<8% DMSO) or one of the concentrations of dantrolene. Times of administration tested were 10 and 5 minutes prior to training, immediately after training or 5, 10, 20 or 25 minutes after training. All groups of chicks were tested 40 minutes post-training. Final sample sizes ranged from 14 to 20 with a mean of 19 for both time of administration studies.

Results

Retention levels for chicks administered 0.01μM (A) or 5mM (B) dantrolene at various times relative to training and tested 40 minutes post-training are shown in Figure 5.9

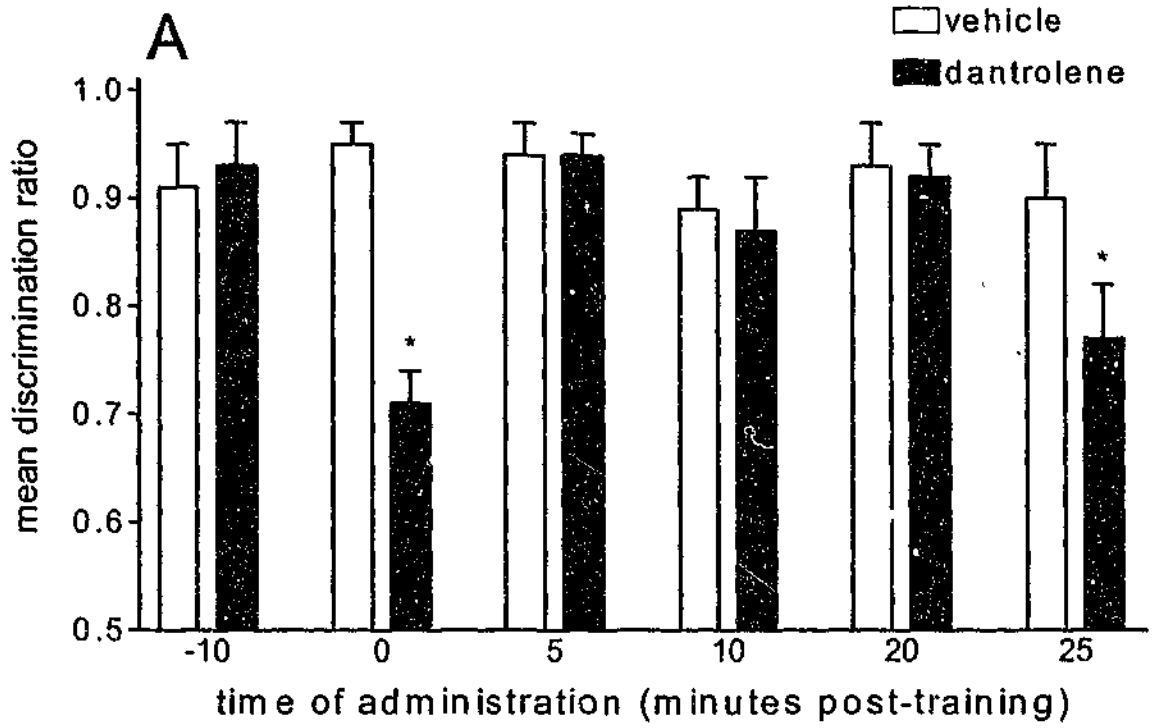


Figure 5.9 Time of administration function for the RyR channel blocker dantrolene. 0.01μM (A) or 5mM (B) dantrolene, compared with saline, was administered at several times relative to training and tested at 40 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Administration of 0.01μM dantrolene (Figure 5.9A) immediately after training and at 25 minutes post-training resulted in an impairment of retention when compared to chicks administered the saline vehicle at the same times relative to training. A two-way ANOVA revealed a significant drug main effect ($F(1,211)=11.76$, $p<.005$), a significant time of administration main effect ($F(5,211)=3.09$, $p<.05$) and a significant drug by time of administration interaction effect ($F(5,211)=3.64$, $p<.005$). Simple main effects analyses showed a significant difference in retention between chicks administered saline and those administered 0.01μM dantrolene immediately post-training ($p<.001$) and at 25 minutes post-training ($p<.05$).

However, chicks given 5mM dantrolene (Figure 5.9B) showed a loss of retention when the drug was administered at all times before and after training, except 25 minutes post-training, when compared to chicks administered the saline vehicle at the same times relative to training. A two-way ANOVA revealed a significant drug main effect ($F(1,211)=99.56$, $p<.001$), a significant time of administration main effect ($F(5,211)=5.01$, $p<.001$) and a significant drug by time of administration interaction effect ($F(5,211)=6.96$, $p<.001$). Simple main effects analyses showed significant differences in retention between chicks administered saline and those administered 5mM dantrolene at all times between 10 minutes before training and 20 minutes post-training (all $p<.005$).

Discussion

In separate experiments, 0.01μM or 5mM dantrolene was administered between 10 minutes prior to training and 25 minutes after training and retention was tested 40 minutes post-training. Only immediately after training and at 25 minutes post-training was retention significantly impaired following administration of 0.01μM dantrolene. In contrast, 5mM dantrolene resulted in a significant loss of retention from 10 minutes before training to 20 minutes post-training.

Importantly, the range of effective administration times with 5mM dantrolene is consistent with the effective times of administration for the NOS inhibitor L-NAME (Rickard et al., 1998). Although Salinska et al. (2001) found administration of the equivalent of 5mM dantrolene effective when administered 10 minutes later than the latest effective time of administration herein, this difference may be attributable to

methodological differences between the two passive avoidance tasks as outlined in Appendix B and by Crowe and Hamalainen (2001).

Curiously, the effective times of administration for 0.01μM dantrolene were found to be immediately post-training and 25 minutes post-training respectively which may imply 0.01μM dantrolene having two temporally distinct actions. The finding that 0.01μM dantrolene impaired retention when administered 25 minutes post-training was inconsistent with the effective range of administration times for the NOS inhibitor L-NAME and was thus considered unlikely to be NO-specific. However, the significant retention loss following administration of 0.01μM dantrolene immediately post-training was considered worthy of further investigation. Further, while the retention function for 0.01μM administered 25 minutes post-training remains unknown it was not pursued as 5mM dantrolene, administered immediately post-training, was ultimately found to be consistent with the action of L-NAME. In addition, previous studies by O'Dowd et al. (1994) into the role of glycogenolytic processes underlying passive avoidance memory determined that 1mM iodoacetate resulted in identical effective times of administration. Subsequent retention studies found administration either immediately post-training or 25 minutes post-training resulted in similar retention functions showing a persistent loss of retention from ITM-B onwards.

While not easy to interpret the difference in range of effective times of administration for the two concentrations of dantrolene tested it is of note that iv administration of dantrolene results in a long elimination half-life of between 2 (Court et al., 1987) and 10 hours (Lerman, McLeod & Strong, 1989) dependent upon the species used. This extended period of elimination is consistent with the higher concentration of dantrolene (5mM) being effective when administered before training up to 20 minutes post-training. In addition, the low concentration of dantrolene (10nM) may be subject to quick elimination or degradation such that it was only effective when administered immediately around the time of channel activation. In this way the lower effective concentration of dantrolene may provide an indication to the time of channel activation not possible with higher concentrations.

5.4.1.3 Time of retention loss study for dantrolene

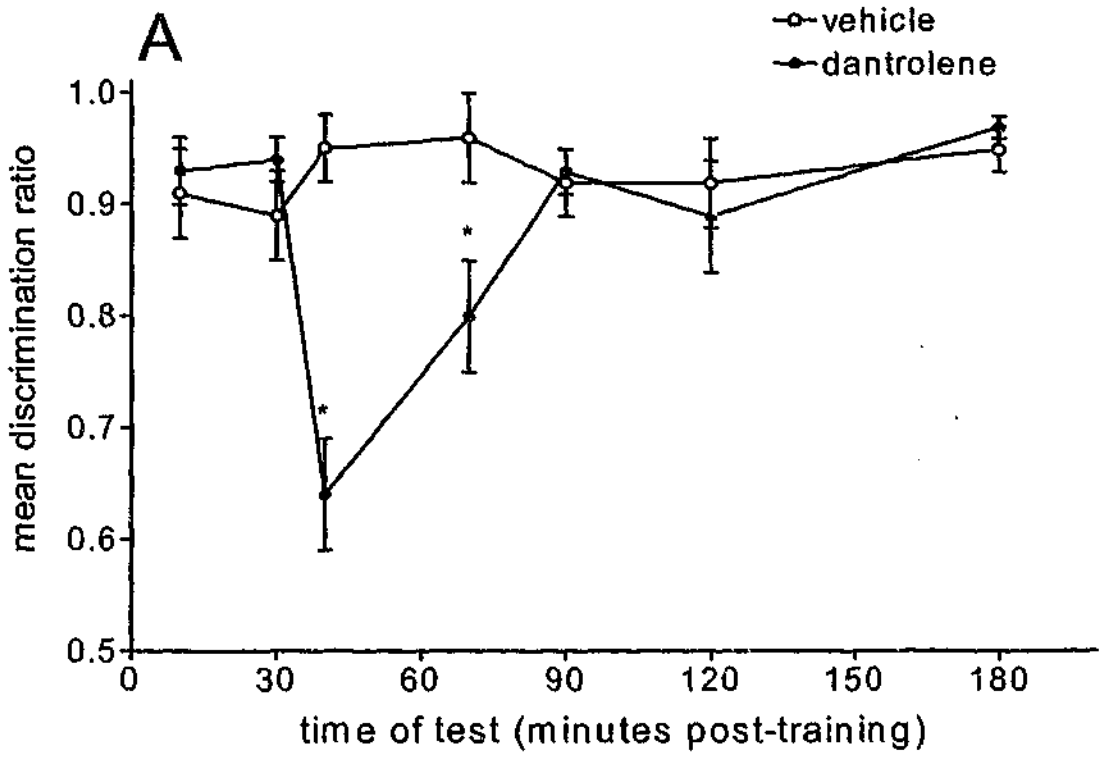
Salinska et al. (2001) observed a significant loss of retention 3 hours post-training when the equivalent of 5mM dantrolene was administered either 30 minutes before or 30 minutes after training, with no effect on retention observed at test 30 minutes post-training. A more comprehensive selection of retention test times is necessary for determining time of retention loss onset and the persistence of retention loss. The studies of Bennett (1999) using the non-specific RyR channel inhibitor, rapamycin, demonstrated a transient loss of retention between 30 and 40 minutes post-training and a persistent loss of retention from 70 minutes onwards. Therefore there exists some behavioural evidence to suggest RyR channels may have a role in memory long before 3 hours post-training and at a time consistent with the action of NO. The present experiment sought to establish retention functions for both 0.01μM and 5mM dantrolene, administered immediately post-training.

Method

Chicks were housed and trained as described in Chapter 2. Two separate retention studies were undertaken, one using 0.01μM dantrolene and the other using 5mM dantrolene. In each retention study, groups of 20 chicks received dantrolene or saline immediately post-training and were tested at 10, 30, 40, 70, 90, 120 or 180 minutes post-training. Group sizes ranged from 15 to 20 per data-point with a mean of 18.

Results

Retention levels for chicks administered 0.01μM (A) or 5mM (B) dantrolene immediately post-training and tested at various times post-training are represented in Figure 5.10.



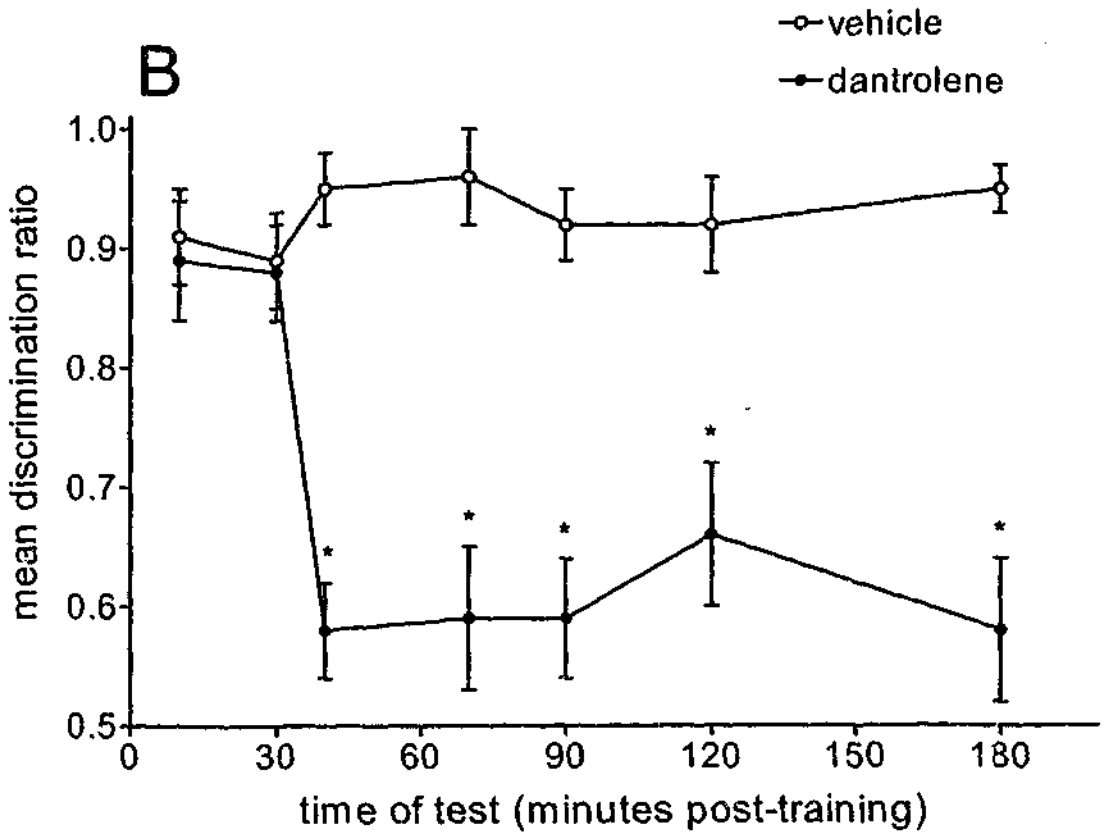


Figure 5.10 Retention function for the RyR channel blocker dantrolene. 0.01μM (A) or 5mM (B) dantrolene, compared with saline, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Administration of 0.01μM dantrolene immediately post-training resulted in a transient loss of retention between 40 and 70 minutes post-training. A two-way ANOVA on the retention function for 0.01μM dantrolene revealed a significant drug main effect ($F(1,235)=8.63$, $p<.005$), a significant time of test main effect ($F(6,235)=4.40$, $p<.001$) and a significant drug by time of test interaction effect ($F(6,235)=6.87$, $p<.001$). Simple main effects analyses revealed a significantly lower mean discrimination ratio for chicks treated with 0.01μM dantrolene compared with the saline vehicle when tested at 40 ($p<.001$) or 70 minutes ($p<.005$) post-training, but not at other times of test.

In contrast, 5mM dantrolene, also administered immediately post-training, resulted in a sustained loss of retention, beginning 40 minutes post-training and persisting until at least 180 minutes post-training. A two-way ANOVA on the retention function for 5mM dantrolene revealed a significant drug main effect ($F(1,236)=99.77$, $p<.001$), a significant time of test main effect ($F(6,236)=4.24$, $p<.001$) and a significant drug by time of test interaction effect ($F(6,236)=7.59$, $p<.001$). Simple main effects analyses showed a significant impairment in retention for those chicks administered 5mM dantrolene, compared with the saline vehicle-treated chicks when tested at all training-test intervals from 40 minutes post-training to 180 minutes post-training, the longest training-test interval used (all $p<.001$).

Discussion

Significant retention losses for 0.01μM dantrolene were observed at 40 and 70 minutes post-training with a restoration of retention by 90 minutes post-training. In contrast, 5mM dantrolene resulted in a persistent loss of retention from 40 minutes post-training until the completion of the experiment 180 minutes post-training.

While both retention functions share some common characteristics with the previous studies of Bennett (1999) who used the non-specific RyR channel inhibitor rapamycin, the differences are attributable to dantrolene's direct and specific action upon the RyR channel. Interestingly, the transient retention loss observed for 0.01μM dantrolene is consistent with the transient retention loss observed from the inhibition of BK_{Ca} channels by IbTX (see Figure 5.7). This may represent a requirement of BK_{Ca} channels for Ca^{2+} derived from ER stores and released by RyR channels as also suggested by the memory studies of Blackwell and Alkon (1995) and in other systems

by Jagger et al. (1998), Ohi et al. (2001), Perez, Bonev, Patlak and Nelson (1999) and Pozo, Perez, Nelson and Mawe (2002). Further, the transient retention loss resulting from administration of 0.01μM dantrolene has a time of onset which is consistent with that found with 100μM ODQ, a specific GC inhibitor, although retention was restored some 20 minutes earlier (70 minutes post-training) following ODQ inhibition. Allweis et al. (1984) interpreted such transient retention losses as an effect upon memory retrieval mechanisms as retention was ultimately restored.

In contrast, administration of 5mM dantrolene results in a persistent loss of retention by 40 minutes post-training, which is consistent with the findings of Salinska et al. (2001). Importantly, the retention loss resulting from the administration of 5mM dantrolene mimics that following NOS inhibition. In addition, the effective times of administration for RyR channel inhibition using 5mM dantrolene also match those for NOS inhibition by L-NAME. It seems possible, therefore, that NO may play a role in memory formation through the direct activation of RyR channels. In turn, RyR channels may release Ca^{2+} into the cytosol which, in turn, allows the activation of various Ca^{2+}-dependent enzymes and other Ca^{2+}-dependent channels such as BK_{Ca} channels (Jagger et al., 1998; Ohi et al., 2001; Perez et al., 1999; Pozo et al., 2002). Therefore, the present results represent the first account of the importance of RyR channels as a mechanism through which NO permits memory formation using passive avoidance learning in the day-old chick.

This conclusion is in contrast to the views of Bernabeu et al. (1995; 1996; 1997) and Izquierdo et al. (2000) who found that inhibitory avoidance learning is consolidated through NO activating GC, which in turn activates PKG. Other studies, such as that of Kendrick et al. (1997), have also suggested NO affects memory formation through the activation of GC. However, the tasks employed by these researchers were done using rats and sheep respectively in contrast to the avian species used herein. Further, although Bernabeu et al. (1995, 1996, 1997) and Izquierdo et al. (2000) used a single trial passive avoidance task, the differences in species and methodology of the task employed make similarities with the present work difficult. This is amplified further by the study of Kendrick et al. (1997) who not only used sheep but also an olfactory recognition task. In this way, species and task differences may account for the different NO-dependent mechanisms implicated in retention. Further, only a very limited

number of test times were used by these researchers. In this way, the experimental designs employed by Bernabeu et al. (1995; 1996; 1997) and Izquierdo et al. (2000) especially may have prevented them from determining specific retrieval processes which were GC-dependent as noted in Chapter 3. Importantly, although RyR channels have been determined to also be activated by PKG (Takasago et al., 1991) there is no resemblance in the retention functions for RyR channel inhibition and those for GC inhibition PKG inhibition. For this reason it can be suggested that GC and PKG act independently of RyR channels in the current paradigm.

This central role for RyR channels in memory formation is somewhat surprising given the lower level of expression in the chick HV. It is, at present, unclear as to why administration of dantrolene into the neostriatal/hyperstriatal complex, containing the IMHV, should result in relatively long lasting retention loss. It is possible that the drug may have dispersed to memory-associated regions of the chick brain proximal to the neostriatal/hyperstriatal complex which have a high concentration of RyR channels. However, this is not the first case of a protein that is poorly expressed in the IMHV, for which an antagonist administered into this brain region resulted in retention loss. The most notable is indeed NOS itself.

SECTION 5 : The effect of impaired peroxynitrite production on passive avoidance learning

NO is highly reactive within biological systems and interacts with both proteins and radicals. One important reaction of NO with other radicals is the binding of superoxide to form peroxynitrite (refer Figures 1.2 & 5.1). Notably, peroxynitrite and NO share a similar reactivity to proteins resulting in nitrosylation of specific amino acid residues, such as tyrosine and tryptophan (Aspée & Lissi, 2000; Beckman et al., 1994; Beckman & Koppenol, 1996). In this way, NO may act on proteins directly to alter their activity, or through the production of peroxynitrite. Peroxynitrite is also a potent and versatile oxidant, as it can oxidise both protein and non-protein thiols (Radi et al., 1991a), protein sulfides (Pryor et al., 1994; Moreno & Pryor, 1992), lipids (Radi et al., 1991b; Darley-Usmar et al., 1992), low-density lipoprotein (Graham et al., 1993) and deoxyribose (Beckman et al., 1990). The functional consequences of this were studied by Di Stasi et al., (1999) using synaptosomes. Not only did the authors reiterate peroxynitrite's general specificity for tyrosine and tryptophan residues, but they also found that peroxynitrite induced tyrosine nitration, amongst other effects, in synaptic vesicles and in the post-synaptic density.

While comparatively little histological evidence exists implicating peroxynitrite activity in memory-related brain regions two studies are of note. At a general level, Di Stasi et al. (1999) found peroxynitrite to be active in synaptosomes including both presynaptic vesicles and the post-synaptic density. With respect to the regional localisation of peroxynitrite, Cha et al. (2000) found that nitrotyrosine immunoreactivity, which is a measure of peroxynitrite production, was concentrated in the pyramidal cells of the CA region of the hippocampus among other regions.

From such studies it may be inferred that peroxynitrite may have a role in memory formation. However, while the action of NO is well defined with respect to memory, the role of peroxynitrite is poorly understood. The beneficial actions of peroxynitrite removal on memory have been shown in a limited number of behavioural studies. One study assessed the role of the peroxynitrite scavenger, deferoxamine,

following head trauma in rats (Long, Ghosh, Moore, Dixon & Dash, 1996). These researchers concluded that deferoxamine protected against neuronal dysfunction and cell death following brain trauma, resulting in improvements in spatial learning when compared to saline-treated rats. However, in this example, it is the *removal* of peroxynitrite which is beneficial to neuronal function and this may have a clinical corollary in Alzheimer's disease, where high levels of peroxynitrite appear to be implicated in the neurodegenerative process (Good, Werner, Hsu, Olanow & Perl, 1996; Matthews & Beal, 1996; Smith, Harris, Sayre, Beckman & Perry, 1997). More importantly, Levin et al. (1998) genetically disrupted superoxide production before subjecting mice to a learning task, thus testing the action of physiological levels of peroxynitrite in memory formation. When the mutant mice were placed in the win-shift 8-arm radial maze to test spatial memory a severe impairment of spatial memory resulted. Interestingly mice engineered to over express superoxide production also displayed noticeable impairments in performing the task, thus suggesting specific concentrations of peroxynitrite must be maintained for proper function. Therefore, while it is well established that high levels of peroxynitrite appear to be detrimental, physiological levels of peroxynitrite appear important in memory formation.

In addition to the limited behavioural evidence suggesting a role for peroxynitrite in memory formation, peroxynitrite has also been characterised to affect cellular processes implicated in memory formation. With respect to the action of peroxynitrite at the synapse, Pellmar, Neel and Lee (1989) discuss the role of deferoxamine, Trolox and DMSO as three scavengers of peroxynitrite, in halting synaptic damage and thus alterations to transmission in hippocampal tissue. In another study, 200μM Trolox improved population spike recovery following hypoxia/reoxygenation-induced synaptic failure (Vlkolinský & Štolc, 1999). In this way, removal of peroxynitrite appears beneficial to the function of neurons.

Peroxynitrite has also been implicated in modulating neurotransmitter release in an extensive study by Trabace and Kendrick (2000). Using *in vivo* microdialysis to see whether NO modulates striatal neurotransmitter release in the rat through cGMP and/or peroxynitrite production, they noted that, when a peroxynitrite scavenger was coperfused with NO-donors glutamate and GABA levels increased, while perfusion of peroxynitrite decreased dopamine and dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid levels. The authors suggest that dopamine, dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid are controlled through

levels of peroxynitrite. In other cellular processes implicated in memory formation Wei, et al. (1996) noted peroxynitrite induced dilation in cerebral arterioles in a dose-dependent fashion. Interestingly this effect was GC-independent but K_{ATP} channel-dependent. Superoxide was also effective in causing vasodilation but, interestingly, appears to work also through a different K^+ channel subtype, namely K_{Ca} channels.

5.5.1 Trolox inhibition of peroxynitrite production

5.5.1.1 Dose response study for Trolox

There are a number of antioxidants which scavenge peroxynitrite in an experimental system, including uric acid, mannitol (mannite), DMSO, sodium benzoate, tocopherol (vitamin E), ebselen (PZ51), Trolox (Chaudière & Ferrari-Iliou, 1999) and metalloporphyrins. However, of these, the metalloporphyrins are unsuitable for behavioural use since they may actually increase the overall cellular nitration and oxidative yield in some circumstances (Crow, 2000).

Of the remaining antioxidants, ebselen and Trolox are both commercially available and often cited as specific to the removal of peroxynitrite without affecting NO levels (re: Trolox - Rajagopalan, M. (Calbiochem), personal communication, 3rd March 2001). Ebselen was shown to be an effective neuroprotective agent following temporary occlusion of the middle cerebral artery of the rat (Dawson, Masayasu, Graham & Macrae, 1995), however, there are a number of pharmacological problems with this drug. While its reaction to peroxynitrite is the fastest of a large range of antioxidants, including Trolox (Seis & Masumoto, 1997), ebselen has a high reactivity for thiols and so may alter enzyme function (Seis & Masumoto, 1997) in addition to any action upon peroxynitrite. Further, according to Wendel, Otter and Tiegs (1986), ebselen can inhibit NADPH-cytochrome P450-reductase at low concentrations *in vitro* but not *in vivo*. This enzyme shares homology with NOS (Bredt et al., 1991) indicating the possibility of a secondary action upon NOS. Further studies have identified secondary actions of ebselen upon several enzymes including NOS (Hattori et al., 1994; Zembowicz, Hatchett, Radziszewski & Gryglewski, 1993), NADPH oxidase

(Wang, Komarov, Siers & de Groot, 1992), H^+/K^+-ATPase (Tabuchi, Ogasawara & Furuhama, 1994), glutathione-S-transferase (Nikawa, Schuch, Wagner & Sies, 1994a, b), papain (Nikawa et al., 1994b), and lipooxygenases (Schewe, Schewe & Wendel, 1994). Therefore, ebselen's lack of specificity and its reactivity with NOS makes this peroxynitrite scavenger a poor choice in the current behavioural studies aimed at determining if NO acts through peroxynitrite formation alone in promoting retention. Finally, ebselen also appears to be somewhat more cytotoxic than Trolox (Kondo et al., 1997).

