Effects of Passive Avoidance Training on Calcium Flux in Chick Forebrain.

Thesis

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Effects of Passive Avoidance Training on Calcium Flux in Chick Forebrain

By

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AHP	After hyperpolarization
AMPA	γ-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	Analysis of variance
AP5	2-amino-5-phosphonopentanoic acid
ARC	Archestriatum
ARM	Amnesia resistant memory
AUG	Augmentation
Ba^{2+}	Barium ions
BDZ	Benzodiazepines
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
$[Ca^{2+}]_i$	Intracellular calcium concentration
$^{45}Ca^{2+}$	Radiolabelled calcium ions
CAM	Calmodulin
CaM Kinase II	Ca^{2+} /calmodulin-dependent kinase type II
cAMP	cyclic AMP
cAMPPDE	cyclic AMP phosphodiesterase
c-fos	Cellular fos mRNA
CGP35348	$GABA_B$ antagonist
c-jun	Cellular jun mRNA
Cl^-	Chloride ions
$[Cl^-]_i$	Intracellular chloride concentration
CNS	Central nervous system
CO_2	Carbon dioxide
CS	Conditioned stimulus
Dantrolene	(1-[(5-[p-Nitrophenyl]furfurylidene)-amino]hydantoin)
DHP	Dihydropyridines
2-DG	2-Deoxyglucose
EEG	Electroencephalography
EGTA	Ethylene glycol-bis[β-aminoether]-N,N,N',N'-tetraacetic acid
EM	Electron micrograph
EPSP	Excitatory post-synaptic potential
FF	Frequency facilitation
FTX	Funnel web toxin
GABA	γ-aminobutyric acid
GC	Glucocorticoid

GSW	Gill-siphon withdrawal reflex
^{3}H-Muscimol	Radiolabelled muscimol
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
H_3PO_4	Orthophosphoric acid
HVA	High voltage activated
5-HT	5-Hydroxytriptamine
IC_{50}	Inhibitory concentration, 50%
IEG	Immediate early gene
i.p.	Intraperitoneal
IPSP	Inhibitory post-synaptic potential
i.v.	Intraventricular
ITM	Intermediate-term memory
^{125}I-ω-CTX GVIA	Radiolabelled ω-conotoxin GVIA
IMHV	Intermediate medial hyperstriatum ventrale
K^+	Potassium ions
$[K^+]_e$	Extracellular potassium concentration
$[K^+]_i$	Intracellular potassium concentration
KCl	Potassium chloride
KRB	Krebs Ringer bicarbonate buffer
LTP	Long-term potentiation
LPO	Lobus parolfactorius
LTD	Long-term depression
LTM	Long-term memory
LVA	Low voltage activated
M-birds	Methylanthranilate-trained birds
M_1-receptors	Muscarinic$_1$-receptors
MeA	Methylanthranilate
MEG	Magnetoencephalography
MK-801	Dizocilpine
$[Mg^{2+}]_i$	Intracellular magnesium concentration
$MgCl_2$	Magnesium chloride
Na^+	Sodium ions
NaCl	Sodium chloride
NaH_2PO_4	Sodium hydrogen phosphate
NCAM	Neural cell adhesion molecule
NDGA	Nordihydroguaiaretic acid
NE	Noradrenaline
NMDA	N-methyl-D-aspartate
Ni^{2+}	Nickel ions
NO	Nitric oxide
O_2	Oxygen

ω-Aga IVA	ω-agatoxin IVA
ω-CTX GVIA	ω-conotoxin GVIA
ω-CTX MVIIC	ω-conotoxin MVIIC
PA	Paleastriatum augmentatum
PAL	Passive avoidance learning
PCP	Phenylcyclidine
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
PLA_2	Phospholipase A_2
PMSF	Phenylmethylsulfonyl fluoride
PTP	Post-tetanic potentiation
PTX	Pertussis toxin
Q-birds	Quiet (untrained) birds
RAM	Radial arm maze
STE	Short-term synaptic enhancement
STP	Short-term potentiation
STM	Short-term memory
TEA	Tetraethylammonium chloride
THSD	Tukeys honestly significant difference
TPCK	N-Tosyl-L-Phenylalanine chloromethyl ketone
TLCK	Tα-P-Tosyl-L-Lysine chloromethyl ketone
US	Unconditioned stimulus
VSCC	Voltage sensitive calcium channels
W-birds	Water-trained birds
^{33}Xe	Radiolabelled xenon

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ABSTRACT

Calcium flux via voltage sensitive calcium channels (VSCC) was studied after passive avoidance training in the day-old chick. Intracellular free calcium concentration $[Ca^{2+}]_i$, after depolarisation with 70mM KCl, was measured using the calcium chelator fura-2/AM in the crude membrane preparations from the IMHV and LPO of chicks. Experiments showed a significant increase in KCl-stimulated $[Ca^{2+}]_i$ in the left IMHV of birds tested immediately after training compared to quiet birds (Q) and water trained birds (W). This increase was sustained in the left IMHV of birds tested 30 minutes after training. There was no significant difference between any of the groups of birds tested at later times. There was no significant increase in KCl-stimulated $[Ca^{2+}]_i$ in the right IMHV of trained birds at any of the times tested. Significant elevation in KCl-stimulated $[Ca^{2+}]_i$ was also detected, in both the left and right LPO, of birds tested 5 minutes after training; which was still evident in birds tested 10 minutes after training. There was no significant difference between any of the treatment groups in the left or right LPO at any of the other times tested.

Addition of different VSCC antagonists; such as ω-conotoxin GVIA (N-type blocker), ω-conotoxin MVIIC (N/P/Q-type blocker), nimodipine (L-type blocker), and ω-agatoxin IVA (P-type blocker), were all shown to inhibit KCl-stimulated $[Ca^{2+}]_i$ elevation in the crude membrane preparation from the IMHV of untrained chicks. Addition of ω-conotoxin GVIA, nimodipine or ω-conotoxin MVIIC into the crude membranes from the left or right IMHV of chicks, tested immediately after training, showed greater inhibition in the left IMHV of trained birds compared to Q- and W-birds. There was no difference in inhibition in the right IMHV between any of the treatment groups .

The GABAergic receptors, $GABA_A$ and $GABA_B$ both inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation in crude membranes prepared from the IMHV of untrained chicks. Furthermore, the $GABA_B$ receptor antagonists were shown to exhibit some atypical properties. $GABA_B$ receptors were shown to evoke greater inhibition on KCl-stimulated $[Ca^{2+}]_i$ elevation in crude membranes prepared from the IMHV of chicks tested 30 minutes after training compared to untrained birds and birds that were tested 5 minutes after training.

Chapter 1

General Introduction

1. Introduction

The ability of an organism to learn and acquire memory for events in its life is a powerful aid to its survival. The study of memory was once limited to psychology, however currently techniques which study cellular mechanisms, neural circuitry and behaviour are also employed. The cellular and biochemical techniques that are used to investigate memory try to determine biochemical and molecular changes that may be taking place in neural tissue following the acquisition and encoding of information. Conversely, studies into the neural circuitry of the brain attempt to determine the area of the central nervous system (CNS) that is activated during acquisition and encoding of information. Finally, the behavioural method used to study memory attempts to determine the ability of a subject to learn and to remember a task.

Much more work needs to be carried out to uncover the mechanisms underlying memory and the location of memory in the CNS. In her introduction for the colloquium titled "Memory: Recording experience in cells and circuits", Goldman-Rakic (1996), indicated that one of the major challenges for researchers investigating mechanisms underlying memory is "the dilemma of localising a complex function in an exquisitely anatomically differentiated central nervous system".

This chapter will cover the different possible levels at which memory can be studied (section 1.1). This will be followed by a brief description of learning (section 1.2) and memory (section 1.3), and some animal (section 1.4) and electrophysiological (section 1.5) models used to study memory. As the model used in this work is the day-old chick, some arguments for using this model to study learning and memory processes (section 1.6) will be presented. Finally voltage sensitive calcium channels (VSCC; section 1.7) will be introduced and mechanisms of modulation by GABAergic receptors will be discussed in section 1.8. Finally the current evidence of the participation of calcium in learning in memory formation is also discussed in section 1.9.

The aim of this thesis is to determine the function of VSCC in certain areas of the chick forebrain particularly the intermediate medial hyperstriatum ventrale (IMHV) and lobus parolfactorious (LPO) following passive avoidance learning (chapter 4), the pharmacological identification of the subtypes of VSCC in the IMHV (chapter 5) and pharmacological experiments to determine GABAergic modulation of VSCC in the IMHV (chapter 6).

1.1. Levels of analysis

Mechanisms of learning and memory can be studied at two levels, one is the cellular and molecular level and the other encompasses a more global approach. The cellular and molecular approach addresses the question of how neurons exhibit history-dependent activity. It was at this level that the neuropsychologist Donald Hebb (1949) proposed a model to explain the creation of persistent synaptic changes (i.e. strengthening of synaptic connections) that occur after an organism has learned and acquired a memory of the new stimulus. His suggestion was:

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

The second approach for studying the mechanisms of learning and memory has been the study of the brain as a whole, which gives a more global level of analysis. Powerful imaging techniques are currently available that enable aspects of brain function to be monitored during the performance of cognitive tasks. These technique gives an insight into the involvement of different brain regions during learning and memory. Positron emission tomography (PET) scanning has proved useful in the study of the dynamic aspects of human brain function (Brownell *et al.*, 1982; Phelps and Mazziotta, 1985). PET studies use isotopes which emit small amounts of radiation that can be detected by sensors. If these isotopes are incorporated into metabolically active compounds, it is possible to trace their path. Thus measurements of the uptake of radiolabelled glucose in the conscious human brain have shown that different brain regions are activated depending on the mental task (for a review see Dudai, 1994). Radiolabelled glucose has also been used in the study of memory formation in animals,

where, for example, pre-training and post-training injections of radiolabelled glucose, [^{3}H]-2-deoxyglucose (2-DG), into the IMHV, LPO and paleostriatum augmentatum (PA) showed regional differences in 2-DG accumulation in chicks tested on the passive avoidance task (Rose and Csillag, 1985). Another imaging technique which has been used in mapping brain activity measures changes in cerebral blood flow (Chein, 1985). This technique, called radioangiography, monitors the concentration of the gamma-emitting isotope of an inert gas, ^{133}Xe, in the blood. In addition, two other brain imaging techniques have also been developed which allow real-time mapping of global brain activity, electroencephalography (EEG) and magnetoencephalography (MEG). The first measures electric fields produced by activated neurons and the second measures the magnetic field as a result of the current in the neuron (for a review see Dudai, 1994).

Thus, in summary, the questions addressed by using these more global approaches to learning and memory allow the experimenter to observe the areas of the whole brain that can be activated during the processes of acquisition, encoding or recall of information.

1.2. Learning

Learning can simply be defined as the processes by which new information about the world is acquired (Bailey *et al.,* 1996). Behavioural studies have allowed scientists to classify learning into at least 3 categories; non-associative, associative and incidental learning.

Non-associative learning occurs when an organism habituates or becomes sensitised to a stimulus. Habituation describes a gradual decrease in an organism's response to a stimulus following repeated exposure. Conversely, sensitisation describes the strengthening of a behavioural response to a previous neutral stimulus following presentation of a potentially threatening stimulus at the same or another site.

Associative learning is subdivided into classical and operant conditioning. Classical conditioning, first described by Pavlov (1927), is observed when a weak conditioning

stimulus (CS) evokes a new behavioural response, or causes an enhancement of a pre-existing response, when the CS is temporarily paired with a strong unconditioned stimulus (US). Operant conditioning, first described by Skinner (1938), occurs when the reinforcing stimulus is dependent on the performance of a certain behaviour of the animal rather than on the application of a CS. The organism consequently remembers the results of its own behaviour and modifies it in accordance with the number of training trials (Dudai, 1994).

Incidental learning is related to the learning of events which have no 'instructive' situations. Thus, in this form of learning an organism acquires information about the world while attending 'incidentally' to sensory inputs. The behavioural consequence of incidental learning is usually latent, and, may manifest as either perceptual learning or insight (Dudai, 1994).

1.3. Memory

Memory is defined as the processes of storing and retrieving information that are acquired through an organisms senses (Dudai, 1994). At present there is still no general agreement for classifying different memory systems. What is evident, however, is that memory is divisible into processes and subprocesses which have heterogeneous forms. Thus, classification of memory systems can be based on either the type of memory (e.g. classical conditioning versus procedural; memory of actions such as riding a bike), the content of memory (e.g. episodic versus semantic), the temporal parameters of memory (e.g. short-term versus long-term), and the level of processing (encoding, retrieval; Goldman-Rakic, 1996). It is evident that the diversity of memory makes it difficult to localise it to a single anatomical structure.

Temporal phases of memory such as short-term memory (STM) and long-term memory (LTM), are frequently used by researchers studying cellular and morphological changes that occur in a variety of animal models (eg. *Aplysia*, *Drosophila*, chick) following particular learning and memory paradigms. This is used as a means of correlating the biological changes in the CNS with time during memory formation. STM registers and retains memory

of cognitive information in a state of ready accessibility usually for a short period of time after the acquisition of the information. The memory may be held by transient changes in the firing properties of particular neurons which may persist from seconds to minutes. Conversely if an organism is subjected to a strong enough stimulus the transient changes in the firing properties of neurons may lead to activation of other biochemical cascades that result in a more stable and longer lasting change in synaptic efficacy. Thus STM may lead to LTM (also known as reference memory), which is the registration and retention of memory to be stored over a long period of time. At the cellular level, LTM requires substantial modifications of cells and their connections such as synaptic strengthening and synaptic remodelling, which leads to information storage that lasts from minutes to years. The interface between STM and LTM is known as consolidation, a process that determines what information will be stored in the brain in a stable form. Recall and recognition are both linked to short- and long-term memories. Recent neurophysiological and neurobiological studies have shown that STM and LTM encompass several subtypes of memories. A psychologically based taxonomy of memory indicates that it can be subdivided into procedural and declarative forms, which are further subdivided into other categories. Description of the psychological taxonomy of memory is beyond the scope of this thesis, however more information on this subject can be obtained from Squire (1995).

Modern behavioural and biological studies have shown that learning and memory are not a unitary process, but a family of distinct processes, each with its own rules (Bailey *et al.*, 1996). In addition, these processes can act in series and/or in a parallel fashion (Goldman-Rakic, 1996) depending on the type of information acquired. In 1966 McGaugh had already proposed a multiple-trace memory-storage model. In his model, memory for a learning trial is held very briefly by a sensory buffer and then by three overlapping stages. Thus, the separate traces begin to form immediately after training for STM, intermediate-term memory (ITM) and LTM, and these traces have successively later peaks. Neuropsychological studies have shown dissociations between STM and LTM in human studies, where, for example, there are reports of impairments in STM while LTM remains intact (Warrington, 1982). Conversely, Scoville and Milner (1957) reported a patient H.M., who suffered from anterograde amnesia, to exhibit impairments of LTM but not STM.

Recently, pharmacological studies showed a dissociation between STM and LTM in rat brain following training on the step-down inhibitory avoidance task (Izquierdo *et al.*, 1998a). Experiments showed that pre-training intracerebral (i.c.) infusion of dopamine (D_1) -receptor agonist, a β-blocker and a 5-HT_{1A} -receptor agonist or antagonist in certain areas of the rat brain disrupted STM without effecting LTM. This strengthened the 'single-trace, dual processes' hypothesis of memory originally proposed by Gold and McGaugh (1975). For example, STM and LTM may share some common pathways that involve glutamate receptors and several kinases in the CA1 region of the hippocampus and ectostriatum (Izquierdo *et al.*, 1998b). In addition STM and LTM may also make use of distinct mechanisms as pharmacological differences were also observed between the two memory systems (Izquierdo *et al.*, 1998a).

Recently, models using *Drosophila* have also highlighted the existence of two distinct forms of memory. Anesthesia resistant memory (ARM) and genuine LTM (DeZazzo and Tully, 1995) were shown to exist in parallel since disruption of one did not disrupt the formation of the other. ARM is insensitive to the protein-synthesis inhibitor cycloheximide and lasts for 4 days and is disrupted by the single-gene mutation *radish*, while LTM is sensitive to cycloheximide and is not disrupted by the single-gene mutation *radish* and lasts up to 7 days after training. Studies that combine the use of genetic mutants with cycloheximide have highlighted several properties of long lasting memory. For example, spaced-training consists of two distinct and functionally independent memory systems, an ARM and genuine LTM. Experiments showed that massed training produced only ARM while spaced-training produce both ARM and LTM, which can exist in parallel. It was also shown that the ARM and LTM systems acted in an additive manner, thus indicating that these systems do not act sequentially. This is because after spaced-training, one-day of training in *radish* flies (LTM only) and cycloheximide fed wild-type flies (ARM only) have similar levels of retention as wild-type flies (ARP + LTM).

1.3.1. Criteria for memory

A set of rules has been developed to ensure that biochemical, molecular or morphological changes detected in the CNS are the result of learning and memory and not simply a result of background activity. The set of criteria that must be met if the changes detected in the brain of an organism are attributed to learning and subsequent memory formation (Rose, 1993; Entingh *et al.*, 1975) is that:

(1) There must be changes in the quantity of the system or substrates, or its rate of production or turnover, in some localised region of the brain during memory formation.

(2) Stress, motor activity or other processes that accompany learning must not, in the absence of memory formation, result in structural or biochemical changes.

(3) If the cellular or biochemical changes are inhibited during the period over which memory formation would normally occur, then memory formation should be prevented and the animal should be amnestic.

(4) Removal of the anatomical site at which the biochemical, cellular and physiological changes occur should interfere with the process of memory formation, depending upon when, in relation to training, the region is removed.

(5) Neurophysiological recordings at the sites of cellular change should detect altered electrical activity from the neurons during, or as a consequence of, memory formation.

(6) The time course of the change must be compatible with the time course of memory formation.

(7) The degree of cellular change should be related to some extent to the strength, or intensity of training.

1.4. Animal models of learning and memory

Several approaches have been used to study memory formation in animals. In addition, several different species of animals have been used to help elucidate the mechanism of learning and memory. Vertebrates such as rodents (rats and mice) and chicks are commonly used in studying learning and memory systems at the behavioural and cellular level. Invertebrates such as *Aplysia* and *Drosophila* have been used to probe the cellular changes that may occur during memory formation following simple forms of learning. Invertebrates offer an alternative neuronal system to the vertebrates because, like *Aplysia*, they have a relatively small repertoire of neural tissue, making it easier to study changes at the synaptic level. On the other hand, prior to the advent of knock out mice, genetically modified *Drosophila* were, and still are, useful for studying the function of molecular systems in different forms of learning. This is because of the relative ease with which certain genes that code for particular proteins, can be removed.

1.4.1. *Aplysia californicus*

The sea-slug, *Aplysia californicus*, has been extensively studied over the past decade to gain a better understanding of the cellular mechanisms associated with certain forms of learning, such as habituation, sensitisation and forms of associative learning where alterations in synaptic efficacy can be directly correlated with behavioural learning. Cellular analysis of learning and memory are possible with *Aplysia* because it has a relatively simple behavioural repertoire which is controlled by easily identifiable motor-neurons and associated sensory synapses (Dudai, 1994). The neurons can be dissected out and studied in isolation thus allowing reductive and simplified steps in analysing the cellular mechanisms of non-associative and associative learning. *Aplysia* has been used in studying two forms of simple defensive reflexes (Kandel and Schwartz, 1982) the Gill-siphon withdrawal (GSW) reflex and the tail withdrawal reflex and their modifications by experience.

The GSW reflex occurs when a stimulus is applied to the siphon, which activates the siphon-sensor and then relays the information to interneurons and the gill and siphon motor neurons

(Dudai, 1994). It is a two-component reflex where the first component consists of contraction and withdrawal of the siphon (siphon withdrawal reflex) and the second component involves the contraction and withdrawal of the gill (gill withdrawal reflex). Both components of the GSW system are controlled by both the central (CNS) and peripheral nervous system (PNS), where the PNS controls the localised response and the CNS controls the overall reflex response (including peripheral responses; Dudai, 1994).

The *Aplysia* preparation has shown that different forms of learning may have different cellular mechanisms. For example during habituation of GSW reflex there is a decrease in presynaptic release of 5-HT, while sensitisation causes an increase in presynaptic release of 5-HT (Kandel and Schwartz, 1982).

1.4.2. *Drosophila melanogaster*

The fruit-fly, *Drosophila melanogaster*, can be trained on several learning paradigms based on olfactory, visual and mechanosensory cues. Studies on *Drosophila* have demonstrated that they are capable of several forms of learning, such as non-associative (sensitisation and habituation) and associative learning (classical and operant conditioning). They are frequently used in genetic studies of learning and memory, which offers a different, powerful and complementary approach to the biochemical studies. Manipulation of their genetic system is based on the assumption that genes encode the macromolecules that allow acquisition, retention and retrieval of neuronal information. Thus, deleting a particular gene that may code for a particular macromolecule important for memory formation should then disrupt learning or memory processes in the mutated organism. Concurrent genetic, biochemical, anatomical and electrophysiological analyses may then reveal the defective gene products and their physiological function. This approach may lead to uncovering identical systems in other organisms. The techniques used to develop *Drosophila* learning mutants are described by Dudai (1994).

The first reliable learning test for *Drosophila* was an olfactory associative-learning task which combined elements of classical and instrumental conditioning (Quinn *et al.*, 1974)

whereby light was used to attract flies into the test tube containing either odorant X or Y. Whenever flies entered tube X but not tube Y they received an electric shock. The flies from tube X would associate that odour with shock while the flies from tube Y were used as control groups. The experiment was repeated with different flies from the same batch but this time, shock was associated with tube Y and tube X was used as control. On test with wild-type flies, two-thirds of flies that experienced shock avoided the odour whereas only one-third of control flies avoided the control odour. The 'learning' index was defined as the fraction of flies avoiding the shock-associated odorant minus the fraction avoiding the control odorant, a value which was about 0.4. This test was originally used for differentiating between the mutant flies. A more robust olfactory learning task has been used subsequently (Tully and Quinn, 1985) which removes the phototactic stimulus (thus removing the operant conditioning component of the learning task) and employed a more rigid classical conditioning procedure. In this apparatus, flies were sequestered into the training compartment (electrified odorant-containing chamber) and trained sequentially to two odorants X and Y. Electric shock was applied during exposure to one of the odorants and the other (not associated with shock) was used as a control. During testing, wild-type flies almost completely avoided the shock-associated odorant, but not the control odorant. Learning indices of 0.9 were measured.

As mentioned previously, *Drosophila* are also capable of learning other tasks such as habituation, sensitisation, as well as classical and operant conditioning in a variety of other paradigms based on olfactory, visual and mechanosensory cues (Dudai, 1994). It should be noted, however, that data gathered from neurogenetic experiments should be interpreted with caution as learning deficits in the memory mutants may not necessarily indicate a specific function of the gene and the molecule for which it codes, since biological systems usually have mechanisms to compensate for such deficits.

1.5. Electrophysiological models of learning and memory

The term 'potentiation' is used in the physiological literature to describe changes in synaptic efficacy with different time courses and different underlying mechanisms. This area of study

makes use of the fact that synaptic connections can display use-dependent synaptic strengthening, similar to that described by Hebbian principles (Hebb, 1949) or, in other words, information storage in the brain as changes in synaptic efficiency.

Activity-dependent synaptic potentiation can occur within milliseconds and persist for hours in anaesthetised animals or *in vitro* hippocampal slices. In the freely moving animal synaptic potentiation can last for days. Different types of synaptic potentiation have a number of mechanistically distinct temporal components:

(a) Post-tetanic potentiation (PTP)
(b) Short-term potentiation (STP)
(c) Long-term potentiation (LTP)

These activity-dependent phases of potentiation are also classified according to the ability of particular N-methyl-D-aspartate (NMDA) receptor antagonists to inhibit their induction (for review see Bliss and Collingridge, 1993). PTP is an NMDA receptor-independent process and is produced by 'weak' tetanic stimulation of neurons. STP is produced by 'intermediate' stimulation and usually decays within 1 hour. LTP was first described by Bliss and Lomo (1973) and is the most extensively studied form of synaptic potentiation and it may be involved in encoding memory (Morris *et al.*, 1986). It was found in all excitatory pathways in several brain regions such as the hippocampus, which is where it is most extensively studied. The expression of LTP is measured as an increase in the size of the synaptic component of the evoked response recorded from individual cells or from a population of neurons. It can be induced in a variety of ways, for example, delivery of a high frequency tetanus to the pathways of interest (trains of 5 - 100 stimuli at 100Hz or more). It can also be induced by a lower stimulus patterns such as theta-burst stimulation (several bursts of 4 shocks at 100Hz delivered at interburst intervals of $200ms^{-1}$) or as primed-burst stimulation (single priming stimulus followed at $200ms^{-1}$ by single 4 shocks at 100Hz). Support for the participation of LTP as a model for memory is strengthened by the fact that theta- and primed-burst stimulation are similar in frequency to the synchronised firing patterns detected in the hippocampus during learning (Otto *et al.*, 1991).

LTP has 3 main properties known as, co-operativity, associativity and input-specificity. Co-operativity describes the existence of an intensity-threshold for induction where the threshold for LTP is dependent on the function of intensity and pattern of tetanic stimulation. Associativity describes the associative functions of LTP, where for example a weak tetanus can be potentiated if it is active at the same time as a strong tetanus applied to a separate but convergent input (for review see Martinez and Derrick, 1996). Associativity in LTP has been described as a cellular analogue of classical conditioning and is an implicit property of Hebbian synapse. Input-specificity describes the observation that inputs that are not active at the time of tetanus do not share in the potentiation induced in the tetanized pathway.

Several amino-acid receptors have been shown to be involved in the induction of LTP. In the hippocampus two distinct forms of LTP have been identified (for review see Nicoll and Malenka, 1995). One involves the activation of the NMDA receptors and leads to an elevation in post-synaptic calcium. The other form of LTP is found at mossy fibre synapses within the hippocampus and is independent of NMDA receptor activation but does require post-synaptic rise in calcium. In the NMDA receptor-dependent form of LTP, induction is dependent on the removal of the Mg^{2+} block from the NMDA receptor coupled ion channel (Bliss and Collingridge, 1993). Thus to trigger LTP the membrane must be sufficiently depolarised, following glutamate activation of α-amino-3-hydroxy-5-methyl-isoxazole (AMPA) receptors, which leads to the removal of the Mg^{2+} block. At the same time glutamate must bind to the NMDA-receptor complex to promote channel opening and allow post-synaptic calcium influx. The properties of co-operativity, associativity and input-specificity occur as a result of the Mg^{2+} block of NMDA channels. For example, weak stimuli that activate only a few afferent fibres fail to induce LTP because the depolarisation produced is not strong enough to remove the Mg^{2+} block. Similarly, associativity can be explained using the same principles except the depolarisation is provided by different afferent fibres. Conversely, input-specificity occurs as a result of the requirement of a particular set of presynaptic terminals with sufficient glutamate to activate the NMDA channel complex.