In contrast, both Vlkolinský and Štolc (1999) and Pellmar et al. (1989) found Trolox to be effective in halting the negative action of peroxynitrite upon synaptic transmission when compared against other antioxidants and was described by Pellmar et al (1989) as "very effective" (p 439). Similarly, Kondo et al. (1997) and McClain, Kalinich and Ramakrishnan (1995) described Trolox as both potent and powerful in its action. While Trolox has been favoured as a peroxynitrite scavenger in research, it is not necessarily the most potent antioxidant (Halliwell, Evans & Whiteman, 1999; Horáková, Štolc, Chromíková, Pekárová & Derková, 1997, 1998; Kondo et al., 1997; Mizuhashi, Ikegaya & Matsuki, 2000; Pannala, Singh & Rice-Evans, 1999; Salgo, Bermúdez, Squadrito & Pryor, 1995; Unchern, Saito & Nishiyama, 1997). Nonetheless, Trolox has a number of advantages over ebselen, in particular being effective *in vivo* (Mickle et al., 1989) and more specific in its action.

Method

Chicks were housed and trained as previously described in Chapter 2. Administration of Trolox occurred immediately after training. Groups of 20 chicks received either the saline vehicle (<1% ethanol) or one concentration of Trolox, being 0.1μM, 1μM, 10μM, 100μM, 200μM, 300μM, 400μM, 500μM, 600μM, 700μM, 750μM, 800μM, 900μM, 1mM and 1.2mM. All groups were tested 120 minutes post-training. The number of chicks representing each concentration ranged from 16 to 38 with a mean of 24.

Results

Retention levels for chicks administered a range of concentrations of Trolox and tested 120 minutes post-training are shown in Figure 5.11.

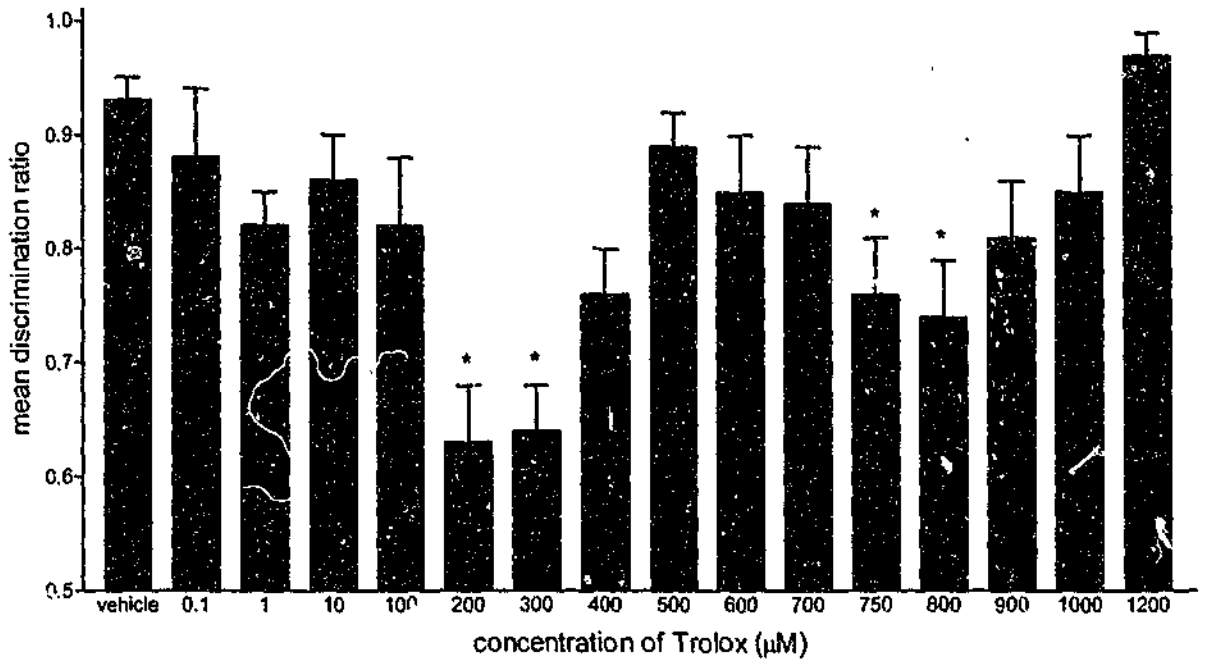


Figure 5.11 Dose response function for the peroxynitrite scavenger Trolox.
Various concentrations of Trolox were administered immediately post-training and retention was tested 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Administration of Trolox at concentrations of 200μM, 300μM, 750μM and 800μM resulted in a reduction of retention levels. A one-way ANOVA revealed a significant concentration effect ($F(15,357)=5.03$, $p<.001$) and Dunnett's post-hoc analysis indicated a significant difference between the retention levels of chicks administered saline and those administered either 200μM ($p<.005$), 300μM ($p<.005$), 750μM ($p<.01$) or 800μM ($p<.05$) Trolox.

Discussion

Impairment of retention occurred when either 200μM, 300μM, 750μM or 800μM Trolox was administered immediately post-training. These two distinct effective

concentration ranges were separated by a range of concentrations which did not inhibit retention.

The effective concentrations were consistent with a number of studies using Trolox in a variety of *in vitro* experimental systems. In such studies, effective concentrations of Trolox ranged from 100μM (Pellmar et al., 1989; Unchern et al., 1997) to 1mM (Salgo et al., 1995) with a number of studies finding a range of 200μM to 500μM effective (Horáková et al., 1997 & 1998; Mizuhashi et al., 2000; Vlkolinský & Štolc, 1999). However, in each of these studies the concentrations of Trolox used were found to be neuroprotective, with cognitive changes not assessed.

As discussed previously, a multimodal dose response function may imply more than one action of Trolox within memory processing. It cannot be ascertained from the current study whether both effective concentration ranges specifically scavenge peroxynitrite, or have other actions in addition to scavenging peroxynitrite.

Finally, the present findings complement those of Levin et al. (1998) who showed that blocking superoxide production, and thus peroxynitrite production, impaired memory formation.

5.5.1.2 Time of administration study for Trolox

Individual time of administration studies were conducted for both 300μM and 800μM Trolox. The different concentrations of Trolox found effective in various experimental systems discussed above suggest that both 300μM and 800μM Trolox could be specific to peroxynitrite removal and, thus, both should be tested for effective administration times in the current paradigm. As no prior behavioural studies exist using Trolox, a broad range of administration times was selected. These times generally reflect the effective times of administration for the NOS inhibitor, L-NAME, which was found to impair retention when administered from before training up to 20 minutes post-training. If NO acts in memory formation through the production of peroxynitrite, then it would be expected that inhibitors of both NOS and peroxynitrite production may share similar effective administration times, save for differences in pharmacokinetics of the drugs.

Method

Chicks were housed and trained as discussed in Chapter 2. As the dose response study for Trolox resulted in a bimodal function, the two optimal concentrations, namely 300μM and 800μM, were investigated further. In each time of administration study, each group of 20 chicks represented one time of administration and received either the saline vehicle (containing <1% ethanol) or the concentration of Trolox specific to the study. Times of administration tested were 10 and 5 minutes prior to training, immediately after training or 2.5, 5, 10, 20 or 25 minutes after training. All groups were tested for retention 120 minutes post-training. Group sizes ranged from 14 to 20 with a mean of 18 for each study.

Results

Retention levels for chicks administered 300μM (A) or 800μM (B) Trolox at times relative to training and tested 120 minutes post-training are shown in Figure 5.12.

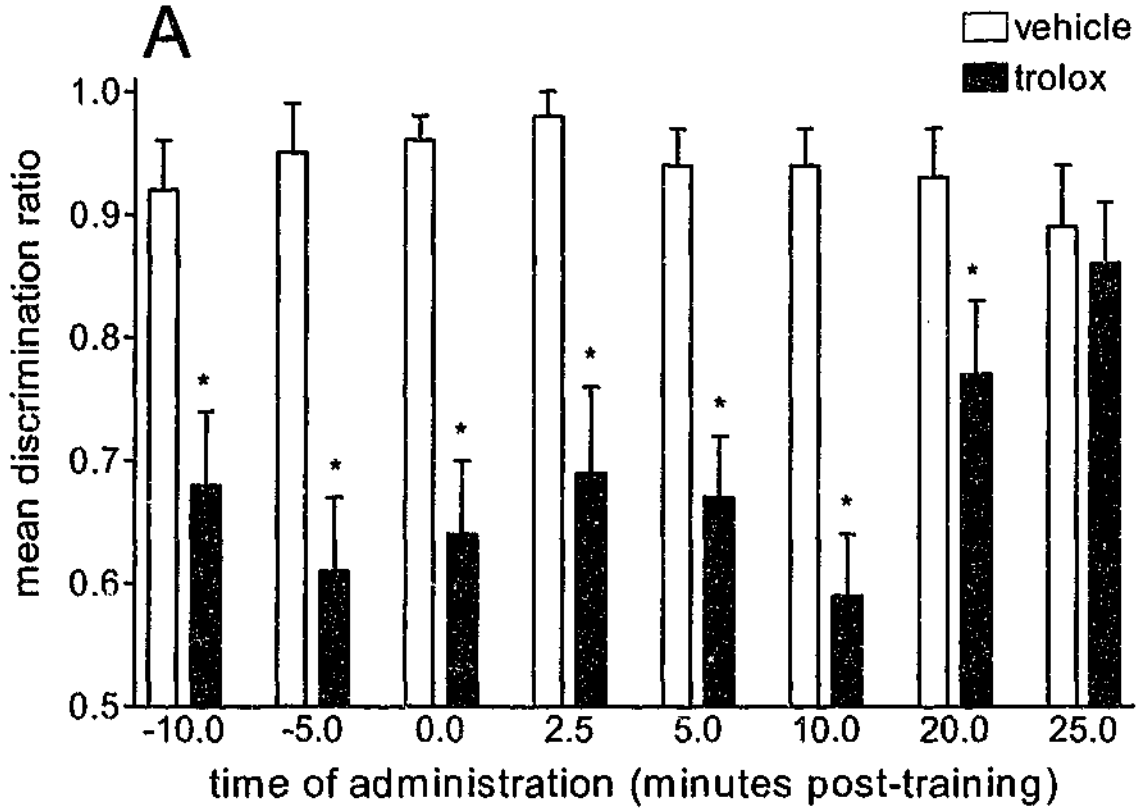


Figure 5.12 Time of administration function for the peroxynitrite scavenger Trolox. 300μM (A) or 800μM (B) Trolox, compared with saline, was administered at several times relative to training and tested at 120 minutes post-training. Each column represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Both 300μM (see Figure 5.12A) and 800μM (see Figure 5.12B) concentrations of Trolox were found to impair retention when given between 10 minutes before training and 20 minutes post-training inclusive. A two-way ANOVA was performed for each concentration followed by simple main effects analyses. The function for 300μM revealed a significant drug main effect ($F(1,273)=105.39$, $p<.001$) and a significant drug by time of administration interaction effect ($F(7,273)=2.29$, $p<.05$). However, the time of administration main effect was not significant ($F(7,273)=1.20$, $p>.05$). Simple main effects analyses revealed a significant difference in retention levels for chicks administered 300μM Trolox at 10 ($p<.005$) and 5 minutes prior to ($p<.001$), immediately after training ($p<.001$), 2.5 ($p<.001$), 5 ($p<.001$), 10 ($p<.001$) and 20 minutes after training ($p<.05$), compared with chicks administered the saline vehicle at the same times.

The function for 800μM revealed a significant drug main effect ($F(1,268)=131.98$, $p<.001$), a significant time of administration main effect ($F(7,268)=2.35$, $p<.05$) and a significant drug by time of administration interaction effect ($F(7,268)=3.01$, $p<.01$). Simple main effects post-hoc analyses revealed a significant difference in retention levels for chicks administered 300μM Trolox at 10 ($p<.001$) and 5 minutes prior to ($p<.001$), immediately after training ($p<.001$), 2.5 ($p<.001$), 5 ($p<.001$), 10 ($p<.001$) and 20 minutes after training ($p<.001$), compared with chicks administered the saline vehicle at the same times.

Discussion

Both 300μM and 800μM Trolox resulted in a significant loss of retention tested 120 minutes post-training when administered between 10 minutes before training and 20 minutes after training.

Importantly, the effective times of administration for both these concentrations match those for L-NAME inhibition of NOS and also 5mM dantrolene inhibition of RyR channels. This may suggest that NO may be acting through peroxynitrite to activate RyR channels in memory formation for the passive avoidance task in the day-old chick. However, differences in drug pharmaco-kinetics makes comparisons between times of administration studies potentially unsafe. Only if the retention functions for 300μM and 800μM Trolox match those following L-NAME or dantrolene administration can such a relationship be clearly suggesting. In such circumstances, the

present findings would certainly support any suggested link between NO, peroxynitrite and RyR channels.

5.5.1.3 Time of retention loss study for Trolox

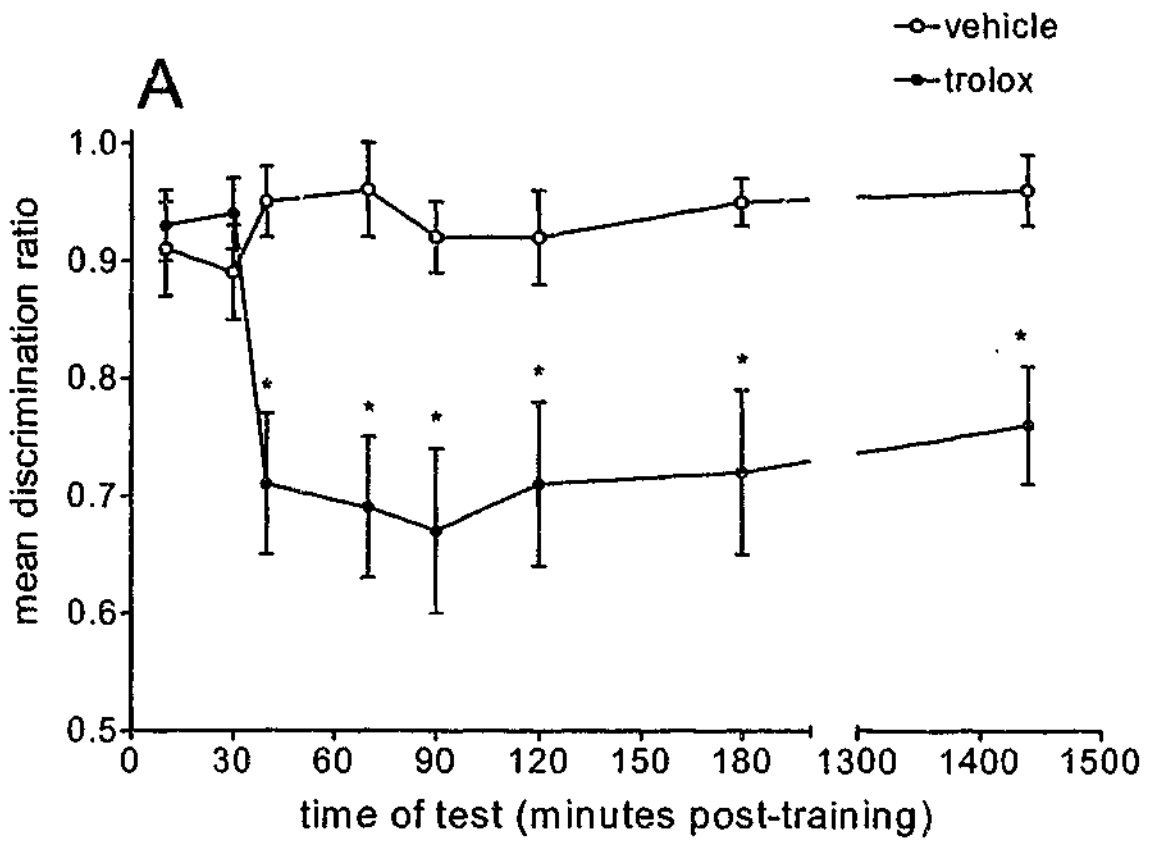
NO is known to form peroxynitrite in conjunction with superoxide radicals and both NO and peroxynitrite share many common biochemical properties (Aspée & Lissi, 2000; Beckman et al., 1994; Beckman & Koppenol, 1996). Further, Levin et al. (1998) has provided behaviour evidence that blocking the production of superoxide, and thus peroxynitrite formation, impairs memory formation for a radial arm maze task. Therefore it is reasonable that NO may affect memory formation through the production of peroxynitrite in passive avoidance learning using the day-old chick. The aim in the present experiment was to determine when retention loss onset occurred following administration of either 300μM or 800μM Trolox administered immediately post-training.

Method

Chicks were housed and trained as described in Chapter 2. Two separate retention studies were undertaken, one for 300μM Trolox and the other for 800μM Trolox. In each retention function study, groups of 20 chicks received Trolox or saline immediately post-training and were tested at 10, 30, 40, 70, 90, 120 or 180 minutes post-training and at 24 hours post-training such that each group administered Trolox had a matched saline vehicle (<1% ethanol) administered group. Group sizes ranged from 15 to 31 for 300μM Trolox with a mean of 18, and from 15 to 23 with a mean of 18 for 800μM Trolox.

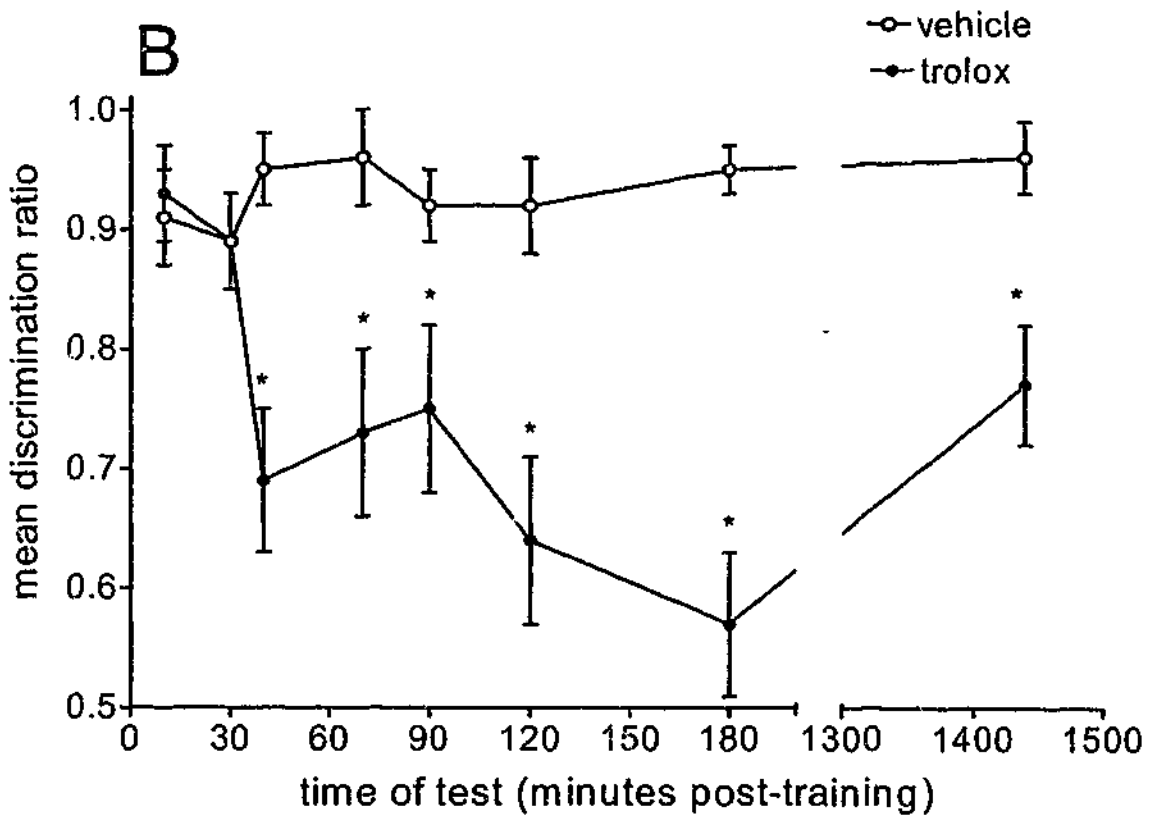
Results

Retention levels for chicks administered 300μM (A) or 800μM (B) immediately post-training and tested at various times post-training are represented in Figure 5.13.



vehicle n =(16) (20)(16) (17) (18) (15) (18) (17)

Trolox n =(19) (19)(18) (20) (16) (17) (17) (31)



vehicle n =(16) (20)(16) (17) (18) (15) (18) (17)

Trolox n =(16) (16)(18) (17) (17) (19) (18) (23)

Figure 5.13 Retention function for the peroxynitrite scavenger Trolox.

300μM (A) or 800μM (B) Trolox, compared with saline, was administered immediately post-training and tested at various times post-training. Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks, * $p<.05$.

Administration of either 300 or 800μM Trolox resulted in a persistent loss of retention from 40 minutes post-training until the completion of the experiment 24 hours post-training. A two-way ANOVA for 300μM Trolox revealed a significant drug main effect ($F(1,277)=45.02$, $p<.001$) and a significant drug by time of test interaction effect ($F(7,277)=3.51$, $p<.005$) but not a significant time of test main effect ($F(7, 277)=1.68$, $p>.05$). Simple main effects analyses revealed significantly lower mean discrimination ratios in chicks treated with 300μM Trolox than in chicks given saline when tested at 40 mins ($p<.005$), 70 mins ($p<.001$), 90 mins ($p<.005$), 120 mins ($p<.005$), 180 mins ($p<.005$) and at 24 hours post-training ($p<.005$).

A similar finding was obtained with 800μM Trolox, a two-way ANOVA revealed significant drug ($F(1,264)=59.57$, $p<.001$) and time of test main effects ($F(7,264)=2.34$, $p<.025$) and a significant drug by time of test interaction effect ($F(7,264)=4.11$, $p<.001$). Simple main effects analyses revealed significantly lower mean discrimination ratios in chicks treated with 800μM Trolox than in chicks given saline and tested for retention loss at 40 mins ($p<.001$), 70 mins ($p<.005$), 90 mins ($p<.05$), 120 mins ($p<.001$), 180 mins ($p<.001$) and at 24 hours post-training ($p<.01$).

Discussion

Administration of either 300μM or 800μM Trolox immediately post-training resulted in a persistent loss of retention from 40 minutes post-training. It should be noted that while there appears to be a partial recovery in retention following administration of 800μM Trolox between 3 and 24 hours post-training, the difference in retention between Trolox and saline-treated chicks remained significant 24 hours post-training. This increase in retention levels may be due to mild behavioural changes that accompany chicks housed overnight and tested more than 24 hours from the time of pre-training, or simply due to sampling error in the 24 hour post-training test group.

These retention profiles are consistent with both the time of onset of retention loss for NOS inhibition using L-NAME, and for 5mM dantrolene inhibition of RyR channels. Further, the retention loss profiles for both 300μM and 800μM Trolox are persistent until the completion of the experiment 24 hours post-training, again mimicking the retention profiles for L-NAME and 5mM dantrolene.

This is the first demonstration of a potential pathway from NO and peroxynitrite to RyR channel activation associated with memory formation processes. These findings

suggests low levels of peroxynitrite play a positive role in cognition which is opposed to the recognised role of peroxynitrite ..eurodegenerative conditions such as Alzheimer's disease. Further, this pathway represents a significant departure from the comprehensive studies of Bernabeu et al. (1995, 1996, 1997) and Izquierdo et al. (2000) which have provided much evidence for a NO, GC and PKG pathway underlying memory formation using an inhibitory avoidance task in rats.

SECTION 6 : Summary of findings

NO is thought to directly activate a number of ion channels. Three channels for which there exists strong evidence for direct activation by NO are CNG channels (Ahmad et al., 1994; Broillet & Firestein, 1996), BK_{Ca} channels (Abdullah & Docherty, 1999; Atucha et al., 1998; Bolotina et al., 1994; Bradley et al., 1998; Cetiner & Bennett, 1993; Chen et al., 1999; R.J. Lang et al., 2000; Mistry & Garland, 1998; Plane et al., 1996; Schmachtenberg & Bacigalupo, 1999; Schmachtenberg & Bacigalupo, 2000; Toki et al., 1998; Zhao et al., 1997) and RyR channels (Aghdasi et al., 1997; Anzai et al., 2000; Stoyanovsky et al., 1997; Xu et al., 1998). Further, these three channels can be localised to memory-related brain regions, have been implicated in memory-related cellular processes, and/or have been directly implicated in memory through the use of behavioural tasks. Therefore, a strong theoretical background exists to support investigation into these three ion channels as possible mechanisms activated by NO and involved in memory processes.

The basic assumption of the present research is that the mechanism(s) activated by NO, corresponding to the effect of L-NAME at 40 minutes post-training, should also, when inhibited, result in an onset of retention loss at 40 minutes post-training. Further, as L-NAME results in a persistent loss of retention then the downstream mechanism, when inhibited, should likewise produce a sustained retention loss. This comes about as each stage of memory formation is reliant on biochemical pathways. If any component of a pathway is inhibited then retention is lost at the time when that pathway was required for the expression of retention.