Calcium influx is an important part of the biochemical cascade of LTP (Nicoll *et al.*, 1988). Previous work has shown that post synaptic calcium influx via NMDA receptor-ion channels

is important for the induction of NMDA-dependent LTP (MacDermott *et al.*, 1986; Jahr and Stevens, 1987; Mayer and Westbrook, 1987). In contrast, the induction of NMDA receptor-independent LTP appears to be dependent on presynaptic calcium influx (see Nicoll and Malenka, 1995). Experimental evidence for the role of calcium in LTP has been shown by several groups. For example, it has already been shown that the induction of LTP can be blocked by intracellular injection of the calcium chelator ethylene glycol-bis[β-aminoether]-N,N,N',N'-tetraacetic acid (EGTA; Lynch *et al.*, 1983). Furthermore, imaging techniques have shown that tetanic stimulation elevates intracellular calcium concentration ($[Ca^{2+}]_i$) in dendritic spines (Regehr and Tank, 1990; Muller and Connor, 1991), partly through activation of synaptic NMDA receptors and also possibly via VSCC. In addition, imaging techniques have shown that the calcium which permeates through NMDA channels is augmented by release of intracellular stores of calcium since inhibitors of intracellular calcium release were found to reduce the calcium transient associated with NMDA receptor-activation (Alford and Collingridge, 1992). It has been proposed that calcium influx via VSCC and calcium permeable AMPA receptors (a subtype of glutamatergic receptors) may also be involved in LTP induction (Bliss and Collingridge, 1993), though evidence for VSCC participation in LTP induction is scarce. The arguments for the possibility of calcium influx occurring via VSCC are based on findings that the area of the hippocampus used for LTP studies, the dendrites of the CA1 pyramidal neurons, have L- and N-type VSCC (Stanton *et al.*, 1989; Westenbroek *et al.*, 1990). Furthermore, Grover and Teyler (1990) found that repetitive activation of L-type VSCC generates an enhancement that mimics LTP. Huang and Malenka (1993) have shown that NMDA receptor-activation can induce LTP, an effect which is not normally influenced by calcium entry via VSCC. However, increased $[Ca^{2+}]_i$ via calcium influx through VSCC has been shown to cause significant changes in synaptic efficacy in CA1 cells that may contribute to long-lasting changes in LTP (Grover and Teyler, 1990; Huang and Malenka, 1993). Although calcium is obviously a necessary factor for LTP it is still unclear whether it is necessary for actually inducing LTP. For example, elevation of intracellular calcium by either evoking calcium currents (Malenka *et al.*, 1989) or by slowly depleting intracellular stores (Harvey and Collingridge, 1992) did not induce LTP.

LTP is maintained in the absence of stimulation (Muller *et al.,* 1992) by mechanisms that include presynaptic glutamate release lasting for several hours (Bliss and Lynch, 1988). In addition, post-translational protein synthesis at the postsynaptic terminals has also been observed during LTP maintenance (Otani *et al.,* 1989).

Several calcium sensitive enzymes such as protease, calpain, phosphatases, phospholipases and kinases, are believed to play a part in converting the probable induction signal, the influx of calcium through NMDA channels, into persistent modifications of synaptic strength (see Bliss and Collingridge, 1993 for a review). For example, experiments have shown that the activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; Fukunaga *et al.,* 1993) and protein kinase C (PKC; Huang *et al.,* 1990) are increased 1-3 hours after the induction of LTP. Furthermore, in the first 1-3 hours after induction, LTP is blocked by selective inhibitors of CaMKII (Ito *et al.,* 1991) and protein kinase C (PKC; Colley and Routtenberg, 1993). Knockout of the gene encoding the αCaMKII isoform, which is heavily enriched in postsynaptic densities, severely impairs, though not always completely blocks, the ability of slices to exhibit LTP (see Bliss and Collingridge, 1993 for a review).

Expression of LTP in terms of increased post-synaptic response generated at potentiated synapses can occur as a result of several factors such as presynaptic modifications, postsynaptic modifications, extrasynaptic changes or morphological modifications. Several calcium-sensitive enzymes have been found to play a part in converting the probable induction signal, i.e. calcium entry through NMDA channels, into persistent modifications of synaptic strength. Experiments using agents that effect enzyme activity have highlighted the possible role of several enzymes in LTP expression. The enzymes involved in these modifications downstream of calcium influx are believed to be proteases, calpain, phosphates, phospholipases, and protein kinases (for review see Bliss and Collingridge, 1993). Kinases, for example, are thought to participate in post-synaptic modifications such as phosphorylation of AMPA receptor linked ion channels which can lead to alterations in the number and / or the properties of ion channels. Release of retrograde messengers may play a part in maintaining potentiated responses by signalling to presynaptic terminals. At present, arachidonic acid (AA) and nitric oxide (NO) have been suggested as candidate retrograde

messengers released during the expression of LTP (for review see Bliss and Collingridge, 1993). For example, Lynch and Voss (1991) had shown that arachidonic acid may be acting as a retrograde messenger. They found that arachidonic acid stimulated hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) in presynaptic terminals and also induced an increase in intrasynaptosomal calcium concentration in synaptosomes prepared from the dentate gyrus 45 minutes after the induction of LTP. It should be noted however that in their review, Bliss and Collingridge (1993) indicate that the problem with both NO and AA is that they do not act as rapid retrograde messengers which would be a necessary property of these messengers during LTP.

Apart from the involvement of calcium in transmitter release in presynaptic terminals and the activation of enzymes (i.e. kinases) in postsynaptic terminals, calcium may also be involved in presynaptic modifications during LTP resulting in persistent changes in transmitter release. Experimental evidence has shown that there is an elevation in $[Ca^{2+}]_i$ in synaptosomes prepared from potentiated dentate gyrus 45 minutes after induction (Lynch and Voss, 1991). It has been proposed that the increase in presynaptic $[Ca^{2+}]_i$ is related to calcium transients associated with each action potential following the induction of LTP. Alternatively LTP may be associated with an increase in the sensitivity to calcium of one or more components of the transmitter release mechanism (Lynch and Bliss, 1986).

1.6. Advantages of using chicks in learning and memory studies.

In the last 30 years the chick has become an attractive animal model for investigating the basis of learning and memory. The young chick model has several advantages over other vertebrate models (Rose, 1993). For example it has a well-organised behavioural repertoire due to it being a prococial animal. It has a large and well developed brain and a relatively high brain to body weight ratio. Chicks can be trained rapidly on a variety of tasks and tested over a range of times enabling the experimenter to study different stages of memory formation. Large numbers of chicks can be tested in a single run allowing the researcher to compare the effects of different agents, doses and times of administration within the same batch of subjects. The day-old chick has an unossified skull which makes it easy to carry out

intracerebral injections directed to a relatively specific area of the brain. The unossified skull also makes the removal of the brain a relatively simple and quick operation. It has a relatively simple blood-brain barrier allowing rapid diffusion of substances injected intraperitoneally (Rose, 1993). The chick can be used to study cerebral asymmetry in relation to learning and memory and also allows learning and memory studies to be conducted on intact animals.

Compared to the obvious advantages noted above the disadvantages of using the chick system are relatively few. However, one of the possible drawbacks of this system could be that specific memory-associated learning changes may be superimposed on changes which may occur naturally due to the young chick's rapid CNS development. Since the chicks, bought from a commercial hatchery, are not completely genetically homogenous, there are several factors that may vary between individuals. Biochemical variability is relatively, large, even in control birds both within and between hatches, where, for example relatively large differences can occur in enzyme activity or metabolic measures in supposedly identical preparations. This can be problematic when the difference in changes the experimenter is trying to determine in trained and control birds is small. This problem can be overcome by the use of large experimental numbers, care in experimental design, replicating measurements, interbatch standardisation and the use of appropriate controls which will allow the neuronal changes to be attributed to learning- and memory-related plasticity. Different strains have been used in different laboratories and it has been suggested that different strains may differ, to some degree, in their behavioural characteristics. Finally it is difficult to determine doses of drugs by i.c. injection because during the injection procedure some of the drugs leaks out rapidly through the needle track (Rose, 1991). Two commonly used chick learning tasks, imprinting (section 1.6.1) and passive avoidance learning (section 1.6.2) are described in more detail below .

1.6.1. Imprinting

Filial Imprinting describes a process in which an organism, during a sensitive period, learns to restrict its social preference to a specific class of objects (Bateson, 1966) and is a powerful form of learning and memory in the chick. It was originally thought that imprinting was a

unitary process (Lorenz, 1935). Subsequent studies on chicks have shown, however, that imprinting is divided into several types, each occurring at different stages in chick development. Filial imprinting is broadly divided into three types: visual, auditory and olfactory. Auditory and olfactory imprinting in the context of filial imprinting develops *in ovo* where the chick embryo demonstrates the ability to learn the hen's vocalisation and odour (Gottlieb, 1965; Tolhurst and Vince, 1976). Visual imprinting in the context of filial imprinting allows the young chick to imprint on the visual characteristics of the hen (Bateson, 1966). In addition to filial imprinting the young chick is also able undergo visual, auditory and olfactory imprinting for the surrounding area. This allows the chick to recognise its surroundings, such as its nest (Bateson, 1964). An area of the chick forebrain, the intermediate medial hyperstriatum ventrale (IMHV), has been shown to be involved in imprinting. Lesions to the IMHV were found to effect a chick's ability to imprint (McCabe *et al.,* 1982). Lesions studies pre- or post-training on an imprinting task had also shown that the IMHV may be important for both acquisition and retention for this task (McCabe *et al,* 1981; McCabe *et al,* 1982).

It soon became apparent that imprinting would be a useful process to help elucidate the mechanisms of learning and memory in the chick. Bateson *et al* (1972) demonstrated that visual imprinting resulted in the incorporation of radioactive uracil into the RNA of cells in the chick forebrain. Subsequent visual imprinting experiments by Bateson *et al.* (1975) had also shown that RNA synthesis was elevated in the IMHV following imprinting. Evidence for the involvement of the IMHV in imprinting was further strengthened by Kohsaka *et al.*(1979) who demonstrated an increase in 2-deoxyglucose (2-DG) uptake into the IMHV following imprinting. Horn *et al.* (1985) had observed asymmetrical neurochemical changes in the IMHV during imprinting. Furthermore, lesioning studies have also shown that the left IMHV is involved in both early and late phases of storage of imprinting memory, but that the right IMHV is only involved in the early storage of imprinting memory (Cipolla-Neto *et al.,* 1982). It has been postulated that the right IMHV may act as a buffer zone for holding memory before it is transferred to another region that may have a larger capacity for storage and perhaps an ability to modify and extend the memory through subsequent experiences, therefore giving greater flexibility for the memory to be used in a different context (Horn and

McCabe, 1990). Horn and Johnson (1989) had already postulated that the right IMHV, as in the mammalian hippocampus, may add 'depth' of memory processing by contributing information during learning.

Analysis of the cellular changes following imprinting is made difficult by the fact that imprinting is confounded by the effects of developmental changes, where for example, there is a relatively large increase in the length of post-synaptic densities of spine synapses in both left and right IMHV in the first days of life (Horn *et al*, 1985). Also, Rogers (1982) suggested that exposure to light alone may be enough to stimulate left hemisphere dominance in chicks. Thus, the possibility exists that changes detected in chicks following imprinting may simply occur as a result of accelerated neuronal maturation instead of acquisition and storage of particular information.

Imprinting also results in several biochemical changes. Strong imprinting in chicks is associated with an increase in NMDA receptor binding in the left IMHV which would indicate lateralized involvement of this sub-type of glutamate receptor in imprinting. This increase may be linked to long term memory formation as it was not detected until 9 hours post-training (Horn and McCabe, 1990; McCabe and Horn, 1991). Interestingly, recent work had shown that administration of glutamate on imprinting may be localised in the right hemisphere (Johnston and Rogers, 1992). Injection of glutamate into the right hemisphere at 1, 3 or 6 hours post-training blocked recall of the imprinting memory. However, similar treatments in the left hemisphere had no effect. At 9 hours after training Johnston and Rogers (1992) found that glutamate had no effect in either hemisphere. Therefore, temporarily elevating glutamatergic mechanisms in the left hemisphere has no significant effect on the encoding of imprinting memory, whereas treatment of the right hemisphere temporarily prevents recall of memory from either hemisphere. Johnston and Rogers concluded that, even though biochemical and morphological changes were observed in the left IMHV, other forebrain regions in the right hemisphere were also involved in recall during these early stages of memory formation.

The α-adrenergic system is also involved in imprinting. Experiments with imprinting a chick on a box resulted in an increase in the concentration of noradrenaline in the chick forebrain immediately after training, and this increase was detected up to 50 hours post-training (Davies *et al.*, 1983). The cholinergic system is also involved in imprinting (Longstaff and Rose, 1981), where concentration of enzymes responsible for the synthesis and breakdown of acetylcholine (ACh) were enhanced following imprinting. Thus, it is likely that there is a complex interrelationship between various neurotransmitter systems involved in imprinting memory formation.

1.6.2. Passive avoidance learning in the chick and stages of memory formation

A second model of learning in the chick uses the abilities of the young bird to learn a one-trial passive avoidance learning (PAL) paradigm. This task has particular advantages when used for general research into memory since it is non-time consuming, easily replicated and provides an accurate and yet simple learning model for investigating the molecular and cellular processes underlying formation and retention of vertebrate memories.

The paradigm developed by Cherkin (1969) is based on the observation that young chicks will spontaneously peck at brightly coloured objects in their field of view and are able to discriminate between aversive and non-aversive substances. Thus, if a chick is presented with a bead coated with an aversive substance such as methylanthranilate (MeA) the chick will peck the bead once and show a strong disgust response and subsequently avoid a similar, but dry, bead for at least 48 hours (Cherkin, 1969; Gibbs and Ng, 1977; Lossner and Rose, 1983). Memory for the task can be blocked by injection of potential amnestic drugs around the time of training or at later times which enables investigation of time-dependent biochemical, molecular and structural changes associated with memory formation.

Dissection of the time course of the biochemical, morphological and molecular changes underlying PAL are perhaps better understood than similar changes following imprinting.

Gibbs and Ng (1977) proposed a three-stage linear model of memory formation following PAL which are separated by transient retention deficits occurring at around 15 and 55 minutes post-training. The three stages are: a short-term memory (STM) phase, a labile, intermediate-term (ITM) memory phase, and a long-term memory (LTM) phase. Thus, Gibbs and Ng proposed that memory for the avoidance task 'moved' through these stages and that if the strength of the stimulus was strong enough the memory trace would be stored for a long-time i.e. LTM.

STM appears to be fully developed within 5 minutes of learning and decays after 10 minutes. It is suggested that this phase of memory formation involves hyperpolarisation associated with changes in K^+ conductance following neural activity. It may also be associated with a reduction in γ-aminobutyric acid (GABA) -mediated inhibition (Clements and Bourne, 1996). ITM is sub-divided into A and B phases which are distinguished according to the susceptibility of ITM(A) but not ITM(B) to inhibition by the uncoupler of oxidative metabolism 2,4-dinitrophenol (Gibbs and Ng, 1984). ITM(A) develops fully within 15 minutes and transfers to ITM(B) at 30 minutes, which decays by 55 minutes post-training at which point it may trigger LTM formation. It has been suggested that ITM involves a phase of hyperpolarization associated with sodium pump activity. LTM develops by 60 minutes post-training and is dependent on an intact ITM and protein synthesis and can last up to 48 hours post-training on the single-trial PAL task.

It should be noted that memory formation is not purely a linear process as suggested by Gibbs and Ng's model (1977). I discussed in section 1.3 earlier observations that different stages of memory formation do 'overlap' (McGaugh, 1996). It is most likely that different stages of memory formation occur in some mechanism that involves a combination of serial and parallel processes.

Recently, Burne and Rose (1997) proposed that differences in training on the weak PAL task (birds tested on beads coated in 10% MeA), but not the strong version (birds tested on beads coated in 100% MeA) of the learning task, can lead to differences in the time-course of memory formation. This difference was found to be dependent on the colour of the training

bead, the sequence of presentation and the form of pre-training trials. Thus, from Gibb's and Ng's laboratory avoidance for a bitter tasting bead was found to persist for less then 30 minutes post-training (Crowe *et al.*, 1990, 1991). However, in Rose's laboratory, avoidance was found to persist for up to 4-6 hours (Sandi and Rose, 1994; Sandi *et al.*, 1995) post-training.

1.6.3. Biochemistry, pharmacology and morphology of learning and memory formation in day-old chicks following passive avoidance learning

Formation of memory for the one-trial passive avoidance task (PAL) is associated with a cascade of time-dependent electrophysiological (Mason and Rose, 1987), biochemical and morphological events in specialised regions of the chick forebrain particularly the IMHV and LPO. The initial changes have been detected primarily in the left IMHV (Rose and Csillag, 1985) but there is a rapid redistribution to other regions, particularly the right IMHV and both hemispheres of the LPO (Patterson *et al*, 1990a). The early changes following PAL are transient and the fact that pharmacological manipulation of certain pathways at around the time of training may induce amnesia at later times indicates that these processes may be more essential for laying down long-term memory for the learning task. The early transient changes in the biochemical cascade that occur at around 30 minutes post-training include calcium influx possibly via N-type calcium channels (Clements and Rose, 1993 ; Clements *et al*, 1995), decrease in $GABA_A$ receptor-modulated synaptic inhibition (Clements and Bourne; 1996) preceding the increase in calcium-dependent release of intracellular glutamate (Daisley and Rose, 1998), translocation of cytosolic protein kinase C (PKC) to pre-synaptic membranes resulting in the phosphorylation of the membrane bound protein B50 (Ali *et al*, 1988 ; Burchuladze *et al*, 1990) and up-regulation of post-synaptic NMDA receptor activity (Stewart *et al*, 1992) in the left IMHV, where both phosphorylation and NMDA receptor upregulation have been shown to decline to control levels within 3 hours post-training. Pre-training injection of the NMDA antagonist, MK-801, induced amnesia in chicks tested 3 or 24 hours post-training (Burchuladze and Rose, 1992). Also the NMDA receptor glycine-binding regulatory site antagonist, 7-chlorokynurenate, produces amnesia for the task when injected pre- or soon after training (Steele and Stewart, 1993). Non-NMDA glutamate

receptors have also been shown to be involved in the consolidation processes for the task, where experiments using both NMDA and non-NMDA receptor antagonists induced amnesia in chicks tested 90 minutes post-training (Rickard *et al.,* 1994). AP5 (competitive NMDA receptor antagonist) was effective when administered pre - or around the time of training and 6,7-Dintroquinoxaline-2,3-dione (DNQX; non-NMDA receptor antagonist) was effective when administered 25 minutes post-training. Protein kinase C (PKC) has been implicated as having a functional role in the Gibbs and Ng (1977) ITM stage of memory formation (Zhao *et al,* 1994), so PKC may be essential for the transition from ITM(A) to ITM(B).

Apart from anterograde signalling from pre- to postsynaptic terminals, retrograde signalling from post- to presynaptic terminals has also been measured in the biochemical cascade associated with PAL. Transmitters that act in this manner are called retrograde messengers. It has been shown that the putative retrograde messenger NO is released immediately post-training, since pre-training injection of nitroarginine, an NO-synthase inhibitor, results in amnesia for the avoidance task (Holscher and Rose, 1993). Arachidonic acid, another putative retrograde messenger, is released between 30 and 75 minutes post-training in the left IMHV (Clements and Rose, 1996) possibly due to an increase in phospholipase A_2 (PLA_2) activity since pre-training injection of nordihydroguaiaretic acid (NDGA; a PLA_2 and lipoxygenase inhibitor) and aristolochic acid (a PLA_2 inhibitor) resulted in amnesia for the avoidance task with an onset time of 1.25 hours post-training (Holscher and Rose, 1994).

One of the intermediate stages of the biochemical cascade following passive avoidance learning in the chick brain is the activation of immediate early genes (IEG), c-fos and c-jun (Anokhin and Rose, 1991a). Activation of *c-fos* and *c-jun* were shown to occur following NMDA receptor activation in the left IMHV of M-birds 1 hour after training (Freeman and Rose, 1995). Most IEG's encode nuclear proteins that are thought to act as transcription factors, activating late gene expression (Angel *et al.,* 1988). Thus it is suggested that following PAL expression of IEG's leads to activation of late structural genes finally resulting in synthesis of structural proteins such as *de novo* glycoprotein synthesis (Rose, 1995). It should be noted, however, that the activation of these early genes does not necessarily lead to synthesis of structural genes. The increased expression of IEG's has been

suggested to be pivotal in the transition between relatively short-term synaptic events and the longer term protein synthesis, which subsequently results in synaptic remodelling that is assumed to underlie long-term memory (for a review see Rose, 1995). PAL is associated with two distinct time windows of protein synthesis. The first occurs within 1 hour of training and involves glycosylation of pre-existing proteins. The second 'wave' of protein synthesis occurs 5.5 - 8 hours post-training and involves *de novo* protein synthesis followed by glycosylation (Freeman *et al.,* 1995). To date, two types of glycoproteins essential for memory formation for the passive avoidance task have been identified, L1 and the neural cell adhesion molecule (NCAM), both of which are members of the family of cell adhesion molecules (Scholey *et al.,* 1995; Mileusnic *et al.,* 1995). Synthesis of these, and possibly of other, cell adhesion molecules may allow for the formation of new synaptic connections or for the strengthening of old synaptic connections which are thought to underlie the encoding of newly acquired information for the storage of memory (Rose, 1995). Corticosteroids have also been shown to participate in long-term memory formation in the IMHV of the chick. Loscertales *et al.* (1997) had shown that pretraining peripheral administration of corticosteroid inhibitors induced amnesia in chicks in a dose-dependent manner when tested 24 hours after training. Furthermore, the facilitation of corticosterone on long-term memory for the PAL task was found to be the result of glycoprotein synthesis and fucosylation occurring at around 5.5 to 8.5 hours after training (Sandi and Rose, 1997).

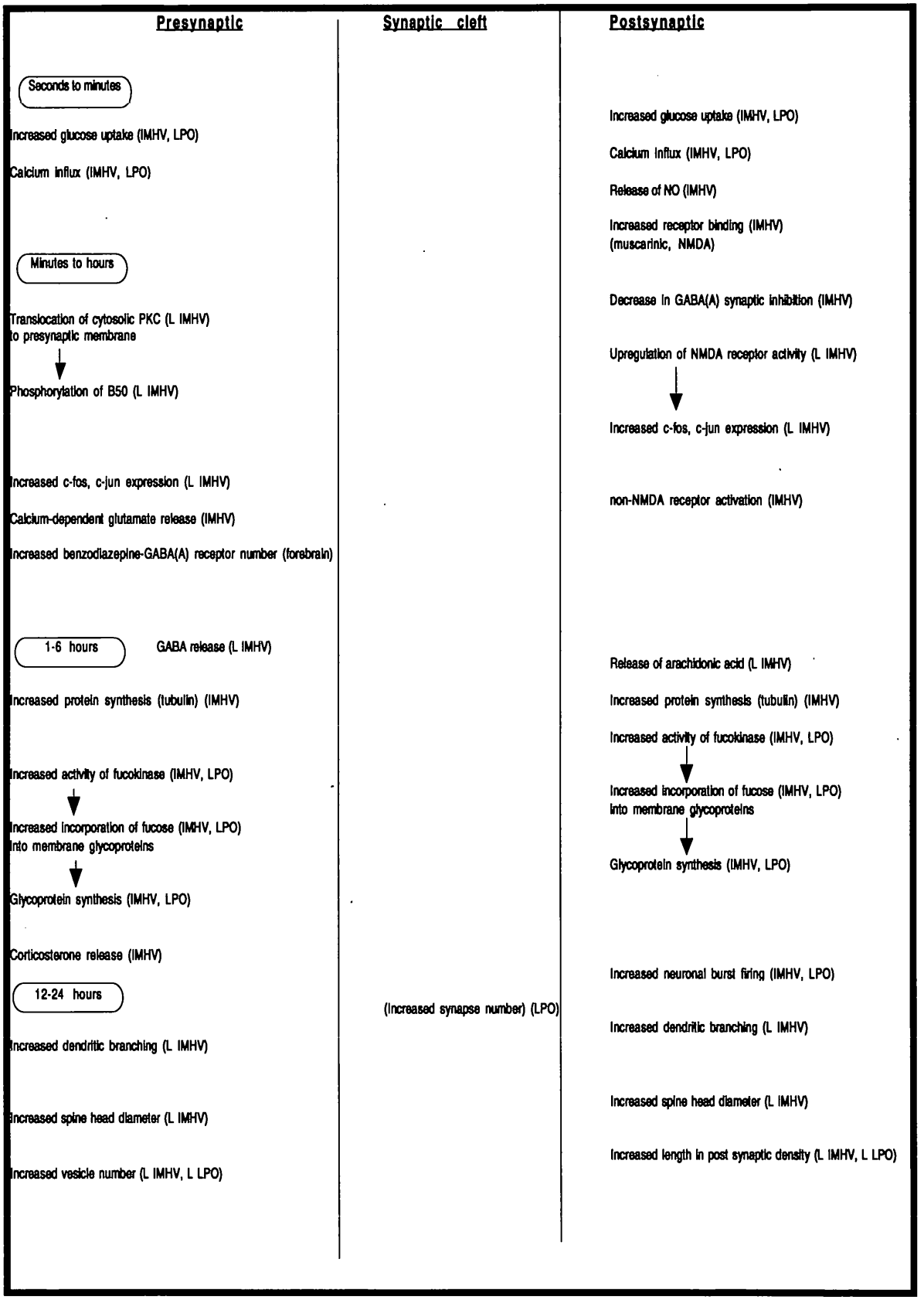
Pharmacological experiments have indicated that the LPO may be involved in the ITM stage of the Gibbs and Ng (1977) three stage model of memory formation following passive avoidance learning. Bilateral administration of ouabain and scopolamine, agents that were found to interfere with ITM when injected bilaterally into the IMHV, also induced amnesia in chicks when administered into the LPO (Serrano *et al,* 1988). Conversely amnestic agents that were found to interfere with STM and LTM when injected into the IMHV did not induce amnesia when administered into the LPO (Serrano *et al,* 1992). Furthermore, bilateral ablation of the LPO 5 minutes before training did not effect avoidance learning in chicks. However, bilateral ablations following training did induce amnesia in chicks (Gilbert *et al,* 1991) thus suggesting the involvement of the LPO in retention and recall but not acquisition of the passive avoidance task.

Morphological studies in birds tested 24 hours post-training had shown significant increases in spine density on large multipolar neurones of all orders in the left LPO of M-birds compared to W-birds. Furthermore, significant increase in spine density was also measured on the 4th and 5th orders branches of the right LPO (Lowndes and Stewart, 1994).

Although studies have shown that the IMHV and LPO are involved in memory formation for the passive avoidance task, anatomical studies have not been able to demonstrate a direct (monosynaptic) connection between them. However, studies have identified a possible connection between the IMHV and LPO via the Archistriatum (Arch; for a review see Stewart 1991). Further support for the participation of Arch in passive avoidance learning was observed following lesions experiments, where pre-training bilateral lesions of the Arch impaired acquisition for the avoidance task (Lowndes and Davies, 1994). Thus the Arch may have a role either on its own for processing information or act as a mediator between the IMHV and LPO following avoidance learning. More recently Csillag *et al.* (1997) found, predominantly, excitatory synaptic connections between the Arch and the LPO indicating possible 'transfer' of information between these areas following avoidance training.

A diagrammatic representation of the biochemical and morphological events that can occur in synaptic terminals in chicks following PAL are shown in figure 1.

Figure 1 Diagrammatic representation of the synaptic cascade in the IMHV and LPO. L = Left, R = Right.



1.6.4. Comparing imprinting and passive avoidance learning

Biochemical and anatomical comparisons between filial imprinting and PAL in the day-old chick for the aversive substance MeA have shown that there are many similarities between the two systems. For example, the IMHV and possibly the LPO are involved in processing learning associated changes for both paradigms (Rose, 1995; Rogers, 1995). There appears to be an asymmetrical distribution of the memory traces in both paradigms, and many of the neurochemical changes underlying the tasks are also similar.

Rogers (1995) speculates that even though imprinting and passive avoidance learning are events that occur early in life they may have more in common with neuroplasticity and changes that are involved in forms of learning that occur in later life.

In his review, Rose (1991), suggests that PAL was a better model for studying memory formation than imprinting, because with PAL the experimenter was able to get precision of timing. In addition, PAL only required one trial for training which resulted in memory formation, the pecking of the bead can be observed and timed, thus allowing the experimenter to dissociate the training experience from the biochemical processes that occur during memory formation when the stimulus is no longer present.

1.7. Voltage sensitive calcium channels

Calcium is required for the function of all cells in the body and extensive studies of neurons have already shown that calcium plays a part in a variety of neuronal functions such as neurotransmitter release, activation of enzyme systems such as kinases and phosphatases and in the cytoarchitecture (Disterhoft et al., 1993). Thus it is not suprising that evidence has accumulated showing that calcium also participates in a variety of 'plastic' changes in the brain, such as during adaptive processes, i.e. learning and development, where changes in transmembrane calcium fluxes have been correlated with changes in neuronal excitability and structural connectivity. For calcium ions to play a part in the cellular biochemical cascade following learning and memory it has to enter the cytosolic medium of the cell either from the

extracellular medium or be released from intracellular stores such as calciosomes, endoplasmic reticulum (E.R.) mitochondria and from calcium binding proteins such as calmodulin, calbindin or paravalbumin. There are at least three major sources of transmembrane calcium influx; (1) VSCC (Augustine *et al.,* 1987), (2) ligand-gated calcium channels (calcium channels coupled to neurotransmitter receptors such as the NMDA-receptor complex; Miller, 1987), and (3) activation of the sodium/calcium exchanger (Rang and Dale, 1991). The work described in this thesis is concerned with calcium influx via VSCC in isolated nerve terminals (synaptosomes and synaptoneurosomes) prepared from two forebrain regions, the IMHV and LPO, of day-old chicks and the relevance of this system following learning and memory formation for the passive avoidance task.

VSCC are found in a variety of excitable cells in vertebrates, invertebrates and even plants. Ion channels, including VSCC, are classified according to their activation and inactivation kinetics, their conductance, their ion specificity and their selectivity to drugs and toxins. Pharmacological and electrophysiological studies (eg. Zhang *et al.,* 1993; Bowman *et al.,* 1993) have shown that there are a variety of subtypes of VSCC which can co-exist on a single neuron. Initial electrophysiological analysis divided these channels into high voltage activated (HVA) channels and low voltage activated (LVA) channels (Dolphin, 1995). LVA channels are activated by small depolarisations and show rapid voltage-dependent inactivation. HVA channels on the other hand are activated by large depolarisations. The classification of VSCC according to electrophysiological and pharmacological studies are shown in Table 1. At present at least six subtypes have been identified: L-type channels (sensitive to 1,4-dihydropyridines), N-type (sensitive to ω-conotoxin GVIA), P-type (sensitive to ω-agatoxin IVA), T-type (no specific blocker but can be blocked by Nickel, amiloride), Q-type (no specific blocker but sensitive to ω-conotoxin MVIIC and high concentration of ω-Aga-IVA) and R-type (no inhibitors known). Electrophysiological studies have also shown that the L-, N- and P-type channels are HVA channels and the Q- and T-types are LVA channels. R-type channels exhibit both HVA and LVA like properties. It is evident from table 1 that electrophysiologically distinct calcium currents can be blocked by an antagonist which is specific for another (i.e. many calcium channels blockers are nonselective). For example the Q-type channel, which has no selective blockers, is blocked

by the 'specific' P-type channel blocker, thus indicating the existence of cross-reactivity of some channel blockers. Also Zhang *et al.*(1993) using cloned and expressed VSCC had found that selective blockers do not necessarily cause complete block in channel activity but did cause a partial inhibition. I have given a more detailed description of the VSCC's in individual subsections (1.7.1 to 1.7.6).

Electrophysiological analysis of the cell bodies of intact neurons have shown that calcium currents are composed of a mixture of rapidly inactivating and non-inactivating components (Fox *et al.*, 1987; Schroder *et al.*, 1990; Sher and Clementi, 1991). Equivalent biochemical studies using synaptosomes have shown that following depolarisation with potassium chloride (KCl) there is a biphasic influx of calcium, where there is a rapid influx immediately after depolarisation which declines to a sustained non-inactivating plateau (Nacshen and Blaustein, 1980; Adam-Vizi and Ashley, 1987; Tibbs *et al.*, 1989). Several groups have suggested that the rapidly activating and non-inactivating VSCC may be coupled to the biphasic calcium influx respectively (Leslie *et al.*, 1983; Nachsen, 1985; Wang *et al.*, 1985; Suszkiw *et al.*, 1986) and it has also been suggested that the rapidly inactivating component of calcium influx may be coupled to neurotransmitter release (Daniell and Lesley, 1986; Adam-Vizi and Ashley, 1987). The plateau, non-inactivating, phase is not as well characterised, however, McMahon and Nicholls (1991) have shown that this phase triggers exocytotic release of glutamate.

Pharmacological characterisation of the rapidly inactivating phase of calcium influx in chicken and rat brain synaptosomes using VSCC blockers have shown large species differences in channel function (Rivier *et al.*, 1987; Suszkiw *et al.*, 1987; Lundy *et al.*, 1991; Scheer, 1990). In chicken synaptosomes the specific N-type blocker, ω-conotoxin GVIA (ω-CTX GVIA), completely blocked potassium stimulated calcium influx for the inactivating phase but only partially inhibited this phase of calcium influx in the rat synaptosomes. This shows that the N-type channel has a dominant role in the inactivating phase of calcium influx in the chicken but not the rat brain. However, in rat synaptosomes this component of calcium influx is sensitive to block by the selective P-type blocker ω-agatoxin-IVA (ω-Aga-IVA; Mintz *et al.*, 1992a).

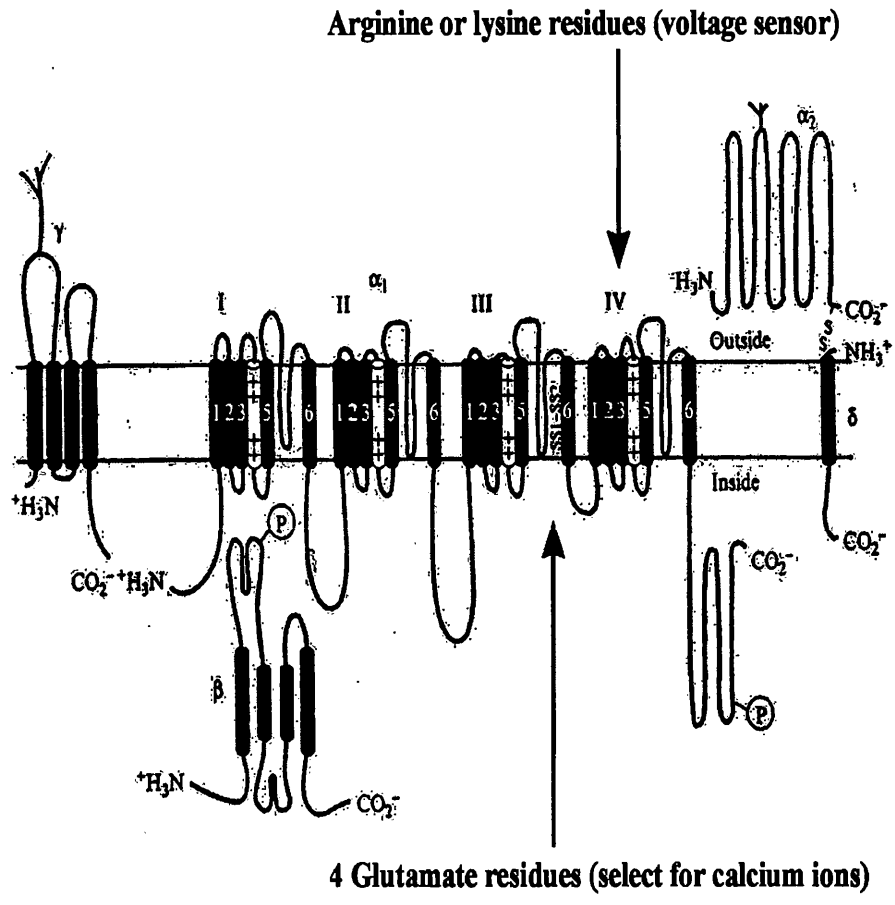
Table 1: Properties of calcium currents from electrophysiological and pharmacological studies (Dolphin, 1995)

Name	Type	Voltage-dependent inactivation during step	Steady-state inactivation V_{50} (mV)	Single-channel conductance (pS)	Pharmacology selective blockers
L	HVA	None (Ca^{2+}-dependent)	-20	24	DHP antagonist
N	HVA	Intermediate	-50	13-20	ω-CTX-GVIA
P	HVA	None	-5	10-18	ω-Aga-IVA
Q	HVA	Intermediate	-45	?	No specific blockers: ω-Aga IVA (>100nM) ω-CTX-MVIIC (also N,P)
R	H/LVA	Fast (τ=20-30 ms)	-15	?	None known
T	LVA	Fast (τ=20-40 ms)	-70	8	No specific blocker: Ni^{2+}, amiloride, phenytoin

Voltage-dependent inactivation during step describes the rate of loss of single channel activity during changes in voltage across channel (τ = time constant of inactivation). Steady-state inactivation V_{50} describes the voltage required to inactivate 50 percent of particular calcium current. *Single-channel conductance* describes the rate calcium ions pass through the channels. *Pharmacology selective blockers* describes drugs which inhibit particular channels.

A variety of techniques have been used to determine the structure and function of VSCC. Chemicals that selectively bind to certain subtypes of channels have been particularly useful in purifying channels. For example the dihydropyridines (DHP), a group of specific blockers of L-type VSCC, and ω-CTX GVIA, a specific blocker of N-type channels, have been used extensively to characterise L- and N-type VSCC respectively (Catterall *et al.*, 1988; McEnery *et al.*, 1991; Witcher *et al.*, 1993a and 1993b). Purification has shown that these channels can be made up of several subunits which have been called, α_1, α_2, β, δ and γ. Analysis of these subunits have shown that there is variation between subtypes and also within subtypes depending on tissue studied (Dolphin, 1995). Figure 2 shows a diagrammatic representation of the putative structure of the cloned VSCC subunits.

Figure 2 Diagrammatic representation of the subunits of the VSCC (taken from Dolphin, 1995)



The α_1 subunit is present in all VSCC and is the primary pore-forming protein (Flockerzi *et al.*, 1986). cDNA clones of skeletal muscle L-type VSCC have shown that the α_1 subunit consists of 1873 amino acids containing 24 α-helical transmembrane segments arranged as 4 repeat domains termed I, II, III and IV linked by intracellular loops (Tanabe *et al.*, 1987). Homology cloning in various tissues has highlighted the existence of additional subtypes of α_1 subunits. They share the same basic domain structure as shown in figure 2 but can vary in tissue distribution, molecular mass and sensitivity to blockers (see Dolphin 1995 for review) and have been labelled A, B, C, D and E (Snutch *et al.*, 1991). C and B subtypes are the best characterised and encode for the DHP-sensitive L-type and ω-CTX GVIA sensitive N-type calcium channels, respectively (Dubel *et al.*, 1992; Witcher *et al.*, 1993b). Functional studies have shown that the 4th transmembrane segment (S4) in each domain always has a cluster of

regularly spaced positively charged arginine or lysine residues which may be acting as the voltage-sensor of the channel (Dolphin, 1995). Evidence for the involvement of these amino acids as voltage-sensors comes from previous experiments on potassium (K^+) and sodium (Na^+) channels. Mutations of one or more of these charged amino acids caused a change in voltage-dependence of currents for these mutated channels. These experiments showed that there was co-operativity between domains so that the charges do not move independently of each other (Catterall, 1993). There is an extracellular hair-pin loop between S5 and S6, in all domains, which bends back into the membrane possibly forming the lining of the pore channel (Heinemann *et al.*, 1992). Binding studies also indicate that the transmembrane helices IIIS6 and IVS6 and the S5/S6 loop are located near each other in the folded structure of the α_1-subunit (Striessnig *et al.*, 1991). Four glutamate residues have been found in the region of the hair-pin loop believed to form part of the pore lining (region ss1-ss2). The glutamate residues may play a part in channel selectivity for calcium ions since mutation of any of the glutamate residues altered the channels' ability to select for calcium but did not effect voltage-dependent gating of the channel for monovalent ions (Kim *et al.*, 1993; Mikala *et al.*, 1993; Sather *et al.*, 1993; Yang and Tsien, 1993; Tang *et al.*, 1993; Yang *et al.*, 1993). Wei *et al.* (1994) have shown that deletion of the C-terminal of the cardiac α_1-subunit increased current opening, indicating a role for this subunit in controlling the probability of channel opening. In addition, the calcium-binding domain of the C-terminal may be responsible for calcium-dependent inactivation (Barbitch *et al.*, 1990). Phosphorylation studies have shown that *in vitro* and in intact tissues both α_1- and β-subunits have several sites that can be phosphorylated by cAMP-dependent protein kinases (Dolphin, 1995).

The other subunits (α_2,β, δ and γ) have a modulatory effect on the α_1-subunit. The β-subunit from the skeletal muscle was the first subunit of this type to be cloned and was found to have a deduced molecular weight of 58 KDa (Ruth *et al.*, 1989). It does not contain any transmembrane segments but consists of 4 α-helical domains, 3 of which contain 4 heptad repeat structures, similar to cytoskeletal proteins. It has been hypothesised that the β-subunit links the α_1-subunit to the cytoskeleton whereby the β-subunit binds to the α_1-subunit on the intracellular loop between domain I and II (Pragnell *et al.*, 1991). Cloning studies have revealed the existence of a wide variety of β–subunit in different tissues. For example, β_{1a} are

prevalent in skeletal muscle (Ruth *et al.*, 1989) and β_{1B} and β_4 is prevalent in the brain (Pragnell *et al.*, 1991; Castellano *et al.*, 1993). Functional studies have shown that co-expression of β subunits with α_1 subunits, in oocytes or cell lines, results in increased current expression. This is usually linked to a shift in voltage dependence of activation of the cell to a more hyperpolarised potential (Neely *et al.*, 1993; Stea *et al.*, 1993) possibly by increased coupling between the movement of gating charge in the α-subunit and channel opening. Co-expression of any of the β-subunits with different α_1 subunits can enhance the calcium current (Castellano *et al.*, 1993) and different β-subunits were found to produce currents with different degrees of inactivation. For example, the β_3 subunits produced the greatest degree of inactivation (Zhang *et al.*, 1993). Following observations that the β-subunits modify calcium currents, it has been suggested that these subunits may be generally important in determining the kinetics of inactivation of native channels (Dolphin, 1995). Immunostaining in rat cultured neurons using antipeptide antibodies with sequences common to most β-subunits have shown greatest immunostaining at the inner surface of the plasma membrane with some cytoplasmic staining. This was taken to show that the subunit is present on the inner membrane surface (Berrow *et al.*, 1995). The degree of staining decreased significantly when cultured cells were injected with antisense DNA oligonucleotides with a complementary sequence to the β-subunits. Parallel electrophysiological experiments also showed a decrease in whole-cell current amplitude and a shift of voltage dependence of activation to a more depolarised potential.

The α_2- δ-subunits has a molecular weight of 125 KDa (Ellis *et al.*, 1988). The δ-subunit is identical to the C-terminal of the α_2 cDNA indicating that the α_2 and δ-subunits are the product of the same gene and are produced by post-translational processing (De Jong *et al.*, 1990). It has been proposed that the α_2-subunit is located in the extracellular domain and is linked to the transmembrane δ-subunit by a disulphide bridge (Jay *et al.*, 1991). The α_2-subunit has phosphorylation sites similar to α_1- and β-subunits however, unlike the α_1- and β-subunits, the α_2-subunit is not phosphorylated under physiological conditions (Dolphin, 1995). Most tissues appear to have the same α_2-δ protein although a splice variant has been found in the rat brain (Kim *et al.*, 1993). The finding that the α_2- δ protein is conserved in

most tissues may indicate an important and large scale function for this protein in the biological systems, thus, resulting in conservatism of structure.

The γ-subunit has a molecular weight of 25 KDa. It contains 4 putative transmembrane domains and appears to be only present in skeletal muscle (Dolphin, 1995).

1.7.1. L-type calcium channels

L-type VSCC are widely distributed in a number of tissues such as the heart, smooth muscle, skeletal muscle and neurons (Speeding and Paoletti, 1992). In neurons, L-type channels are located preferentially on the soma, dendrites, and presynaptic terminals, usually in clusters in the membrane (Atchison and O'Leary, 1987; Niesen *et al.*, 1987). At present there is no definitive evidence for their role in neurotransmitter release (Nachshen and Blaustein, 1980; Miller, 1987). Skeletal L-type channels are the most widely studied of the VSCC, and molecular analysis has shown that they consist of all the subunits, α_1, α_2, β, δ and γ (Dolphin, 1995). It should be noted, however, that the γ -subunit has been found only in skeletal muscle L-type channels.

Voltage-gated L-type channels can be distinguished from the other types of calcium channels by their sensitivity to dihydropyridines (DHP) and other organic calcium channel modulators, such as phenylamines and benzothiazepines (Glossman and Striessnig, 1990). The binding sites of the drugs are localised exclusively on the channel-forming α_1-subunit. DHP binding sites have been found to be accessible only from the extracellular side of the membrane (Kass *et al.*, 1991). Detailed photolabelling analysis of the DHP binding domain using the radiolabelled DHP molecules [^{3}H](+)-PN200-110 and [^{3}H]-azidopine, molecules which correspond approximately to the S5/S6 loop, transmembrane helix S6 of domain III and transmembrane helix S6 of domain IV, have shown that the DHP binding centre is closely associated with the transmembrane IIIS6 region and adjacent residues, which are preferentially labelled by [^{3}H](+)-PN200-110 (Striessnig *et al.*, 1991). The S5/S6 loop of domain III may be the primary site of interactions with the long side chains of azidopine-like substituted DHP such as diazipine (Glossman and Striessnig, 1990). The model proposed by

Striessnig *et al.* (1991) suggests that the extracellular end of transmembrane helices IIIS6 and IVS6, and the adjacent residues, are involved in forming the DHP binding site. Experimental evidence indicates that the β-subunit modulates DHP binding to the α_1-subunit since a number of co-expression studies have shown that the expression of β-subunits increased the number of DHP receptors, although it has not been found that this is not due to increased expression of the α_1-subunit protein (Neely *et al.*, 1993). More recently Berrow *et al.* (1995) found that the DHP agonist was less effective in promoting calcium currents following β-antisense oligonucleotide injection.

Homology cloning of different tissues for L-type channels have revealed the existence of 4 additional subtypes of α_1-subunits which all have the same basic domain structure shown in figure 2 (Hui *et al.*, 1991; Starr *et al.*, 1991; Dubel *et al.*, 1992). Two distinct clones which code for DHP-sensitive channels, have been termed C and D (Snutch and Reiner, 1992). The C-class is found predominantly in cardiac tissue and in smaller amount in other tissues such as the brain. The D-class has 70% homology with the C-class clones but it is more prevalent in the brain, neuroendocrine tissue and pancreatic islets. Williams *et al.* (1992) found that the α_1-subunit of the D-class channels are reversibly inhibited by the N-type channel blocker ω-CTX-GVIA. The inhibition of the D-class by ω-CTX GVIA supports previous suggestion by Fox *et al.* (1987) that this supposedly selective N-type blocker was able to block both L- and N-channels and also accounts for the reversible inhibition of the antagonist in several tissues including cerebellar granule neurons (Pearson *et al.*, 1993).

1.7.2. N-type calcium channels

The N-type channel is a high threshold VSCC and is distinguished by its sensitivity to the neurotoxin ω-CTX GVIA, which is a potent and selective inhibitor of the N-type VSCC (Nowycky *et al.*, 1985). The toxin is extracted from the venom of the marine-snail *Conus geographus*. It is one of the best characterised of the family of peptide toxins and it consists of 27 amino acids with 3 disulphide bonds (Olivera *et al.*, 1984).

The N-type channel is somewhat homologous to cardiac L-type channels consisting of 3 tightly associated subunits α_1, α_2–δ and β which co-purify with a 95-100 KDa subunit which has yet to be identified (Witcher *et al.*, 1993a; Witcher *et al.*, 1993b). Cloning studies of the α_1-subunits have shown that the neuron specific B-type clone (α_{1B}) is specific for N-type channels since it was irreversibly blocked by ω-CTX GVIA when expressed in oocytes (Dubel *et al.*, 1992). The N-type channel has been shown by some groups to co-purify with various other synaptic proteins such as synaptotagmin, syntaxin and HPC-1 (Leveque *et al.*, 1992; Leveque *et al.*, 1994), and it has been proposed that synaptotagmin may link the N-type channel to the secretory apparatus associated with synaptic vesicles. Purification and immunoprecipitation studies have shown that the N-type, ω-CTX GVIA-sensitive channels consist of the β_3 subtype (Witcher *et al.*, 1993b).

The N-type channels are thought to be primarily responsible for regulating calcium influx linked neurotransmitter release in avian neurons (Feldman *et al.*, 1987) and synaptosomes (Lundy *et al.*, 1989). Comparisons between species has shown that 0.3μM ω-CTX GVIA was most potent in inhibiting the stimulated increase in $[Ca^{2+}]_i$ in the frog and chick brain, but only partially inhibited increase in $[Ca^{2+}]_i$ in the rat brain thus possibly indicating a large population of N-type channels in the frog and chick brain (Susckiw *et al.*, 1987). One of the earliest pieces of evidence showing that N-type channels may be linked to neurotransmitter release was shown using fluorescently-tagged ω-CTX GVIA. In these experiments increased binding of ω-CTX GVIA was detected in pre-synaptic terminals, particularly in the active zone of frog neuromuscular synapse (Robitaille *et al.*, 1990). ω-CTX GVIA also blocked the control and increased amplitude end-plate potential (EPP) in frog-neuromuscular junction, an effect which was specific to the facilitation and augmentation components of stimulation-induced increase in transmitter release (Zengel *et al.*, 1993). There is evidence that N-type, but not L-type, channels are involved in synaptic transmission in rat hippocampal CA3 and CA1 regions (Kamayia *et al.*, 1988; Dutar *et al.*, 1989; O'Reagan *et al.*, 1991; Rascol *et al.*, 1991), since ω-CTX-GVIA but not dihydropyridines blocked synaptic transmission. This was taken as further evidence for the involvement of N-type channels in transmitter release. Even though ω-CTX-GVIA is specific for N-type channels it has been shown to inhibit both N- and L-type VSCC in the dorsal root ganglion neurons (McCleskey *et al.*, 1987).

However, in the CA3 and CA1 regions ω-CTX-GVIA did not appear to have any effect on L-type channels (Rijnhout *et al.,* 1990). More detailed analyses in rat hippocampal CA1 neurons has shown that ω-CTX GVIA partially inhibited excitatory post-synaptic potential (EPSP) but totally blocked the inhibitory post-synaptic potential (IPSP; Potier *et al.,* 1993). This observation lead Potier and co-workers to conclude that N-type channels are closely linked to controlling the release of inhibitory neurotransmitters. Furthermore, these authors found that ω-CTX GVIA blocked excitatory synaptic transmission by mechanisms independent of a Pertussis toxin (PTX)-sensitive G-protein. On the other hand ω-CTX GVIA blocked the inhibitory synaptic transmission by mechanisms dependent on PTX-sensitive G-proteins. It should be noted however, that other subtypes of VSCC other than the N-type channels may also be responsible for controlling release of excitatory amino-acids via a system not sensitive to the effects of the G-protein inhibitor PTX (Potier *et al.,* 1993).