Of the three NO-activated cation channels tested only the inhibition of RyR channels using 5mM dantrolene mimicked the retention function produced by L-NAME administration. Reinforcing this was a range of effective times of administration extending from before training up to 20 minutes post-training, which is also consistent with L-NAME administration. Notably, even although Gibbs et al. (1996) have previously observed effective administration times for the glutamine synthetase inhibitor, methionine sulfoximine, to extend from between 5 and 20 minutes post-training, effective administration times from before training to 20 minutes after training have only been observed once before within this

paradigm, namely the inhibition of BK_{Ca} channels with IbTX. Therefore, it is notable in itself that two drugs with possibly different pharmaco-kinetic properties should result in both the same retention function and uncommon range of effective times of administration. Taken together, although the primary evidence for a relationship between NO and RyR channels comes from common retention functions, the consistency of effective administration times between L-NAME and dantrolene (5mM) further suggests that NO acts upon RyR channels.

In contrast, the retention function following the administration of 10nM dantrolene immediately post-training yielded a transient retention loss between 40 and 70 minutes post-training. The specific BK_{Ca} channel blocker, IbTX (50nM), also transiently blocked retention from 40 to 70 minutes post-training. Finally, administration of 900μM verapamil, most likely blocking CNG channels, resulted in a transient retention loss from 90 to 120 minutes post-training. From these investigations it is suggested that NO activates RyR channels. Therefore intracellular calcium release is an important process in the ITM-B stage of memory formation and therefore in the consolidation of longer-term stages of memory formation (see Figure 5.14).

As NO may act upon proteins directly, or through the formation of another radical, peroxynitrite, behavioural studies were conducted using either 300μM or 800μM Trolox to scavenge this radical. The retention function for each Trolox concentration displayed a persistent loss of retention from 40 minutes onwards, consistent with L-NAME inhibition of NOS and also showed consistent effective times of administration with L-NAME. These findings are consistent with NO acting upon RyR channels either directly or through the formation of peroxynitrite (see Figure 5.14).

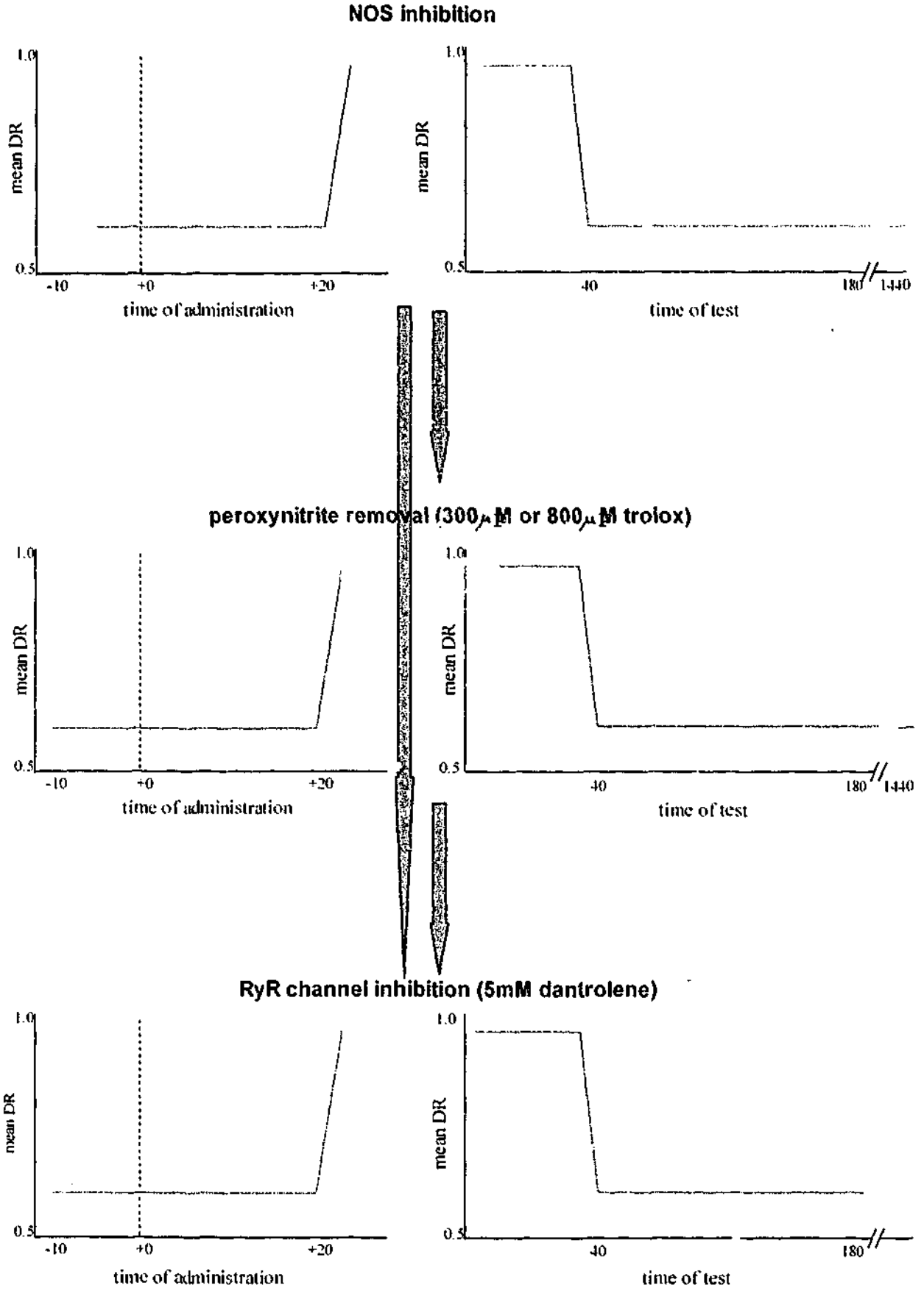


Figure 5.14 Diagrammatic representation of both time of administration (left) and retention loss (right) functions for NOS inhibition, peroxynitrite removal and RyR channel inhibition. Retention level marked in blue. NO appears to activate RyR channels directly or through the production of peroxynitrite

CHAPTER 6 -

SUMMARY AND IMPLICATIONS OF THE FINDINGS AND FUTURE DIRECTIONS OF RESEARCH

SECTION 1: Major research aims

SECTION 2: Findings

6.2.1 Summary of major findings

6.2.2 Implications of the findings

SECTION 3: Limitations of the present research programme

SECTION 4: Future directions of research

Chapter 6 – Summary and Implications of the Findings and Future Direction of Research

SECTION 1 : Major Research Aims

Nitric oxide has been widely recognised as an important molecule underlying memory formation. However, only limited evidence exists as to the biochemical mechanism(s) through which NO acts upon memory formation. Therefore, the current research programme investigated the involvement of a number of mechanisms which had been identified in previous biochemical, electrophysiological and behavioural studies, as candidate mechanisms. Pharmaco-behavioural studies were used to determine the mechanism(s) through which NO maintains the ITM-B stage and, therefore, consolidates the LTM stage within the Gibbs and Ng three stage model of memory formation (Gibbs & Ng, 1979a). A discrimination variant of a single trial passive avoidance task developed for the day-old chick was used.

The mechanisms investigated were: activation of PKG through GC; activation of *mono*(ADP-ribosyl) transferase; activation of CNG, BK_{Ca} and RyR channels; and finally, the production of peroxynitrite radicals.

SECTION 2 : Findings

6.2.1 Summary of major findings

Interference with each of the pathways through which NO may play a role in memory processing resulted, in each case, in disturbances to retention. However, the effects differ with respect to when, relative to training, the interference must be instituted, when retention losses first appear, and how long the losses last. Figure 6.1 summarises the major findings with respect to these characteristics for each pharmacological treatment used. The findings may be divided into those associated with retention losses that persist for at least 24 hours and may, thus, reflect interference with long-term memory consolidation and those associated with transient retention losses.

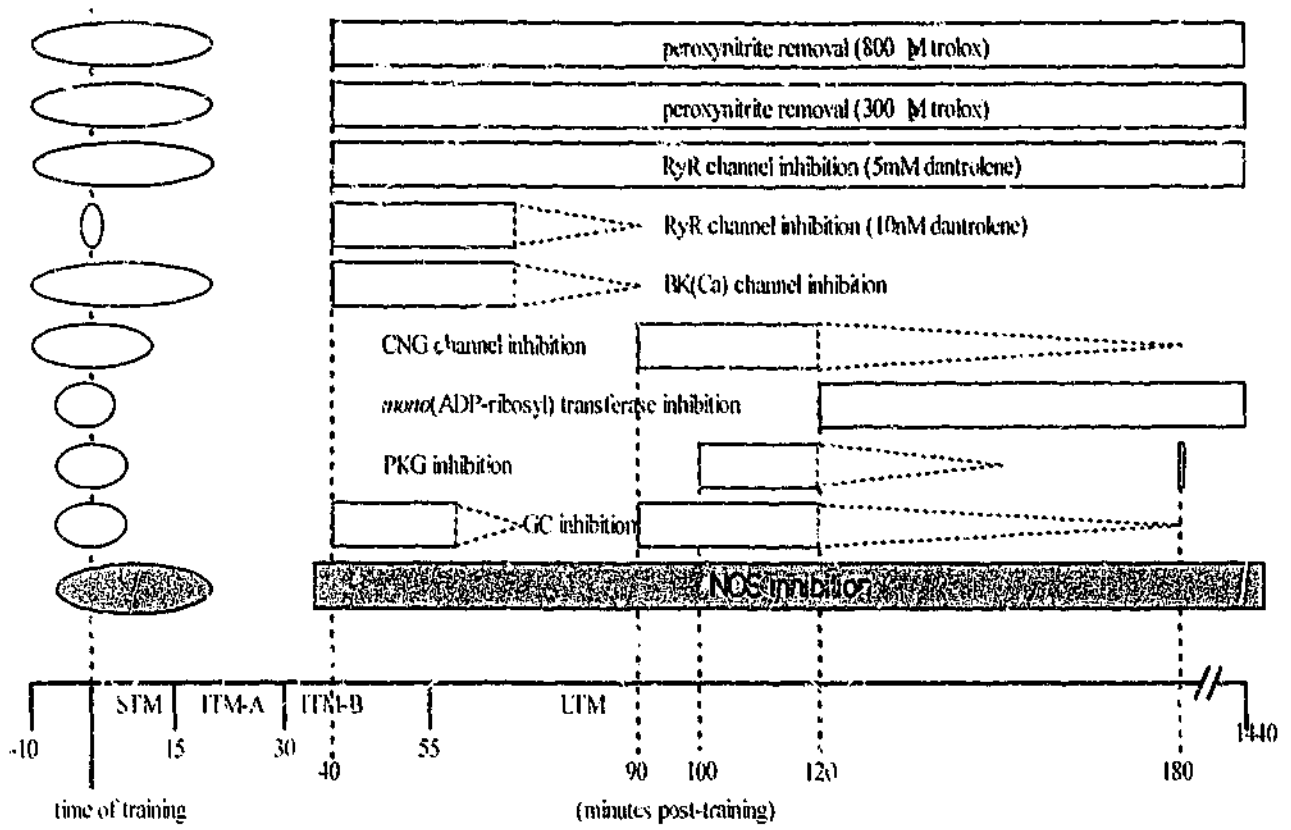


Figure 6.1 A summary of both the effective administration times and periods of retention loss for each mechanism studied in the present research.

Effective administration times, represented as ellipses, and periods of retention loss, represented as bars, are set against the x-axis representing time post-training. Some bars show a progressive narrowing which is intended to suggest a restoration of retention sometime between the times of test indicated by the extremities of the narrowing. Vertical hatched lines indicate the time of retention loss onset for each mechanism and these times are placed within the three stages of the Gibbs and Ng model (Gibbs & Ng, 1979a) indicated by the blue text associated with the x-axis.

6.2.1.2 Treatments inducing long-lasting retention loss

Previous research has clearly established that NO directly activates RyR channels in a number of different systems. The present findings demonstrate that antagonists of either NOS or RyR channels (with 5mM dantrolene) resulted in a persistent retention loss from 40 minutes post-training. In addition, these antagonists shared the same range of effective administration times. RyR channels have been implicated in memory formation, both directly and through investigations of the cellular processes thought to underlie memory formation. The present findings are consistent

with the view that NO activates RyR channels, leading to the consolidation of the LTM stage. In addition, although Gibbs et al. (1996) have previously observed effective administration times for the inhibitor of glutamine synthetase, methionine sulfoximine, to extend from 5 minutes post-training to 20 minutes post-training, effective administration times extending from before training to 20 minutes after training has only been observed once within this paradigm, namely the inhibition of BK_{Ca} channels reported herein. The fact that the inhibition of NOS and the inhibition of RyR channels by 5mM dantrolene should share this uncommon range of effective administration times strongly suggests that the role of NO in long-term memory consolidation is through the activation of RyR channels.

Removal of peroxynitrite using a pharmacological scavenger also resulted in a retention function and an effective time of administration function similar to those found with NOS and RyR channel inhibition. This is the first behavioural demonstration of the memory-related effects of peroxynitrite removal. As peroxynitrite shares a number of common biochemical properties with NO it is possible that NO activates the RyR channel directly or through the production of peroxynitrite.

Blockade of *mono*(ADP-ribosyl) transferase activity also resulted in a retention loss which persisted until at least 24 hours post-training , although, in contrast to the effects of RyR channels and peroxynitrite inhibition, the onset of the loss was at 120 mins post-training and not at 40 mins. Retention was inhibited only when *mono*(ADP-ribosyl) transferase inhibitors (MSB or NOVO) were administered around the time of training, which is in contrast to that for L-NAME inhibition of NOS. This study represents the first behavioural demonstration of the importance of *mono*(ADP-ribosyl) transferase in memory processing.

6.2.1.3 Treatments inducing transient retention losses

The other NO-dependent mechanisms investigated in this thesis were sensitive to inhibition at times different from that for NOS antagonists, and resulted in transient retention losses only. While these mechanisms cannot be excluded as being NO-dependent within the current paradigm, the persistent loss of retention from 40 minutes

post-training following NOS inhibition makes it impossible to deduce, from these studies alone, whether NO is required at times later than the ITM-B stage.

Previous investigations into the role of NO in memory formation have focussed on the activation of GC and in turn PKG (Bernabeau et al., 1995, 1996, 1997; Izquierdo et al., 2000; Kemenes et al., 2002; Serrano et al , 1994). These studies have established a relationship between NO, GC and PKG in memory formation using both pharmaco-behavioural techniques and techniques measuring enzyme activity following training. However, in the present investigations, inhibition of PKG by H-8 resulted in a period of transient retention loss that was significant by 100 minutes post-training, with recovery at the latest 150 minutes post-training. Furthermore, the effective times of administration for the PKG inhibitor, H-8, were close to the time of training, extending no later than 5 minutes post-training. Similarly, inhibition of GC, which has also been implicated in other memory tasks, resulted in two temporally distinct transient retention losses centred at 40 and 120 minutes post-training, respectively. While the earlier period of retention loss was consistent with the time of onset following NOS inhibition the latter, was consistent with the transient retention loss observed following PKG inhibition. This pattern of retention loss clearly does not match the persistent retention loss following NOS inhibition (Hölscher & Rose, 1992, 1993 Rickard et al., 1998). Interestingly, inhibitors of GC and PKG established similar effective times of administration but these were different from the effective times of administration of the isoform non-specific NOS inhibitors (see Chapter 3).

In addition to RyR channels, NO is also known to directly activate specific cation channels, including CNG channels and BK_{Ca} channels. In the present study, inhibition of CNG channels by verapamil resulted in retention loss when the drug was administered between 10 minutes before training and 10 minutes after training. Immediate post-training blockade of CNG channels induced a transient loss of retention between 90 and 120 minutes post-training. In contrast, blockade of BK_{Ca} channels by IbTX resulted in a transient retention loss between 40 and 70 minutes post-training. The effective range of administration times from 10 minutes before training up to 20 minutes post-training, was similar to those for L-NAME inhibition of NOS. The period of retention loss was similar to that observed with the inhibition of RyR channels by 10nM dantrolene.

6.2.2 Implications of the findings

The primary aim of this thesis was to isolate, using pharmaco-behavioural studies, the specific neurochemical pathway(s) responsible for the role of NO in memory formation. Inhibition of NO production through suppressing NOS activity by such agents as L-NAME has been shown in previous studies to result in a relatively permanent loss of memory. As indicated earlier, NO may operate through a number of pathways. In the interpretation of the findings that follows, it is assumed that inhibition of any of the mechanisms in a given pathway should result in, at least, a similar time of onset of retention loss if that pathway is relevant to memory processing. Furthermore, where more than one agent is used to inhibit a given mechanism, it would be reasonable to expect that the effective times of administration of the different agents should not be too discrepant, within the limits imposed by the pharmaco-kinetics of each agent. However, the time of administration function may differ for agents affecting different mechanisms, even within the same pathway. Detailed discussion of the implications of each of the findings has been presented in the relevant sections in the preceding chapters. The intention here is to provide a broad integrated view.

At a general level, there are a number of common features amongst the retention functions for the different mechanisms investigated in this thesis (summarised in Figure 6.1). Specifically, the onset of retention loss appears to occur only during the ITM-B stage and/or within the glutamate receptor-dependent phase of LTM (90 minutes post-training or later). While, difficult to interpret, it is possible that these two periods of memory formation are NO-dependent. However, although retention loss onset appears confined to either the ITM-B stage or after the consolidation of LTM, both transient and persistent retention losses result. Therefore, it may be that NO is responsible for activating both memory formation and retrieval processes.

The main finding of this thesis is that NO appears to be required for passive avoidance learning in the day-old chick through the activation of RyR channels either directly or through the action of peroxynitrite leading to the consolidation of LTM. This finding is important in itself, but also suggests a mechanism other than activation of GC as a means by which NO consolidates memory formation. Indeed, these findings

are the first to clearly demonstrate a role for NO in memory formation other than through the activation of GC.

6.2.2.1 Nitric oxide promotion of long-term memory consolidation

The importance of NO-activated RyR channels in long-term memory consolidation following passive avoidance learning in the chick is consistent with a range of other behavioural findings, including those of Salinska et al. (2001) who used a variant of the present task. In addition, Kouzu et al. (2000) and Ohnuki and Nomura (1996) used RyR3 knock-out studies and pharmaco-behavioural studies, respectively, to observe impaired avoidance learning, amongst other forms of learning, for rodents. In addition, RyR channel blockade or disruption induced impairment of spatial learning (Kouzu et al., 2000; Ohnuki & Nomura, 1996) and contextual fear conditioning in mice (Kouzu et al., 2000). Disruption of RyR channel function has also been observed to impair classical conditioning in *Hermissenda crassicornis* (Blackwell, 2002; Blackwell & Alkon, 1999).

The major findings in this thesis are also consistent with a range of studies implicating RyR channels in several mechanisms thought to underlie memory formation. For example, while the RyR3 isoform may have a role in post-synaptic events (Balschun et al., 1999) a number of studies have suggested a role for presynaptic RyR channel-dependent intracellular calcium stores in neurotransmitter release for a range of neurons. Such studies have identified neurons from *Aplysia* (Chameau, van de Vrede, Fossier & Baux, 2001; Mothet et al., 1998), cell lines capable of affecting a response from striated myotubules (Ronde, Dougherty & Nichols, 2000) and Purkinje cells of rats (Llano et al., 2000) as being at least somewhat dependent upon RyR channel-activated presynaptic calcium stores for neurotransmitter release. In addition Ouyang et al. (1997) identified the presence of RyR channels in the developing chick cerebellum from pre-hatch to two weeks post-hatch. Although different isoforms of the RyR channel differed in their distribution they found consistent immunocytochemical labelling of both the axon and presynaptic boutons of the parallel fibres in the molecular layer and the mossy fibre terminals associated with the granular layer glomeruli. Padua, Nagy and Geiger (1996) have

shown that RyR channels appear to be enriched in the presynaptic terminals of rat hippocampal tissue. This is important not only because the hippocampus is an area associated with memory formation, but because NO is also a candidate retrograde messenger in LTP (O'Dell et al., 1991), a process identified in the hippocampus and thought to underlie memory formation. Therefore, it is possible that one function of NO in memory formation is to activate RyR channels, leading to the enhancement of presynaptic neurotransmitter release that has been proposed to underlie LTP expression (Bliss et al., 1986).

In addition, RyR channels may affect memory processing through the release of non-LTP associated neurotransmitters, as well as facilitating memory processes through cerebral vasodilation. For example, Huang and Zhou (1996) have reported that the RyR channel agonist, caffeine, was slightly effective in enhancing [^{3}H]noradrenaline release, although dantrolene was ineffective in attenuating noradrenaline release. While certainly not unequivocal, these findings may be important since it has been previously established that NO potentiates noradrenaline release and that noradrenaline is vital to the consolidation of LTM in passive avoidance learning in the neonate chick (see Chapter 1). Finally, RyR channels have been shown to have a broad localisation within smooth muscle, and to be necessary in alteration of arterial diameter amongst other roles (Knot, Standen & Nelson, 1998; Neylon, Richards, Larsen, Agrotis & Bobik, 1995). Specifically, RyR channels have been identified as important in the induction of vasodilation in cerebral arteries (Jagger, Stevenson & Nelson, 1998; Knot et al., 1998; Lohn et al., 2001; Perez et al., 1999). Therefore, RyR channels may be involved in memory formation through modulating cerebral vasodilation. This is consistent with the findings of Rickard (1995) that SNP and prazosin, both vasodilators, were effective in promoting the continuation of the memory trace beyond the ITM-B stage following weakly reinforced learning in the chick. NO may, therefore play a role in memory formation through the activation of RyR channels, leading to an up-regulation of the level of presynaptic neurotransmitter release associated with LTP, increased release of noradrenaline to facilitate reinforcement, or through the promotion of cerebral vasodilation, or through all of the above.

6.2.2.2 *The role of nitric oxide in memory stage-specific retrieval*

While persistent retention losses may be considered to be due to the blockade of memory formation processes, transient retention losses have, in contrast, traditionally been considered to be due to interference with memory retrieval processes. The first study detailing such transient retention losses in the chick trained on the single trial passive avoidance task was by Allweis et al. (1984), in which either anoxia or hypoxia, induced soon after training, was shown to result in a transient loss of retention spanning the whole ITM stage, with the greatest loss of retention occurring during ITM-B. Their findings are similar to findings reported here for GC, BK_{Ca} channel and RyR channel (using 10nM dantrolene) inhibition and may suggest that ITM-B has a critical role in retrieval processes.

Not only does the onset of transient retention loss following the inhibition of GC, BK_{Ca} channels and RyR channels (with 10nM dantrolene) occur within ITM-B, but the retention loss from GC inhibition does not extend beyond the beginning of the LTM stage. In addition, recovery from the memory loss resulting from BK_{Ca} channel or RyR channel inhibition occurs by 90 minutes post-training at the latest. This time of recovery is generally consistent with the time of retention loss onset following the inhibition of glutamate receptors (Rickard et al., 1994b, 1995) and tyrosine kinase activity (Whitechurch et al., 1997) and with the later transient retention loss following GC inhibition. This may indicate the onset of a second phase within the LTM stage defined by Gibbs and Ng (1979a).

Interestingly, the common retention functions obtained with BK_{Ca} channel inhibition and RyR channel inhibition by 10nM dantrolene are reflected in the physical association of these two channels which underlies a cellular process known as calcium induced calcium release (CICR) (Akita & Kuba, 2000). Briefly, CICR occurs through calcium influx, associated with voltage-gated calcium channels, activating RyR channels to release calcium from intracellular stores (Berridge, 1998). This amplification of calcium levels results in the up-regulation of calcium-dependent enzymes and channels. One channel to be up-regulated by this process is the BK_{Ca} channel as identified in a number of studies using neuronal (Merriam, Scornik & Parsons, 1999) or smooth muscle tissue (Lee & Earm, 1994). Importantly, this

association between RyR channels and BK_{Ca} channels has also been observed in the rat hippocampus (Uneyama, Munakata, Akaike, 1993).

Finally, CNG channel inhibition results in a transient retention loss from 90 minutes post-training and lasting until at least 120 minutes post-training. The time of offset of this transient loss coincides with the time of retention loss *onset* following *mono*ADP-ribosylation blockade and may represent the onset of a third phase of the LTM stage. While it is speculation that memory retrieval stages broadly align with memory formation stages, the concept of a limited number of retrieval stages, possibly tied to memory stages, has been suggested by Summers (1998).

In addition to similarities in retention losses for each of the mechanisms investigated within this thesis, each drug tested showed an effective administration range from about 5 minutes before training to 5 minutes after training or from 10 minutes before training up until 20 minutes post-training. Only the inhibition of CNG channels using verapamil resulted in a different range of effective administration times. This consistency in effective administration times was unexpected as each drug is pharmacologically distinct and might have been expected to show different pharmaco-kinetic characteristics. As each mechanism tested may be NO-dependent, such limited administration times may suggest two periods during which NO-dependent processes are activated - around the time of training and by 20 minutes post-training. This speculation is reinforced by the effective times of administration for the specific nNOS inhibitor, NPLA, compared to either the eNOS-specific inhibitor, DPI, or the isoform non-specific inhibitor L-NAME. NPLA must be administered close to the time of training if it is to effectively block retention by 40 minutes post-training while DPI or L-NAME can be administered up until 20 minutes post-training and result in retention loss also by 40 minutes post-training (Rickard et al., 1999; Rickard & Gibbs, in preparation – a).

SECTION 3 : Limitations of the present research programme

The neonate chick model has several advantages for investigating the sequence of cellular events that are involved in memory formation from the time of learning. As described by Ng and Gibbs (1989), the neonate chick is biologically well-prepared for the single trial task used here and this overcomes the possibility of overlapping memory traces and the confounding effects upon retention testing associated with multiple trials. It, therefore, allows detailed investigation when, after learning, various cellular processes are necessary for memory processing. Further, any sensory-motor effects of a particular drug are easily identified using the discrimination variant of the task. Finally, absence of an ossified skull in the day-old chick also permits drug administration into specific brain regions without the need for cannulation.

Despite the above advantages, there are limitations to the model. The immaturity of the neonate chick brain may be problematic. Chick synapses are known to undergo maturation for several weeks post-hatch (Rostas & Jeffrey, 1981; Rostas et al., 1984; Wienberger & Rostas, 1988a, b). This was noted both in age-dependent alterations to the post-synaptic density and in the pattern of expression of particular enzymes studied. Therefore, some caution should be exercised when generalising the current findings to memory formation and retrieval in mature animals. Finally, there is also the issue of generalisability of findings to mammals, particularly humans. However, in the present case, NO has been found to underlie memory processing in both vertebrate and invertebrate species and possibly does so through a limited and conserved set of biochemical interactions.