Recently, the non-selective Q- , L- and N-channel blocker ω-conotoxin MVIIC (ω-CTX MVIIC), a 26 amino acid peptide that is naturally found in the venom of the cone snail, *Conus magus* (Hillyard *et al.* 1992) and completely blocks P-type channels in purkinje cells, was also shown to reversibly inhibit cloned α_{1B} (N-type) channels expressed in HEK 293 cells (Grantham *et al.,* 1994). Conversely the N-type specific blocker, ω-CTX GVIA, irreversibly inhibited the channel. ω-CTX MVIIC was observed to have a greater rate of inhibition than ω-CTX GVIA in these cells. This is in agreement with previous work by Hillyard *et al.,* (1992) who found that ω-CTX MVIIC displaced ^{125}I- ω-CTX GVIA binding in rat brain membranes and that the non-specific blocker also attenuated the inhibitory effect of ω-CTX GVIA on calcium-currents in hippocampal neurons.

1.7.3. P-type calcium channels

The P-type VSCC was first described by Llinas *et al.* (1989). Llinas and co-workers observed that the HVA calcium currents recorded in cerebellar purkinje cells were insensitive to both ω-CTX GVIA and DHP's but were very sensitive to funnel web-toxin (FTX), a low molecular weight toxin fraction purified from the venom of the funnel web spider

(*Agelenopsis aperta*), and to ω-agatoxin IVA (ω-Aga IVA), a peptide toxin purified from the same funnel Web spider venom (Mintz *et al.*, 1992a). The electrophysiological parameters of P-type channels are very closely related to N-type channels (Llinas *et al.*, 1989).

Little is known about the specific structure of P type channels, and the identity of the corresponding P-channel identified, electrophysiologically, following inhibition with ω-Aga IVA is still unknown. Cloning of the α_{1E}-subunit, and subsequent expression in oocytes have shown that the α_{1E} current is partially inhibited by 200nM ω-Aga IVA, and more potently inhibited by ω-CTX MVIIC (Zhang *et al.*, 1993). This has led to the suggestion that the α_{1E} clone could be part of the P- or Q-type currents in cerebellar purkinje (Mintz *et al.*, 1992a) and granule (Zhang *et al.*, 1993) cells respectively. Mori *et al.* (1991) defined, from cDNA clones, the primary structure of a calcium channel with similar pharmacological characteristics to the P-channels. Mori and his co-workers observed that activity of these channels was dramatically increased when co-expressed with α_2 and β subunits. This was taken to indicate the possible existence of these subunits in natural P-channels. Electrophysiological analysis has shown that these channels appear to have little voltage-dependent inactivation (Spedding and Paoletti, 1992).

Regan *et al.* (1991) have suggested that these channels may form a large proportion of calcium channels in the rat brain. Furthermore, it has also been suggested that P-channels may be responsible for neurotransmitter release in different cell types in the brain (Hillman *et al.*, 1991). It has been proposed that the ω-Aga IVA sensitive P-type channel may be involved in modulating ω-CTX GVIA resistant excitatory post-synaptic potential (EPSP) in the rat hippocampal neurons (Potier *et al.*, 1993). This proposition is based on previous findings that ω-Aga IVA inhibits calcium influx into rat brain synaptosomes much more efficiently than the specific N-type blocker ω-CTX GVIA (Mintz *et al.*, 1992). In addition this toxin was also shown to inhibit calcium-dependent, ω-CTX GVIA resistant glutamate release from rat synaptosomes (Toselli and Taglietti, 1990).

1.7.4. T-type calcium channels

T-type or transient- channels are activated by low frequency depolarisation and show rapid voltage-dependent inactivation. As a result they are also termed low voltage activated (LVA) channels (Dolphin, 1995). The single channel conductance of T-type VSCC are low, usually in the range of 9 to 11 pS^{-1} in 110mM Ba^{2+} (Dolphin, 1995). These channels have been found in a variety of tissues such as in the atrioventricular node, the specialised conducting tissue of the heart (Tseng and Boyden, 1989), smooth muscle cells (Loirand *et al.,* 1989) and neurons (Nowcyky *et al.,* 1985). LVA currents have also been detected at the early stages of development in skeletal muscle and can co-exist with L-currents in many tissues (Dolphin, 1995). Recent molecular studies have identified neuronal T-type channels (Perez-Reyes *et al.,* 1998). Furthermore, the subtypes α_{1G} and α_{1H} subunits were found to encode two different isoforms of T-type calcium channels

The lack of specific T-type channel blockers and the lack of high-affinity probes has hindered the structural definition of these channels. However, they can be inhibited by non-specific blockers such as nickel (Ni^{2+}), amiloride and phenytoin (Dolphin, 1995).

1.7.5. Q-type calcium channels

Inhibition of L-, N- and P- currents, in many neuronal cells still leaves a substantial calcium current (Zhang *et al.,* 1993). The non-selective blocker ω-CTX MVIIC, which has the ability to block N- and P-currents was also found to be effective in blocking the additional current termed Q-type, in cerebellar granule neurons. This Q-current was also blocked with high concentrations (>100nM) of ω-Aga IVA.

Q-type channels are high voltage activated channels with an intermediate voltage-dependent inactivation rate. Electrophysiological analysis indicates that it has similar characteristics to N-type channels. There is no known data for its single channel conductance and there are no known specific blockers for this channel although, as indicated above, it is blocked by the N-

and P-type channel blocker ω-CTX MVIIC and by high concentration of the P-type channel blocker ω-Aga IVA.

1.7.6. R-type calcium channels

The R-type channels have been classified following observations that despite the addition of all blockers to N, L, P, Q and T- channels, there was still a small component of calcium current in cerebellar granule neurons (Zhang *et al.,* 1993). It is activated by both high and low voltages thus; possibly indicating the existence of subtypes of R-type channels. There are no known pharmacological agents that block this channel. They have been identified purely from electrophysiological experiments (Dolphin, 1995).

1.8. GABA receptors

GABA receptors are known to interact with other neurotransmitter systems in the brain in regulating learning and memory formation in various animals (for a review see Decker and McGaugh, 1991). There is ample evidence in the literature showing GABAergic modulation of VSCC (for a review see Dolphin, 1995). In chapter 6 I describe experiments to determine the effects of various GABA agonists and antagonist on VSCC in the IMHV of chicks. In this section I will describe the different subtypes of GABA receptors and then give some evidence for the effect of GABA on memory. Finally, I will discuss current findings in the mechanisms of GABA modulation of VSCC.

The ubiquitous neurotransmitter γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the CNS. GABA receptor analyses have so far confirmed the existence of two subtypes of the receptor, $GABA_A$- and $GABA_B$-receptors. The $GABA_A$ -receptor is a heteromeric chloride gated ion channel located predominantly in the post-synaptic terminals of neural cells. Activation of $GABA_A$ receptors opens the ion channel leading to chloride ion influx into cells inducing membrane hyperpolarisation (Borman, 1988). The $GABA_A$ receptor also has additional binding sites for benzodiazepines (BDZ; Mohler and Okada,

1977), picrotoxin (Maksay and Simonyi, 1987), neuropeptides (Majewska *et al.*, 1986) and glycine. These sites have the ability to modulate the activity of $GABA_A$ -receptors.

$GABA_B$-receptors, which are not as well characterised as $GABA_A$-receptors, belong to the family of metabotropic receptors. They are coupled to a variety of effectors such as calcium and potassium channels, via pertussis-toxin sensitive G-proteins (Nicholls, 1994). They are less abundant than $GABA_A$-receptors and are localised in pre- or post-synaptic terminals of neural cells. The coupling of $GABA_B$-receptors to G-proteins enables it to influence a host of cellular and physiological functions (Mott and Lewis, 1994). Although $GABA_A$-receptors outnumber $GABA_B$-receptors in the whole brain, there are region specific differences where $GABA_B$-receptors outnumber $GABA_A$-subtypes in areas such as the cerebellum, amygdala, globus pallidus and temporal cortex of the rat brain (Bowery *et al.*, 1987).

1.8.1. Effects of the GABAergic system on memory

The amygdala which has been shown to be involved in memory storage processes is rich in $GABA_A$ and $GABA_B$ receptors. Post-training inter-amygdala injections of muscimol ($GABA_A$ agonist) decreases retention of inhibitory avoidance in rats, while injections of $GABA_A$ antagonist bicuculline enhances retention for the task (Brioni *et al.*, 1989). This was one of the earlier pieces of evidence indicating that the GABAergic system in the amygdala may function to down-regulate memory consolidation. In addition post-training intra-amygdala injection of the $GABA_B$ agonist baclofen impairs memory retention (Castellano *et al.*, 1989). This may indicate that both $GABA_A$ and $GABA_B$ systems may down regulate memory formation by the processes of inhibitory modulation of adjacent neurons. Following the discovery of endogenous benzodiazepines (BDZ) in the brain (De Blas *et al.*, 1987), experiments were conducted to determine the function of $GABA_A$ modulators on memory. Pre-training administration of the BDZ antagonist flunarizine enhanced retention of habituation to buzzer and passive avoidance (Izquierdo *et al.*, 1990) and active avoidance (Lal *et al.*, 1988) in rats. It should be noted that flunarizine enhanced retention only in animals trained on stressful or anxiogenic tests such as avoidance tasks or habituation to buzzer. Animals trained on less stressful tasks, such as open field behaviour did not exhibit

retention deficits following BDZ administration (Izquerdo *et al.,* 1990), however, animals injected with the $GABA_A$ inhibitor picrotoxin did show retention deficits for open field behaviour. It has been hypothesised that the differences on memory retention for the different tasks is due to the different mode of action of BDZ and picrotoxin, where flunarizine acting on the modulatory site of $GABA_A$, attenuates the sensitivity of $GABA_A$ receptors to agonists while picrotoxin blocks the chloride channels of the $GABA_A$ complex. Izquierdo and Medina (1991) proposed that $GABA_A$ receptors involved in memory modulation in the medial septum, amygdala and hippocampus of the rat may be regulated by endogenous BDZ which is released from the same brain structures. The amount released may depend on the degree and / or stress associated with each task. The $GABA_A$ and $GABA_B$ receptors may have differential effects on memory formation depending on the stress associated with the task. Contrary to the effects seen with $GABA_A$ receptor stimulation, post-training administration of baclofen decreased retention in rats trained on the non-stressful radial arm maze (RAM; Stackman and Walsh, 1994), further implying a possible mechanism for GABAergic modulation in the maintenance and / or retrieval of spatial working memory.

Experiments have shown that the GABAergic system may have an effect on memory by modulating other neurotransmitter systems. For example, muscimol was found to block the memory enhancing effect of the opioid antagonists naloxone and naltrexone (Castellano *et al.,* 1989). In addition picrotoxin and bicucculine attenuated the memory-impairing effects of the opoid peptide antagonists, β-endorphins (Castellano *et al.,* 1993). Administration of muscimol or benzodiazepine agonists alone impaired spatial memory (Brioni *et al.,* 1990; Stackman and Walsh, 1992) and also impaired reinforced alternation and inhibitory avoidance in rats (Nagahara and McGaugh, 1992). In addition muscimol and benzodiazepine agonists were also found to reduce both acetylcholine (Ach) turn over (Blacker *et al.,* 1986) and high affinity choline transport (HACt) turn over (Walsh *et al.,* 1986). Intrioni-Colison and Baratti (1986) showed that opioids influence memory storage by effecting noradrenaline (NE) release and in later experiments also showed that the GABAergic systems influence NE release in the amygdala (Intrioini-Collison *et al.,* 1994). Taken together, the GABAergic and opoid systems may interact in modulating memory storage, possibly via a common effect on

NE release. In the chick brain, the β-adrenergic agonist increased retention for the weak learning passive avoidance task and it has been suggested that it may extend the period between intermediate term memory (ITM) and long-term memory (LTM; Crowe *et al.*, 1990; Crowe *et al.*, 1991). In addition pre-training injections of scopolamine (cholinergic receptor antagonist) and piperizine (muscarinic [M_1] antagonist) rendered chicks amnesiac on tests 15 and 30 minutes following training on the passive avoidance task (Patterson *et al.*, 1990). Thus it would seem most probable that modulation between adrenergic, cholinergic and GABAergic systems plays some part in memory modulation in the chick as has been described in the rat.

It has recently been proposed that the early time course of the biochemical changes following passive avoidance learning in day-old chicks may be controlled by subtle modifications in the depolarisation state of neurons in the IMHV. Specifically, the decrease in $GABA_A$ mediated synaptic inhibition triggers the time-dependent biochemical cascade (Clements and Bourne, 1996) but that this system is not required prior to the 2nd wave of glycoprotein synthesis. The experiments showed that pre-training injections of muscimol decreased retention for the passive avoidance task when chicks were tested 30 minutes and 3 hours post-training. In contrast, 30 minutes pre- but not 4 hours post-training administration of the $GABA_A$ antagonist bicucculine increased retention in chicks tested on the weak learning avoidance task. Thus the results by Clements and Bourne (1996) may show a direct or indirect interaction of the GABAergic system with cholinergic and noradrenergic systems during acquisition and / or retention of the passive avoidance task in chicks.

1.8.2. GABAergic modulation of voltage sensitive calcium channels

VSCC can be differentially modulated by intracellular G-proteins and second messengers as a means of controlling calcium influx. Over the years many hormones and neurotransmitters, acting via seven transmembrane G-protein coupled receptors such as muscarinic, cholinergic, GABAergic, opioid, somatostatin, prostaglandin, adrenergic and adenosine, have been linked to inhibition of native calcium currents (see Hill, 1994 for a review). High

voltage activated (HVA) but not low voltage activated (LVA) calcium currents have been shown to be modulated by the GABAergic system. The inhibition of HVA calcium currents have several similar characteristics:

(1) Activation of G-protein-coupled receptors results in the reduction of peak current amplitude, accompanied by an apparent slowing of activation and inactivation kinetics of the current (see Zamponi and Snutch, 1998 for a review).

(2) Modulation of VSCC by metabotropic receptors leads to decreased transmitter release, via activation of the heterotrimeric G-protein, $G\alpha_0$ or in some instances via $G\alpha_i$ and G_q (see Zamponi and Snutch, 1998 for a review). The inhibitory effect of metabotropic receptors can be attenuated by the $G\alpha_0$ and G α_i G-protein inhibitor pertussis toxin.

(3) Calcium current inhibition appears to occur via membrane-bound pathways rather than via diffusible cytoplasmic messengers (see Hill, 1994 for a review).

(4) Membrane-bound G-protein-dependent inhibition is strongly voltage dependent. Thus, G-protein-dependent inhibition of VSCC can be temporarily removed by the application of strong depolarisation, resulting in an apparent voltage-dependent unblock of the channel. The exact stoichiometry of G-protein inhibition of VSCC and subsequent recovery is still unknown.

Neuronal N-type, ω-CTX GVIA-sensitive VSCC has been shown to be modulated by G-protein linked receptors (Hill, 1994). In addition, a variety of transmitters such as opioids, GABA and somatostatin have also been shown to block P/Q-type currents (Currie and Fox, 1997; Bourinet *et al.*, 1996; Zhang *et al.*, 1996). Molecular studies have shown that G-protein modulation occurs on the α_1-subunit of VSCC (Zamponi and Snutch, 1998). Detailed analysis using cloned calcium channels has revealed that G-proteins selectively modulate different subtypes of calcium channel α_1-subunits. For example, α_{1A} and α_{1B} subtypes were inhibited by G-proteins in a manner identical to that for native P/Q-type and N-type currents, respectively (Bourinet *et al.*, 1996; Zhang *et al.*, 1996), whereas α_{1c}, L-

type currents were not inhibited. In some studies the α_{1e} subunit was inhibited by G-proteins only in the absence of co-expressed β-subunit (Toth *et al.*, 1996).

As a result of the existence of the numerous subtypes of G-protein subunits (α, β and γ), analysis of the mechanism of G-protein inhibition of VSCC has led to conflicting results. A general description of the subtypes of the G-protein subunits is beyond the scope of this thesis. However, general reviews can be found including Nicholls (1994) and Dolphin (1995). In brief, G-proteins act as the link between receptors and the effector enzymes (i.e. the adenylate cyclase/cAMP system, the phospholipase C/inositol phosphate system) or ion channels (regulation of ion channels such as VSCCs). G-proteins, given this name because of their interaction with the guanine nucleotides, GTP and GDP, consist of three subunits, α, β and γ. Guanine nucleotides bind to the α-subunit, which has enzymatic activity, catalysing the conversion of GTP to GDP. The β and γ subunits are very hydrophobic and remain attached to the cytoplasmic surface of the membrane as a Gβγ complex. G-proteins are freely diffusible in the plane of the membrane and are able to interact with several different receptors. Binding of an agonist to a receptor leads to a conformational change in its structure causing it to have a high affinity for the αβγ complex. Association of the complex causes GDP, bound to the α-subunit, to dissociate and be replaced with GTP, which in turn causes the Gα-GTP subunit to dissociate from the βγ complex. The 'activated' Gα-GTP subunit diffuses in the membrane and can associate with various enzymes and ion channels, causing either activation or inactivation of the particular effector protein. The process is terminated by GTPase activity of the α-subunit, leading to the conversion of GTP to GDP. The resulting Gα-GDP dissociates from the effector protein and reassociates with the βγ complex, completing the cycle. Mechanisms of this type in general result in amplification of the original signal because a single agonist-receptor complex is capable of activating several G-protein molecules, which in turn are able to remain associated with the effector enzyme to produce many molecules of product.

Techniques such as exogenous expression of G_α subunits, or the inhibition of the G_α subunit using antisense oligonucleotides and antibodies have led to the idea that $G_{\alpha o}$ and / or $G_{\alpha i}$

subunit are involved in calcium channel inhibition (Dolphin, 1995 for a review). However, more recently some studies have shown that in the absence of agonist, the co-expression of $G_{\beta\gamma}$ subunit but not G_{α} subunits mimicked transmitter-induced inhibition of N- and P/Q-type currents (Herlitze *et al.*, 1996; Ikeda, 1996). Recently, studies have shown that the β subunit of G-proteins may also have a modulatory role on VSCC. Expression of different combinations of $G_{\beta\gamma}$ ($G_{\beta1\gamma2}$, $G_{\beta1\gamma3}$, $G_{\beta1\gamma7}$) subunits were equally effective in reducing calcium currents. Radiolabel studies of the G-protein subunits have shown that $G_{\beta\gamma}$ but not $G_{\alpha o}/G_{\alpha i}$ bind to the α_{1A} and α_{1B} cytoplasmic linker between domains I and II of the α_1 subunit of VSCC (Zamponi *et al.*, 1997). The region on the I-II linker of the VSCC bound by $G_{\alpha\beta}$ overlaps one of the sites that binds to the modulatory β-subunit of VSCC. This could be indicative of the attenuating effect of β-subunits on G-protein inhibition of VSCC (Campbell *et al.*, 1995). Chimeric and point mutation studies on the I-II linker region have also shown an interaction of this site with G-proteins leading to N- and P/Q-type channel modulation (Page *et al.*, 1997; Herlitze *et al.*, 1997). It should be noted that some experiments have shown regions other than the I-II linker on VSCC to be important in G-protein modulation. For example, domain I and the carboxy terminus of α_{1A}, α_{1B} and α_{1E} showed large amounts of binding with G-proteins (Zhang *et al.*, 1996; Qin *et al.*, 1997).

1.9. Evidence for the role of calcium in learning and memory

In the final section of chapter 1 the evidence for the role of calcium in synaptic plasticity and learning and memory will be discussed.

1.9.1. Synaptic plasticity and calcium

As mentioned previously by Hebb (1949), neurons exhibit history-dependent activity, where signalling between neurons may lead to strengthening of connections. At the 'whole organism level' these plastic changes in synaptic efficacy contribute to behavioural plasticity. The main form of communication between neurons is dependent on calcium mediated transmitter release (Augustine, 1987). Calcium has been shown to participate in several forms of synaptic plasticity ranging from short-term synaptic plasticity (Zucker, 1994) to

long-term potentiation (Bliss and Collingridge, 1993). Neurons that undergo activity dependent short-term synaptic changes following stimulation are said to be expressing short-term synaptic enhancement (STE). It can be categorised into 3 types, frequency facilitation (FF), augmentation (AUG) and post-tetanic potentiation (PTP). FF describes synaptic enhancement that occurs within a few seconds of stimulation and decays within 1 second, AUG describes enhancement of synaptic transmission after a few seconds of synaptic activation and decays with a time-course ranging from a few to tens of seconds. PTP typically requires higher frequency of activation for up to a few minutes and decays with a time-course of minutes.

Several experimental results have lent support to the residual calcium hypothesis which states that STE is dependent on activation of calcium entering nerve terminals during activation. For example, Katz and Miledi (1968) have demonstrated the importance of extracellular calcium in the induction of synaptic enhancement. In addition Kamiya and Zucker (1994) and Zangel *et al.* (1994) have also shown that increasing the intracellular calcium concentration resulted in increased synaptic strength. Atluri and Regehr (1996), using calcium imaging techniques, had also shown a strong correlation between increased intracellular calcium concentration during activation and synaptic enhancement.

An action potential at pre-synaptic terminals causes phasic transmitter release solely by elevating presynaptic calcium concentration locally and briefly to levels as high as 100μM at release sites of the active zone. This leads to rapid binding of multiple calcium ions to low affinity sites near calcium channels resulting in exocytotic release of neurotransmitter-containing vesicles (Zucker, 1994). The different forms of STE plasticity have different calcium kinetics. For example, augmentation and potentiation are induced as a result of residual calcium acting with slow kinetics (300 ms) at high affinity sites. This leads to enhanced neurotransmitter release following an action potential (Delaney *et al.*, 1989; Delaney and Tank, 1994). On the other hand facilitation is induced due to residual calcium acting at a different site with much faster kinetics (Yamada and Zucker, 1992).

Calcium dependent STE have been observed in inhibitory synapses between interneurons L30 (inhibitory) and L29 (excitatory) in the abdominal ganglion of *Aplysia* (Fischer and Calrew, 1995). This form of STE is involved in regulating the siphon-withdrawal reflex through recurrent inhibition onto L29 interneurons. Recently Fischer *et al.* (1997) carried out further studies to determine the calcium dependence of the three types of STE in *Aplysia* using isolated L30 and L29 interneurons. Experiments were carried out to determine the role of residual pre- and post-synaptic calcium on the maintenance of L30 STE. These experiments were carried out following recent observations by Bao *et al.* (1997) of the existence of a novel form of calcium-dependent postsynaptic enhancement in *Aplysia* following potentiation of synaptic transmission. Fischer and co-workers discovered that buffering presynaptic calcium resulted in a decrease of FF, AUG and PTP on postsynaptic L29 interneurons. On the other hand postsynaptic calcium buffering on postsynaptic L29 interneurons did not affect the induction of FF, AUG or PTP on presynaptic interneurons. The results were thus interpreted to mean that STE in L30 neurons is dependent on presynaptic free calcium for its maintenance but does not require elevation of post-synaptic calcium for induction. It should be noted that participation of postsynaptic calcium in synaptic plasticity has already been briefly discussed in section 1.5.

1.9.2. Behavioural and biochemical evidence for the role of calcium in learning and memory

Behavioural analysis into the function of calcium in learning and memory involves disrupting the 'movements' of these ions using agents that block calcium channels prior to or after training an animal model on a learning task. The pivotal role of calcium in many neuronal functions has led to the hypothesis that aberrant calcium metabolism is, at least in part, linked to age related memory loss. Evidence for the participation of calcium in cellular mechanisms of learning and memory formation has come from observations that aberrant calcium metabolism occurs in older animals, including humans with difficulty in learning new associations (Khachaturian, 1989). Intracellular calcium concentration in normal cells are tightly regulated (Scott *et al.*, 1991) by a variety of buffering systems. The buffering systems can vary from calcium-binding proteins to processes that trigger the sequestration of

free calcium into intracellular stores such as the endoplasmic reticulum (Disterhoft *et al.*, 1993). The 'calcium hypothesis of aging' proposes that cells in the aging brain have 'lost' the regulatory mechanisms that keep intracellular calcium concentration levels within tight constraints thus leading to an excess in $[Ca^{2+}]_i$ concentration (Khachaturian, 1989). The abnormal calcium buffering leads to increased intracellular calcium concentration, approximately one order of magnitude higher than normal. The consequences of large $[Ca^{2+}]_i$ are deleterious, resulting in effects such as membrane deterioration and eventually cell death. Experimental evidence for an altered calcium metabolism in aging has come from a variety of studies. For example, electrophysiological studies has shown that aging neurons exhibit increased after-hyperpolarization (AHP) partly as a result of increased $[Ca^{2+}]_i$. AHP in normal cells is important in controlling central neuron excitability. However, in abnormal neurons excess AHP will lead to decreased cellular excitability and possibly learning deficits (Disterhoft *et al.*, 1993). If this were so, then blocking calcium influx would decrease AHP and consequently increase the learning ability of memory impaired animals.

Eye blink conditioning experiments in rabbits have shown that nimodipine, an L-type VSCC blocker, facilitates learning in aging rabbits (Straube *et al.*, 1990). For example, nimodipine was found to increase acquisition of eye-blink conditioning in aging rabbits to the same degree as untreated young controls (Deyo *et al.*, 1989). In addition, comparisons between treated aged rabbits and vehicle control aged rabbits showed that the treated rabbits were significantly better in learning the eye-blink conditioning task compared to controls (Deyo *et al.*, 1989). Interestingly Deyo and co-workers observed that nimodipine did not enhance acquisition of the task in treated young rabbits when compared to young controls. This finding lends further support to the notion that abnormal calcium metabolism in aging animals leads to impairment of associative learning. Subsequent *in vivo* electrophysiological studies have shown that intraventricular (i.v.) administration of nimodipine in awake young and aged rabbits causes an age- and concentration- dependent enhancement of the spontaneous firing rate of pyramidal neurons (Thompson *et al.*, 1990). The drug was also found to be most effective on aging rabbits and at a concentration previously used to increase eye-blink learning in the aged animals (Deyo *et al.*, 1989). However, analysis with other L-type blockers, nifedipine and flunarizine did not show any enhancement. Biochemical

analysis has shown that there is a negative correlation between KCl-stimulated $^{45}Ca^{2+}$ influx in synaptosomes from aged rats and their ability to learn an 8-arm radial maze task (Blanco *et al.*; 1994). Furthermore, Blanco and co-workers found significant deficiencies in the calcium storing ability in synaptic mitochondria of aged, memory impaired rats compared to non-impaired rats. Experiments on aged non-human primates have also shown that nimodipine significantly improves certain memory tasks (LeVere and LeVere; 1994) when compared to control.

It was subsequently proposed that nimodipine exerted its 'nootropic' effect as a result of its vasodilatory effect (Haws *et al.*, 1983), resulting in increased cerebral blood flow. However, *in vitro* experiments in hippocampal slice preparation have also shown that nimodipine decreases the slow AHP in aging neurons which, in untreated slices from aged animals was greater in its peak and amplitude compared to slices from young animals (Moyer *et al.*, 1992). Similarly, it has been reported that following trace conditioning in young rabbits there was a decrease in calcium activated postsynaptic post-burst AHP and in spike-frequency accommodation (Moyer *et al.*, 1996; Thompson *et al.*, 1996). The calcium action potential was found to last longer in aging neurons compared to young neurons from the CA1 region of the hippocampus. In addition low concentrations of nimodipine were found to decrease the depolarising plateau of the calcium action potential in the CA1 neurons from the aging rabbit. Thus this data would appear to indicate that nimodipine is at least in part increasing the learning capabilities of the aging rabbits by directly blocking calcium influx via L-type VSCC. Calcium influx via ligand-gated channels may also be involved in the acquisition of eye-blink conditioning, since blockers of NMDA channels PCP and MK-801 were shown to decrease the animals ability to remember the task on test (Disterhoft *et al.*, 1997).

Experiments in several laboratories have shed some light into the role of calcium in learning and memory in young normal animals. The chick has been used to study the role of calcium in the brain after training the animal on the passive avoidance task. Previous work has already shown that 95% of all VSCC in presynaptic terminals from neurons in the chick

forebrain are of the N-type, ω-CTX GVIA-sensitive, channels (Bowman *et al.*, 1993). Furthermore, these VSCC are believed to be solely responsible for regulating neurotransmitter-linked calcium flux in avian neurons, since these channels appear to be most concentrated in the active zone of pre-synaptic terminals (Sher and Clementi, 1991 ; Robitaille *et al.*, 1990). Thus the N-type channel may be the primary VSCC involved in the calcium-dependent glutamate release that occurs within 30 minutes post-training (Daisley and Rose 1994). Behavioural analysis by Clements *et al.* (1995) has shown that the ω-CTX GVIA was effective in inducing amnesia in chicks when tested 30 minutes and 3 hours post-training following bilateral and unilateral injections in the IMHV 5 minutes pre- or 5 minutes post-training on the passive avoidance task. Surprisingly, there was no amnestic effect with the L-type blockers nimodipine, nifedipine or amlodipine, in chicks tested on the avoidance task. Bilateral injection of ω-CTX GVIA 5 minutes pre-training was also found to severely impair acquisition in an appetitive discrimination task (visual discrimination task). However, despite this impairment there appeared to be enough learning and consolidation that by the time of the retention test 30 minutes and 3 hours post-training there were no significant differences between the ω-CTX GVIA and saline groups. Nifedipine did not affect acquisition or retention in chicks tested on the visual discrimination task.

The data collected by Clements *et al.* (1995) contradicts previously published data by Deyo *et al.* (1990), who found that intraperitoneal (i.p.) administration of nimodipine at low doses, increased retention in chicks tested on a visual discrimination task compared to controls. At high doses of nimodipine however, the chicks made more errors in acquisition and retention of the task compared to controls. They had concluded from their data that nimodipine had a dose-dependent effect on facilitation in learning but no effect on retention in young subjects. When nifedipine was injected intracerebally in 4 day-old chicks prior to training on a visual discrimination task retention but not acquisition was impaired when the chicks were tested 24 hours later (Deyo *et al.*, 1992). This data is consistent with that of Clements *et al.* (1995) described previously and with other data accumulated by Edmonds *et al.*(1990), who also found that nifedipine administered to *Aplysia* disrupted a form of plasticity, namely modulation of transmitter release by tonic depolarisation of the sensory neuron. In contrast to

the nifedipine and ω-CTX GVIA data, chronic administration of nimodipine was found to enhance acquisition of reversal training in young and aged rats tested on a spatial learning task (McMonagle-Struko and Fanelli, 1993). Even though nimodipine has been shown to be able to affect directly neuronal tissue it is still possible that some of the memory enhancing effects of drugs such as nimodipine are a result of increased blood flow to relevant areas of the brain. Amlodipine (another L-type VSCC blocker) was found to increase performance in mice tested on a variety of tasks such as single-trial passive avoidance task, conditioned emotional response and discrimination performance task (Quatermain *et al.*, 1993). Additionally nimodipine was also found to facilitate recovery of learned brightness discrimination in a Y-maze in rats with lesioned visual cortex (LeVere *et al.*, 1989) and it also prevented performance deficits in rats with lesions to the medial septal lobe when tested on the Morris water maze (Bannon *et al.*, 1993). Intrahippocampal infusion of nifedipine was found to enhance memory retention in rats given the drug 0 minutes post-training when tested on a step down inhibitory avoidance task 24 hours later (Quevedo *et al.*, 1998)

As mentioned earlier VSCCs play a vital role in the NMDA-independent form of LTP. Experiments have now shown that LTP induced by the K^+ channel blocker tetraethylammonium chloride (TEA) at the synapses in rat hippocampal slices consists of calcium influx via NMDA receptor channels and VSCCs (Huber *et al.*, 1995). Furthermore, this group showed that the mechanisms underlying VSCC and NMDA receptor components of TEA LTP are different and do not share a common saturable mechanism since occlusion experiments demonstrated that saturation of VSCC-dependent TEA LTP did not reduce NMDA-receptor-dependent TEA LTP. Recently, experiments have been carried out to determine the function of the other subtypes of VSCCs in different learning and memory models. For example, Wang *et al.* (1997a) has shown that the induction of NMDA-independent LTP in the dentate gyrus from rat hippocampal slices was dependent on the activation of LVA VSCCs such as the T- and R-type channels. They found that, in the presence of Ni^{2+}, a blocker of LVA calcium channels, but not nimodipine, induction of LTP was inhibited. Long-term depression (LTD), a form of synaptic plasticity describing the long lasting activity-dependent reduction in excitatory glutamatergic transmission, was shown to

be dependent on calcium influx via LVA but not HVA VSCCs and calcium release from intracellular stores (Wang *et al.*, 1997b). The induction of LTD in the dentate gyrus of rat hippocampal slices was significantly inhibited by Ni^{2+} but not by nifedipine or by the NMDA receptor antagonist D(-)-2-amino-5-phosphonopentanoic acid (AP5).

More recently there has been some evidence showing an interaction between the hormonal system, such as the corticosterones, and calcium in certain learning tasks. Furthermore, corticosteroid receptor activation has been shown to influence voltage dependent calcium conductance (Kerr *et al.*, 1992; Karst *et al.*, 1994) and homeostasis (Elliot and Sapolsky, 1993) in CA1 hippocampal cells. Glucocorticoid (GC) hormones which are known to be elevated during stress are known to influence memory function (for review see McGaugh; 1989). Dachir *et al.* (1995) had studied the effects of nimodipine on the behaviour of rats treated with GC releasing pellets. Their results suggested that blocking calcium influx with nimodipine may be useful in counteracting certain prolonged stress-related cognitive impairments.

Further evidence supporting the role of calcium in learning and memory has come from the analysis of calcium-dependent protein kinases that are suspected to play a part in the biochemical cascade of memory formation. Calcium is known to activate, either directly or indirectly, a variety of intracellular protein kinases such as the enzyme calcium/calmodulin-dependent protein kinase II (CAMKII). This enzyme is directly activated by the binding of calcium ions to the regulatory protein calmodulin (CAM) forming a calcium/calmodulin complex (Ca^{2+}/CAM) which subsequently activates CAMKII (Jodar and Kaneto, 1995). Protein kinase A (PKA) can be activated by either G-protein activation following receptor stimulation or by cyclic AMP (cAMP). The second pathway of PKA activation occurs when the Ca^{2+}/CAM complex activates cyclic AMP phosphodiesterase (cAMP PDE) which catalyses cAMP activation of PKA.

Pre-training injections of non-specific protein kinase inhibitors of cAMKII into the chick forebrain induced amnesia in birds tested 15 minutes post-training, which corresponds to the hypothesised ITM stage of memory formation Serrano *et al.* (1994), however these results

suggesting a role for cAMKII in ITM should be interpreted with caution since the non-specific protein kinase inhibitors may be effecting other systems too. More recently it has been shown that administration of the specific cAMKII inhibitor, KN-62, into the IMHV inhibited memory formation for the passive avoidance task at earlier times after training, possibly in the STM stage of memory formation (Zhao *et al.*, 1996). In these experiments *in vitro* measurements of soluble cAMKII activity of samples from M-birds were no different to samples from control groups. However, there was increased activity in membrane bound cAMKII in samples of M-birds compared to control groups where there was substantial increase in cAMP activity in samples isolated from birds tested at 10 minutes post-training, which was maintained in birds tested at 70 minutes post-training. Administration of KN-62 between 20 minutes pre-training and 5 minutes post-training induced amnesia in chicks tested 180 minutes post-training. Furthermore, addition of KN-62 immediately post-training induced amnesia in birds tested between 5 and 180 minutes post-training. Lateralized activity of cAMKII was also detected, since in the right hemisphere KN-62 was only effective in inducing amnesia when given up to 2.5 minutes post-training whereas in the left hemisphere amnesia was still detected in chicks given the drug up to 5 minutes post-training on test 180 minutes post-training.

It is apparent from the summary of previous work from several groups that calcium has an important role in the biochemical and electrophysiological processes associated with learning and memory formation in different animal models. The conflicting data on the effects with different VSCC antagonists in learning and memory may indicate that: (1) there may be variation in activation of these VSCC at different time points along the biochemical and electrophysiological cascade of events, (2) the difference in biophysical properties of these drugs will result in different interactions with the VSCC's at different sites on the neuron and various other tissues, (3) variation between species and even strain and age may lead to variable effects with antagonists, (4) differences in rates of metabolism between different antagonist will led to different times of antagonistic effects and (5) the route of drug administration may also effect the potency of antagonist.

1.10. Aims of Study

The aim of this study is divided into three parts:

(1) Determine a time-course of calcium influx, via VSCC, in the IMHV and LPO of day-old chicks trained on the passive avoidance task.

(2) Carry out pharmacological identification of the subtypes of VSCCs present in the IMHV of day-old chicks, and then determine if the different subtypes of VSCCs express differential activity dependent inhibition with specific blockers immediately after training chicks on the passive avoidance task.

(3) Pharmacologically analyse the modulation of the GABAergic system on VSCCs in untrained chicks, followed by experiments to determine if the degree of modulation varies between untrained chicks, chicks tested 5 minutes and 30 minutes after training on the passive avoidance task.

Chapter 2

Methods and Materials

2. Methods

2.1. Animals

Chicks (Ross I Chunky) of both sexes were used in all experiments. The chicks were hatched on site and placed in a communal incubator on a 12 hour light-dark cycle at 38-40°C until they were 24-36 hours old. All animal experimental work was carried out under Home Office Licence and with the approval of the local Animal Ethical Committee. For the one trial passive avoidance experiments, chicks were placed in pairs into 20 x 25 x 20 cm aluminium pens, each illuminated with a 25 watt red light and left to acclimatise for 1 hour at 28-30°C before being trained on the one-trial passive avoidance task. Chick crumb was scattered in each pen. Small bowls of water were also placed in those pens containing chicks to be tested 3 or 6 hours after training.

2.2. One-trial passive avoidance training

The one-trial passive avoidance task used in our laboratory has been described previously (Lossner & Rose, 1983; Burchuladze & Rose, 1992). Briefly, chicks were pretrained by allowing them to peck at a small (2mm diameter) white bead. The bead was presented 3 times, for 10 seconds each, with 5 minutes intervals between presentations, and only chicks which had pecked on at least two presentations (generally >95%) were subjected to the training procedure. Ten minutes after the last pretraining trial, chicks were offered a 5mm diameter chrome bead dipped in either water (W) or 100% solution of the aversive, bitter-tasting substance methyl anthranilate (M; Cherkin, 1969). Those chicks that did not peck at the chrome bead were excluded from further analysis. Chicks which were neither pretrained on the white bead nor a chrome bead formed a third, quiet (Q) control group. M-trained birds that showed a disgust response (vigorous shaking of head, wiping of beak on the cage floor) upon pecking the bead or water-trained birds that avidly pecked the bead were subsequently tested (Lossner and Rose, 1983). Trained birds were tested at one of several times after training: immediately (no more than 90s; time = 0), 5 minutes, 10 minutes, 30 minutes and 6hours, by offering them a similar, but dry, chrome bead for 10

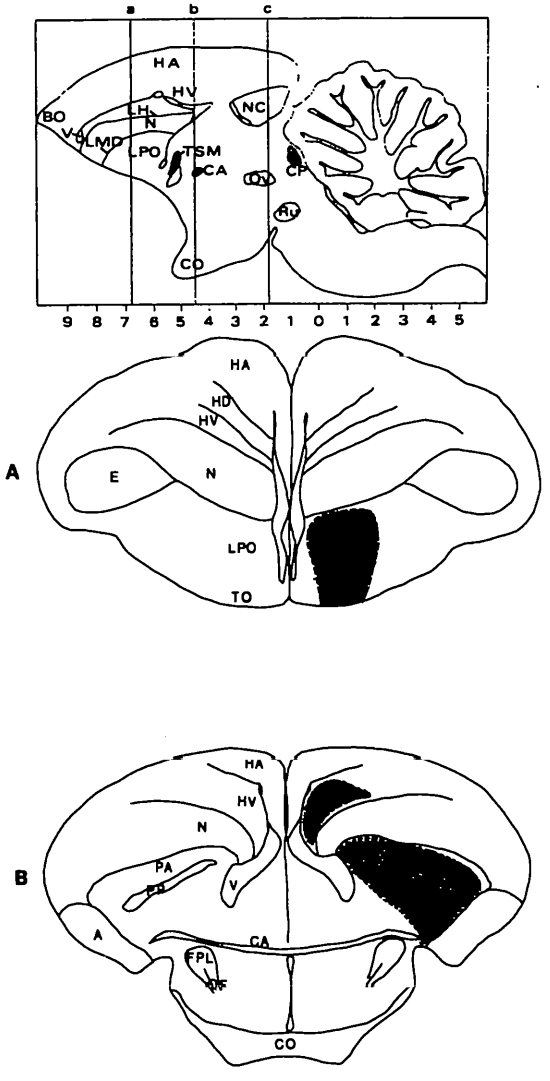
seconds. Chicks which had pecked the M-coated bead avoided the chrome bead on test; W-trained birds pecked the bead on test. Only birds giving the correct response at test, avoiding if M-trained or pecking if W-trained (>80%), were taken for analysis. Each bird was trained and tested only once. Immediately after testing, chicks were killed by decapitation and the IMHV or LPO of the left and right hemispheres was dissected out using a brain mould according to the method of Rose and Csillag (1985) and Bullock *et al.* (1987). As earlier evidence points to a differential role for the two hemispheres (Rose, 1991; Steele *et al.*, 1995) left and right IMHV and LPO were treated independently in experiments that were carried out to determine temporal changes in calcium fluxes following training.

2.3. Preparation of crude membrane fraction (synaptosome and synaptoneurosome)

Synaptoneurosomes were prepared from the left and right IMHV or LPO of day-old Ross I chunky chicks as described by Murakami *et al.* (1986). The chicks were killed by decapitation and the brain removed from the skull. To standardise the dissection technique, the brains were placed in a specially designed solid resin chick brain mould with 3 slots, each being the width of a thin razor blade. The design of the brain mould enables quick and easy dissection of the left and right IMHV and LPO from the chick brain (see figure 3). The IMHV and LPO were dissected out on ice. After dissecting the IMHV or LPO they were placed separately in 500µl ice-cold sucrose solution (0.32M, pH 7.4). All subsequent steps were carried out at 4°C. The tissue was homogenised (8 strokes in a hand-held Jencon teflon/glass homogeniser with a 0.25mm shear) and the homogeniser was rinsed with 500µl sucrose. The homogenate was centrifuged at 1000 g (1,000 RCF, EBA 12R, 1412 fixed angle rotor) for 5 minutes at 4°C. The supernatant S_1 was retained and centrifuged for a further 20 minutes at 15000 g (15, 000 RCF, EBA 12R,1412 fixed angle rotor) at 4°C. Finally the supernatant, S_2, was discarded and the crude synaptosomal pellet, P_2, was resuspended in an appropriate volume of calcium-free ice-cold physiological Krebs Ringer bicarbonate buffer, KRB, (composition of KRB buffer in mM : NaCl, 120 ;

KCl, 2.5 ; NaH_2PO_4, 1.2 ; $NaHCO_3$, 25 ; Glucose, 10 ; Hepes: [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5 ; $MgCl_2$, 1.2 ; pH, 7.4) to give an approximate protein concentration of 1mg/ml and saturated with O_2/CO_2 (95%/5%) gas.

Figure 3 Schematic drawing of coronal slices of day-old chick brains prepared using a brain mould. The position and angle of cuts is shown in the sagittal scheme (stereotaxic co-ordinates after Youngren and Phillips, 1978). 1(B)The first slice represented by face A (taken at level "a") shows the location for dissecting LPO. 1(C) The second slice, represented by face B (taken at level "b") was used for dissecting IMHV. The dissected regions are represented by shading. Taken from Rose and Csillag (1985).



2.4. Electron microscopic study of crude membrane fraction

P_2 samples were initially fixed in suspension (in a volume sufficient to cover the pellet) by incubation in fixative (2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4) for 10 minutes at room temperature. Samples were then centrifuged (Beckman microfuge 12) for 3 minutes at 5585 g and fixed for a further 20 minutes. The pellet was dislodged using cocktail sticks and the fixative solution removed. Samples were washed 3 times (5 minutes each time) at room temperature in 0.1M sodium cacodylate buffer. After the third wash samples were transferred to a petri dish containing 0.1M sodium cacodylate buffer (in volume sufficient to cover samples) and cut into 4 roughly equal segments. After cutting, the samples were post-fixed by replacing the sodium cacodylate buffer with 1ml of osmium tetraoxide (OsO_4) in sodium cacodylate buffer (1 volume double strength buffer and 1 volume 2% OsO_4) for 1 hour at room temperature. The samples were then washed a further 3 times as described above. After the 3rd wash the segments were stained by placing in (excess volume) 2% uranyl acetate and incubated for 2 hours. Following the fixation and staining procedure the samples were dehydrated by sequentially incubating the samples in increasing concentrations of ethanol (1.5mls; 30 %, 50%, 70%, 90%, 100%; volume made up in distilled water). The samples were incubated once for 5 minutes in all concentrations of ethanol except for the final (100% ethanol) dehydration phase, where samples were incubated 3 times for 15 minutes each. After the final dehydration phase the samples were infiltrated and embedded according to the London Resin (L.R.) white resin processing method. The infiltration procedure was carried out by incubating the samples in 1.5mls of L.R. / ethanol mixture (L.R. : ethanol 100% ; 1:1 volume) for 4 hours on a rotating mixer. After incubating the samples for 4 hours they were removed from the L.R. / ethanol mixture and reincubated in fresh L.R. / ethanol mixture (1.5mls, 1:1 volume) for a further 1 hour on a rotating mixer. The 1 hour incubation phase was repeated a further 3 times. Finally the samples were embedded in fresh L.R. white resin in gelatine capsules with the lids on to exclude oxygen (resin will not polymerise if exposed to oxygen) and left to polymerise at 60°C for 22-24 hours. The samples were then sectioned on a ultramicrotome (Ultracut E) to a thickness of 80nm. Sections were examined using (JEOL JEM 1010) microscope. The image was

stored on a Macintosh Quadra using the NIH. IMAGE program and photographed at magnifications of 8000, 15000 and 20000, for the determination of purity of the P_2 preparation.

2.5. $^{45}Ca^{2+}$ Studies

2.5.1. KCl-stimulated $^{45}Ca^{2+}$ uptake studies in synaptoneurosomes prepared from the IMHV of untrained day-old chicks

The method was based on the procedure described by Blaustein (1975) and is used to determine $^{45}Ca^{2+}$ uptake into synaptoneurosomes prepared from the IMHV and LPO of one day old chicks. The synaptoneurosomal pellet prepared from the day-old Ross I Chunky chicks was resuspended in 400µl of Krebs Ringer bicarbonate buffer (KRB; composition of buffer in mM : NaCl, 120 ; KCl, 2.5 ; NaH_2PO_4, 1.2 ; $NaHCO_3$, 25 ; Glucose, 10 ; Hepes: [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5 ; $MgCl_2$, 1.2 ; $CaCl_2$, 2 ; pH, 7.4). Fifty microlitre aliquots of synaptoneurosomes were pre-incubated at 42°C under a constant stream of O_2/CO_2 (95% / 5%) for 15 minutes in eppendorf tubes. Following pre-incubation, 50µl of KRB buffer containing $^{45}Ca^{2+}$ (1µl/ml, specific activity 1.8mCi/ml, Amersham) was added to the samples which were incubated for a further 1 minute. The reaction was terminated by addition of 1ml of ice-cold KRB buffer containing 10µM ouabain and the samples were placed on ice. Samples were transferred to a filtration manifold (Millipore) washed extensively with ice-cold KRB buffer (20ml) under continuous filtration. Filters (0.45µm Millipore) were transferred to scintillation vials and dissolved in 4ml scintillation fluid (Beckman Ready-safe) and $^{45}Ca^{2+}$ uptake was determined by scintillation counting (Beckman LS 1701) for 3 minutes. Protein concentrations were determined by the method of Bradford (Bradford, 1976; section 2.6.).

To investigate KCl-stimulated $^{45}Ca^{2+}$ uptake, aliquots (50µl) of pre-incubated synaptoneurosomes were incubated in duplicate with 50µl Krebs containing $^{45}Ca^{2+}$ (1mCi/ml) or 50µl Krebs containing $^{45}Ca^{2+}$ (1mCi/ml) and 140mM KCl (final

concentration 70mM KCl) for 1 minute before the reaction was terminated as described above.

2.5.2. The effect of ω–conotoxin GVIA on KCl-simulated $^{45}Ca^{2+}$ uptake in synaptoneurosomes prepared from the IMHV of untrained day-old chicks

The crude synaptoneurosomal pellet of the one day old Ross I Chunky chicks was resuspended in an appropriate volume of KRB. Fifty microlitre aliquots of synaptoneurosomes were pre-incubated with either 10µl of KRB or 10µl of KRB containing increasing concentrations of ω-conotoxin GVIA (final concentration; 6nM, 60nM or 600nM) for 15 minutes at 42°C under a constant stream of O_2/CO_2 (95% / 5%). $^{45}Ca^{2+}$ uptake into synaptoneurosomes was examined in the presence or absence of KCl (70 mM) as described above (section 2.5.1).

2.5.3. KCl-stimulated $^{45}Ca^{2+}$ uptake studies in synaptoneurosomes prepared from the IMHV of chicks trained on the passive avoidance task

Synaptoneurosomes were prepared from left and right IMHV of water-trained birds that pecked the bead or from M-birds that avoided the bead. $^{45}Ca^{2+}$ uptake into synaptoneurosomes in the presence or absence of KCl (70mM) was examined as previously described in section 2.5.1.

2.6. Determination of protein concentration

To determine the concentration of protein in synaptoneurosomal preparations the Bradford method was used (Bradford, 1976). Stock solution of Bovine Serum Albumin (BSA; 1mg/ml) was used to make up the protein standards. Stock solution of BSA was serially diluted in distilled water to make up the appropriate concentration of standards. A range of standards of varying concentrations were made up, (Concentration of standard in µg/ml; 0, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700), by serial dilutions.

Measurement of protein concentration was carried out in 96 well microtitre plates. The first column was used as blank (no proteins added). The next column was used to measure absorbance of the standards in duplicate. The last column was used to measure synaptoneurosomal protein concentration in duplicate. In each well 10µl of appropriate sample was added, thus in the blank wells 10µl of distilled water was added, in the wells used as standards 10µl of the relevant standard was added and in the sample wells 10µl of synaptoneurosomal samples were added. In all the wells, 30µl of sodium hydroxide (10mM) and 250µl of Bradford dye, were added. Bradford dye was made up of 95 percent ethanol (50ml), Coomassie blue (G250; 100mg) and phosphoric acid (H_3PO_4; 100ml). Mixture was made up in 1 litre of distilled water mixed thoroughly and filtered through 2 sheets of filter paper (Whatman general purpose filter paper, Grade 1).

Solutions in the 96 well plate were then measured for absorbance in a multiscan plate reader (Labsystems multiscan plus) at 595nM wavelength using the programme for protein measurements. Standard curves were drawn to determine the concentration of proteins in synaptoneurosomal samples.

Bradford dye and sodium hydroxide (10mM) were stored at 5°C and all standards were stored at -20°C.