The use of specific inhibitors bilaterally administered into the neostriatal/hyperstriatal complex containing the IMHV may also be problematic. Excluding issues surrounding drug specificity and sensory-motor effects which were controlled for to a large extent by the use of a discriminative variant of the task and alternative antagonists in a number of studies, a couple of concerns remain. First, the work of Bennett (1999) has especially noted the importance of unilateral injections, suggesting hemispheric differences are an important feature in understanding memory

formation processes in the day-old chick. This work was furthered recently with respect to NOS by Rickard and Gibbs (in preparation - a) in which L-NAME was found to inhibit retention if injected into the left hemisphere up to 5 minutes post-training but to inhibit retention if injected into the right hemisphere between 10 and 25 minutes post-training. Because bilateral injections were used in the current research, the potential lateralisation of any of the NO-related mechanisms identified as involved in memory cannot be ascertained. Secondly, administration of drugs into only the neostriatal/hyperstriatal complex may also be problematic. The role of this region in memory processing in the chick is well established however, all stages of passive avoidance memory formation most likely rely on other brain regions as well. Most notable of these is the LPO, which has been discussed by both Rose (1991) and Rosenzweig et al. (1991). Interestingly, however, Rickard and Gibbs (in preparation - b) showed that administration of L-NAME into the LPO had no effect upon retention. Thus, the LPO may not be involved in NO-dependent memory processes.

Finally, intracranial injections may be problematic with respect to the spread of the drug following administration. Rosenzweig et al. (1991) describe a number of difficulties associated with intracranial administration of drugs. They expressed concern over the leak of drugs from the site of injection out through the needle track. However, radio-labelled leucine and ouabain did not diffuse widely within the brain tissue itself. Greenberg et al. (1997) also found that the NOS antagonist L-NNA demonstrated little diffusion even several hours post-administration using both topical and icv administration into the rat brain. It is likely, therefore, that the site of action of NO-dependent memory processes are within, or very close to, the neostriatal/hyperstriatal complex. In addition, we have found in our laboratory that injection of india ink also results in very little diffusion from the injection site (N.S. Rickard, personal communication, 5th July 2002). Although india ink may not have the same diffusion characteristics of the drugs used in the current study, this observation does, at least, suggest that the injection procedure is suitably gentle with regard to expulsion of liquid from the needle point.

SECTION 4 : Future directions of research

The current studies prompt a number of further investigations. The work of Bennett (1999) and Rickard and Gibbs (in preparation - a) suggest the possibility of hemispheric differences in the expression of NO-activated mechanisms. It would be worth investigating possible lateralised effects of the treatments used here. Although unlikely for reasons discussed in Chapter 3, the two transient retention losses found following the administration of the GC inhibitors ODQ and LY83583 may prove to be hemisphere-specific.

As the day-old chick has not yet reached synaptic maturity, as discussed above, the generalisability of the present findings to adult chicks may be limited. It would be of value to determine if RyR channels and peroxynitrite formation could account for the role of NO in passive avoidance learning in older chicks. A passive avoidance task developed for older chicks is the 'pebble floor task', which also makes use of the chicks natural propensity to peck small round objects (Andrew, 1991). On a broader level, the temporal relationships between the mechanisms studied here should also be tested in mammalian species such as rats. The single trial inhibitory avoidance task used by Bernabeu et al. (1995, 1996, 1997) and by Izquierdo et al. (2000) would be ideal for this purpose as it has already been used effectively to study the relationship between NOS, GC and PKG.

Weakly reinforced learning has, in the past, been used to clarify mechanisms necessary for the induction of the ITM-B stage. By itself, weakly reinforced training fails to lead to the formation of the ITM-B or LTM stage in the Gibbs and Ng model (Gibbs & Ng, 1979a). When weakly reinforced learning is coupled with a spontaneous NO-donor such as SNP (Rickard et al., 1994a), the ITM-B and LTM stages are formed. This is strong evidence for a role for NO in ITM-B induction. The methodology would also be useful for clarifying the role of RyR channels in memory formation if agonists could be obtained that are specific to RyR channel-dependent intracellular calcium release. Although agonists such as caffeine have previously been used to open RyR channels, this agonist for example, is not appropriate as it also activates adenosine receptors, as well having a generalised action upon arousal. Other pharmaco-behavioural methods that have previously been used to identify relationships between

molecules may be appropriate. For example, to determine whether NO activates RyR channels, NO production could be inhibited and then the resulting memory loss challenged by a specific RyR channel agonist. If retention is restored, then it may be argued that NO does indeed activate RyR channels. Taken together, the above studies may be useful for corroborating the present evidence relating the activation of RyR channels to NO.

Pharmaco-behavioural studies alone, for reasons of drug action specificity, are not sufficient for drawing unequivocal conclusions regarding which cellular events are proximally associated with the behaviour being studied. Corroborative evidence from measurements of changes in cellular activity can materially strengthen the conclusions from such studies. It would be useful, therefore, to identify the time post-training when NOS activation and calcium release from RyR channels occur, making use of the high degree of temporal specificity observed with the passive avoidance task for the day-old chick. As NO is a highly labile radical NOS activation should overlap the period of calcium release if NO indeed acts upon RyR channels. The time of NOS activation post-training can be ascertained by commercially available assay kits such as those from Caymen Chemicals, or by the radioactive method favoured by Bernabeu et al. (1995) in their behavioural studies. However, temporal measurement of calcium release from RyR-dependent stores may not be simple. Even so, a number of methods exist for observing calcium flux and, in some cases, these have been applied to the action of the RyR channel. For example, Mothet et al. (1998) have used the fluorescent dye rhod-2 to observe the action of the RyR channel with respect to controlling neurotransmitter release. It may be appropriate to inject rhod-2 dye before training and fix the level of calcium release at various times post-training by immersion of the chick head in liquid nitrogen, a standard technique to halt biochemical processes. By observation of the decrease in calcium in the endoplasmic reticulum, the level of intracellular calcium release may be measured at various times post-training without the confounding effects of calcium entry from extra-cellular stores as is known to happen during the STM stage (Gibbs et al., 1979).

In addition to studies focusing on the relationship between NO and RyR channels, the studies undertaken in this thesis also prompt other investigations. For example, the common times of temporary retention loss for two of the three classes of proteins activated by GC, namely CNG channels and PKG, coincide with the later transient retention loss following GC inhibition, occurring by about 90 minutes post-

training and showing restoration of retention by no later than 180 minutes post-training. Therefore, it is reasonable to suggest that inhibition of the third class of GC-activated protein, namely PDEs, may result in a transient retention loss coinciding with the earlier transitory retention loss following GC inhibition, from 40 to 60 minutes post-training. It may be hypothesized that retention loss following PDE inhibition would occur 40 minutes post-training. This can be easily tested in future studies using the same pharmaco-behavioural methods as in the current research.

In addition, GC is known to be activated by a number of molecules, most notably NO and CO. As the retention functions following the inhibition of NOS and GC do not match, it is possible that, in this memory paradigm, CO rather than NO is responsible for activating GC. While the role of physiological levels of CO in memory formation remains controversial, it would nevertheless be of interest to investigate this role of CO in the present context. Preliminary findings in our laboratory suggest that CO inhibition may indeed produced transient retention losses at the same time as observed with GC inhibition (unpublished data).

With regard to the transient retention losses observed throughout this research, it has been assumed that they reflect impairment of memory retrieval processes. There exists a variant of the present task which uses a 'reminder' presentation of the aversive bead to test memory retrieval (Summers, 1998). In brief, the aversive learning experience is a red bead dipped in the aversant MeA while the reminder, presented at various times post-training, is a dry red bead which the chicks are not allowed to peck. Drug administration occurs immediately following the reminder to ensure that the effects, if any, are on retrieval, rather than on memory formation processes. The use of this task in conjunction with administration of those pharmacological agents found to cause transient retention losses may shed light on the role of NO-dependent mechanisms on memory retrieval processes.

Finally, future research should be applied to clinical conditions. The use of NO in pharmaceuticals to date has revolved around its capacity as a vasodilator in such applications as angina medications. Related to this is the pharmaceutical sildenafil (Viagra), which is a PDE class V inhibitor acting to increase NO-derived cGMP levels in the corpora cavernosa of the penis (Boolell et al., 1996). Interestingly, sildenafil has also been shown to act to improve memory using a single trial step-through avoidance task in mice (Baratti & Boccia, 1999).

However, with respect to the central findings of this thesis, if inhibition of RyR channels impairs retention in healthy animals, including the chick, then activation of these channels may improve retention in conditions where memory loss is a major factor. While specific agonists need to be developed which can both cross the blood-brain barrier and are cell permeable, one common agonist is caffeine. Although there exists some evidence suggesting caffeine can aid memory enhancement (Gevaerd, Takahashi, Silveira & Da Cunha, 2001; Hogervorst, Riedel, Kovacs, Brouns & Jolles, 1999; Jarvis, 1993; Miller & Miller, 1996; Warburton, Bersellini & Sweeney, 2001; Warburton, 1995), the action of caffeine is complex, being an agonist of adenosine receptors and being capable of increasing arousal generally. In addition, the major findings of this thesis suggest that a biologically relevant level of peroxynitrite is necessary for memory formation. While high levels of peroxynitrite have been associated with memory-associated disorders such as Alzheimer's disease (Good et al., 1996; Matthews & Beal, 1996; Smith et al., 1997) suppression of peroxynitrite below physiological levels may indeed also result in memory impairment

In conclusion, the single trial passive avoidance task for the day-old chick has proved a particularly useful paradigm by which to investigate nitric-oxide related mechanisms in memory formation. Previous research has largely indicated that GC and PKG were likely to be the downstream mechanisms of NO activation in memory processes. The current research has uncovered a much more critical role for RyR channels and peroxynitrite in NO-related memory formation for this task. This should not be entirely surprising given that NO is an essential precursor chemical of peroxynitrite production, and both NO and peroxynitrite can directly activate RyR channels. It also suggests that the roles of GC and PKG may be associated with stage-related retrieval mechanisms rather than with memory formation *per se*. The failure to detect such transient changes in previous studies may be attributed in large measure to the failure to sample sufficient training-retention intervals with single trial tasks or to the difficulty of tracking such changes in multiple trial tasks that likely generate overlapping memory processes.

This thesis has presented the first pharmaco-behavioural evidence that NO may be involved in memory formation through the production of peroxynitrite and the activation of RyR channels. These novel findings provide new directions for future research aimed at exploring the underlying cellular mechanisms of memory formation. In particular, the possibility that activation of the NO-RyR channel pathway influences

neuronal processes implicated in memory, such as LTP, is exciting. The general presynaptic action of NO observed by O'Dell et al (1991) and the role of presynaptic RyR channel-dependent intracellular calcium stores in LTP noted by Mothet et al. (1998), suggest that the candidate retrograde messenger NO could, for instance, influence neurotransmitter release via release of calcium from RyR channel-dependent intracellular stores. While the process remains unknown, the current findings provide strong evidence that the function of NO in long-term memory formation for at least one memory task involves RyR channels and peroxynitrite formation, rather than GC and PKG activity.

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DOCTORAL THESIS
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SURNAME: Edwards

OTHER NAMES: Thomas Mark

ADDRESS: (valid for the next six months) *25 Berrima Rd*
Donvale, 3111

TELEPHONE: (BH) 9905 3900 (AH) 98487783

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NITRIC OXIDE-ACTIVATED MECHANISMS UNDERLYING MEMORY FORMATION USING A PASSIVE AVOIDANCE TASK FOR THE DAY-OLD CHICK

(Volume two)

Submitted by

Thomas M. Edwards BSc(Hons)

A thesis submitted in total fulfilment of the requirements for the degree of

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Department of Psychology

School of Psychology, Psychiatry & Psychological Medicine

Faculty of Medicine

Monash University

Clayton, Victoria, 3800

Australia

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APPENDICES

Appendix A – statistical tables for:

1. L-NAME
2. H-8
3. ODQ
4. LY83583
5. Menadione sodium bisulfite
6. Novobiocin
7. Verapamil
8. Iberiotoxin
9. Dantrolene
10. Trolox

Appendix B – Differences in method between two variants of the passive avoidance task for day-old chicks

Appendix C - Conformation of retention loss associated with the time of training and not the time of administration for H-8 and ODQ

Appendix D – Papers and Abstracts Published in Support of this Thesis

APPENDIX A - Statistical tables for behavioural studies

A.1 - L-NAME

A.1.1 Retention study for L-NAME

Table A.1.1.1 – Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	saline	.91	.04	16
	L- NAME	.88	.05	12
30	saline	.89	.04	20
	L-NAME	.92	.03	18
40	saline	.95	.03	16
	L-NAME	.74	.05	16
60	saline	.89	.05	15
	L-NAME	.71	.04	20
70	saline	.96	.04	17
	L-NAME	.75	.06	17
120	saline	.92	.04	15
	L-NAME	.76	.04	17
180	saline	.95	.02	18
	L-NAME	.72	.05	29
1440	saline	.96	.03	17
	L-NAME	.70	.06	23

Table A.1.1.2 – Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	11.48	291	.04		
Drug effect	1.74	1	1.74	44.13	.000
Time of test effect	.27	7	.04	.99	.437
Interaction effect	.66	7	.09	2.38	.022

Table A.1.1.3 – Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.01	1	.01	.29	.588
30	.01	1	.01	.21	.646
40	.35	1	.35	8.79	.003
60	.32	1	.32	8.24	.004
70	.34	1	.34	8.68	.003
120	.34	1	.34	8.70	.003
180	.65	1	.65	16.38	.000
1440	.68	1	.68	17.14	.000

A.2 - H-8

A.2.1 – Dose response study for H-8

Table A.2.1.1 - Summary data

Concentration of H-8 (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
saline	.95	.02	17
.01	.91	.03	17
1	.91	.04	15
100	.88	.05	17
150	.69	.06	17
200	.59	.06	15
250	.67	.06	17
300	.69	.07	17
500	.84	.06	17

Table A.2.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between Groups	2.277	8	.285	6.361	.000
Within groups	6.264	140	.045		
Total	8.541	148			

Table A.2.1.3 - Dunnett's test post-hoc analysis

Concentration of H-8 (μM)	*Significance (p<.05)*
.01	.995
1	.995
100	.881
150	.003
200	.000
250	.001
300	.003
500	.476

A.2.2 – Time of administration study for H-8

Table A.2.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	saline	.92	.04	20
	H-8	.87	.04	20
-5	saline	.96	.02	18
	H-8	.69	.06	18
+0	saline	.96	.02	18
	H-8	.63	.06	19
2.5	saline	.98	.02	19
	H-8	.76	.05	17
5	saline	.94	.03	19
	H-8	.79	.07	18
10	saline	.94	.03	18
	H-8	.97	.02	16
20	saline	.93	.04	19
	H-8	.94	.02	20

Table A.2.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	6.99	245	.03		
Drug effect	1.30	1	1.30	45.42	.000
Time of administration effect	.69	6	.12	4.06	.001
Interaction effect	1.09	6	.18	6.38	.000

Table A.2.2.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.02	1	.02	.69	.407
-5	.67	1	.67	21.95	.000
+0	1.01	1	1.01	33.01	.000
2.5	.46	1	.46	14.91	.000
5	.23	1	.23	7.38	.007
10	.00	1	.00	.06	.809
20	.00	1	.00	.08	.775

A.2.3 – Retention study for H-8

Table A.2.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	saline	.91	.04	16
	H-8	.92	.04	16
20	saline	.92	.04	14
	H-8	.90	.05	17
30	saline	.89	.04	20
	H-8	.93	.02	18
40	saline	.95	.03	16
	H-8	.93	.02	38
60	saline	.89	.05	15
	H-8	.83	.07	15
70	saline	.96	.04	17
	H-8	.87	.04	18
80	saline	.87	.05	15
	H-8	.88	.04	19
90	saline	.92	.03	18
	H-8	.82	.06	16
100	saline	.93	.04	16
	H-8	.74	.05	27
120	saline	.92	.04	15
	H-8	.68	.05	38
150	saline	.91	.05	15
	H-8	.90	.04	17
180	saline	.95	.02	18
	H-8	.82	.04	34
1440	saline	.96	.03	17
	H-8	.95	.03	28

Table A.2.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	17.96	490	.04		
Drug effect	.48	1	.48	12.98	.000
Time of test effect	.88	12	.07	1.99	.023
Interaction effect	.81	12	.07	1.85	.038

Table A.2.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.00	1	.00	.01	.919
20	.00	1	.00	.05	.826
30	.01	1	.01	.39	.535
40	.01	1	.01	.23	.635
60	.03	1	.03	.91	.341
70	.07	1	.07	1.78	.183
80	.00	1	.00	.00	.969
90	.09	1	.09	2.45	.118
100	.48	1	.48	12.98	.000
120	1.28	1	1.28	34.88	.000
150	.00	1	.00	.01	.917
180	.25	1	.25	6.75	.010
1440	.00	1	.00	.09	.762

A.3 - ODQ

A.3.1 – Dose response study for ODQ

Table A.3.1.1 - Summary data

Concentration of ODQ (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
vehicle	.95	.02	17
0.01	.92	.04	18
0.1	.88	.05	16
1	.83	.05	19
10	.80	.06	17
50	.81	.05	16
100	.64	.05	17
150	.83	.05	17
200	.89	.04	20
500	.93	.03	19

Table A.3.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between groups	1.2703	9	.1411	3.9915	.000
Within groups	5.8701	166	.0354		
Total	7.1404	175			

Table A.3.1.3 - Dunnett's test post-hoc analysis

Concentration of ODQ (μM)	*Significance (p<.05)*
0.01	1.000
0.1	.825
1	.296
10	.134
50	.158
100	.001
150	.336
200	.940
500	1.000

A.3.2 – Time of administration study for ODQ

Table A.3.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	vehicle	.92	.04	20
	ODQ	.85	.04	17
-5	vehicle	.96	.02	18
	ODQ	.73	.07	14
+0	vehicle	.96	.02	18
	ODQ	.72	.07	17
2.5	vehicle	.98	.02	19
	ODQ	.74	.06	18
5	vehicle	.94	.03	19
	ODQ	.80	.05	17
10	vehicle	.94	.03	18
	ODQ	.83	.05	14
20	vehicle	.93	.04	19
	ODQ	.94	.03	18
25	vehicle	.91	.05	19
	ODQ	.91	.05	15

Table A.3.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	8.54	264	.03		
Drug effect	1.15	1	1.15	35.66	.000
Time of administration effect	.27	7	.04	1.21	.299
Interaction effect	.69	7	.10	3.05	.004

Table A.3.2.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.04	1	.04	1.18	.279
-5	.41	1	.41	12.52	.000
+0	.51	1	.51	16.74	.000
2.5	.55	1	.55	18.09	.000
5	.18	1	.18	6.04	.015
10	.11	1	.11	3.63	.058
20	.00	1	.00	.01	.929
25	.00	1	.00	.00	.974

A.3.3 – Retention study for ODQ

Table A.3.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	vehicle	.91	.04	16
	ODQ	.95	.03	17
20	vehicle	.92	.04	14
	ODQ	.94	.03	16
30	vehicle	.89	.04	20
	ODQ	.97	.02	18
40	vehicle	.95	.03	16
	ODQ	.74	.05	32
50	vehicle	.94	.03	14
	ODQ	.76	.05	31
60	vehicle	.89	.05	15
	ODQ	.80	.04	35
70	vehicle	.96	.04	17
	ODQ	.90	.03	35
80	vehicle	.88	.05	15
	ODQ	.79	.05	18
90	vehicle	.92	.03	18
	ODQ	.76	.04	36
100	vehicle	.93	.04	16
	ODQ	.65	.07	18
120	vehicle	.92	.04	15
	ODQ	.67	.08	13
180	vehicle	.95	.02	18
	ODQ	.94	.03	18
1440	vehicle	.96	.03	17
	ODQ	.90	.04	26

Table A.3.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	17.63	498	.04		
Drug effect	1.13	1	1.13	31.88	.000
Time of test Effect	1.39	12	.12	3.28	.000
Interaction effect	1.33	12	.11	3.12	.000

Table A.3.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.01	1	.01	.39	.533
20	.00	1	.00	.06	.802
30	.05	1	.05	1.41	.235
40	.59	1	.59	15.89	.000
50	.48	1	.48	12.93	.000
60	.17	1	.17	4.69	.031
70	.01	1	.01	.23	.635
80	.06	1	.06	1.67	.197
90	.45	1	.45	12.00	.001
100	.72	1	.72	19.36	.000
120	.40	1	.40	10.84	.001
180	.00	1	.00	.07	.789
1440	.01	1	.01	.36	.550

A.3.4 – ODQ test of dose-dependency measured 40 minutes post-training

Table A.3.4.1 - Summary Data

Concentration of ODQ (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
vehicle	.88	.06	14
1	.79	.04	18
10	.88	.05	17
100	.63	.07	17
500	.79	.06	16
1000	.89	.04	17

Table A.3.4.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between groups	.787	5	.157	3.272	.009
Within groups	4.473	93	.0481		
Total	5.260	98			

Table A.3.4.3 - Dunnett's test post-hoc analysis

Concentration of ODQ (μM)	*Significance (p<.05)*
1	.725
10	1.000
100	.016
500	.807
1000	.998

A.3.5 – ODQ test of dose-dependency measured 70 minutes post-training

Table A.3.5.1 - Summary data

Concentration of ODQ (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
vehicle	.92	.04	16
1	.90	.04	15
10	.97	.02	18
100	.96	.03	18
500	.89	.04	19
1000	.92	.03	20

Table A.3.5.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between groups	.081	5	.016	.765	.577
Within groups	2.106	100	.021		
Total	2.187	105			

A.4 -- LY83583

A.4.1 – Dose response study for LY83583

Table A.4.1.1 - Summary data

Concentration of LY 83583 (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
vehicle	.93	.02	32
.01	.83	.04	19
1	.72	.06	16
10	.82	.04	35
40	.84	.03	35
70	.91	.02	32
100	.73	.04	36
150	.86	.04	36
200	.84	.05	20
250	.86	.05	15
400	.97	.02	15

Table A.4.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between Groups	1.355	10	.135	3.445	.000
Within groups	11.009	280	.039		
Total	12.364	290			

Table A.4.1.3 - Dunnett's test post-hoc analysis

Concentration of LY83583 (μM)	*Significance (p<.05)*
.01	.470
1	.007
10	.153
40	.436
70	1.000
100	.000
150	.641
200	.562
250	.914
400	.998

A.4.2 – Time of administration study for LY83583

Table A.4.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	vehicle	.92	.04	20
	LY83583	.82	.05	17
-5	vehicle	.95	.02	18
	LY83583	.85	.04	16
+0	vehicle	.96	.02	18
	LY83583	.69	.07	15
2.5	vehicle	.98	.02	19
	LY83583	.82	.04	19
5	vehicle	.94	.03	19
	LY83583	.77	.07	15
10	vehicle	.94	.03	18
	LY83583	.85	.05	18
20	vehicle	.93	.04	19
	LY83583	.95	.04	19

Table A.4.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	7.49	232	.03		
Drug effect	.95	1	.95	29.57	.000
Time of administration effect	.28	6	.05	1.43	.202
Interaction effect	.37	6	.06	1.91	.080

Table A.4.2.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.08	1	.08	2.58	.110
-5	.07	1	.12	2.24	.136
+0	.53	1	.53	16.33	.000
2.5	.21	1	.21	6.46	.012
5	.25	1	.25	7.62	.006
10	.07	1	.07	2.29	.132
20	.00	1	.00	.01	.936

A.4.3 – Retention study for LY83583

Table A.4.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
30	vehicle	.89	.04	20
	LY83583	.94	.05	17
40	vehicle	.95	.02	17
	LY83583	.74	.05	35
50	vehicle	.95	.03	14
	LY83583	.77	.07	17
60	vehicle	.89	.05	15
	LY83583	.82	.05	17
70	vehicle	.96	.04	17
	LY83583	.90	.03	18
90	vehicle	.92	.03	18
	LY83583	.85	.06	17
120	vehicle	.92	.04	15
	LY83583	.60	.05	38
180	vehicle	.95	.02	18
	LY83583	.87	.04	18

Table A.4.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	12.38	295	.04		
Drug effect	1.02	1	1.02	24.25	.000
Time of test effect	.83	7	.12	2.82	.007
Interaction effect	.92	7	.13	3.12	.003

Table A.4.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
30	.02	1	.02	.38	.540
40	.66	1	.66	15.15	.000
50	.24	1	.24	5.53	.019
60	.04	1	.04	.97	.326
70	.02	1	.02	.55	.459
90	.05	1	.05	1.18	.279
120	2.23	1	2.23	51.00	.000
180	.06	1	.06	1.37	.242

A.5 – Menadione Sodium Bisulfite

A.5.1 – Dose response study for menadione sodium bisulfite

Table A.5.1.1 - Summary data

Concentration of MSB (μM)	*Mean discrimination ratio*	*Standard error of the mean*	Sample size
saline	.93	.03	17
1	.92	.04	18
10	.93	.03	18
100	.91	.03	15
200	.76	.04	19
250	.63	.05	19
300	.77	.05	17
400	.86	.06	16
500	.91	.04	14

Table A.5.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between Groups	1.595	8	.199	5.669	.000
Within groups	5.065	144	.035		
Total	6.661	152			

Table A.5.1.3 - Dunnett's test post-hoc analysis

Concentration of MSB (μM)	*Significance (p<.05)*
1	1.000
10	1.000
100	1.000
200	.043
250	.000
300	.170
400	.828
500	1.000

A.5.2 – Time of administration study for menadione sodium bisulfite

Table A.5.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	saline	.92	.04	20
	MSB	.85	.05	19
-5	saline	.95	.04	18
	MSB	.79	.06	14
+0	saline	.96	.02	18
	MSB	.73	.06	19
2.5	saline	.98	.02	19
	MSB	.73	.06	17
5	saline	.94	.03	19
	MSB	.83	.06	16
10	saline	.94	.03	18
	MSB	.87	.05	20
20	saline	.93	.04	19
	MSB	.96	.02	17