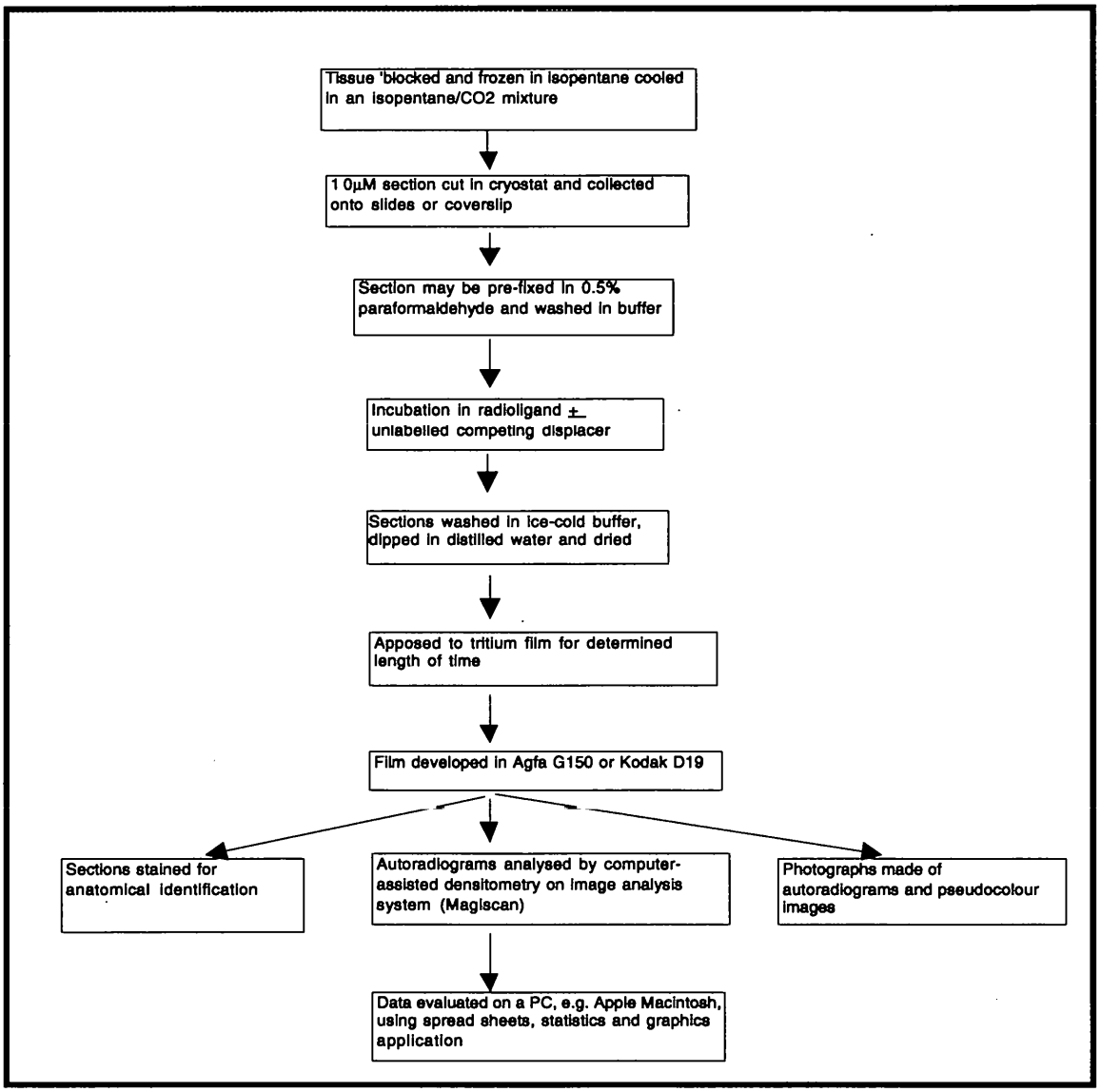
2.7. Autoradiography

2.7.1. Tissue preparation

Day-old chicks were trained on a water- or MeA-coated bead and tested at various times after training (30 minutes, 6 and 24 hours). Brains were removed and divided into two hemispheres using a brain mould for guidance and frozen in an isopentane/CO_2 mixture. A summary of the protocol described in this section is shown in figure 4. Brain segments were wrapped in foil, coded and stored air-tight at -80°C prior to use. Serial coronal sections of 10µm thickness were cut on a cryostat (Reichert-Jung Cryocut E) at -20°C and placed onto poly-L-lysine-coated (0.01%) 22mm^2 coverslips. Twelve sections were taken

from anterior forebrain containing LPO and posterior forebrain containing IMHV. The sections were dried in a cool airstream and stored at -80°C until use. Prior to radioligand binding the sections were brought to room temperature before incubating with ^{3}H-PN-200-110. The sections were put in racks and incubated at room temperature in ice-cold Tris-citrate buffer (HEPES: [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5mM ; Tris, 5mM ; EGTA: ethylene glycol-bis[β-aminoether]-N,N,N',N'-tetraacetic acid, 0.1mM ; NaCl, 100mM ; Sucrose, 120mM ; Tween-20, 0.05% ; BSA: Bovine Serum Albumin, 0.1% ; Lysozyme, 0.2mg/ml ; Leupeptine, 20mg/ml ; Phenylmethylsulfonyl Fluoride (PMSF), 0.5mM ; Nα-$_{P}$-Tosyl-$_{L}$-Lysine Chloromethyl Ketone (TLCK), 10μg/ml ; N-Tosyl-$_{L}$-Phenylalanine Chloromethyl Ketone (TPCK), 25μg/ml ; pH 7.4) containing 50pM of ^{3}H-PN-200-110 for 20 minutes to determine specific binding. Non-specific binding was determined by incubating serial brain sections in Tris-citrate buffer containing 50pM of ^{3}H-PN-200-110 and 250nM of unlabelled PN-200-110. At the end of the incubation period, the sections were washed 3 times for 20 minutes in ice-cold washing buffer (HEPES: [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5mM ; NaCl, 160mM ; $CaCl_2$, 1.5mM ; BSA, 0.1% ; Tween-20, 0.05%, pH 7.4). After the final wash the samples were immersed very briefly in rinsing buffer (ice-cold distilled water ; Tween 20, 0.05%) to remove buffer salts and left to dry under a stream of cold air from a fan. The coverslips with the sections were then glued onto a cardboard sheet together with sections of standards made from brain paste containing a range of known concentrations (fmol/ml-1; 2.13, 6.32, 13.47, 22.89, 32.79, 110.64) of ^{3}H-PN-200-110. The samples and standards were then opposed to a photographic film, LKB [^{3}H]Ultrafilm (Pharmacia, Milton Keynes, UK) secured between 2 A1 metal plates and left in the dark for 5 days. After exposure, the films were developed in AGTA G150 developer at 20°C for 4 minutes then fixed in Ilford Hypam fixative for 3 minutes followed by washing for 1 hour in distilled water.

Figure 4 **Flow chart showing the protocol for in vitro tritium film autoradiography of binding of radioligand to ion channels or neurotransmitter receptors (taken from Stewart and Bourne, 1992).**



2.7.2. Densitometry and image analysis

Densitometry of the film autoradiogram was performed on a Joyce-Loebl Magiscan MD image analysis system (see figure 5). Densitometric analysis for the measurement of receptor binding levels using the magiscan is divided into several stages. The light source and video was switched on about an 1 hour prior to use to allow the system to warm up. A perspex ruler was placed above the diffuser, of the light box, which is illuminated by a standardised light source of 4 tungsten (12 watt) lamps. A calibration line length of 10 was entered into the computer. The image of the ruler was captured onto the computer and the light pen was used to calibrate a length of 10cm. Before making any measurements on the autoradiograms, background measurements of the photographic paper was carried out. This is done by obtaining a reference image from a blank, and homogenous area of the autoradiographic film. An autoradiogram was placed on the diffuser box and a matt-black perspex shield, with an aperture slightly larger then the autoradiogram was placed over the photographic paper, to minimise the effects from stray-light from the light box. The light pen was used to draw around the blank area. The background level is automatically recorded by the computer, and was automatically subtracted (pixel-by-pixel) from the grey level of the autoradiograms during analysis.

Autoradiograms of the 6 standards were then measured. The standards first undergo a process of image enhancement, to emphasise the differences in the grey scale between different areas of the autoradiogram. The aperture on the camera is set to give a peak grey level in the range between 59 to 61. Next the wedge on the standards are corrected to enhance the image of the standards where the lowest standard, (standard with the smallest concentration of (^{3}H)-PN-200-110 binding), was pale in colour and the highest standard, (standard with the greatest concentration of (^{3}H)-PN-200-110 binding), was dark red (i.e. the colours are ordered in spectral sequence, where red indicates the highest grey value and black the lowest). After setting the wedge to the experimenters liking, the standards were measured starting with the lowest standard. The light pen was used to draw around each standard and the appropriate activity was entered into the computer (activity of standards; 2.13, 6.32, 13.47, 22.89, 32.79, 110.64). The computer then draws a standard curve to be

used for the analysis of the sample autoradiograms. Before making measurements on the sample autoradiograms, it is usually common practice to adjust the wedge of the image analysis system in order to assist identification of the different brain regions, thus allowing the experimenter to observe maximal differential labelling on the autoradiograms. Finally different brain areas were analysed by using the light-pen to draw around specific areas of the brain on the autoradiographic image. The absolute value of ligand bound in specific brain areas is automatically computed as fmol/mg^{-1} tissue and stored. It is important to have a suitable coding system for the data collected. Figures 6 to 9 shows examples of specific and non-specific binding of the L-type VSCC antagonist ^{3}H-PN 200 110 in the IMHV and LPO respectively.

Figure 5 Schematic diagram of the components of the magiscan (Taken from Stewart and Bourne, 1992).

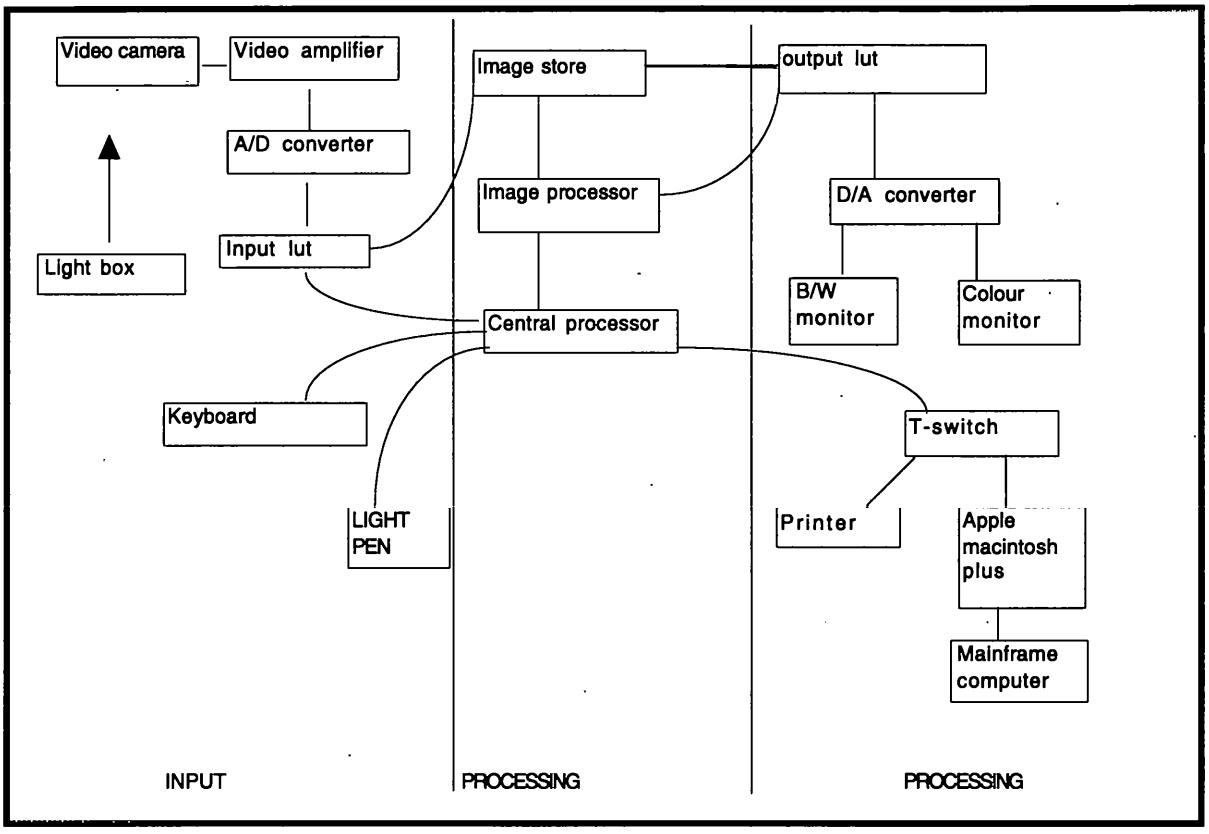


Figure 6 **Autoradiogram of specific binding of ^{3}H-PN 200 110 in the IMHV.**

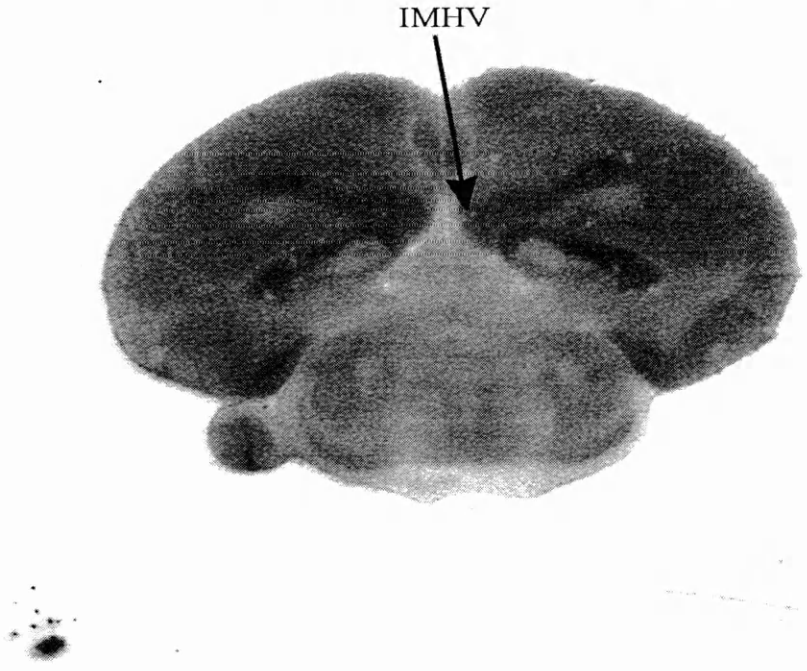


Figure 7 **Autoradiogram of non specific binding of ^{3}H-PN 200 110 in the IMHV.**

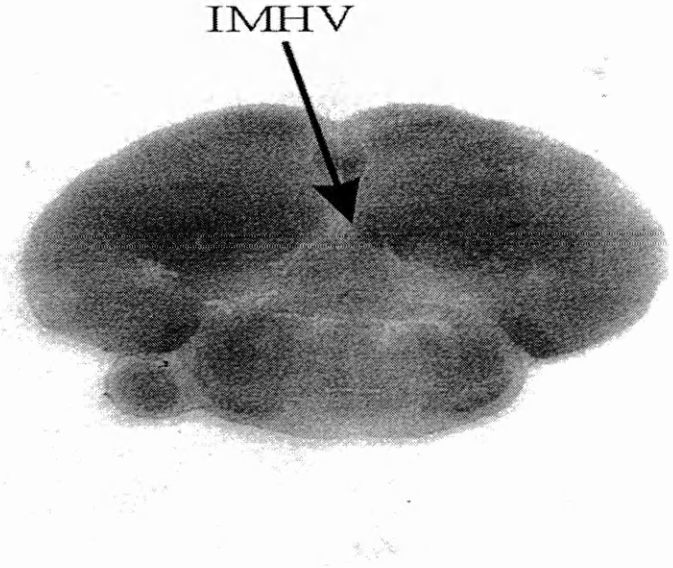


Figure 8 **Autoradiogram of specific binding of ^{3}H-PN 200 110 in the LPO**

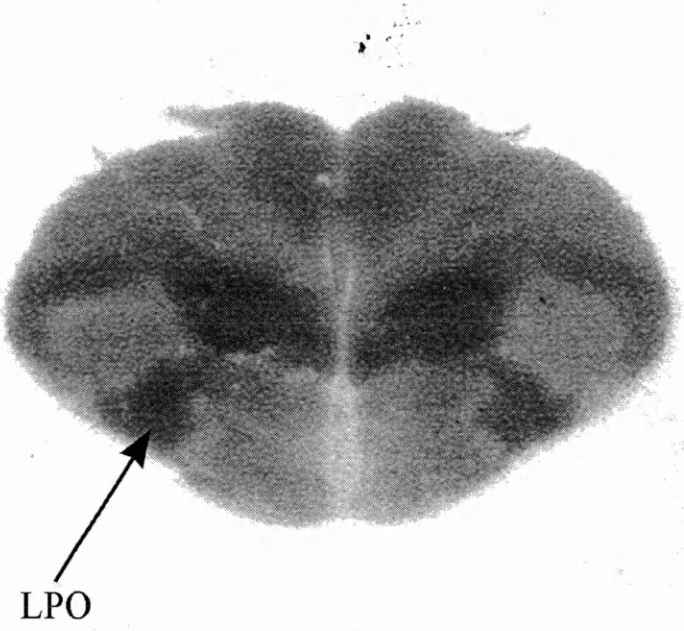
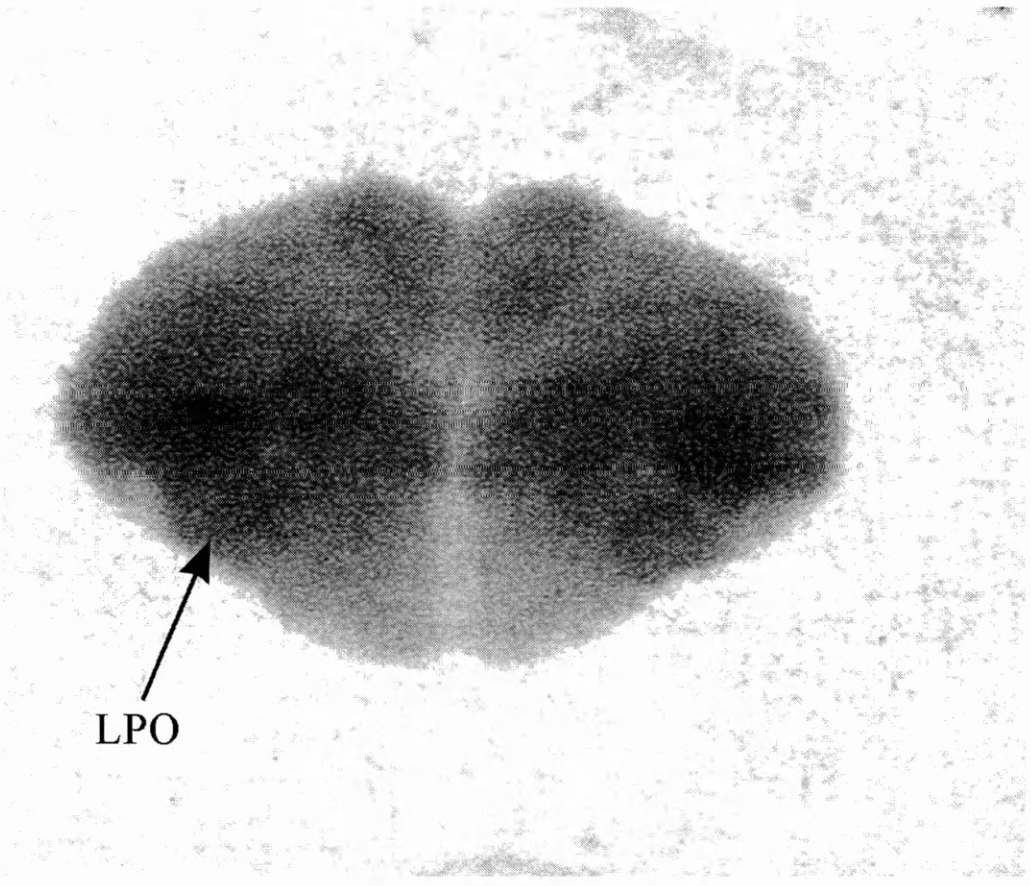


Figure 9 **Autoradiogram of non specific binding of ^{3}H-PN 200 110 in the LPO**



2.8. Calcium measurements using Fura-2/AM

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured using fura-2/AM based fluorimetry. Synaptoneurosomes (protein concentration 1mg/ml) constituted in KRB buffer (composition of buffer in mM : NaCl, 120 ; KCl, 2.5 ; NaH_2PO_4, 1.2 ; $NaHCO_3$, 25 ; Glucose, 10 ; Hepes: [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5 ; $MgCl_2$, 1.2 ; pH 7.4) were incubated for 30 minutes at 30°C in the dark with fura-2/AM (final concentration approximately 1.7μM). The samples were then incubated for a further 10 minutes at 30°C in the dark after adding an appropriate volume of KRB buffer (composition of buffer in mM : NaCl, 120 ; KCl, 2.5 ; NaH_2PO_4, 1.2 ; $NaHCO_3$, 25 ; Glucose, 10 ; Hepes: [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5 ; $MgCl_2$, 1.2 ; pH 7.4) to wash off excess Fura-2/AM. Following the second incubation the samples were centrifuged at 12, 500 g (12, 500 RCF, EBA 12R, 1412 fixed angle rotor) for 5 minutes at 4°C. The supernatant, containing excess fura-2/AM, was discarded and the pellet resuspended in an appropriate volume of KRB to give an approximate protein concentration of 1mg/ml. The recording and calibration of the fura-2/AM fluorescence signal was performed according to the method of McMahon and Nicholls (1991). Fluorescence measurements were made in a fluorescence spectrometer (Perkin Elmer fluorescence spectrometer, model 3000) where fluorescence was measured at two excitation wavelengths of 340 and 380nm and at a constant emission wavelength of 510nm. Values for $[Ca^{2+}]_i$ were calculated according to Grynkiewicz *et al.* (1985) after background (autofluorescence) subtraction using the equation:

$$[Ca^{2+}]_i = K_D\,(R - R_{min} / R_{max} - R)\,(S_{f2} / S_{b2})$$

where K_D is the calibration constant (224nm). R is the fluorescence intensity ratio of the excitation wavelengths at 340 and 380nm (340/380nm), R_{max} and R_{min} are the fluorescence intensity ratio of samples in the presence of saturating concentration of Ca^{2+} and absence of Ca^{2+} respectively, S_{f2} (the proportionality co-efficient of free fura-2/AM) and S_{b2} (the proportionality co-efficient of bound fura-2/AM) are measures of the fluorescent intensities of fura-2AM in calcium free(1mM EGTA) and calcium saturated

(5mM Ca^{2+}) buffer solutions, in the absence of synaptoneurosomes, respectively at 380nm. To determine K_D first make up two KRB solutions A and B, where buffer A contains 10mM EGTA and buffer B contains 10mM EGTA, 10mM calcium chloride and fura-2. For both buffer solutions three excitation spectra are generated under varying conditions (measure relative fluorescence against excitation wavelength ranging from 300nm to 400nm). Curve *a* was generated following addition of fura-2 (pentapotassium salt) to the experimental solutions containing CaEGTA/EGTA in ratios 0.9 to 0.1 (buffer A) and 0.6 to 0.4 (buffer B). Curve *b* was calculated by addition of excess calcium chloride to saturate EGTA and fura-2, and curve *c* was measured in the buffers following the addition of EGTA (1mM)/KOH (pH>8) to ensure that all the fura-2 was in the calcium free form. The excitation scans were then used to calculate K_D using the equation described above. In this instance R is measured from the spectral curve *a* for a particular solution scan. R_{max} and R_{min} are measured from curves *b* (calcium-saturated) and c (calcium-free) respectively, S_{f2} and S_{b2} will be measured and $[Ca^{2+}]_i$ is already known (for a review see Williams and Fay, 1990).

The advantage of using the ratiometric system is that it enables greater accuracy in measuring $[Ca^{2+}]_i$ because it decreases:

(1) The effects of uneven dye loading in synaptoneurosomes

(2) The effects of dye leakage from the synaptoneurosomes

(3) The effects of photobleaching

(4) Problems associated with measuring $[Ca^{2+}]_i$ in cells of unequal thickness

To measure synaptoneurosomal calcium concentrations, 50µl aliquot of samples were placed into a quartz cuvette (path length, 4mm) and 200µl of KRB buffer was added (composition of buffer in mM : NaCl, 120 ; KCl, 2.5 ; NaH_2PO_4, 1.2 ; $NaHCO_3$, 25 ; Glucose, 10 ; Hepes: [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5 ; $MgCl_2$, 1.2 ; $CaCl_2$, 2 ; pH 7.4). Final protein concentration was 200µg/ml. Before each measurement the sample was thoroughly mixed using plastic stirring rods (Sarstedt) and the fluorescence measured at excitation wavelengths 340 and 380nm and emission wavelength 510nm.

Depending on the protocol, fluorescence measurements of samples were taken at 5 or 6 experimental points (5 repeats); (1) in the unstimulated synaptoneurosomal sample, (2) after addition of antagonists (during antagonists experiments in chapter 5) or $GABA_{A/B}$ antagonist or agonist (during GABAergic experiments in chapter 6), (3) after stimulation with 70 mM KCl, (4) after addition of 2% (v/v) triton-X100 to calculate R_{max} and (5) after the addition of EGTA (3M; ethylene glycol-bis[β-aminoether]-N,N,N',N'-tetraacetic acid) to calculate R_{min}.

KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes was measured 20-30 seconds after the application of KCl, during the so called plateau phase of KCl-stimulated calcium influx (Bowman *et al.,* 1993). The results in chapter 4 were expressed as the percentage increase in $[Ca^{2+}]_i$ following KCl stimulation compared with basal unstimulated $[Ca^{2+}]_i$. In the experiments to determine the effects of VSCC antagonists (chapter 5), and $GABA_{A/B}$ agonist and antagonist on KCl-stimulated elevation in $[Ca^{2+}]_i$; the results were expressed as percentage increase in $[Ca^{2+}]_i$ following KCl stimulation compared to unstimulated $[Ca^{2+}]_i$ following incubation with VSCC antagonist or $GABA_{A/B}$ agonist or antagonist.

2.9. Statistical analysis and materials

A two-tailed t-test (in Microsoft Excel, version 4) was performed on the preliminary data from the experiments to determine the effects of KCl-stimulation on $^{45}Ca^{2+}$ in synaptoneurosomes (chapter 3). The statistical package *Statistica* (version 5 for the P.C) was used in all other data analysis in this thesis. In chapter 4 Statistical analysis was performed on the time-course of KCl-stimulated increase in $[Ca^{2+}]_i$ in the IMHV and LPO of birds tested at particular times after training. To determine the effects of training and time, analysis of variance (ANOVA) was used, and where significant differences were found, further analysis was performed using a post-hoc Tukey's honestly significant difference (THSD) test.

One-way ANOVA was used for the following:

(1) to compare the effects of different antagonists added in combination or separately on KCl-stimulated $[Ca^{2+}]_i$ elevation in the IMHV of untrained chicks (chapter 5).

(2) to analyse the effects of different antagonists on KCl-stimulated $[Ca^{2+}]_i$ elevation in the IMHV of chicks tested immediately after training (chapter 5).

(3) to determine the effects of adding $GABA_B$ agonist and antagonist alone or in combination on KCl-stimulated increase in $[Ca^{2+}]_i$ (chapter 6).

(4) to analyse the effects of some GABAergic agonist on KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes from the IMHV of chicks tested at 5 and 30 minutes after training (chapter 6).

2.10. Materials

Baclofen	Tocris
Bovine Serum Albumin (BSA)	Sigma
Bicuculline	Tocris
Calcium Chloride ($CaCl_2$)	BDH
Carbon Dioxide (CO_2)	BOC
EGTA	Sigma
Ethanol (C_2H_5OH)	Hayman
Fura- 2/AM	Calbiochem
γ-Aminobutyric Acid (GABA)	Sigma
Gelatine Capsule	Agar Scientific
Glucose	BDH
HEPES	Sigma
Isopentane	MERCK
Coomasie Blue	Sigma
Ortho-Phosphoric Acid (H_3PO_4)	BDH
Leupeptine	Sigma
London Resin (L.R.)	Agar Scientific
Lysozyme	Sigma
Magnesium Chloride ($MgCl_2$)	BDH
Methyl Anthranilate (MeA)	Sigma
Muscimol	Tocris
Nimodipine	Bayer
Osmium tetraoxide (OsO_4)	Agar Scientific
Paraformaldehyde	Agar Scientific
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma
Potassium Chloride (KCl)	BDH
Radiolabelled Calcium ($^{45}Ca^{2+}$)	Amersham
Saclofen	Tocris
Sodium Cacodylate	Agar Scientific
Sodium Chloride (NaCl)	BDH
Sodium Hydrogen Peroxide (NaH_2PO_4)	BDH
Sodium Hydroxide (NaOH)	BDH
Sodium Hydroxide Carbonate ($NaHCO_3$)	BDH
Sucrose	BDH
Nα-$_P$-Tosyl-$_L$-Lysine Chloromethyl Ketone (TLCK)	Sigma
N-Tosyl-$_L$-Phenylalanine Chloromethyl Ketone (TPCK)	Sigma
Trizma	Sigma
Tween-20	BDH
ω-Agatoxin IVA (ω-Aga IVA)	Calbiochem
ω-Conotoxin GVIA (ω-CTX GVIA)	Sigma
ω-Conotoxin MVIIC (ω-CTX MVIIC)	Sigma
O_2/CO_2	BOC
Urynyl Acetate	MERCK

ω-conotoxin GVIA, ω-conotoxin MVIIC and ω-Aga IVA were prepared in the incubation buffer. However, nimodipine was initially dissolved in 100% ethanol and then serially diluted in incubation buffer to make up the desired concentration. Baclofen, muscimol and

saclofen were made up in sodium hydroxide in equal volumes (1:1) and subsequently diluted to desired concentrations. Bicuculline was dissolved in 100% ethanol; then serially diluted in the incubation buffer to make up to the desired concentration. GABA was dissolved in distilled water. The final concentration of ethanol in all solutions was always less than 0.05% and the final concentration of sodium hydroxide in all solutions was always less than 0.1%. Stock solution of fura-2AM was dissolved in dimethyl sulfoxide (DMSO). Fura-2AM and nimodipine were protected from light and all solutions were stored at -20^{o}C.

Chapter 3

Preliminary Studies

3. Introduction

The main aim of my thesis was to determine a time-course of calcium influx in the intermediate medial hyperstriatum ventrale (IMHV) and lobus parolfactorius (LPO) of day-old chicks trained on the passive avoidance task. I used the crude membrane preparation consisting of synaptosomes and synaptoneurosomes as an *in vitro* model of the synapse, to perform my experiments. To do this I initially used radiolabelled calcium ($^{45}Ca^{2+}$) to determine changes in calcium influx. However, as will be discussed later in this chapter, I was unsuccessful in obtaining consistent results with this technique. Thus, I subsequently measured changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in synaptoneurosomal preparation using the fluorescent calcium indicator fura-2/AM.

In the first part of this chapter I will briefly describe evidence for the viability of crude membranes as an *in vitro* model of the synapse. Then I will describe some results I collected with experiments using $^{45}Ca^{2+}$. Finally, I will briefly describe the viability of using fura-2/AM to measure $[Ca^{2+}]_i$ in birds trained on the passive avoidance task and give some preliminary data.

3.1. Crude synaptic membranes containing synaptosomes and synaptoneurosomes

As mentioned above my membrane preparations consisted of synaptosomes which are predominantly presynaptic nerve endings and synaptoneurosomes which are isolated presynaptic terminals attached to postsynaptic membranes. These membrane fractions are prepared from brain homogenates. It should be noted that my crude membrane fraction will be called synaptoneurosome in the rest of the thesis.

Synaptosomes were originally used for studying chemical and morphological characteristics of vertebrate presynaptic terminals (DeRobertis *et al.*, 1962; Gray and Whittaker, 1962) which retain many of their metabolic (Bradford, 1969, 1970; Bradford and Thomas, 1969), osmotic (Marchbanks, 1967; Keen and White, 1970; 1971) and alkali metal ion transport

(Ling and Abdel-Latiff, 1968; Bradford, 1969, Escuetta and Appel, 1969; Blaustein and Weismann, 1970) properties usually seen in more intact tissue preparations. All this suggests that during the preparative homogenisation processes, the pinched-off terminals reseal and retain intracellular contents such as mitochondria, synaptic vesicles, soluble cytoplasmic enzymes and even small inorganic ions. Figure 10 is low magnification (x8000) electron micrograph (EM) of a crude synaptic membrane preparation from the chick IMHV showing the existence of synaptosomes and synaptoneurosomes. Figures 11 and 12 are high magnification (x15000 and x20000 respectively) EM of synaptosomes and synaptoneurosomes respectively from the chick IMHV. Blaustein and Goldring (1975) provided further evidence for the functional integrity of membranes, as indicated by their observation of the ability of membranes to accumulate and retain K^+ ions against a concentration gradient. This was in support of earlier work which showed that membranes are much more permeable to K^+ ions then sodium (Na^+) ions (Keen and White, 1971). Both these observations indicate that membranes may have a resting membrane potential similar to K^+-diffusion potential as seen in most resting intact nerve cells. Thus by increasing the extracellular K^+ ion concentration it is possible to depolarise synaptosomal and synaptoneurosomal membranes, as shown by Blaustein and Goldring (1975). They used a series of 3,3'-dialkyl1-2,2'-oxacarbocyanine, fluorochrome dyes, as an indicator of membrane potentials (V_m) where, previously, fluorescence had been shown to change linearly with voltage, when membranes of squid giant axons were depolarised or hyperpolarised. Depolarisation caused an increase in fluorescence (Cohen *et al.*, 1974) with a linear relationship, where fluorescence increased with increasing concentration of extracellular K^+ ions.

It should be noted, however, that the crude membrane fraction preparations I used (synaptosomes and synaptoneurosomes), while enriched with nerve endings, also had significant proportion of contaminants such as free mitochondria, myelin and fragmented membranes, usually in the form of apparently empty vesicles.

Table 2 shows the average percentage of protein recovered during the preparation of the crude membranes from the chick IMHV.

Figure 10 Electron micrograph of crude membrane preparation from the IMHV of untrained day-old chick (Low Magnification: x8000).

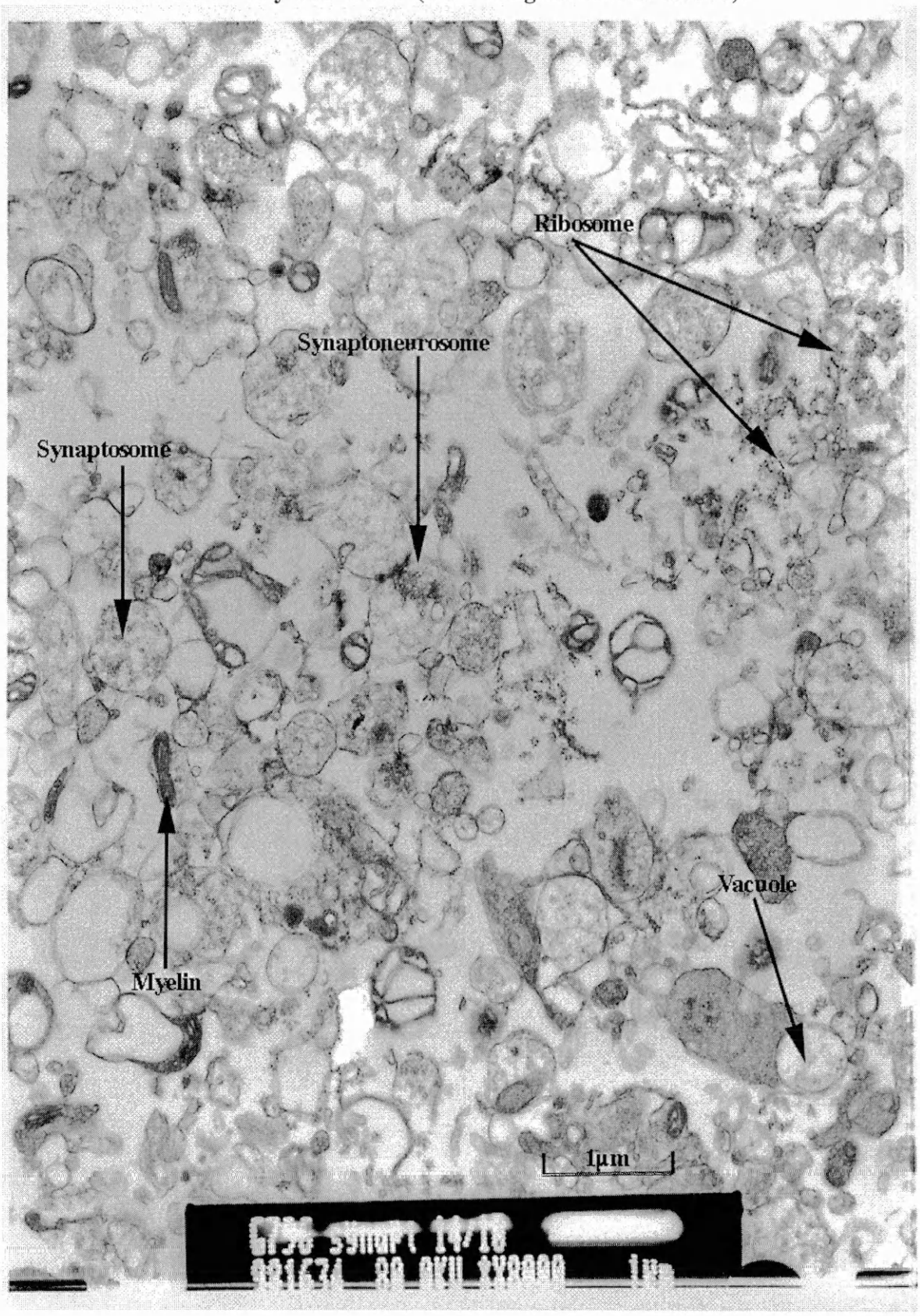


Figure 10 Low power electron micrograph of the crude membrane preparation from the chick IMHV. The picture shows the presence of synaptosomes, synaptoneurosomes, myelin, empty vacuoles and ribosome's.

Figure 11 Electron micrograph of synaptosomes from the IMHV of untrained day-old chick (High Magnification: x15,000).

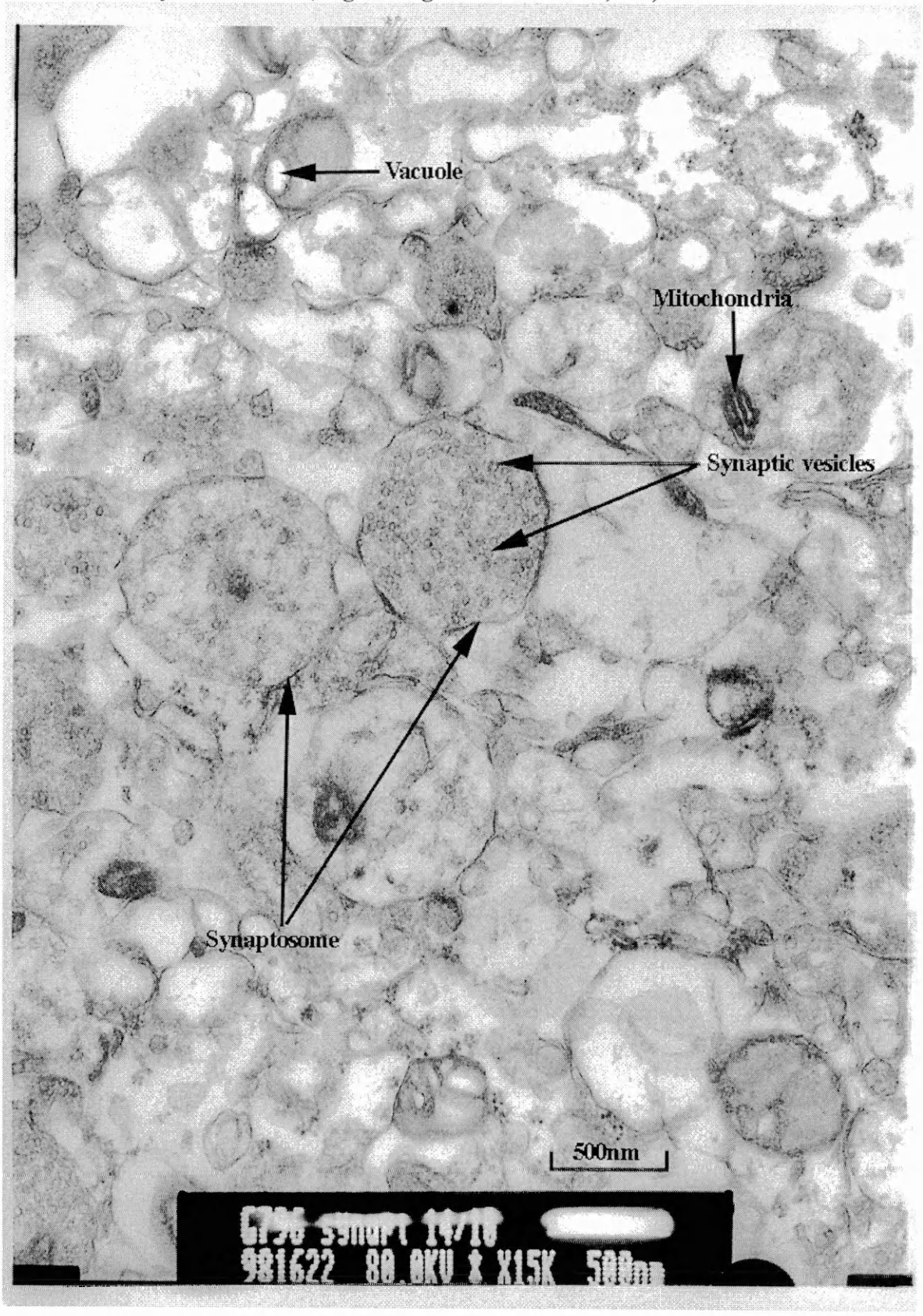


Figure 11 High power electron micrograph of a synaptosome preparation from the chick IMHV. The picture shows the presence of synaptosomes, synaptic vesicles, mitochondria and empty vacuoles.

Figure 12 Electron micrograph of synaptoneurosome from the IMHV of untrained day-old chick (High Magnification: x20,000).

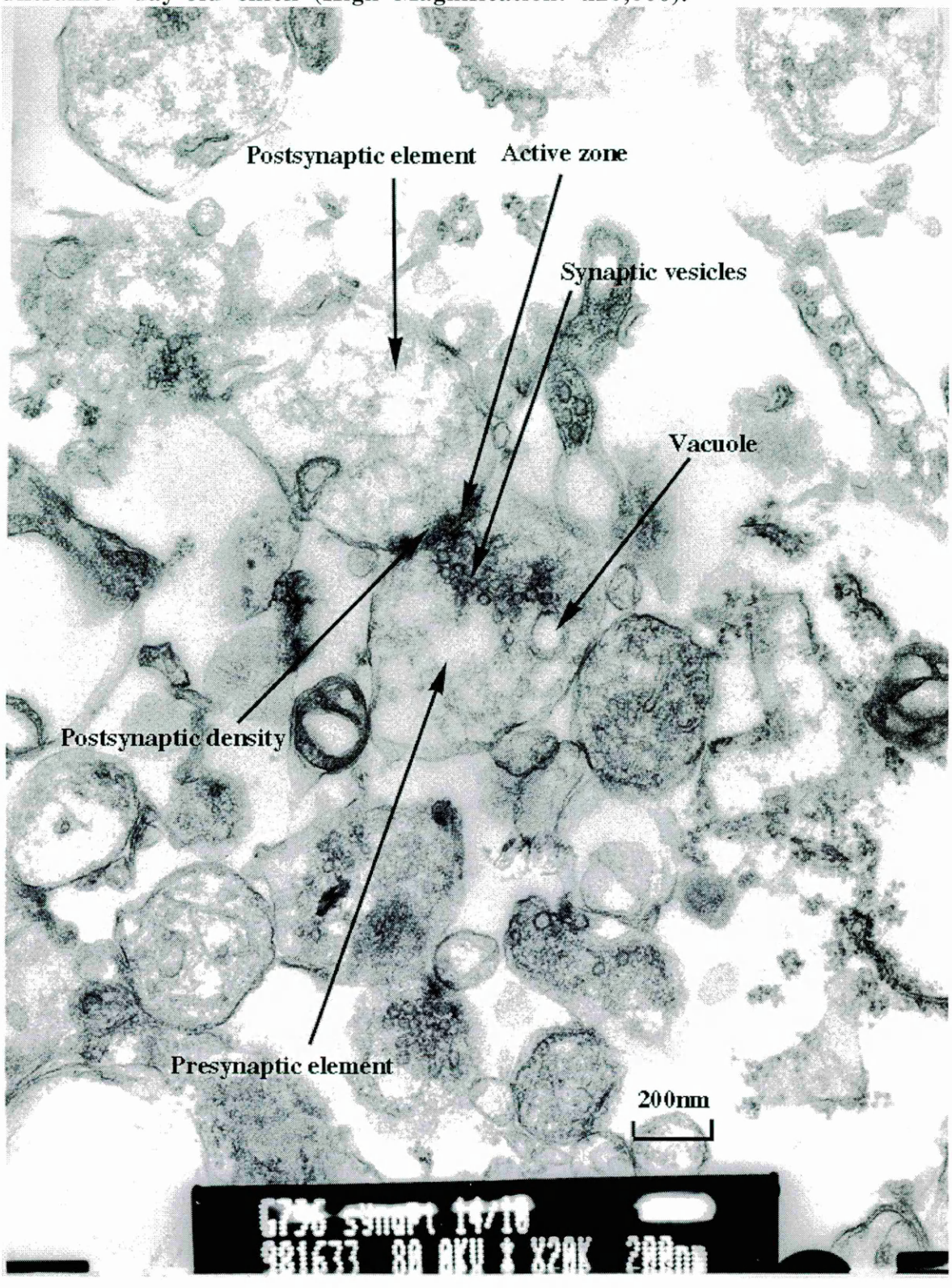


Figure 12 High power electron micrograph a synaptoneurosome preparation from the chick IMHV. The picture shows the presence of presynaptic element, postsynaptic element, synaptic vesicles, postsynaptic density, active zone and vacuoles.

Table 2: **Percentage recovery of proteins in the crude membrane from the IMHV.**

	Tissue Weight (mg)	Protein Concentration after 1st Homogenisation (mg/ml)	Final Protein Concentration (mg/ml)	% Loss
1	33	1.4	0.7	50
2	44	1.5	1.1	27
3	36	1.6	0.8	50
4	38	1.5	0.9	40
5	45	1.5	1	33
6	44	1.5	1	33
7	25	1.4	1	29
8	37	1.5	1.2	29

Average loss of protein = 35%

3.2. $^{45}Ca^{2+}$ experiments

Extensive physiological studies have already shown that high concentrations of extracellular K^+ - ions can depolarise membranes leading to $^{45}Ca^{2+}$ influx (Blaustein, 1975), and that there is a sigmoidal relationship between increasing extracellular potassium ion concentration ($[K^+]_o$), and relative increases in $^{45}Ca^{2+}$ uptake in this preparation. Experiments also confirmed that stimulation of terminals did not render them irreversibly leaky to calcium. Membranes are relatively permeable to chloride ions (Cl^-) and it was suggested that they might swell up when external sodium chloride (NaCl) was replaced with KCl (Keen and White, 1971). This therefore led some to suggest that the observed K^+ stimulated calcium uptake in membranes incubated in Cl^-containing medium might be the result of passive redistribution of calcium due to bulk flow of water and solutes into the nerve terminals. However, the evidence for the ability of K^+ to stimulate calcium influx is strengthened by the fact that membranes are relatively impermeable to calcium (Keen and White, 1970), and that

replacement of extracellular chloride ion ($[Cl^-]_o$) by impermeant anions such as sulphate (Keen and White, 1971) and methylsulphate (Hutter and Noble, 1960) did not abolish K^+stimulated calcium influx (Blaustein, 1975). Since reverse exocytosis or pinocytosis were not the cause of calcium influx, since significant KCl-stimulated $^{45}Ca^{2+}$ but not [^{3}H] mannitol and [^{14}C]inulin (agents that are taken up by presynaptic terminals by processes such as pinocytosis) influx was detected in presynaptic terminals, would suggest that calcium influx following depolarisation with KCl is a real and reversible effect.

As shown in figure 10, and discussed previously, crude membrane preparations are contaminated with organelles such as 'free' mitochondria even though these contaminants are able to accumulate calcium via an energy-dependent process. It has been shown that uptake in these organelles is not significantly altered by relative changes of $[K^+]_o$ and $[Na^+]_o$ in the incubation medium (Drahota and Lehninger, 1965; Lazarewicz *et al.*, 1974), thus indicating that most of the KCl-stimulated calcium influx in synaptosomal preparations take place in synaptic endings.

Taking into account studies described above and more recent studies by Lundy *et al.* (1994), Maubecin *et al.* (1995) and Pocock *et al.* (1992), groups who used KCl-stimulated $^{45}Ca^{2+}$ influx in membranes to study VSCC function, I initially decided to try the same method to study calcium influx via VSCC in synaptoneurosomes prepared from the IMHV and LPO of day-old chicks. I was unable to get consistent results, although I did finally manage to obtain KCl-stimulated calcium influx in membranes. Because of the great variability in the data, I decided to turn to the newer technique using fura-2/AM to measure calcium concentration in synaptoneurosomes.

3.2.1. Preliminary $^{45}Ca^{2+}$ data

In this section I have shown some preliminary data on the effects of KCl-stimulation on $^{45}Ca^{2+}$ influx in synaptoneurosomal preparations.

Figure 13 Effect of 70mM KCl on $^{45}Ca^{2+}$ influx in crude synaptic membranes prepared from the left and right IMHV of untrained day-old chicks.

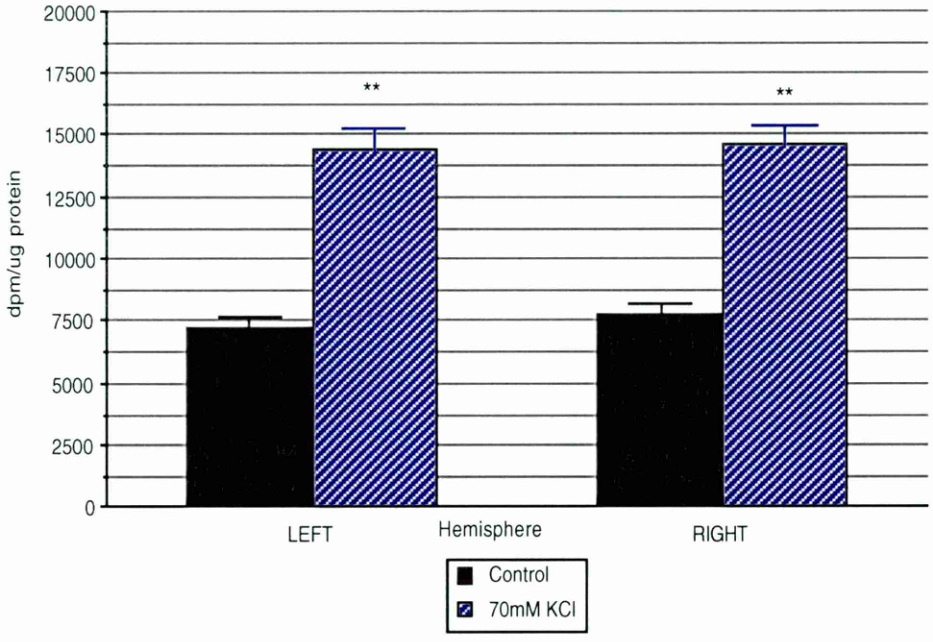


Figure 13 Effect of 70mM KCl on $^{45}Ca^{2+}$ influx in synaptoneurosomes prepared from the left and right IMHV of untrained chicks. Results are means ± s.e.m. expressed as dpm/µg protein. (n=7 in duplicate). ** = $p < 0.025$

Figure 14 Effect of 70mM KCl on $^{45}Ca^{2+}$ influx in crude synaptic membranes prepared from the left and right IMHV of day-old chicks tested 5 minutes post-training.