Table A.5.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	7.71	239	.03		
Drug effect	1.00	1	1.00	31.05	.000
Time of test effect	.24	6	.04	1.26	.277
Interaction effect	.53	6	.09	2.75	.013

Table A.5.2.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.04	1	.04	1.24	.266
-5	.22	1	.22	6.89	.009
+0	.52	1	.52	16.08	.000
2.5	.55	1	.55	16.93	.000
5	.12	1	.12	3.57	.060
10	.05	1	.05	1.59	.209
20	.00	1	.00	.10	.753

A.5.3 – Retention study for menadione sodium bisulfite inhibition

Table A.5.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	saline	.91	.04	16
	MSB	.87	.05	16
30	saline	.89	.04	20
	MSB	.96	.03	18
40	saline	.95	.03	16
	MSB	.92	.04	19
60	saline	.96	.04	17
	MSB	.92	.04	19
90	saline	.92	.03	18
	MSB	.94	.04	18
110	saline	.92	.04	16
	MSB	.86	.06	14
120	saline	.92	.04	15
	MSB	.74	.05	31
180	saline	.95	.02	18
	MSB	.72	.07	16
1440	saline	.96	.03	17
	MSB	.81	.05	25

Table A.5.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	11.33	309	.04		
Drug effect	.32	1	.32	8.77	.003
Time of test effect	.42	8	.05	1.43	.181
Interaction effect	.79	8	.10	2.68	.007

Table A.5.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.01	1	.01	.35	.557
30	.04	1	.04	1.21	.273
40	.01	1	.01	.15	.702
60	.01	1	.01	.20	.655
90	.01	1	.01	.14	.710
110	.03	1	.03	.71	.401
120	.55	1	.55	14.71	.000
180	.46	1	.46	12.29	.001
1440	.24	1	.24	6.45	.012

A.6 – Novobiocin

A.6.1 – Dose response study for novobiocin

Table A.6.1.1 - Summary data

Concentration of novobiocin (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
saline	.93	.03	33
.1	.90	.04	15
1	.87	.04	19
10	.89	.06	16
50	.83	.06	19
100	.80	.06	18
250	.93	.03	15
350	.79	.07	15
500	.80	.04	33
650	.67	.07	19
800	.78	.05	20
1000	.88	.04	17

Table A.6.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between Groups	1.238	11	.113	2.412	.007
Within groups	10.591	227	.047		
Total	11.828	238			

Table A.6.1.3 - Dunnett's test post-hoc analysis

Concentration of novobiocin (μM)	*Significance (p<.05)*
0.1	1.000
1	.981
10	1.000
50	.690
100	.377
250	1.000
350	.307
500	.136
650	.001
800	.129
1000	.998

A.6.2 – Time of administration study for novobiocin

Table A.6.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	saline	.92	.04	20
	novobiocin	.90	.05	18
-5	saline	.95	.04	14
	novobiocin	.81	.05	18
+0	saline	.96	.02	18
	novobiocin	.68	.06	17
2.5	saline	.98	.02	19
	novobiocin	.64	.06	16
5	saline	.94	.03	19
	novobiocin	.87	.04	17
10	saline	.94	.03	18
	novobiocin	.86	.05	15
20	saline	.93	.04	19
	novobiocin	.77	.06	16

Table A.6.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	7.57	234	.03		
Drug effect	1.53	1	1.53	47.33	.000
Time of test effect	.35	6	.06	1.80	.099
Interaction effect	.72	6	.12	3.71	.002

Table A.6.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.01	1	.01	.15	.695
-5	.21	1	.21	6.41	.012
+0	.68	1	.68	20.61	.000
2.5	.95	1	.95	28.69	.000
5	.05	1	.05	1.42	.234
10	.06	1	.06	1.90	.169
20	.22	1	.22	6.59	.011

A.6.3 – Retention study for novobiocin

Table A.6.3.1 - Summary data

Time of test (minutes post-training)	*Drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	saline	.91	.04	16
	novobiocin	.99	.01	19
30	saline	.89	.04	20
	novobiocin	.93	.03	17
40	saline	.95	.03	16
	novobiocin	.98	.01	17
70	saline	.96	.04	17
	novobiocin	.95	.03	19
90	saline	.92	.03	18
	novobiocin	.96	.02	20
110	saline	.92	.04	16
	novobiocin	.79	.05	17
120	saline	.92	.04	15
	novobiocin	.62	.05	32
180	saline	.95	.02	18
	novobiocin	.62	.07	17
1440	saline	.96	.03	17
	novobiocin	.68	.06	29

Table A.6.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	11.10	320	.03		
Drug effect	.65	1	.65	18.76	.000
Time of test effect	1.62	8	.20	5.83	.000
Interaction effect	2.25	8	.28	8.10	.000

Table A.6.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.07	1	.07	1.73	.190
30	.02	1	.02	.40	.527
40	.01	1	.01	.21	.650
70	.04	1	.04	.91	.342
90	.02	1	.02	.62	.431
110	.14	1	.14	3.74	.054
120	1.69	1	1.69	43.63	.000
180	.93	1	.93	23.97	.000
1440	1.12	1	1.12	28.77	.000

A.7 - Verapamil

A.7.1 - Dose response study for verapamil

Table A.7.1.1 - Summary data

Concentration of verapamil (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
saline	.94	.02	38
.01	.94	.02	19
.1	.90	.04	17
1	.93	.03	20
10	.92	.04	19
100	.90	.03	18
300	.87	.04	17
500	.91	.04	20
700	.80	.05	17
800	.73	.04	36
900	.65	.04	37
1000	.89	.03	38
1200	.99	.01	19
1500	.90	.03	18

Table A.7.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between Groups	3.309	13	.255	8.30	.000
Within groups	9.816	320	.03067		
Total	13.125	333			

Table A.7.1.3 - Dunnett's test post-hoc analysis

Concentration of verapamil (μM)	*Significance (p<.05)*
.01	1.000
.1	.999
1	1.000
10	1.000
100	.998
300	.915
500	1.000
700	.085
800	.000
900	.000
1000	.918
1200	.957
1500	1.000

A.7.2 – Time of administration study for verapamil

Table A.7.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	saline	.92	.04	20
	verapamil	.77	.05	19
-5	saline	.96	.02	18
	verapamil	.64	.07	14
+0	saline	.96	.02	18
	verapamil	.64	.05	19
5	saline	.94	.03	19
	verapamil	.75	.06	16
10	saline	.94	.03	18
	verapamil	.71	.03	20
20	saline	.93	.04	19
	verapamil	.84	.03	20

Table A.7.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	6.01	208	.03		
Drug effect	2.58	1	2.58	89.33	.000
Time of test effect	.21	5	.04	1.43	.215
Interaction effect	.40	5	.08	2.80	.018

Table A.7 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.20	1	.20	6.96	.009
-5	.80	1	.80	27.35	.000
+0	.94	1	.94	32.29	.000
5	.33	1	33	11.33	.001
10	.54	1	.54	18.50	.000
20	.07	1	.07	2.52	.114

A.7.3 – Retention study for verapamil

Table A.7.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	saline	.91	.04	16
	verapamil	.86	.05	19
30	saline	.89	.04	20
	verapamil	.89	.04	19
40	saline	.95	.03	16
	verapamil	.86	.05	20
70	saline	.96	.04	17
	verapamil	.92	.03	18
90	saline	.92	.03	18
	verapamil	.70	.05	20
120	saline	.92	.04	15
	verapamil	.65	.04	38
180	saline	.95	.02	18
	verapamil	.86	.04	34

Table A.7.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	9.37	274	.03		
Drug effect	.85	1	.85	24.80	.000
Time of test effect	.74	6	.12	3.61	.002
Interaction effect	.61	6	.10	2.96	.008

Table A.7.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.02	1	.02	.68	.411
30	.00	1	.00	.00	.952
40	.07	1	.07	1.94	.165
70	.01	1	.01	.38	.539
90	.50	1	.50	13.81	.000
120	1.58	1	1.58	43.81	.000
180	.08	1	.08	2.18	.141

A.8 - Iberiotoxin

A.8.1 - Dose response study for iberiotoxin

Table A.8.1.1 - Summary data

Concentration of verapamil (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
saline	.90	.03	19
1	.96	.02	19
10	.80	.05	17
50	.67	.05	17
100	.76	.06	19
250	.77	.05	20
500	.97	.02	16

Table A.8.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between Groups	1.351	6	.225	5.996	.000
Within groups	4.507	120	.038		
Total	5.859	126			

Table A.8.1.3 - Dunnett's test post-hoc analysis

Concentration of verapamil (μM)	*Significance (p<.05)*
1	.857
10	.432
50	.002
100	.141
250	.168
500	.798

A.8.2 - Time of administration study for iberiotoxin

Table A.8.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	saline	.92	.04	20
	iberiotoxin	.71	.05	20
-5	saline	.96	.02	18
	iberiotoxin	.67	.05	19
+0	saline	.96	.02	18
	iberiotoxin	.62	.05	19
5	saline	.94	.03	19
	iberiotoxin	.58	.06	19
10	saline	.94	.03	18
	iberiotoxin	.54	.05	18
20	saline	.93	.04	19
	iberiotoxin	.66	.04	38
25	saline	.91	.05	19
	iberiotoxin	.85	.03	38
30	saline	.90	.03	14
	iberiotoxin	.95	.02	29

Table A.8.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	11.42	319	.04		
Drug effect	4.36	1	4.36	121.95	.000
Time of test effect	.96	7	.14	3.84	.001
Interaction effect	1.60	7	.23	6.39	.000

Table A.8.2.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.41	1	.41	10.69	.001
-5	.79	1	.79	20.72	.000
+0	1.08	1	1.08	28.48	.000
5	1.26	1	1.26	33.18	.000
10	1.45	1	1.45	38.13	.000
20	1.15	1	1.15	30.36	.000
25	.00	1	.00	.13	.724
30	.06	1	.06	1.45	.229

A.8.3 - Retention study for iberiotoxin

Table A.8.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	saline	.91	.04	16
	iberiotoxin	.95	.04	15
30	saline	.89	.04	20
	iberiotoxin	.96	.02	16
40	saline	.95	.03	16
	iberiotoxin	.71	.05	20
70	saline	.96	.04	17
	iberiotoxin	.83	.06	19
90	saline	.92	.03	18
	iberiotoxin	.95	.02	18
120	saline	.92	.04	15
	iberiotoxin	.91	.03	16
180	saline	.95	.02	18
	iberiotoxin	.93	.03	18

Table A.8.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	5.41	228	.02		
Drug effect	.09	1	.09	3.65	.057
Time of test effect	.32	6	.05	2.26	.039
Interaction effect	.64	6	.11	4.49	.000

Table A.8.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.01	1	.01	.37	.544
30	.04	1	.04	1.66	.199
40	.60	1	.60	24.59	.000
70	.16	1	.16	6.37	.012
90	.01	1	.01	.36	.552
120	.00	1	.00	.04	.845
180	.00	1	.00	.15	.702

A.9 - Dantrolene

A.9.1 – Dose response study for dantrolene

Table A.9.1.1 - Summary data

Concentration of dantrolene (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
vehicle	.94	.03	19
.001	.88	.04	19
.01	.61	.06	19
.1	.66	.06	18
1	.76	.04	20
100	.91	.03	19
400	.92	.05	19
700	.90	.05	19
1000	.90	.04	20
5000	.77	.05	20

Table A.9.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between Groups	2.460	9	.273	.685	.000
Within groups	7.306	183	.040		
Total	9.766	192			

Table A.9.1.3 - Dunnett's test post-hoc analysis

Concentration of dantrolene (μM)	*Significance (p<.05)*
.001	.860
.01	.000
.1	.000
1	.019
100	.994
400	.998
700	.962
1000	.964
5000	.029

A.9.2 – Time of administration study for 0.01μM dantrolene

Table A.9.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	vehicle	.92	.04	20
	dantrolene	.93	.04	18
+0	vehicle	.96	.02	18
	dantrolene	.71	.03	20
5	vehicle	.94	.03	19
	dantrolene	.94	.02	18
10	vehicle	.94	.03	18
	dantrolene	.87	.05	20
20	vehicle	.93	.04	19
	dantrolene	.92	.03	20
25	vehicle	.90	.05	14
	dantrolene	.78	.05	19

Table A.9.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	5.33	211	.03		
Drug effect	.30	1	.30	11.76	.001
Time of test effect	.39	5	.08	3.09	.010
Interaction effect	.46	5	.09	3.64	.004

Table A.9.2.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.00	1	.00	.05	.832
+0	.62	1	.62	23.41	.000
5	.00	1	.00	.03	.853
10	.04	1	.04	1.66	.199
20	.00	1	.00	.03	.870
25	.15	1	.15	5.74	.017

A.9.3 – Retention study for 0.01μM dantrolene

Table A.9.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	vehicle	.91	.04	16
	dantrolene	.93	.03	19
30	vehicle	.89	.04	20
	dantrolene	.94	.02	18
40	vehicle	.95	.03	16
	dantrolene	.64	.05	20
70	vehicle	.96	.04	17
	dantrolene	.80	.05	18
90	vehicle	.92	.03	18
	dantrolene	.93	.02	20
120	vehicle	.92	.04	15
	dantrolene	.89	.05	18
180	vehicle	.95	.02	18
	dantrolene	.98	.01	16

Table A.9.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	5.42	235	.02		
Drug effect	.20	1	.20	8.63	.004
Time of test effect	.61	6	.10	4.40	.000
Interaction effect	.95	6	.16	6.87	.000

Table A.9.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.00	1	.00	.17	.680
30	.03	1	.03	1.14	.286
40	1.02	1	1.02	40.79	.000
70	.23	1	.23	9.22	.003
90	.00	1	.00	.06	.808
120	.01	1	.01	.26	.611
180	.00	1	.00	.07	.793

A.9.4 – Time of administration study for 5mM dantrolene

Table A.9.4.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	vehicle	.92	.04	20
	dantrolene	.53	.05	20
+0	vehicle	.96	.02	18
	dantrolene	.59	.07	19
5	vehicle	.94	.03	19
	dantrolene	.72	.06	18
10	vehicle	.94	.03	18
	dantrolene	.62	.05	19
20	vehicle	.93	.04	19
	dantrolene	.59	.06	19
25	vehicle	.90	.05	14
	dantrolene	.97	.02	20

Table A.9.4.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	7.99	211	.04		
Drug effect	3.77	1	3.77	99.56	.000
Time of test effect	.95	5	.19	5.01	.000
Interaction effect	1.32	5	.26	6.96	.000

Table A.9.4.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	1.46	1	1.46	35.17	.000
+0	1.26	1	1.26	30.35	.001
5	.47	1	.47	11.37	.000
10	.99	1	.99	23.80	.000
20	1.15	1	1.15	27.70	.000
25	.14	1	.14	3.29	.071

A.9.5 – Retention study for 5mM dantrolene

Table A.9.5.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	vehicle	.91	.04	16
	dantrolene	.89	.05	18
30	vehicle	.89	.04	20
	dantrolene	.93	.04	19
40	vehicle	.95	.03	16
	dantrolene	.58	.04	18
70	vehicle	.96	.04	17
	dantrolene	.59	.06	17
90	vehicle	.92	.03	18
	dantrolene	.59	.05	20
120	vehicle	.92	.04	15
	dantrolene	.66	.06	20
180	vehicle	.95	.02	18
	dantrolene	.58	.06	19

Table A.9.5.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	8.66	236	.04		
Drug effect	3.66	1	3.66	99.77	.000
Time of test effect	.93	6	.16	4.24	.000
Interaction effect	1.67	6	.28	7.59	.000

Table A.9.5.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.00	1	.00	.07	.798
30	.01	1	.01	.21	.645
40	1.20	1	1.20	30.35	.000
70	1.19	1	1.19	30.11	.000
90	1.07	1	1.07	27.07	.000
120	.62	1	.62	15.62	.000
180	1.31	1	1.31	33.10	.000

A.10 - Trolox

A.10.1 – Dose response study for Trolox

Table A.10.1.1 - Summary data

Concentration of Trolox (μM)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
vehicle	.93	.02	38
.1	.88	.06	18
1	.82	.03	34
10	.86	.04	18
100	.82	.06	18
200	.63	.05	19
300	.64	.04	37
400	.76	.04	19
500	.89	.03	34
600	.85	.05	18
700	.84	.05	19
750	.76	.05	32
800	.74	.05	19
900	.81	.05	19
1000	.85	.05	19
1200	.97	.02	16

Table A.10.1.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between groups	3.226	15	.215	5.034	.001
Within groups	15.253	357	.043		
Total	18.479	372			

Table A.10.1.3 - Dunnett's test post-hoc analysis

Concentration of Trolox (μM)	*Significance (p<.05)*
.1	.997
1	.263
10	.930
100	.485
200	.001
300	.001
400	.034
500	.996
600	.878
700	.807
750	.008
800	.017
900	.350
1000	.819
1200	>.999

A.10.2 – Time of administration study for 300μM Trolox

Table A.10.2.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10	vehicle	.92	.04	20
	Trolox	.68	.06	19
-5	vehicle	.95	.04	14
	Trolox	.61	.06	18
+0	vehicle	.96	.02	18
	Trolox	.64	.06	19
2.5	vehicle	.98	.02	19
	Trolox	.69	.06	17
5	vehicle	.94	.03	19
	Trolox	.67	.05	18
10	vehicle	.94	.03	18
	Trolox	.59	.05	20
20	vehicle	.93	.04	19
	Trolox	.77	.06	19
25	vehicle	.90	.05	14
	Trolox	.86	.05	18

Table A.10.2.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	11.61	273	.04	105.39	.000
Drug effect	4.48	1	4.48	1.20	.304
Time of test effect	.36	7	.05	2.29	.028
Interaction effect	.68	7	.10		

Table A.10.2.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10	.53	1	.53	12.29	.001
-5	.97	1	.97	22.78	.001
+0	.98	1	.98	23.03	.001
2.5	.78	1	.78	18.20	.001
5	.67	1	.67	15.73	.001
10	1.20	1	1.20	28.04	.001
20	.25	1	.25	5.81	.017
25	.00	1	.00	.04	.834

A.10.3 – Retention study for 300μM Trolox

Table A.10.3.1 - Summary data

Time of test (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	vehicle	.91	.04	16
	Trolox	.93	.03	19
30	vehicle	.89	.04	20
	Trolox	.94	.03	19
40	vehicle	.95	.03	16
	Trolox	.71	.06	18
70	vehicle	.96	.04	17
	Trolox	.69	.06	20
90	vehicle	.92	.03	18
	Trolox	.67	.07	16
120	vehicle	.92	.04	15
	Trolox	.71	.06	17
180	vehicle	.95	.02	18
	Trolox	.72	.07	17
1440	vehicle	.96	.03	17
	Trolox	.76	.05	31

Table A.10.3.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	11.97	277	.04		
Drug effect	1.95	1	1.95	45.02	.000
Time of test effect	.51	7	.07	1.68	.113
Interaction effect	1.06	7	.15	3.51	.001

Table A.10.3.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.01	1	.01	.30	.582
30	.03	1	.03	.65	.423
40	.51	1	.51	11.60	.001
70	.71	1	.71	16.27	.000
90	.50	1	.50	11.34	.001
120	.37	1	.37	8.38	.004
180	.46	1	.46	10.50	.001
1440	.43	1	.43	9.89	.002

A.10.4 – Time of administration study for 800µM Trolox

Table A.10.4.1 - Summary data

Time of administration (minutes post-training)	*drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
-10.0	vehicle	.92	.04	20
	Trolox	.58	.05	18
-5.0	vehicle	.95	.04	14
	Trolox	.67	.06	17
+0.0	vehicle	.96	.02	18
	Trolox	.64	.06	15
2.5	vehicle	.98	.02	19
	Trolox	.69	.07	19
5.0	vehicle	.94	.03	19
	Trolox	.69	.05	18
10.0	vehicle	.94	.03	18
	Trolox	.58	.06	19
20.0	vehicle	.93	.04	19
	Trolox	.53	.06	19
25.0	vehicle	.89	.05	14
	Trolox	.90	.04	18

Table A.10.4.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	11.18	268	.04		
Drug effect	5.50	1	5.5	131.98	.000
Time of test effect	.69	7	.10	2.35	.024
Interaction effect	.88	7	.13	3.01	.005

Table A.10.4.3 - Simple main effects post-hoc analysis

Time of administration (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
-10.0	1.04	1	1.04	24.16	.000
-5.0	.57	1	.57	13.19	.000
+0.0	.83	1	.83	19.32	.000
2.5	.81	1	.81	18.85	.000
5.0	.61	1	.61	14.09	.000
10.0	1.24	1	1.24	28.77	.000
20.0	1.54	1	1.54	35.69	.000
25.0	.01	1	.01	.13	.721

A.7.5 – Retention study for 800µM Trolox

Table A.10.5.1 - Summary data

Time of test (minutes post-training)	*Drugs*	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
10	vehicle	.91	.04	16
	Trolox	.93	.04	16
30	vehicle	.89	.04	20
	Trolox	.89	.04	16
40	vehicle	.95	.03	16
	Trolox	.69	.06	18
70	vehicle	.96	.04	17
	Trolox	.73	.07	17
90	vehicle	.92	.03	18
	Trolox	.75	.07	17
120	vehicle	.92	.04	15
	Trolox	.64	.07	19
180	vehicle	.95	.02	18
	Trolox	.57	.06	18
1440	vehicle	.96	.03	17
	Trolox	.77	.05	23

Table A.10.5.2 - Two-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Within cells	10.85	264	.04		
Drug effect	2.45	1	2.45	59.57	.000
Time of test effect	.67	7	.10	2.34	.025
Interaction effect	1.18	7	.17	4.11	.000

Table A.10.5.3 - Simple main effects post-hoc analysis

Time of test (minutes post-training)	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
10	.01	1	.01	.16	.688
30	.00	1	.00	.01	.943
40	.59	1	.59	13.79	.000
70	.47	1	.47	11.01	.001
90	.27	1	.27	6.34	.012
120	.75	1	.75	17.63	.000
180	1.35	1	1.35	31.65	.000
1440	.35	1	.35	8.19	.005

Appendix B – Differences in method between two variants of the passive avoidance tasks for day-old chicks

Difference	*Method used by Salinska et al. (2001) and others*	*Method used in the present research (Ng & Gibbs, 1991)*
Strain of chick used	Day-old Ross 1 Chunky chicks	Day-old white Leghorn x black Australorp chicks
Sex of chick used	Male and female	Male only
Laboratory illumination	Red light	Full spectrum light
Pre-training procedure	2mm diameter white bead presented 3 times	Chrome bead presented twice followed by presentation of a red and then blue bead
Training procedure	5mm diameter chrome bead used	Presentation of an identical red bead to that used in pre-training
Drug administration procedure	Use of head-holder	Performed freehand
Measurement of retention	Percentage of chicks avoiding the aversive bead	Mean discrimination ratio as a comparison of pecking between an aversive and non aversive bead. Exclusion of chicks which did not peck the nonaversive bead prior to analysis.

Appendix C – Conformation of retention loss associated with the time of training and not the time of administration for H-8 and ODQ

Retention function for H-8 tested relative to the time of administration

The occurrence of a temporary retention loss after the formation of the LTM stage (Gibbs & Ng, 1979a) has not previously been observed. Further, such results are inconsistent with those of Serrano et al. (1994). To establish that H-8 affects memory processes directly it was necessary to establish that its effects upon retention were relative to the time of test and not the time of administration. High retention levels at test 120 minutes post-training, for a time of administration 10 minutes post-training, are in contrast to the loss of retention observed 110 minutes post-training, when administered immediately post-training (see Chapter 3), suggest the action of H-8 is relative to the time of training this had yet to be clearly established.

Method

Chicks were housed and trained as stated in Chapter 2. Groups of 20 chicks were administered either 154mM saline or 200μM H-8 24 hours post-training and tested at 90, 100, 120, 130 and 150 minutes post-administration and retention levels observed. The final number of chicks representing a single data-point is less than 20 as chicks may refuse to train or peck at the non-aversive bead during testing. In either case such chicks are excluded from analysis. The range of chicks representing data-points in this study extends from 10 to 16 with a mean of 13.

The time of administration was chosen to be sufficiently remote from the time of training to allow a clear temporal association between either the time of training or administration and the time of retention loss. Further, administration 24 hours post-

training takes into account the circadian rhythms of the chick (Aschoff, von Saint Paul, 1976) and so is particularly appropriate. In comparison, the post-administration times of test were chosen based upon the times of retention loss discussed in section 3.3.1.3.

Results

Retention levels for chicks administered either 200μM H-8 or saline 24 hours post-training and tested at various times following administration is described in Figure C.1.

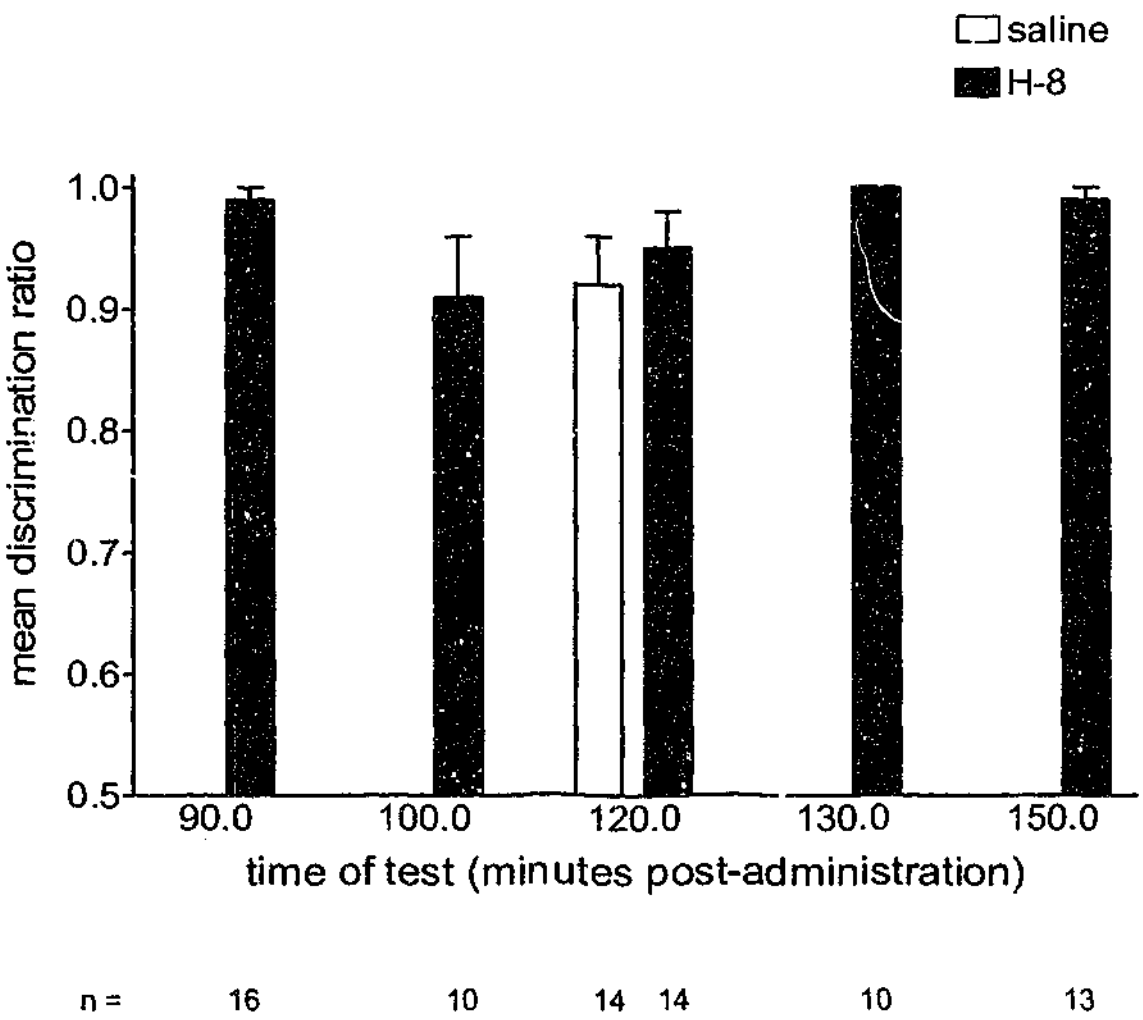

Figure C.1 Retention function for 200μM H-8, compared with saline, administered 24 hours post-training and tested at various times post-administration.
Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks.

Retention tests at 90, 100, 120, 130 and 150 minutes post-administration revealed no loss of retention when compared to the administration of saline. A one-way

ANOVA confirmed this by revealing a non-significant effect ($F(5,71)=1.344$, $p>.05$), see Table C.1 and C.2.

Table C.1 - Summary data

Time of test (represented in minutes post-24 hours after training)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
Vehicle 120	.92	.04	16
90	.99	.01	10
100	.91	.05	14
120	.95	.03	14
130	1.00	.00	10
150	.99	.01	13

Table C.2 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between groups	.099	5	.019	1.344	.256
Within groups	1.054	71	.0148		
Total	1.154	76			

Discussion

The present study found that retention loss did *not* result by 100 minutes following administration 24 hours post-training. In conjunction with the retention study

for H-8, described in section 3.3.1.3, the present retention study suggested the onset of retention loss is linked to the time of learning and thus the action of H-8 is specific to memory processing. This interpretation is further reinforced as the discrimination measure did not exclude large numbers of chicks in either retention studies using H-8 inferring an absence of sensory-motor effects.

Retention function for ODQ relative to the time of drug administration

To more clearly establish the effect of ODQ upon memory processes it was necessary to determine that the retention losses were temporally linked to the training experience and not to the time of dug administration. If ODQ affected memory processes directly then retention should not be impaired if tested at specific times following a delayed time of drug administration. Comparison between the ineffective time of administration at 10 minutes post-training, when tested 120 minutes post-training, and the effective loss of retention at 110 minutes post-training following ODQ administration immediately post-training suggests that the drug effects memory processes directly. However this had yet to be shown conclusively.

Method

Chicks were housed and trained as described in Chapter 2. Groups of 20 chicks were administered either 100μM ODQ or the vehicle 24 hours after training. The time of administration was chosen to be sufficiently different from the time of training so as to detect retention loss relative to either time. Further, as chicks possess circadian rhythms administration 24 hours post-training excluded time-of day effects as a reason for retention loss.

Due to the exclusion of chicks which did not train or peck the non-aversive bead during testing from the analyses the range of chick numbers which represented the data-points was from 9 to 16 with a mean of 13. Testing of ODQ administered groups occurred either 40, 50, 90, 100 and 120 minutes post-administration which, based upon the retention function discussed in section 3.4.1.3, would have been expected to show a

retention loss if the onset of retention loss was specific to the time of administration. A single group administered the vehicle was tested 40 minutes post-administration.

Results

Figure C.2 describes retention levels for chicks administered 100µM ODQ 24 hours post-training and tested 40, 50, 90, 100 and 120 minutes post-administration. These were compared to a single group of chicks administered the vehicle 24 hours post-training and tested 40 minutes post-administration.

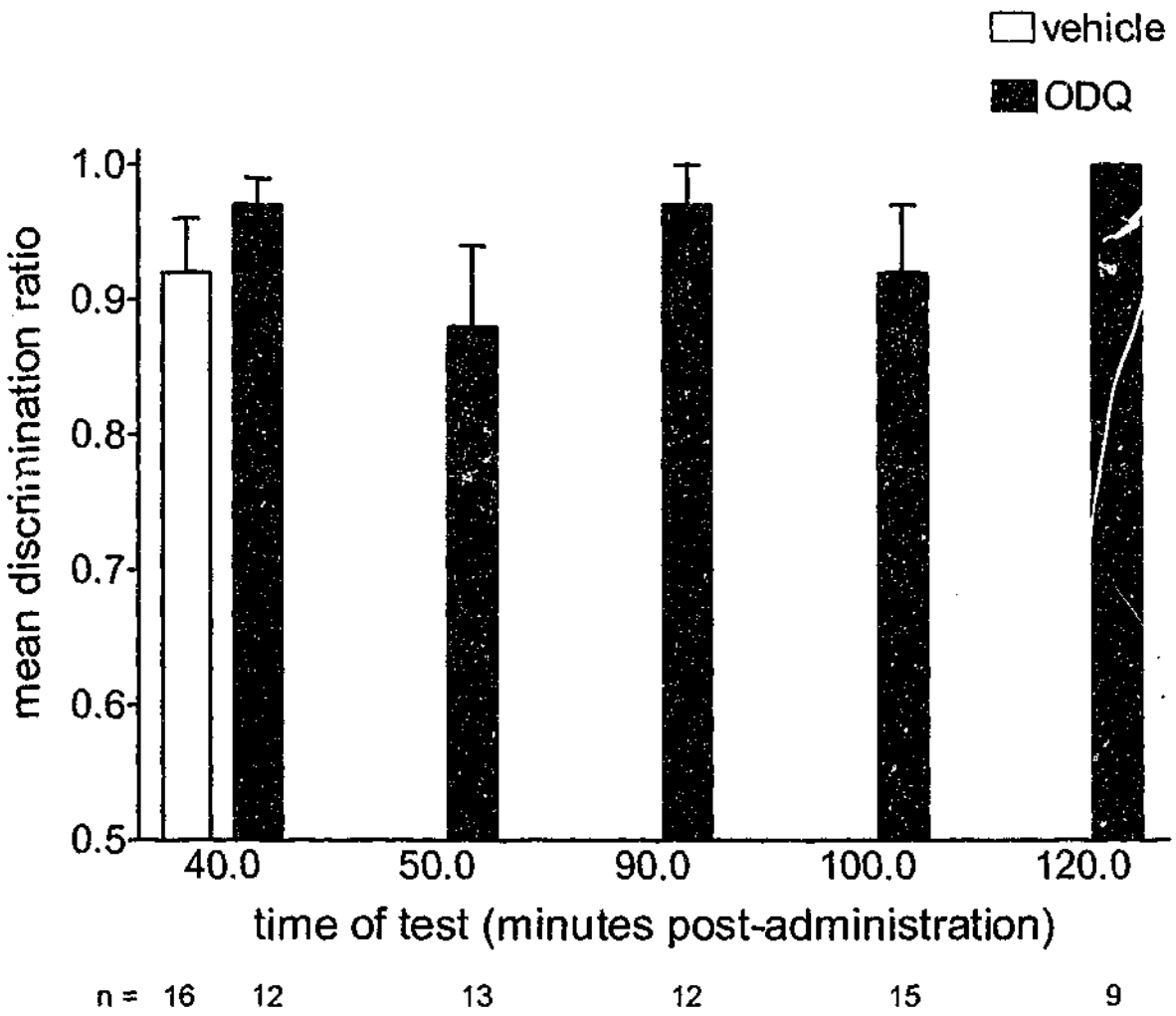

Figure C.2 Retention function for 100µM ODQ, compared with vehicle saline (<1% DMSO), administered 24 hours post-training and tested at various times post-administration.
Each point represents the mean discrimination ratio (+/- SEM) for a separate group of chicks.

Figure C.2 describes no difference in retention levels between the chicks administered the vehicle and those administered 100µM ODQ. A one-way ANOVA

was performed and revealed no significant difference in retention levels between those groups administered ODQ 24 hours post-training and the group administered the vehicle ($F(5,71)=1.062$, $p>.05$), see Table C.3 and C.4.

Table C.3 - Summary data

Time of test (represented in minutes post-24 hours after training)	*Mean discrimination ratio*	*Standard error of the mean*	*Sample size*
Vehicle 40	.92	.04	16
40	.97	.02	12
50	.88	.06	13
90	.97	.	12
100	.92	.05	15
120	1.0	.00	9

Table C.4 - One-way ANOVA

	Sum of squares	*Degrees of freedom*	*Mean squares*	*F value*	*Significance of F value*
Between groups	.111	5	.022	1.062	.389
Within groups	1.490	71	.0218		
Total	1.601	76			

Discussion

The initial retention study using 100μM ODQ administered immediately post-training suggested a loss of retention centered at 40 minutes and 120 minutes post-training respectively. However, as administration of the drug occurred immediately post-training it could not be established if the effect of the drug was related to the training experience or the time of drug injection. If ODQ had an effect upon non-memory functions then retention would be expected to be impaired when tested at 40 minutes and 120 minutes post-administration since the temporary retention losses observed in the initial retention study using ODQ would shift with respect to the time of administration. In the present study ODQ was administered 24 hours after training and at least 22 hours after the consolidation of long-term memory (Ng & Gibbs, 1988). As no loss of retention was observed for any groups tested at various times post-administration, this suggests that the effect of ODQ is related specifically to the training experience.

Appendix D – Papers and Abstracts Published in Support of this Thesis

Peer reviewed papers:

Edwards, T.M. & Rickard, N.S. (2002). Inhibition of *mono*ADP-ribosylation prevents long-term memory consolidation of a single-trial passive avoidance task in the day-old chick. *Neurobiology of Learning and Memory, 78,* 192-198. doi: 10.1006/nlme.2001.4043.

Edwards, T.M., Rickard, N.S. & Ng, K.T. (2002). Inhibition of guanylate cyclase and protein kinase G impairs retention for the passive avoidance task in the day-old chick. *Neurobiology of Learning and Memory, 77,* 313-326. doi: 10.1006/nlme.2001.4021.

Papers in preparation:

Edwards, T.M. & Rickard, N.S. The role of cation channels in passive avoidance memory formation. (research paper)

Edwards, T.M. & Rickard, N.S. Peroxynitrite is necessary for the consolidation of long-term memory formation in the day-old chick. (brief report)

Edwards, T.M. & Rickard, N.S. The role of nitric oxide in memory formation: guanylyl cyclase and beyond. (review)

Articles appearing in non-reviewed university publications:

Edwards, T.M. (2002). Biochemistry of memory – is NO the answer? In: J. Nieman (Ed.), *Compass* (pp. 17-18). Melbourne, Monash University Post-graduate Association.

Conference abstracts supporting this thesis:

Edwards, T.M., Ng, K.T., & Rickard, N.S. (1998). Guanylyl cyclase inhibition prevents maintenance of long-term memory in the day-old chick. *Proceedings of the Australian Neuroscience Society, 9*, 196.

Edwards, T.M. & Rickard, N.S. (2002). Inhibition of the ryanodine receptor-related calcium channel prevents the formation for long-term memory in the day-old chick. *Proceedings of the Australian Neuroscience Society, 13*, 235.

Edwards, T.M., Rickard, N.S., & Ng, K.T. (1998). Two distinct roles for nitric oxide in memory formation in the day-old chick. *Nitric Oxide (Archives of Biochemistry and Biophysics – Part B), 2(2)*, 80.

Edwards, T.M., Rickard, N.S., & Ng, K.T. (1999). mono(ADP-ribosyl) transferase inhibition prevents maintenance of long-term memory formation in the day-old chick. *Proceedings of the Australian Neuroscience Society, 10*, 202.

Edwards, T.M., Rickard, N.S., & Ng, K.T. (2000). The complex role of nitric oxide in passive avoidance memory formation in the day-old chick. *Nitric Oxide (Archives of Biochemistry and Biophysics – Part B), 4(3)*, 265.

Conference abstracts related to this thesis

Edwards, T.M , Jacka, K., Moutsoulas, P., Rickard, N.S., & Ng, K.T. (2000). Measurement of activated nitric oxide synthase following a training experience for day-old chicks. *Proceedings of the Australian Neuroscience Society, 11*, 229.

Neurobiology of Learning and Memory 78, 192–198 (2002)
doi:10.1006/nlme.2001.4043

BRIEF REPORT

Inhibition of *mono*ADP-Ribosylation Prevents Long-Term Memory Consolidation of a Single-Trial Passive Avoidance Task in the Day-Old Chick

T. M. Edwards and N. S. Rickard

Department of Psychology, Monash University, 3800 Victoria, Australia

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The cytosolic posttranslational protein-modifying mechanism of *mono*ADP-ribosylation has been implicated in long-term potentiation, a synaptic model of memory formation. The current study investigated the effect of inhibiting *mono*(ADP-ribosyl) transferase on memory for the passive avoidance task in day-old chicks (white Leghorn–black Australorp). Various doses of novobiocin or menadione sodium bisulfite were administered intracranially at different times before or after training. Control chicks were administered saline at matched times. Novobiocin (650 μM) or menadione sodium bisulfite (250 μM) administered between 5.0 min pretraining and 2.5 min posttraining was found to cause a persistent loss of retention from 120 min posttraining. These data provide the first demonstration that *mono*ADP-ribosylation is required for the maintenance of long-term memory. Furthermore, the temporal characteristics of the memory loss caused by *mono*ADP-ribosylation inhibition appears to exclude this mechanism as a downstream effect of the well-established nitric oxide activity previously shown to occur within 40 min of passive avoidance training.

Key Words: ADP-ribosylation; memory formation; day-old chick; passive avoidance; menadione sodium bisulfite; novobiocin.

ADP-ribosylation is an important posttranslational mechanism of protein modification involving the transfer of ADP-ribose from NAD to specific proteins (Shall, 1995; Ueda & Hayaishi, 1985). ADP-ribosylation can be divided into two classes: *mono*ADP-ribosylation, which is cytosol specific, and *poly*ADP-ribosylation, which is nucleus specific. A

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Address correspondence and reprint requests to N. S. Rickard, Department of Psychology, Monash University, 900 Dandenong Road, Caulfield East 3145, Victoria, Australia. E-mail: n.rickard@sci.monash.edu.au.

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role for *mono*ADP-ribosylation has been demonstrated in a range of functions, including modification of cell shape, vasodilation, and synaptic plasticity. By contrast, *poly*ADP-ribosylation is necessary for DNA repair, cell differentiation, and RNA synthesis.

Long-term potentiation (LTP) is considered a synaptic model of memory formation, whereby the efficacy of the postsynaptic response is enhanced following tetanic stimulation. A role for *mono*ADP-ribosylation in hippocampal LTP has been demonstrated via pharmacological studies. Administration of three different inhibitors of ADP-ribosylation (nicotinamide, luminol, and vitamin K) blocked the induction of LTP (Schuman, Merffet, Schulman, & Madison, 1992). Interestingly, the inhibitor nicotinamide was found to act at the presynaptic terminal, which is also thought to be the site of action of nitric oxide (a potential mediator of ADP-ribosylation) in LTP. Finally, a specific inhibitor of the *poly* form of ADP-ribosylation (benzamide) failed to block LTP, suggesting that the *mono* form is involved in LTP (Schuman et al., 1992). A role for ADP-ribosylation in LTP induction has also been confirmed by Schuman, Merffet, Schulman, and Madison (1994) and by Kleppisch et al. (1999). It should be noted, however, that Duman, Terwilliger and Nestler (1993) observed an overall reduction in ADP-ribosylation in hippocampal tissue following LTP induction.

A role for ADP-ribosylation in memory processing has yet to be demonstrated. The current study uses a passive avoidance task for the day-old chick to investigate whether pharmacological inhibition of *mono*(ADP-ribosyl) transferase impairs memory formation. The importance of ADP-ribosylation in synaptic plasticity has been confirmed in chicks as well as in rodents. For instance, the ADP-ribosylation inhibitor, nicotinamide (1 mM), was found to overcome synaptic depression induced in an in vitro slice preparation of the chick intermediate medial hyperstriatum ventrale (Barcellos, Bradley, Burns, & Webb, 2000).

Day-old white Leghorn–black Australorp cockerels were trained in pairs on the single-trial passive avoidance task described in detail by Ng and Gibbs (1988). Chicks were presented with a red bead coated in the taste aversant, methylanthranilate (MeA), for 10 s. Menadione sodium bisulfite (MSB, Sigma, Castle Hill, Australia), novobiocin (NOVO, Sigma), or saline was administered intracranially in 10-μl volumes into each hemisphere freehand using a Hamilton repeating dispenser syringe. The neostriatal–hyperstriatal complex of the chick brain was targeted because of the importance of several regions in this area for passive avoidance memory formation (Rose & Csillag, 1985). Both inhibitors are potent and highly specific to *mono*(ADP-ribosyl) transferase in vitro (Banasik, Komura, Shimoyama, & Ueda, 1992). When the effect of both drugs are considered together, they provide convergent evidence for *mono*(ADP-ribosyl) transferase specificity since their non-specific effects are different. That is, MSB also acts by increasing superoxide concentrations (Georgellis, Tsirigotis, & Rydstrom, 1988) while NOVO also acts by inhibiting topoisomerases (Sekiguchi, Stivers, Mildvan, & Shuman, 1996). Chicks were tested by presentation of a dry red bead (previously aversive) and a dry blue bead (previously nonaversive), and the number of pecks at each bead was recorded. Retention for each chick was measured by a discrimination ratio (number of pecks at the blue bead relative to number of pecks at both beads) after the exclusion of chicks that did not train or did not peck the nonaversive blue bead during testing. A mean discrimination ratio was calculated for each data point represented by approximately 20 chicks. Injections and

testing occurred at various times relative to training as determined by the purpose of the experiment.

Dose–response studies. To determine whether ADP-ribosylation is required for memory formation for this task, various concentrations of the inhibitors, MSB (1–500 μM) and NOVO (0.1–1000 μM), were administered to different groups of chicks immediately after training. Control chicks were administered physiological (154 mM) saline immediately after training. Retention was tested 120 min later, a time at which long-term memory is well established.

MSB and NOVO each inhibited retention when tested at 120 min posttraining (see Fig. 1). A one-way analysis of variance (ANOVA) indicated a significant MSB dose effect, $F(8, 144) = 5.67, p < .001$, and Dunnett's post hoc tests revealed a significant difference between retention levels of chicks administered saline and those administered 200 μM MSB ($p < .05$) and 250 μM MSB ($p < .001$). A separate one-way ANOVA revealed

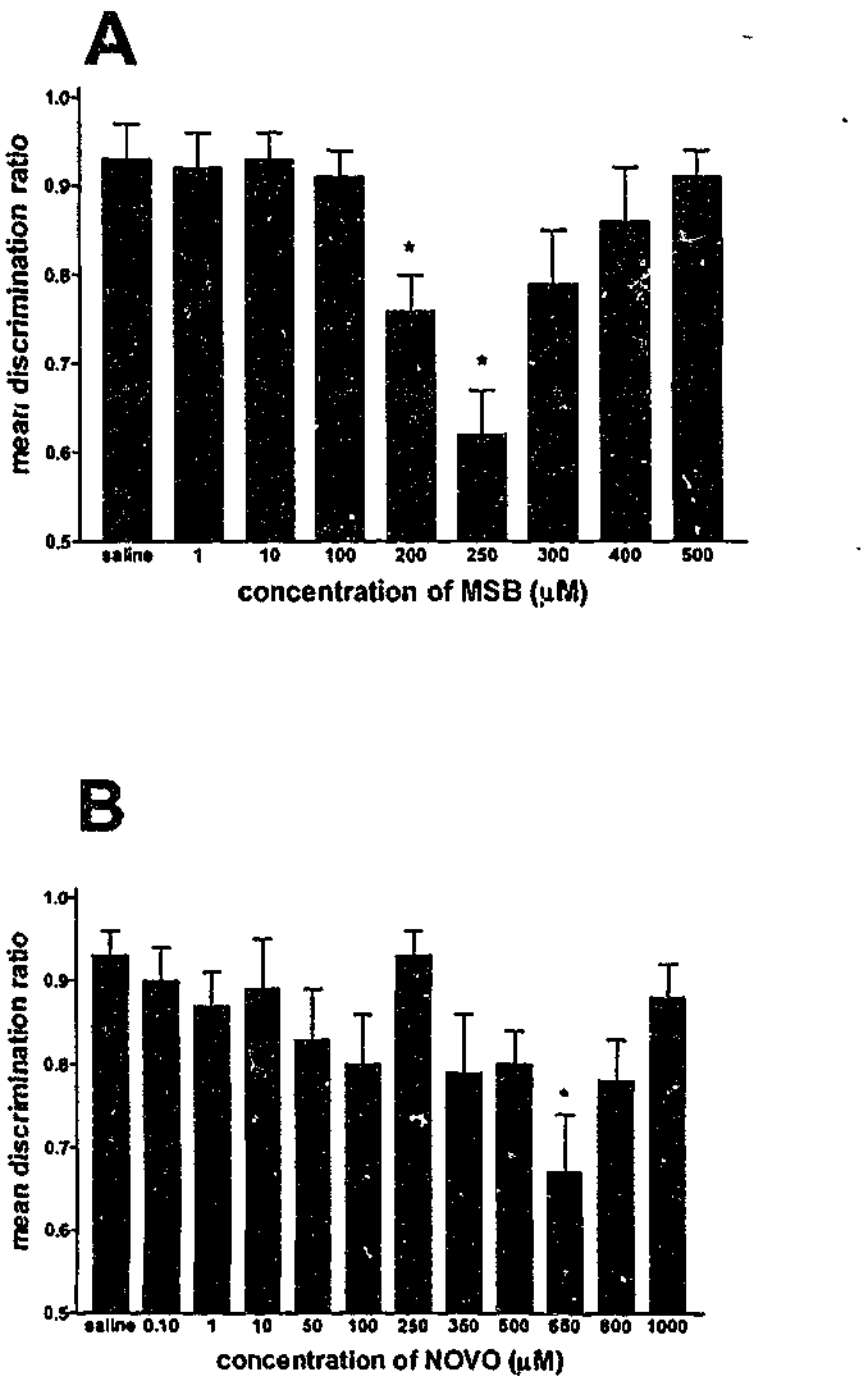

FIG. 1. The effect on retention of various doses of (A) MSB or (B) NOVO, administered immediately after training and tested 2 h posttraining. Each column represents the mean discrimination ratio (+/− SEM) for a separate group of chicks. $^*p < .05$.

that the NOVO dose effect was also significant, $F(11, 227) = 2.41$, $p < .01$. Post hoc Dunnett's tests revealed that only the 650 μM concentration was found to yield significantly different retention levels from saline ($p < .005$).

Time of administration studies. The effective times of administration for *mono*(ADP-ribosyl) transferase inhibitors were investigated by administering 250 μM MSB, 650 μM NOVO, or saline to different groups of chicks at various times between 10 min prior to training and 20 min posttraining. A separate two-way ANOVA (drug by time of administration) was performed for each ADP-ribosylation inhibitor. A significant drug effect (MSB: $F(1, 239) = 31.97$, $p < .001$; NOVO: $F(1, 234) = 48.81$, $p < .001$) and a significant interaction effect (MSB: $F(6, 239) = 2.88$, $p < .05$; NOVO: $F(6, 234) = 3.84$, $p < .005$) was observed for each inhibitor (see Fig. 2). Simple main effects analyses demonstrated a significant difference in retention levels between chicks administered MSB and saline at 5 min prior to training ($p < .01$), immediately posttraining ($p < .001$), and 2.5 min after training ($p < .001$). Similarly, NOVO administration yielded significantly lower

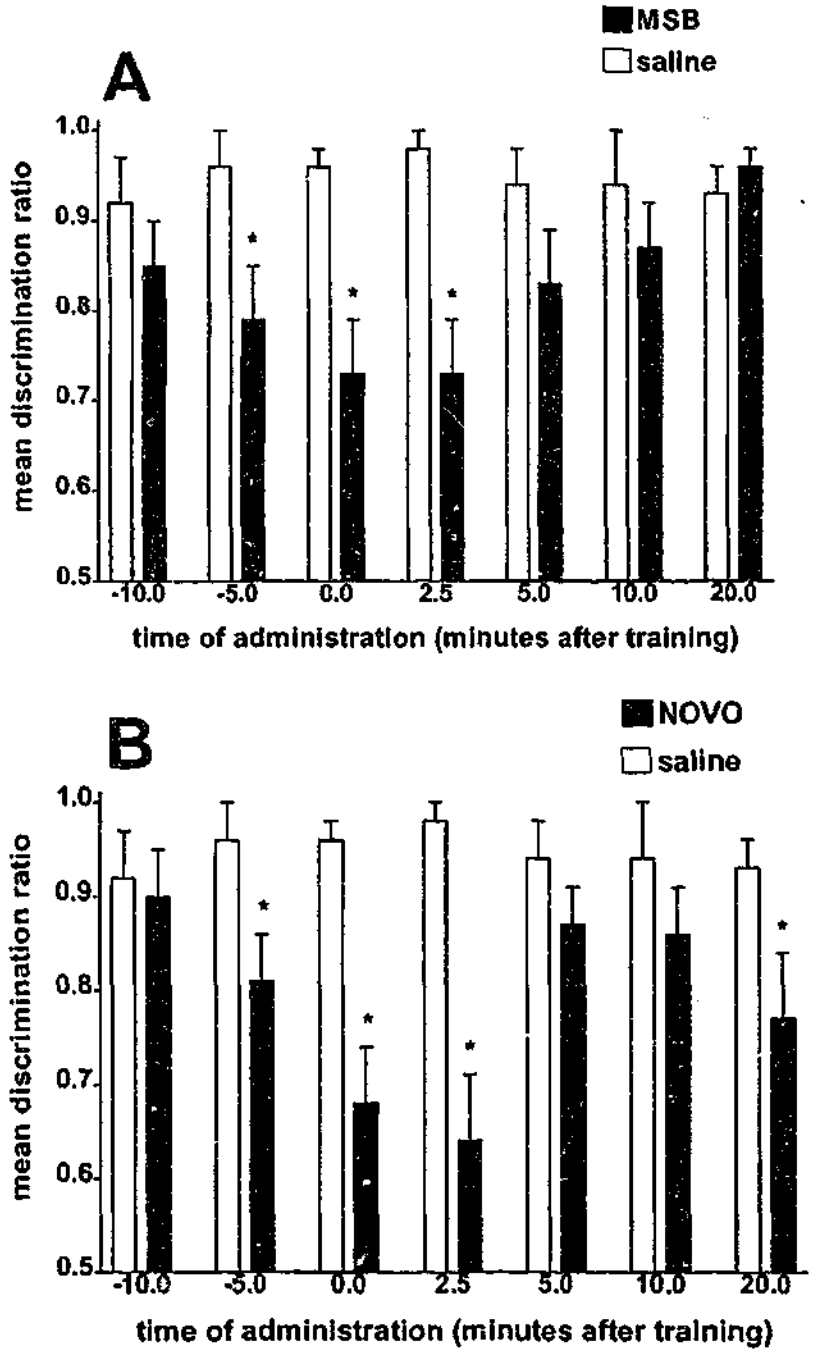

FIG. 2. The effect on retention of (A) 250 μM MSB or (B) 650 μM NOVO, compared to saline, administered at various times relative to training and tested at 2 h posttraining. Each column represents the mean discrimination ratio (+/− SEM) for a separate group of chicks. *$p < .05$.

retention levels than did saline at 5 min prior to training ($p < .05$), immediately after training ($p < .001$), and 2.5 min after training ($p < .001$). A significant difference between retention levels of chicks administered NOVO at 20 min posttraining and their matched controls ($p < .05$) that was not observed with MSB may reflect sampling error due to small sample sizes.

Retention functions. To determine the time of retention loss onset, 250 μM MSB, 650 μM NOVO, or saline was administered immediately posttraining in separate studies. Chicks were tested for retention at a range of times from 10 min posttraining to 24 h posttraining. For each drug, retention loss was first observed at 110 min posttraining and retention was significantly different from that of matched controls from 120 min onward (see Fig. 3). A two-way ANOVA was performed for each retention study. A significant drug effect, $F(1, 332) = 11.10$, $p < .001$, and interaction effect, $F(8, 332) = 3.02$, $p < .005$, was

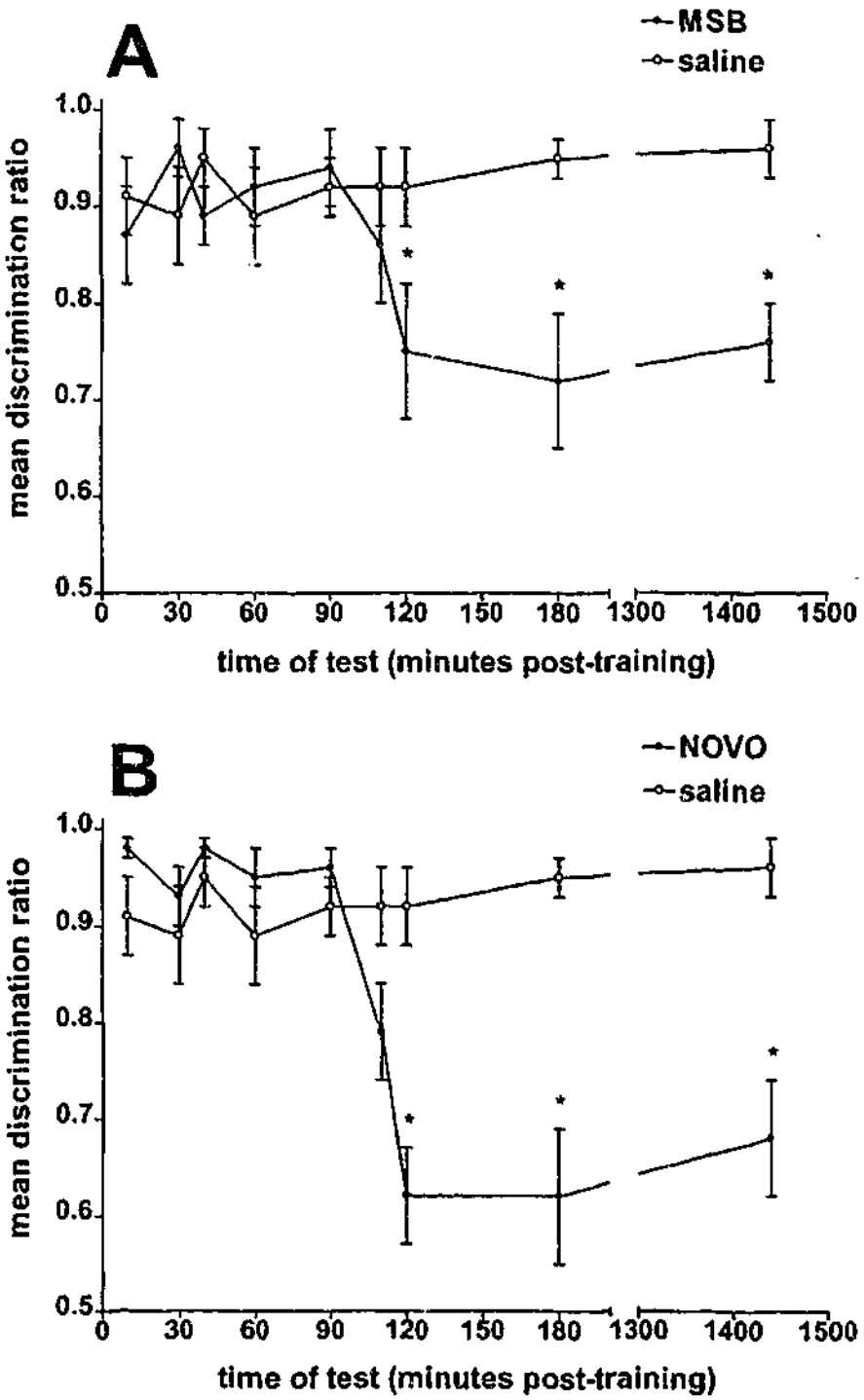

FIG. 3. The retention function of (A) 250 μM MSB or (B) 650 μM NOVO, compared to saline, administered immediately posttraining and tested at various times posttraining. Each point represents the mean discrimination ratio (+/− SEM) for a separate group of chicks. *$p < .05$.

observed for MSB. NOVO produced a significant drug effect, $F(1, 320) = 18.76$, $p < .001$; time of test effect, $F(8, 320) = 5.83$, $p < .001$; and interaction effect, $F(8, 320) = 8.10$, $p < .001$. Simple main effects post hoc analyses demonstrated a significant difference between retention for chicks administered either drug and the matched controls at 120 min (MSB, $p <. 05$; NOVO, $p < .001$), 180 min (MSB, $p < .001$; NOVO, $p < .001$), and 24 h posttraining (MSB, $p < .001$; NOVO, $p < .001$).

The current findings present the first demonstration that administration of *mono*(ADP-ribosyl) transferase inhibitors close to the time of learning causes long-term memory loss. Retention function studies revealed that memory loss following *mono*ADP-ribosylation inhibition was first significant at 2 h posttraining and that the memory loss persisted for at least 24 h. These findings indicate that *mono*ADP-ribosylation may be necessary for the maintenance of long-term memory. This onset time of amnesia does not coincide with any known stage of memory within the Gibbs–Ng model of memory formation and is some 60 min after the formation of the protein synthesis-dependent LTM stage (Ng & Gibbs, 1988).

The effective dose of each inhibitor was extremely specific, which is unusual within the passive avoidance paradigm and cannot be readily explained. If ADP-ribosylation is required for memory formation, then it would be expected that a wider range of doses would sufficiently inhibit the mechanism to impair memory processing. Nevertheless, the observed effects are unlikely to be explained by nonspecific pharmacological effects of the drugs since very similar effects were observed with two *mono*ADP-ribosylation inhibitors that act via different biochemical pathways. Nonspecific behavioral effects are also unlikely to account for the memory loss since no behavioral disturbances were observed in the chicks, and the measure of retention incorporates a control for nonmnemonic effects by excluding chicks who do not continue to peck normally at the previously nonaversive bead. Therefore, despite the irregularity of the dose–response functions, the effect of NOVO and MSB would seem to be specific to memory formation. By contrast, the "U-shaped" dose–response functions are quite common in this paradigm (Rickard, Poot, Gibbs, & Ng, 1994; Whitechurch, Ng, & Sedman, 1997) and others (see Calabrese & Baldwin, 2001) and may represent the triggering of feedback mechanisms to remove the inhibitor.

The target proteins of *mono*ADP-ribosylation in memory formation cannot be ascertained from the current study, although the temporal characteristics of its involvement are similar to those of several other LTP-related mechanisms that are necessary for the maintenance of LTM (Rickard et al., 1994; Whitechurch et al., 1997). Interestingly, a number of studies have suggested that nitric oxide may activate *mono*(ADP-ribosyl) transferase (Brune & Lapetina, 1989; Kleppisch et al., 1999). However, the temporal characteristics of memory loss induced by inhibition of *mono*ADP-ribosylation are inconsistent with those observed following NOS inhibition. Memory for this task has previously been shown as susceptible to NOS inhibition up to 20 min posttraining (Rickard, Ng, & Gibbs, 1998), and the onset of memory loss occurs around 40 min posttraining (Hölscher & Rose, 1992, 1993; Rickard et al., 1998; Rickard, Gibbs, & Ng, 1999). By contrast, *mono*ADP-ribosylation inhibitors are effective for a much narrower time window around the time of training, and the onset of memory loss occurs considerably later. Taken together with our previous findings, these results indicate that neither *mono*ADP-ribosylation nor cGMP production/PKG activity (Edwards & Rickard, 2002) follow nitric oxide activity 40 min after passive avoidance training.

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Inhibition of Guanylate Cyclase and Protein Kinase G Impairs Retention for the Passive Avoidance Task in the Day-Old Chick

T. M. Edwards, N. S. Rickard, and K. T. Ng

Department of Psychology, Monash University, 3800, Australia

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Nitric oxide (NO) is a highly labile chemical messenger which has previously been implicated in memory processes in a variety of learning paradigms and species. However, there is only limited evidence to suggest which enzymes are acted upon by NO during the formation of memory. The present study investigates the role of guanylate cyclase (GC) and protein kinase G (PKG) in a form of passive avoidance learning known to be dependent on nitric oxide activity. It was determined that *in vivo* pharmacological inhibition of GC using either 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one or 6-anilino-5,8-quinolinedione resulted in two transitory memory retention deficits centred around 40 and 120 min posttraining, respectively. In contrast, inhibition of PKG with *N*-[2-(methylamino)ehtyl]-5-isoquinoline-sulfornamide hydrochloride (H-8) resulted in a single temporary retention loss centered at 120 min posttraining. These temporary retention losses appear to be specific to memory since they were dose-dependent and could not be explained by nonspecific performance effects. Further, these results suggest that these agents inhibit memory retrieval rather than formation, since memory is subsequently available. The current findings indicate that guanylyl cyclase mediates two memory retrieval processes, the latter of which appears to be PKG-dependent. In contrast, since inhibition of NO results in a permanent retention loss, it is suggested that NO is required for memory formation through GC-independent processes.

Nitric oxide (NO) is a labile chemical messenger which has come to prominence due to its broad localization (Förstermann & Dun, 1996) and the wide range of cellular processes which it mediates including many within the central nervous system (Brenman & Bredt, 1996). Importantly, NO is thought to influence brain function through such processes as cerebrovasodilation (Iadecola, Zhang, & Xu, 1993; Raszkiewicz, Linville, Kerwin,

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Address reprint requests to Nikki Rickard, Psychology Department, Monash University, Caulfield East, VIC. 3145. Australia.

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Wagenaar, & Arneric, 1992), long-term potentiation (Böhme, Bon, Stutzmann, Doble, & Blanchard, 1991; O'Dell, Hawkins, Kandel, & Arancio, 1991), cerebellar long-term depression (Crepel & Jaillard, 1990), and neurotransmitter modulation (Rabin, 1996; Kano, Shimizu-Sasamata, Huang, Moskowitz, & Lo, 1998; Prast & Phillipu, 1992; Lonart, Wang, & Johnson, 1992), each of which is thought to impact upon memory formation.

Numerous behavioral studies using a wide variety of species and behavioral tasks have suggested that NO is a mediator of memory formation. For instance, a number of studies have shown that pharmacological inhibition of nitric oxide synthase (NOS) results in an impairment of spatial learning using such tasks as the Morris water maze (Chapman, Atkins, Aleen, Haley, & Steinmetz, 1992) or the radial arm maze (Suzuki, Ikari, Hayashi, & Iguchi, 1996; Zou, Yamada, Tanaka, Kameyama, & Nabeshima, 1998). However, several studies have also shown NO inhibition to have no effect on memory using the Morris water maze (Blokland et al., 1999), while Bannerman, Chapman, Kelly, Butcher, and Morris (1994) suggested any inhibition may be due to subtle sensorimotor effects resulting from the pharmacological inhibitors utilized. Furthermore, using the same task, Hölscher, Canevari, and Richter-Levin (1995) demonstrated that both NO and arachidonic acid must be inhibited to completely impair spatial learning.

Additional insight into the role of NO in memory processes has been possible via studies using the temporally well-defined passive avoidance task paradigm for the day-old chick (Hölscher & Rose, 1992, 1993; Rickard, Gibbs, & Ng, 1994a, 1999; Rickard, Ng, & Gibbs, 1998). This paradigm also provides a useful control for sensorimotor disturbances by using a discrimination index to measure retention. Loss of memory for the passive avoidance task in the day-old chick has been consistently observed by 40 min posttraining. This memory loss persisted until the completion of each experiment, providing strong evidence for a role for NO in memory formation.

Despite considerable evidence for NO involvement in at least some forms of memory processing, the function for which it is required is not yet known. However, the majority of NO-mediated physiological processes are thought to result from activation of guanylate cyclase (GC) and in turn cGMP activation of protein kinase G (PKG). This three-step linear pathway is also assumed to be a prominent pathway by which NO acts in memory processing (Bernabeu, Schmitz, Faillace, Izquierdo, & Medina, 1996; Bernabeu, Schroder, Quevedo, Cammarota, Izquierdo, & Medina, 1997; Izquierdo, Vianna, Barros, Mello e Souza, Ardenghi, Sant' Anna, Rodrigues, Medina, & Izquierdo, 2000). Evidence for both GC and PKG activation in memory formation has been shown in a number of studies. Kendrick, Guevara-Guzman, Zorilla, Hinton, Broad, Mimmack, and Okhura (1997) found that olfactory memory was impaired either by administration of a NOS inhibitor, L-nitroarginine (L-NArg) or by the administration of the specific GC inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) directly into the olfactory bulb. Using a step-down inhibitory avoidance task for rats, Bernabeu et al. (1996, 1997) found that infusion of the GC inhibitor LY 83583 into the rat hippocampus immediately after training impaired retention when tested at 24 h posttraining. Administration of the cGMP analogue 8-Br-cGMP enhanced retention when administered immediately after training (Bernabeu et al., 1996). However, neither the inhibitor nor the activator affected retention when administered at later times.

Biochemical assays also revealed that hippocampal cGMP levels were increased immediately and 30 min after training, but not at 3 h posttraining (Bernabeu et al., 1997). Using

the same task, Izquierdo et al. (2000) found that in addition to long-term memory loss (24 h posttraining), memory was also impaired by GC inhibition when tested during short-term memory (1.5 h posttraining). In contrast, administration of the PKG inhibitor 8*R*,9*S*11*S*-(-)-9-methoxycarbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7*b*,11*a*-trizadibenzo-(*a*,*g*)-cycloocta-(*c*,*d*,*e*)-trinden-1-one (KT5823) into the CA1 region of the hippocampus immediately after training caused memory loss when tested at only 24 h posttraining and not at 1.5 h posttraining. These findings suggest that GC is active during both short-term and long-term memory processes, whereas PKG is active only during long-term memory processes. It is worth noting, however, that biochemical assays revealed PKG activity levels to be significantly increased immediately after training, but not at 30 or 120 min after training (Bernabeu et al., 1997). Moreover, the behavioral findings from this paradigm must be treated with some caution since the task involves repeated testing of animals. The multiple memory formation traces that result can, therefore, confuse interpretation of the temporal parameters of memory loss. In this context, it is interesting that the role of PKG in memory formation has also been studied using a single-trial passive avoidance task for the day-old chick (Serrano, Beniston, Oxonian, Rodriguez, Rosenzweig, & Bennett, 1994). This paradigm involves a brief training trial, and since the task is simple and quick, different animals can be tested for each time point. Serrano et al. (1994) found that posttraining administration of the PKG inhibitor *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide hydrochloride (H-8) resulted in memory loss after 60 min posttraining. This time point coincides with the formation of the long-term memory stage in this model and is therefore consistent with the behavioral findings in the rat model (Izquierdo et al., 2000).

Given that both GC and PKG have been implicated in memory formation, it seems reasonable to infer that the role of NO in memory may be to stimulate these enzymes. Nonetheless, this pathway has yet to be clearly demonstrated in behavioral studies. In this case, it would be expected that inhibition of any of these three enzymes would result in memory loss around the same time. In the current study, we provide evidence for independence between the roles of NO, GC, and PKG in memory processing.

MATERIALS AND METHODS

Animals

Day-old white-Leghorn black-Australorp cockerels were obtained from a local hatchery on the morning of each experiment. Upon arrival chicks were randomly sorted into pairs to eliminate isolation stress and each pair was placed in a wooden holding pen (18 × 25 × 20 cm) for the duration of the experiment. One chick of each pair was marked upon its head with a permanent felt tip marker for the purposes of identification. The floor of each pen was scattered with crushed poultry food prior to occupation by the chicks. The chicks did not receive water additional to that which could be removed from the beads during pretraining. Each pen was illuminated and heated by a suspended 25-W globe with further heating provided by a wall-mounted fan heater. The laboratory temperature was maintained between 22 and 26°C. Each data point was represented by 10 pairs of chicks housed in two adjacent rows of five adjoining pens.

Task Procedure

The single-trial passive avoidance task has been described in detail elsewhere (Ng & Gibbs, 1991). Pretraining consisted of two presentations of a chrome bead (2 mm in diameter) and one presentation each of a red and a blue glass bead (5 mm in diameter). Each pretraining bead was coated in water to encourage pecking and was attached to a 34-cm wire that was held at the other end. Beads were presented for approximately 10 s each or until chicks pecked the beads. Training commenced approximately 1 h after the completion of pretraining. Chicks were presented for 10 s with an identical red bead to that used in pretraining but dipped in a taste aversant, methyl anthranilate (MeA). Once it was pecked, chicks typically displayed a disgust response denoted by head shaking and beak wiping. Discrimination retention tests were conducted at specific times posttraining depending on the purpose of the experiment. On these trials, the red and blue glass beads presented during pretraining were again successively presented to each pair of chicks for 10 s, except that the beads were dry.

The number of pecks at each bead, and the latency to first peck, during each trial was recorded for each chick using a hand-held recorder. Retention of each chick was measured using a discrimination ratio (DR) and then a mean DR was calculated for each group of chicks. Defined as the number of pecks to the blue bead during testing divided by the total number of pecks to both the blue and red beads during testing, the DR is a more sensitive measure of memory than discrete avoidance. A mean DR score of 1.0 indicates perfect discrimination between the previously aversive and nonaversive colored beads, while a mean DR of 0.5 indicates no discrimination (amnesia). Those chicks that did not peck the red bead during training or the nonaversive blue bead during testing were excluded from further analysis. Generally the total number of excluded birds was no greater than 15%.

Drugs and Injections

Drugs were prepared during the hour interval between pretraining and training. ODQ (Caymen) was selected as it is the most commonly used potent inhibitor of GC. LY 83583 (Cayman) was used to confirm any effects observed with ODQ since it inhibits GC via different mechanisms than does ODQ (Mülsch, Luckhoff, Pohl, Busse, & Bassenge, 1989). Unlike methylene blue which is thought to inhibit GC in a similar way as ODQ (Mayer, Brunner, & Schmidt, 1993; Schrammel, Behrends, Schmidt, Koesling, & Mayer, 1996), LY 83583 potentially provides convergent evidence for the effects of inhibiting GC that cannot be attributed to GC-nonspecific effects of the drugs.

ODQ and LY 83583 were first dissolved in dimethyl sulfoxide (DMSO) and then serially diluted to the desired concentration in 154 mM saline. The final concentration of DMSO was less than 1% (v/v). Further, in all experiments using ODQ or LY 83583 the control vehicle was 154 mM saline containing < 1% DMSO. The PKG inhibitor H-8 (ICN) was made up in 154 mM saline only and then serially diluted to the desired concentration. Each drug was administered freehand using a Hamilton repeating dispenser syringe that delivered 10-μl volumes into each hemisphere. The syringe was fitted with a 27-gauge needle inside a plastic sleeve that regulated the depth to 3.5 mm. The target of each injection was the neostriatal/hyperstriatal region located 3.5 mm below the lateral borders of the fontanelle, which can be felt through the chick's skin. This region of the

chick brain is a major area implicated in memory formation for the passive avoidance task (Rose & Csillag, 1985; Sedman, O'Dowd, Rickard, Gibbs, & Ng, 1992). The drug dose, time of administration, and time of retention test varied according to the design of each experiment (details included under Results).

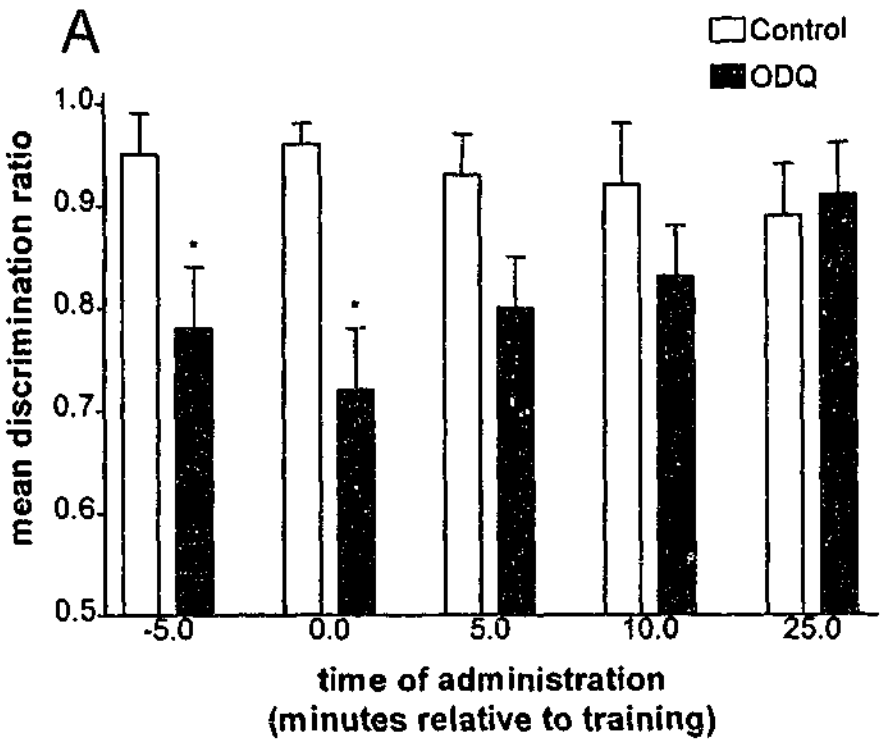
RESULTS

The Effect of GC Inhibition on Memory

Dose response functions for the GC inhibitors ODQ and LY 83583. To determine if GC is required for memory, the effects of two different GC inhibitors on retention were investigated. Concentrations of ODQ ranging from 0.01 to 500 μM, and concentrations of LY 83583 ranging from 10 to 400 μM, were used. Each group of chicks was administered one concentration of either ODQ or LY 83583 or the vehicle (saline containing $<$ 1% DMSO) as a control immediately after training. All chicks were tested 120 min posttraining, a time at which long-term memory is considered established (Ng & Gibbs, 1991). GC inhibition impaired retention when tested 2 h after training, and for each drug, the only effective concentration was 100 μM (data not shown). A one-way ANOVA indicated a significant dose effect for ODQ ($F(9, 166) = 3.99$, $p < .001$), and Dunnett's posthoc tests revealed a significant difference ($p < .05$) between retention levels of chicks administered the 100 μM concentration and those administered the vehicle. The dose effect was also significant for LY 83583 ($F(10, 180) = 3.018$, $p < .001$), with the posthoc Dunnett's test revealing a significant difference between retention of chicks administered 1 μM ($p < .05$) and 100 μM ($p < .005$) and those of control chicks. Such "U"-shaped dose response functions have previously been observed within our model (Rickard et al., 1994d; Whitechurch, Ng, & Sedman, 1997) and could be explained by additional negative feedback mechanisms being triggered at high doses of an antagonist.

Time of administration function for the GC inhibitors ODQ and LY 83583. To determine the period during which GC inhibitors were effective, chicks were administered 100 μM ODQ, 100 μM LY 83583, or the vehicle at a range of times pre- and posttraining. Due to practical constraints on experimenter availability across different experimental days, the same time points could not be tested for each drug. ODQ was administered 5 min before, immediately after, and 5, 10, and 25 min after training, while LY 83583 was administered 5 min pretraining, immediately after, and 5 and 10 min posttraining. Since NO inhibitors impair retention when administered as early as 30 min prior to training, the effect of earlier pretraining inhibition of GC was also investigated by administering LY 83583 also at a range of times between 10 and 50 min prior to training. At each time of administration for each drug, a control group was administered the vehicle. Retention for control and drug-treated chicks was measured 120 min posttraining (see Fig. 1).

Retention appeared to be impaired only if either drug was administered close to the time of training. Separate two-way ANOVAs were performed for each drug because the administration times were not matched. In each case, a significant drug effect was observed (ODQ: $F(1, 139) = 14.45$, $p < .001$; LY 83583: $F(1, 294) = 18.08$, $p < .001$). However, neither the time of administration effect nor the interaction between drug and time of administration was significant. While performing posthoc tests in the absence of significant interaction effects is controversial, it has been argued that posthoc tests are justified when



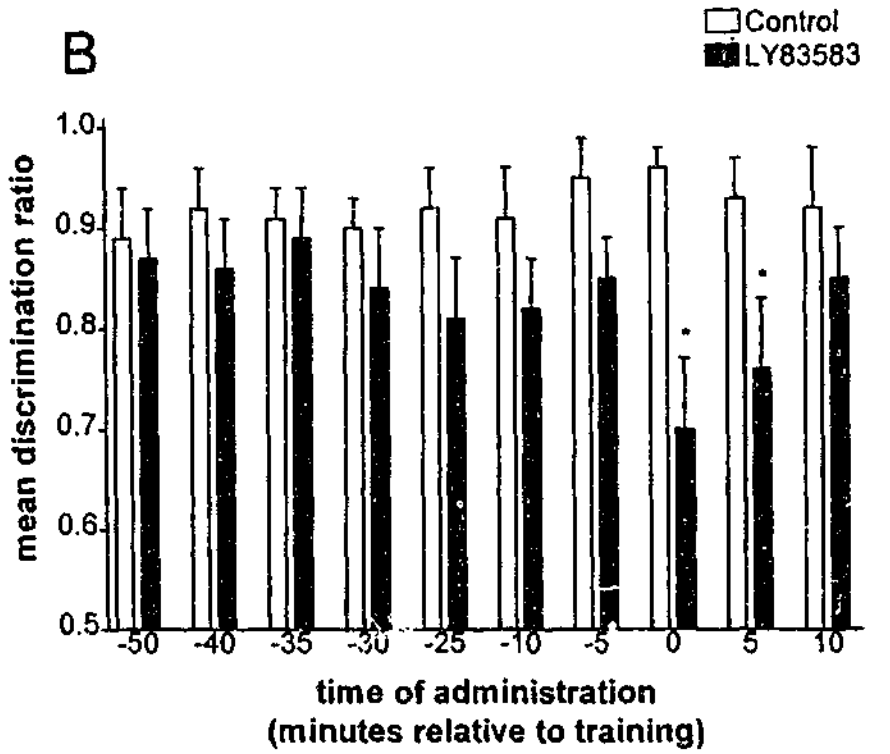


FIG. 1. The effect of guanylate cyclase inhibitors or the vehicle (control), using (A) 100 μM ODQ or (B) 100 μM LY83583, administered at various times relative to training on retention tested 120 min posttraining. Each column represents the mean DR (±3EM) for a separate group of chicks. $^*p < .05$.

many nondifferences occur between treatments (Howell, 1982), as is apparent in these data. In addition, the small sample sizes (ranging from 12 to 19 chicks per group) in these experiments may have reduced the power of the tests somewhat. Simple main effects analyses confirmed that retention levels of chicks administered ODQ at 5 min before ($p < .01$) or immediately after ($p < .001$) training were considerably lower than those of control chicks administered the vehicle at the same time. Similarly, simple main effect analyses revealed that chicks administered LY 83583 immediately ($p < .001$) or 5 min ($p < .05$) after training showed significantly lower retention than did controls.

Retention functions for the GC inhibitors ODQ and LY 83583. To determine when retention deficits first became apparent, 100 μM ODQ, 100 μM LY 83583, or the vehicle was administered immediately posttraining and chicks were tested at a range of times between 10 min and 24 h posttraining (see Fig. 2).

From Fig. 2 it can be seen that administration of either drug resulted in two transitory retention deficits, one centred around the 40-min time point and the second around the 120-min time point. A two-way ANOVA on the ODQ data revealed a significant drug effect ($F(1, 492) = 31.39$, $p < .001$), a significant time of test effect ($F(12, 492) = 3.23$, $p < .001$), and a significant interaction effect ($F(12, 492) = 2.90$, $p < .005$). A simple main effects posthoc analysis indicated that retention levels of ODQ-treated chicks were significantly different from those of controls at 40 ($p < .001$), 50 ($p < .001$), 60 ($p < .05$), 90 ($p < .005$), 100 ($p < .001$), and 120 ($p < .005$) min posttraining. A two-way

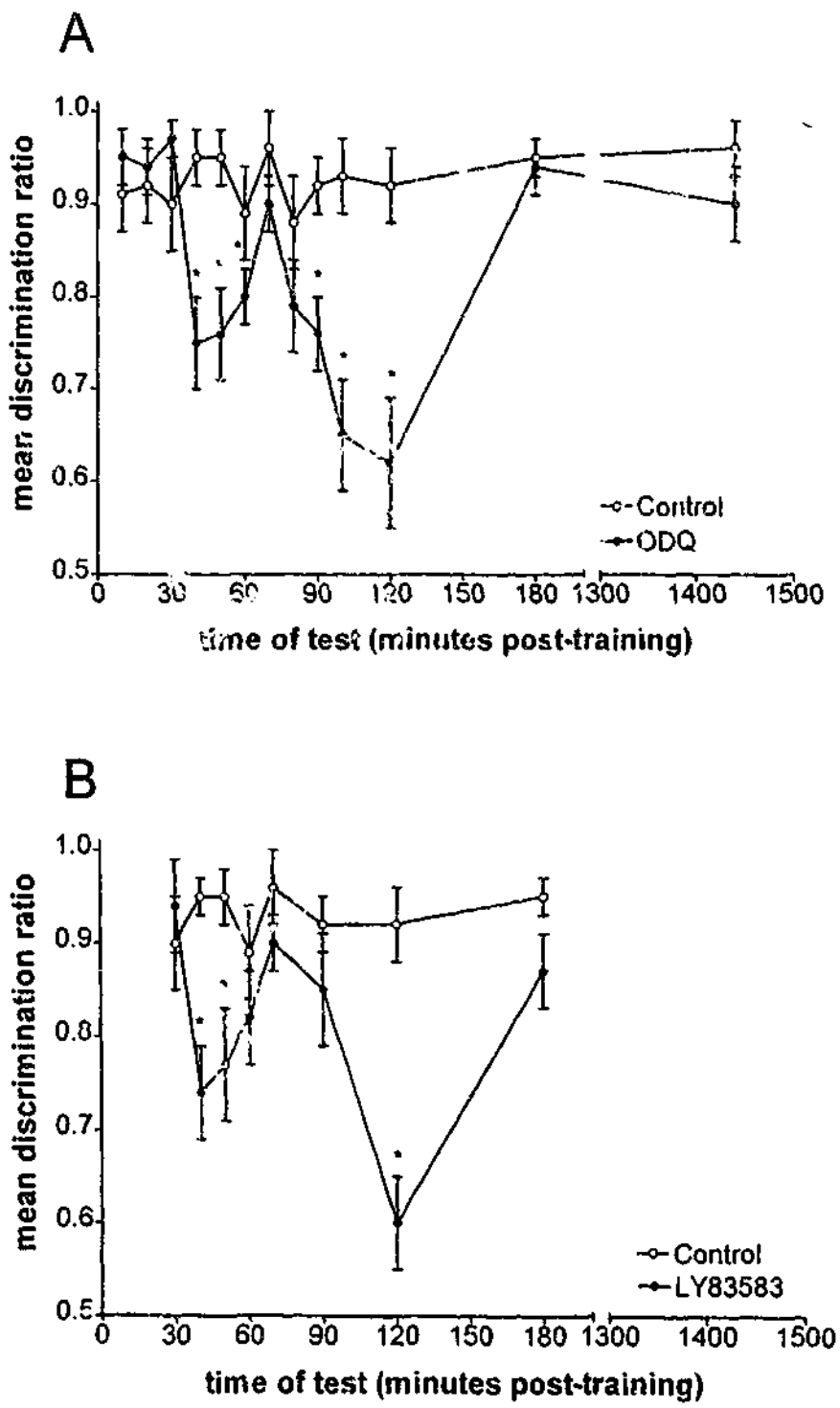


FIG. 2. Retention functions resulting from the vehicle (control) or guanylate cyclase inhibitors, using (A) 100 μM ODQ or (B) 100 μM LY83583, administered immediately after training. Each point represents the mean DR (±SEM) for a separate group of chicks. *$p < .05$.

ANOVA on the LY 83583 data also revealed a significant drug effect ($F(1, 289) = 23.55$, $p < .001$), a significant time of test effect ($F(7, 289) = 2.76$, $p < .01$), and a significant interaction effect ($F(7, 289) = 2.81$, $p < .01$). A simple main effects analysis demonstrated a significant difference between retention levels of LY83583-administered groups and those of the control groups at 40 ($p < .001$), 50 ($p < .05$), and 120 ($p < .001$) min posttraining.

Since GC inhibition results in transient rather than permanent retention loss, it is possible that the drugs temporarily impair some aspect of performance 40 and 120 min after administration. To test whether the observed effects of GC inhibitors could be attributed to nonspecific effects of the drugs, chicks were injected with 100 μM ODQ 24 h after training. Retention was then tested 40, 50, 90, 100, and 120 min after drug administration. If the previously observed transient retention losses are due to nonspecific effects of the drug rather than on memory-related processes, the same retention losses should be observed, since the effects would not be temporally dependent on learning. Retention loss was not observed for any group (data not shown), indicating that the GC-mediated mechanisms inhibited by ODQ are likely to be required specifically for memory processes.

The Effect of PKG Inhibition on Memory

Dose response function for the PKG inhibitor H-8. To determine whether PKG was also required for memory, the effect of the PKG inhibitor H-8 was investigated. Concentrations of H-8 ranging from 0.01 to 500 μM were administered to different groups of chicks immediately after training, and a control group was administered the vehicle saline at the same time. Retention of all chicks was tested 120 min posttraining. Inhibition of PKG impaired retention when tested 2 h after training (data not shown). This effect appears to be dose-dependent, with 200 μM the most effective amnestic concentration. A one-way ANOVA indicated a significant dose effect ($F(8,140) = 6.36$, $p < .001$), and a Dunnett's posthoc analysis revealed a significant difference ($p < .05$) between the retention levels of control chicks and chicks administered 150, 200, 250, or 300 μM H-8.

Time of administration function for the PKG inhibitor H-8. To determine the period during which H-8 was effective, 200 μM H-8 or the vehicle was administered to different groups of chicks at various times between 10 min prior to and 10 min after training. Retention was measured 120 min posttraining (see Fig. 3).

Figure 3 shows that H-8 impaired retention when administered at times close to training. A two-way ANOVA revealed a significant drug effect ($F(1, 154) = 21.92$, $p < .001$), a significant time of administration effect ($F(4, 154) = 2.72$, $p < .05$), and a significant interaction effect ($F(4, 154) = 4.84$, $p < .005$). Posthoc simple main effects analyses confirmed that chicks administered H-8 5 min prior to training ($p < .001$), immediately after training ($p < .001$), or 5 min after ($p < .05$) training demonstrated significantly lower retention levels than chicks administered the vehicle at the same times.

Retention function for the PKG inhibitor H-8. To determine when the PKG inhibitor-induced retention loss first occurred, 200 μM H-8 or the vehicle was administered to chicks immediately posttraining, and retention tested at various times from 10 min to 24 h after training (see Fig. 4).

PKG inhibition resulted in a single transient retention deficit around 120 min posttraining. A two-way ANOVA revealed a significant drug effect ($F(1, 486) = 12.56$, $p < .001$),

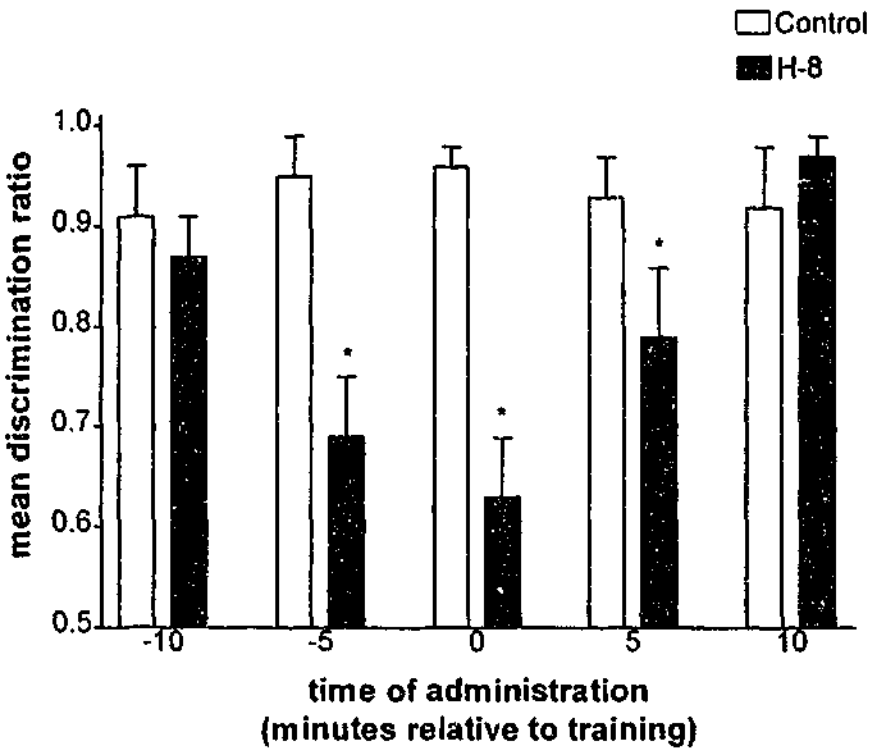


FIG. 3. The effect of the PKG inhibitor 200 μM H-8 or the vehicle (control), administered at various times relative to training on retention tested 120 min posttraining. Each column represents the mean DR (±SEM) for a separate group of chicks. *$p < .05$.

a significant time of test effect ($F(12, 486) = 1.94, p < .05$), and a significant interaction effect ($F(12, 486) = 1.78, p < .05$). Posthoc simple main effects analyses confirmed that retention levels of H-8-treated chicks were significantly lower than those of control chicks at 100 min ($p < .001$) and 120 min ($p < .001$) posttraining, but also at 180 min ($p < .05$) posttraining.

To ensure that the transient retention deficit was not due to nonspecific effects of the drug on performance, chicks were administered 200 μM H-8 24 h after training and tested at 90, 100, 120, 130, and 150 min posttraining. Retention loss was not observed for any group (data not shown), indicating that the PKG-mediated mechanisms inhibited by H-8 are required specifically for memory processes.

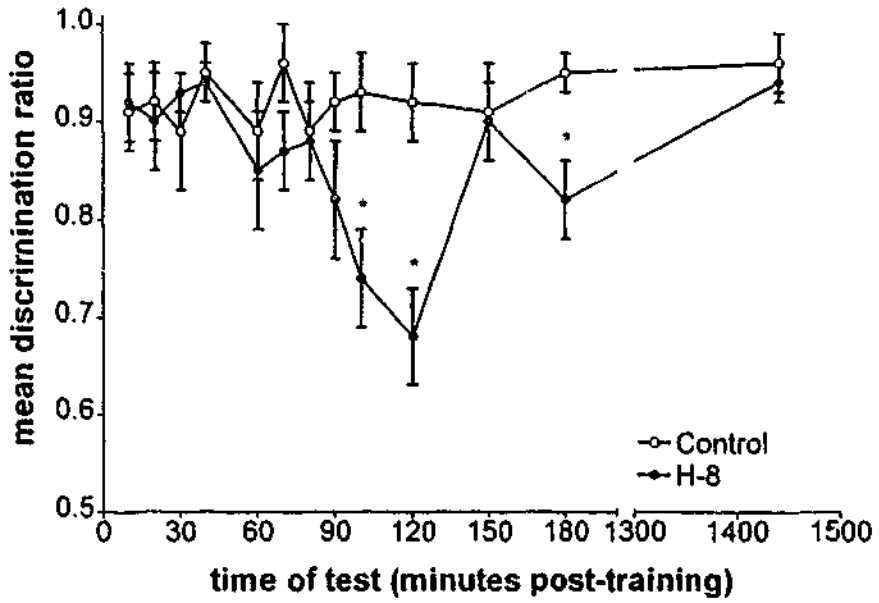


FIG. 4. Retention function resulting from the vehicle (control) or the PKG inhibitor 200 μM H-8 administered immediately after training. Each point represents the mean DR (±SEM) for a separate group of chicks. *$p < .05$.

DISCUSSION

The relationship between NOS, GC, and PKG has been well characterised for numerous physiological processes (Lincoln, Cornell, Komalavilas, & Boerth, 1996). Moreover, NOS (Hölscher & Rose, 1992, 1993; Rickard et al., 1998, 1999; Kendrick et al., 1997), GC (Kendrick et al., 1997; Bernabeu et al., 1996, 1997; Izquierdo et al., 2000), and PKG (Serrano et al., 1994; Bernabeu et al., 1997; Izquierdo et al., 2000) have each been implicated in memory formation. Nevertheless, the conjecture that GC and PKG activities underlie the NO activity required for memory formation has yet to be confirmed. In the current study, it was hypothesized that if learning-induced NO activity stimulated GC and PKG, then inhibition of either GC or PKG would result in permanent memory loss at the same time posttraining as does NO inhibition. Previous studies have shown that NO inhibition results in permanent memory loss for the passive avoidance task in the day-old chick around 40 min after training (Hölscher & Rose, 1992, 1993; Rickard et al., 1998, 1999). The current results indicate, however, that inhibition of neither GC nor PKG resulted in permanent memory loss. In contrast, GC inhibition resulted in temporary retention losses at around 40 and 120 min posttraining, while PKG inhibition resulted in a single temporary retention loss around 120 min posttraining. Therefore, while GC and PKG appear to play a role in memory processing, they are not essential for long-term memory formation. Furthermore, the current findings suggest that GC and PKG may not be the memory formation mechanisms up-regulated by NOS activity following passive avoidance training in the chick.

In the current study, memory loss following GC inhibition was first observed around 40 min posttraining, and memory loss from PKG inhibition was first observed considerably later, around 120 min posttraining. A three-stage model of memory formation has been proposed by Gibbs and Ng (1977; Ng & Gibbs, 1991) and confirmed by other researchers (Rosenzweig, Bennett, Patterson, & Mizimori, 1993) for the passive avoidance paradigm for the day-old chick. Behavioral and pharmacological studies provide evidence for a short-term memory stage which lasts for about 15 min after training, an intermediate stage, which lasts from about 20 to 55 min posttraining, and a long-term memory stage, which is formed by 60 min posttraining and lasts indefinitely (Ng & Gibbs, 1991). The current data, therefore, suggest that while GC inhibitors must be administered close to the time of learning, the effect on retention is during the intermediate and long-term memory stages. While PKG inhibition must also occur close to the time of learning within this paradigm, retention of only the long-term memory stage is affected.

These findings are consistent with previous research that indicated that GC might be active earlier in the memory formation process than is PKG. In the rat, GC inhibition at the time of learning caused memory loss when tested at either 1.5 h posttraining (short-term memory; Izquierdo et al., 2000) or 24 h posttraining (long-term memory, Bernabau et al., 1997; Izquierdo et al., 2000). In contrast, PKG inhibition immediately after training resulted in only long-term memory loss, with short-term memory remaining intact (Izquierdo et al., 2000). Similarly, PKG inhibition blocked long-term memory formation in the chick, while having no effect on short-term memory (Serrano et al., 1994). Taken together, these findings suggest that GC plays a role in short-term and long-term memory stages, while PKG plays a role in only longer term memory stages. The reason PKG inhibitors must be administered around the time of learning even though memory loss

does not occur for some time is not clear, although clearly the finding is consistent across each of these laboratories.

It is of interest that Serrano et al. (1994) found that PKG inhibition 5 min prior to training resulted in memory loss 1 h earlier than observed in the current study, despite using a similar task in the same species. This inconsistency may be explained by differences in task procedure or chick strain, but is most likely to have been due to the higher dose of the PKG inhibitor used by Serrano et al. (16.7 mM compared with 200 μM used in the current study). The drug H-8 preferentially inhibits PKG (K_i = 0.5 μM), but at higher doses also inhibits PKA (K_i = 1.2 μM). We have previously shown that inhibition of PKA results in memory loss for this task at 60 min posttraining (Zhao, Polya, Wang, Gibbs, Sedman, & Ng, 1995), which is consistent with the effect observed by Serrano et al. (1994).

The mechanisms for which GC and PKG are involved in passive avoidance learning cannot be determined from the current results. It is clear, however, that the transient amnestic effects we observed following GC or PKG inhibition are not the same as the permanent memory loss observed 40 min posttraining following NOS inhibition in this paradigm (Hölscher & Rose, 1992, 1993; Rickard et al., 1998, 1999). This would suggest that a three-step linear pathway from NO to GC and PKG does not underlie memory formation for this task. While not sufficient, it remains possible that the temporary retention loss caused by GC inhibition at 40 min posttraining is in some way related to the permanent memory loss induced by NOS inhibition. Not only is the activation of GC by NO well characterized in the literature but since inhibition of either enzyme results in retention loss by 40 min posttraining, it seems likely that NOS and GC are mechanisms of the same biochemical cascade necessary for retention at this time.

The transient effect of GC and PKG inhibitors on retention around 120 min after training could also be related to NO activity. However, given that NOS inhibition results in permanent memory loss at 40 min posttraining, the effect of NOS inhibitors on any subsequent NO activity would be masked by the earlier persistent effect. Retention for the passive avoidance task is thought to be dependent on ionotropic (Rickard, Poot, Gibbs, & Ng, 1994b) and metabotropic (Rickard & Ng, 1995) glutamate receptors and tyrosine kinase activity (Whitechurch et al., 1997), from 90 min after training. Each of these mechanisms is also known to be involved in long-term potentiation (LTP), a phenomenon which models the strengthening of synapses in a neuronal network (Malenka & Nicoll, 1999). Since NO is also regarded as a likely retrograde messenger in LTP, the relationship between these mechanisms during long-term memory would be an area worthy of further investigation.

The current findings that both GC and PKG are involved in memory processing and that GC appears to be involved at an earlier stage than PKG are broadly consistent with previous research. In contrast, however, the effects observed in the current study were transient, followed by complete recovery of retention. It is difficult to compare the longevity of the amnestic effects of these drugs with previous studies since most have only used one test time, and recovery after amnesia has not been tested. While Izquierdo et al. (2000) found retention was impaired at both 1.5 and 24 h posttraining, they interpreted these time points as representing short-term and long-term memory. Since long-term memory in their rat model appears to begin between 3 and 6 h posttraining (Izquierdo et al., 1999), whereas in the chick, long-term memory begins at 1 h posttraining, the 24-h

time point in the rat could be comparable to the 2-h time point in the chick. Similarly, Serrano et al. (1994) did not test any time points later than 120 min after passive avoidance training in the chick. Later time points corresponding to established or late long-term memory have not been tested in previous studies investigating the effect of GC or PKG inhibition on rat memory, so it is difficult to know whether the effects observed in previous studies were permanent or transient.

Interpretation of transient retention losses within the passive avoidance paradigm is difficult, given the putative sequential dependence of the memory formation stages within this model (Gibbs & Ng, 1977; Ng & Gibbs, 1991). Temporary retention losses have, nonetheless, been observed in this model with several other treatments. For instance, exposure to a hypoxic or an anoxic environment soon after training was found to cause a temporary memory loss between 20 and 50 min after training (Allweis, Gibbs, Ng, & Hodge, 1984). The authors concluded that hypoxia and anoxia might impair retrieval rather than formation of memories, since memory clearly persisted in the absence of its expression. In this context, the current findings could indicate that GC and PKG are necessary for the recall of information at specific times, rather than the formation of memory. The disparity in the permanence of the retention functions following NOS and GC inhibition may, therefore, indicate that NO mediates memory retrieval processes through GC and PKG while also activating memory formation processes through another mechanism such as ADP ribosylation or nitrosylation.

The transience of the effect is unlikely to be due to methodological issues. The temporal specificity of the effect with regard to training was demonstrated, with no effects observed 40 or 120 min after injection of the drug 24 h after training. Second, a wide range of doses and administration times was tested and thus it is unlikely that the transience is simply due to using a nonoptimal dose or administration time. A second drug also replicated the effects observed with ODQ, even though LY 83583 blocks GC via a different mechanism. Finally, the discrimination index used in this version of the passive avoidance task ensures chicks that avoid the previously aversive bead are not doing so because of nonspecific effects of the drug by including only those chicks that continue to peck at a neutral bead. Nonspecific effects of the drug on performance are also reduced by direct intracranial administration of the drugs.

The current data support a role for GC and PKG in memory retrieval rather than memory formation. While the mechanisms for which these enzymes are required are unclear, GC and PKG clearly play roles in retention of the passive avoidance task in day-old chicks. These roles appear to be brief and transient and therefore not essential for long-term memory formation. We are currently investigating alternative downstream pathways of NO activation that may be responsible for the persistent memory loss previously observed following NOS inhibition.

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