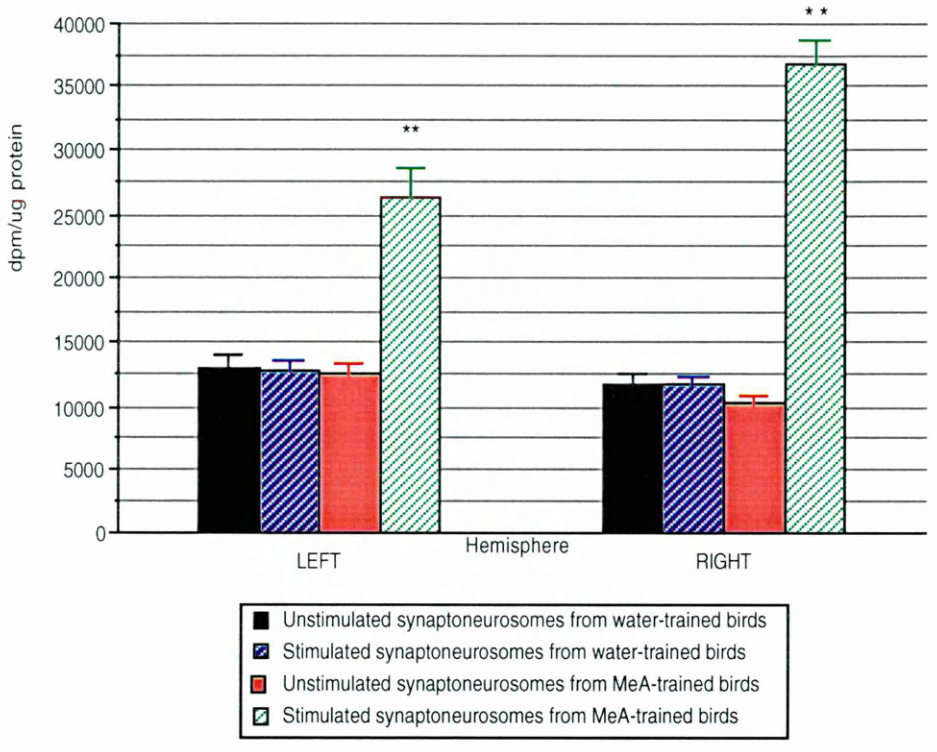


Figure 14 Comparison of the effect of 70mM KCl on $^{45}Ca^{2+}$ influx in synaptoneurosomes prepared from the left and right IMHV of water- and MeA-trained birds tested 5 minutes post-training. Results are means ± s.e.m. expressed as dpm/μg protein. (n=18 in duplicate). ** = $p < 0.025$

Figure 15 Effect of 70mM KCl on $^{45}Ca^{2+}$ influx in crude synaptic membrane prepared from the left and right IMHV of day-old chicks tested 30 minutes post-training.

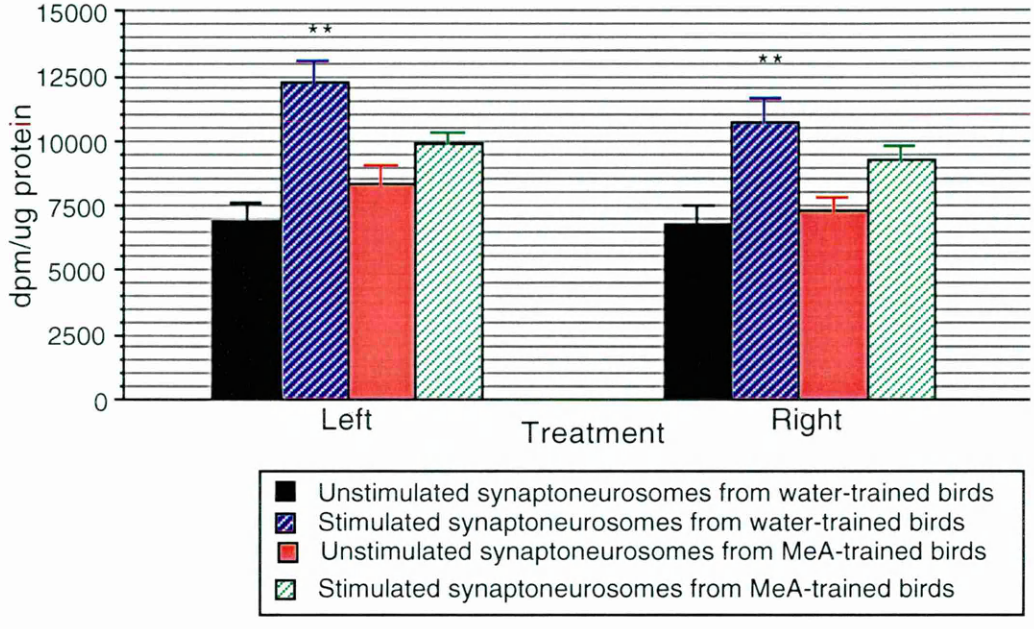


Figure 15 Comparison of the effects of 70mM KCl on $^{45}Ca^{2+}$ influx in synaptoneurosomes prepared from the left and right IMHV of chicks tested 30 minutes post-training. Results are means ± s.e.m. expressed as dpm/µg protein. (n=18 in duplicate). ** = $p < 0.025$

Figure 16 Effect of varying concentration of ω-CTX GVIA on 70mM KCl stimulated $^{45}Ca^{2+}$ influx in crude synaptic membrane prepared from the IMHV of untrained chicks.

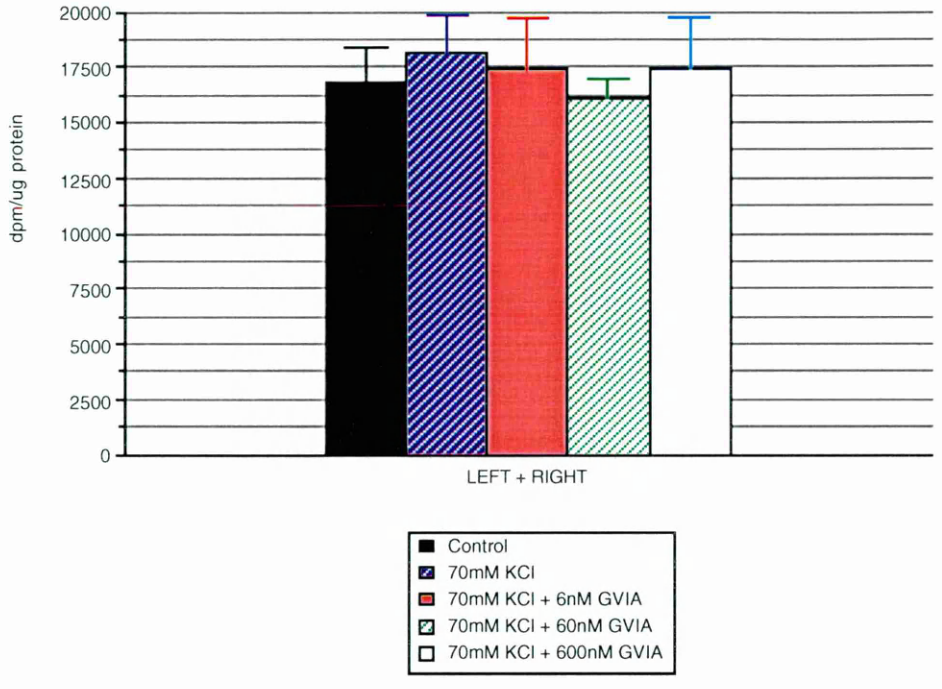


Figure 16 Comparison of the effects of the N-type VSCC blocker ω-CTX GVIA on KCl-stimulated $^{45}Ca^{2+}$ influx in synaptoneurosomes prepared from the IMHV of untrained birds. Results are means ± s.e.m. expressed as dpm/μg protein. (n=6 in duplicate).

3.3. Fura-2/AM

The fluorescent calcium indicator fura-2/AM is a divalent metal ion chelator which changes its fluorescent properties upon complexation (Neher, 1995). It belongs to a family of indicators with 8 co-ordinated tetracarboxylate chelating sites with stilbene chromophores with an ethylenic linkage of the stilbene into a heterocyclic ring which increases the quantum efficiency and photochemical stability of the fluorophore. Fura-2/AM belongs to a newer breed of indicators which is the preferred choice for calcium analysis over its predecessor quin-2. This is because it shows much stronger fluorescence, wavelength shifts upon calcium binding, somewhat weaker affinity for calcium (thus allowing larger concentrations of $[Ca^{2+}]_i$ to be measured), and better selectivity against magnesium and heavy metals (Grynkiewicz *et al.*, 1985).

The fluorescent intensity of fura-2/AM changes between its calcium bound and unbound states. Thus measurements of the ratio of fluorescence at 340nm and 380nm, the fluorescence of the indicator in bound and unbound states respectively, enables the experimenter to calculate $[Ca^{2+}]_i$ (as shown in the calculation in chapter 2; Grynkiewicz *et al.*, 1985).

The initial plan was to measure the absolute calcium concentrations in membranes from birds in either of the three test groups, thus give a more accurate indication of calcium influx in the IMHV and LPO. However, as seen in table 4-7 of the appendix, comparisons between different treatments was not possible because of relatively large variations in absolute $[Ca^{2+}]_i$ within the sample batches. This gave larger standard errors, as a result of which statistical analysis did not show any significant differences. An explanation for the relatively large variations in $[Ca^{2+}]_i$ in synaptoneurosomal samples could be due to slight variations in the preparation process. For example, Verhage *et al.* (1988) found differences in synaptosomal $[Ca^{2+}]_i$ depending on the fraction used. Furthermore, the authors suggested that 'empty' membrane structures present in synaptosomal /synaptoneurosomal preparations may accumulate indicators such as fura-2/AM as a result of esterase activity but do not have the ability to regulate $[Ca^{2+}]_i$.

3.3.1. Preliminary fura-2/AM results

I carried out some preliminary experiments with fura-2/AM to determine the best concentration of KCl to obtain maximal calcium influx. Furthermore I have shown some absolute values of basal $[Ca^{2+}]_i$ to determine viability of my membrane preparation containing synaptosomes and synaptoneurosomes.

Figure 17 Effect of increasing concentration of KCl on $[Ca^{2+}]_i$ elevation in crude synaptic membrane in the IMHV of untrained day-old chicks.

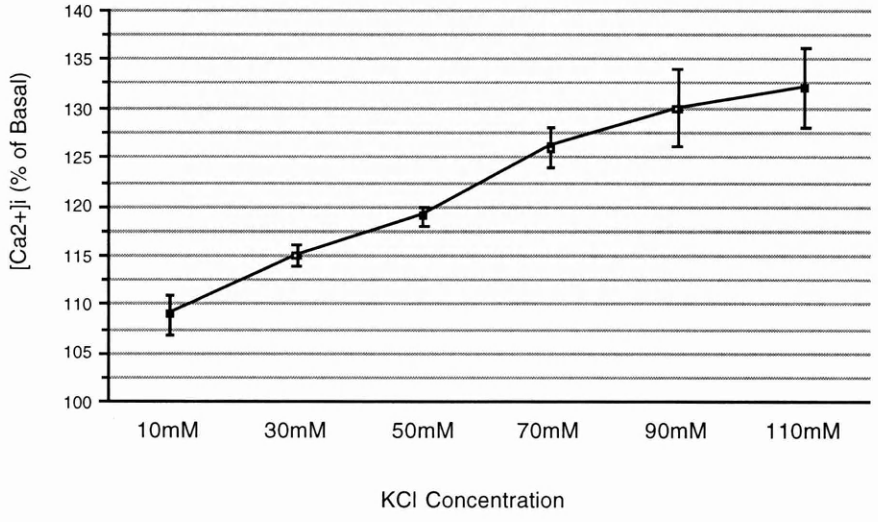


Figure 17 Dose-response curve to determine the effects of increasing concentrations of KCl on $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the IMHV of untrained chicks. Results are means ± s.e.m. (n=3 in duplicate).

Table 3: **Individual intracellular basal calcium concentration in crude synaptic membranes prepared from the IMHV of untrained day-old chicks.**

[Ca2+]i (nM)	
176.1	224.9
167.7	235.1
190.7	225.8
158.6	197.8
163.2	222.1
202.0	227.0
212.6	238.6
178.3	243.2
201.4	274.5
182.4	164.7
442.5	151.7
176.8	144.4
195.9	139.1

Mean = 205 $\pm$ 2.3

3.4. Discussion

The low magnification EM picture show that the crude membrane preparation from the chick IMHV possesses a mixture of synaptosomes, synaptoneurosomes and contaminants such as free mitochondria, glia and membrane fragments. The high EM shows the integrity of the synaptosomes and synaptoneurosomes which have resealed after being 'pinched off' from neurons during the preparation of the samples. Furthermore, Table 2 shows that 63% of the initial protein sample is retained during the preparation of the crude membranes.

The graphs in figures 13 to 16 show some of my early experiments with $^{45}Ca^{2+}$ uptake in synaptosomal preparations. In figure 13 I have shown significant increase in $^{45}Ca^{2+}$ in membranes stimulated with 70mM KCl compared to unstimulated samples from untrained chicks. I then proceeded to attempt to study the effect of passive avoidance training on KCl-stimulated $^{45}Ca^{2+}$ influx in birds tested 5 minutes and 30 minutes post-training. In these studies, controls were taken as birds trained on a water-coated bead (W-birds) and test birds

were taken as birds trained on methylanthranilate coated beads (M-birds). Figure 14 shows the results from chicks tested 5 minutes post-training where comparisons between unstimulated (W and M) and stimulated (70W and 70M) synaptoneurosomes taken from W-birds and M-birds shows increased $^{45}Ca^{2+}$ influx in KCl-stimulated membranes from M-birds but not in W-birds. Conversely in birds tested 30 minutes post-training (figure 15) I saw significant increased calcium influx in KCl-stimulated synaptoneurosomes from W-birds compared to unstimulated samples but no difference between stimulated and unstimulated synaptoneurosomes taken from M-birds. As mentioned earlier, it was apparent that I was not getting consistent influxes with $^{45}Ca^{2+}$ following depolarisation with 70mM KCl as previously shown (Blaustein, 1975).

I then decided to try one final experiment using this method, as is shown in figure 16. Samples were divided into 5 groups. One group was used as a control, another was incubated with 70mM KCl, and the remaining groups were incubated with different concentrations of the N-type voltage sensitive calcium channel antagonist ω-CTX GVIA prior to addition of 70mM KCl. The results show no difference between any of the sample groups. I was unable to fully explain the lack of stimulation in most of my samples although one possible explanation could be that the concentration of KCl used could have been too high since Blaustein (1975) had shown very high concentration of K^+-ions may affect calcium influx. However, since I managed to get significant stimulation in some experiments, though not consistently, and the fact that Blaustein did get stimulated calcium influx in his synaptosomal samples with 70mM KCl, I decided that this could not be the explanation. Thus, since, my results were inconsistent I decided to try using the fluorescent calcium indicator, fura-2/AM, for the rest of my studies.

Figure 17 shows a dose-response study to determine the effect of increasing concentrations of KCl on calcium influx in synaptoneurosomes using the fura-2/AM method. The graph shows an almost linear increase in KCl-stimulated $[Ca^{2+}]_i$ elevation when KCl concentration was increased from 10 to 110mM. The graph begins to plateau at KCl concentrations of 90 and 110mM. This is in some agreement with previous findings by Blaustein (1975), who found that membranes with low intracellular synaptosomal K^+-ions ($[K^+]_i$ when exposed to

high concentrations of KCl ($[K^+]_o$ >100 mM) showed a decreased amount of stimulated calcium influx compared to synaptosomes exposed to lower concentrations of KCl. I used low $[K^+]_i$ concentration in the incubation medium (2.5mM compared 5mM for previous experiments e.g. Lundy *et al.*, 1992) which could account for the plateau in synaptoneurosomes exposed to 90 and 110mM KCl. An interesting feature of the graph is the relatively low degree of calcium influx in synaptoneurosomes following stimulation with 70mM KCl (~25%) compared to previous observations by various groups using synaptosomes. For example both Bowman *et al.* (1993) and Grantham *et al.* (1994), using fura-2AM, found that even with 20mM KCl they were able to measure approximately 100% increases in calcium influx in synaptosomes during the plateau phase of KCl-stimulated biphasic calcium cascade. Similarly Pocock *et al.* (1992), using the $^{45}Ca^{2+}$ uptake method found approximately a 150% increase in calcium influx in synaptosomes stimulated with 50mM KCl. The main difference between my study here and that of Bowman *et al.* (1993), Grantham *et al.* (1994) and Pocock *et al.* (1992) is that I had used crude synaptoneurosomal fractions compared to purer synaptosomal fractions used by the others. Verhage *et al.* (1988) had shown that measurements of $[Ca^{2+}]_i$ using the fura-2AM method varied depending on the purity of the synaptosomal preparation used. Thus, it is possible that some amount of fura-2AM will be taken up by the impurities in my crude fraction such as myelin, free mitochondria and fragments of membranes, resulting in a smaller concentration of fluorescent dye available to measure accurately the $[Ca^{2+}]_i$ in the synaptoneurosomal fractions. In addition, fura-2AM binding to calcium has been observed to be temperature dependent, where cooler temperatures resulted in lowered interaction between fura-2AM and calcium (personal communications with E.J.Salinska). In my experimental set up I had observed that the room temperature in the laboratory was usually slightly below 20^oC. It should be noted, however, that minor temperatures should only cause slight variations in calcium measurements. The age of the animal used has also been shown to affect stimulated calcium influx. Kavanagh *et al.* (1995) observed that $^{45}Ca^{2+}$ accumulation in 3-4 day-old chick forebrain prisms following stimulation with quisqualic acid (AMPA receptor agonist) varied with age. I used 1 day-old chicks while Bowman *et al.* (1993) and Grantham *et al.* (1994) used 14 day-old chicks and Pocock *et al.* (1992) used 2 day-old chicks.

Finally table 3 gives absolute values of $[Ca^{2+}]_i$ from individual synaptoneurosomal samples prepared from the IMHV of untrained day-old chicks. The average value was 205nM, which is similar to previous estimates of $[Ca^{2+}]_i$ in neurons (~180nM; Disterhoft *et al.*, 1993) thus indicating that my preparation at least, in terms of calcium concentration is normal. I standardised my data by representing the change in $[Ca^{2+}]_i$ following KCl-stimulation, as a percentage of basal $[Ca^{2+}]_i$,as seen in the graphical format in figure 15 and in subsequent figures in chapter 4, 5 and 6.

Chapter 4

Time-Course of KCl-stimulated $[Ca^{2+}]_i$ Elevation in Crude Synaptic Membranes Prepared from the IMHV and LPO of Day-Old Chicks following Passive Avoidance Learning

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4. Introduction

Memory formation for the one-trial passive avoidance task is associated with a cascade of time-dependent electrophysiological, biochemical and morphological events in particular areas of the chick brain (Rose, 1993). It is postulated that one of the early biochemical events following avoidance learning is increased calcium influx in the IMHV and possibly, the LPO of the chick brain. Calcium has the ability to activate several enzymes in neurons and has a crucial role in the development of synaptic plasticity. The mechanism of calcium activated plasticity varies with different forms of learning (for a review see Jodar and Kaneto, 1995). In some cases, it is a primary signal that initiates the biochemical cascade necessary for synaptic strength; and in others, it acts as a second messenger following the activation by chemical or electrical signals.

Recent work in our laboratory have shown that there is an enhanced calcium-dependent release of glutamate, and GABA, 30 minutes and 1 hour post-training respectively in the left IMHV of M-trained chicks (Daisley and Rose, 1998). Furthermore, Clements *et al.* (1995) observed that pretraining bilateral injections of the N-type VSCC blocker ω-conotoxin GVIA induced amnesia in chicks tested on the avoidance task 30 minutes or 3 hours post-training.

4.1. Aim of experiments

The aim of the experiments in this chapter was to determine a time-course of calcium influx, via VSCC in certain areas of chick brain following training. In order to do this, I designed an experiment to establish a time course of potassium chloride (KCl)-evoked changes in intracellular calcium concentration $[Ca^{2+}]_i$ in synaptoneurosomes from the IMHV and LPO of day-old chicks trained on the passive avoidance task. Chicks were divided into three groups; quiet untrained controls (Q), water-trained controls (W) and methyl anthranilate test group (M).

4.2. Time-course of calcium influx in the IMHV and LPO

4.2.1. Basal $[Ca^{2+}]_i$

The mean basal $[Ca^{2+}]_i$ in synaptoneurosomes was similar in the left and right IMHV and LPO of all treatment groups (Tables 4 to 7 in the appendix). There was no significant difference in basal $[Ca^{2+}]_i$ between experimental groups or time points after training in either hemisphere of the IMHV or LPO. Due to the relatively large variation in basal $[Ca^{2+}]_i$ between the individual preparations, the increase in $[Ca^{2+}]_i$ following KCl stimulation was calculated as a percentage of basal level for each sample. $[Ca^{2+}]_i$ was measured at 1 minute after the addition of KCl. At this time the initial peak in $[Ca^{2+}]_i$, characteristic of KCl stimulation had decayed to a plateau (Bowman *et al.,* 1993; R.C. Bourne and E.J. Salinska, unpublished observations).

4.2.2. Training induced changes in KCl-stimulated increase in $[Ca^{2+}]_i$ in the IMHV

Addition of KCl (70mM) to the synaptoneurosomal samples from (Q) birds resulted in an average increase in $[Ca^{2+}]_i$ of 14 $\pm$ 0.4% and 15 $\pm$ 0.6% in the left and right IMHV respectively, compared to the basal, unstimulated levels (n=35, Figure 18 and 19). Two-way ANOVA showed a time and training interaction in the left ($F_{10,91}$=3.24, p=0.001) but not the right IMHV ($F_{10,11}$=0.49, p=1).

In the synaptoneurosomes prepared from the left IMHV of birds tested immediately after training, on the MeA-coated bead, 70 mM KCl evoked a significant, 33.1 $\pm$ 3.5% increase in $[Ca^{2+}]_i$ compared to basal. KCl-stimulation of synaptoneurosomes from the Q- and W-birds resulted in a 14.2 $\pm$ 1.7% and 19 $\pm$ 2% increase respectively in $[Ca^{2+}]_i$. One-way ANOVA of the percent increase in $[Ca^{2+}]_i$ following KCl-stimulation, immediately after training showed a significant training effect ($F_{2,16}$=14.62, p=0.0002). Post-hoc analysis using Tukeys honestly significant difference(THSD) analysis showed that the increase in $[Ca^{2+}]_i$ in M-birds was significantly greater then W- and Q-birds (THSD; P=0.03). The data from the right IMHV did not show any significant time and training interaction, and

therefore it was not possible to perform further statistical analysis on these data. It should be noted, however, that there was a small, but not statistically significant, increase in the W- (24.1 ± 2.9%) and M- (25.6 ± 2.9%) birds compared with Q-birds (17.1 ± 1.2%).

A significant training effect was also observed in the left IMHV of chicks tested 5 minutes post-training ($F_{2,17}$=4.32, P=0.03) where, KCl-stimulated increase in $[Ca^{2+}]_i$ persisted in M-birds 5 minutes post-training (23.9 ± 1%). Post-hoc analysis did not show significant difference between W- and M-birds or between W- and Q-birds. However, there was significant difference between Q- and M-birds (THSD; P=0.03).

A significant training effect was also measured in birds tested ten minutes post-training ($F_{2,29}$=6.69, p=0.004), where, KCl-stimulated increase in $[Ca^{2+}]_i$ in Q-, W- and M-birds was 12 ± 1.4%, 16.7 ± 1.6% and 20 ± 1.1% respectively. Post-hoc analysis only showed significant difference between M- and Q-birds (THSD; p=0.003). There was no statistically significant difference between W- and M-birds.

Thirty minutes post-training there was still a significant training effect ($F_{20,20}$=4.56, p=0.02). Post-hoc analysis showed only significant difference between M-birds compared to Q-birds (THSD; p=0.008).

One-way ANOVA did not show any training effect in birds tested 3 or 6 hours post-training. Therefore, further analysis was not carried out between any of the 3 groups of birds.

Although post-hoc analysis did not show any significant training effect in W-birds, there appears to be a trend showing some degree of experience related increases in $[Ca^{2+}]_i$ following KCl stimulation. Experiments were carried out to determine, whether the act of pecking at the chrome bead, and the possible appetitive effect of W-training on the birds, resulted in greater KCl-stimulated increase in $[Ca^{2+}]_i$ compared to Q-birds. Chicks were pre-trained as described in chapter 2, and then trained on a dry-chrome bead (D). In chicks tested immediately, or 5 minutes post-training there was no significant time and training

interaction in KCl-stimulated increase in $[Ca^{2+}]_i$ between Q and D-trained birds in the left ($F_{1,14}$=3.18, p=0.1) or right IMHV ($F_{1,15}$=0.26, p=0.7).

4.2.3. Training induced changes in KCl-stimulated increase in $[Ca^{2+}]_i$ in the LPO

Two-way ANOVA of KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the LPO showed that there was a significant time and training interaction in both the left LPO ($F_{10,91}$=3.24, p=0.001) and the right LPO ($F_{10,96}$=2, p=0.04; figure 20 and 21)

One-way analysis of KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the left or right LPO of Q-, W- and M-birds, tested immediately after training, did not show any significant difference between the groups of birds. Therefore, no further statistical analysis was carried out.

At 5 minutes post-training analysis of the percentage increase in KCl-stimulated $[Ca^{2+}]_i$ indicated a significant training effect in the left LPO ($F_{2,14}$=5.76, p=0.02). Post-hoc analysis showed that the increase in M-birds (35.3 $\pm$ 2.1%) was significantly greater than in both the Q- and W-birds (25.8 $\pm$ 2.8% and 26.1 $\pm$ 1.6% respectively; THSD; p=0.04). A significant training effect was also observed in the right LPO of birds tested 5 minutes post-training ($F_{2,14}$ =5.6, p=0.02). Post-hoc analysis showed that the $[Ca^{2+}]_i$ increase in M-birds (36.9 $\pm$ 2.8%) was significantly greater than in Q- (24.8 $\pm$ 2.3%) and W- (25.6 $\pm$ 3.7%) birds (THSD; p=0.04).

There is a significant training effect in synaptoneurome prepared from the left LPO ($F_{2,19}$=17.51, p=0.0001) and right LPO ($F_{2,19}$=8.18, p=0.003) of birds tested 10 minutes post-training. Post-hoc analysis in the left LPO showed a significantly greater increase in KCl-stimulated $[Ca^{2+}]_i$ in M-birds (31.6 $\pm$ 2.1%) compared to Q- (23.1 $\pm$ 1.9%) and W- (16.8 $\pm$ 1.2%) birds (THSD; p=0.03). Furthermore, in the right LPO the KCl-stimulated increase in $[Ca^{2+}]_i$ in M- (28.3 + 2%) and W-birds (29.5 $\pm$ 2.7%) was significantly greater then Q-birds (18.5 $\pm$ 1.3%; THSD; p=0.03).

There was no significant difference in KCl-stimulated increase in $[Ca^{2+}]_i$ in the left or right LPO of Q-, W- and M-birds when tested at 30 minutes, 3- or 6 hours post-training. However, it should be noted, that in the right LPO, KCl-stimulated increase in $[Ca^{2+}]_i$ in M-birds (23.8 $\pm$ 1.8%) was slightly greater then Q- (17.6 $\pm$ 2.9%) and W-birds (18.8 $\pm$ 1.8%).

Figure 18 KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptosomes prepared from the left IMHV of Q-birds and W- and M- trained birds tested at specific times after training.

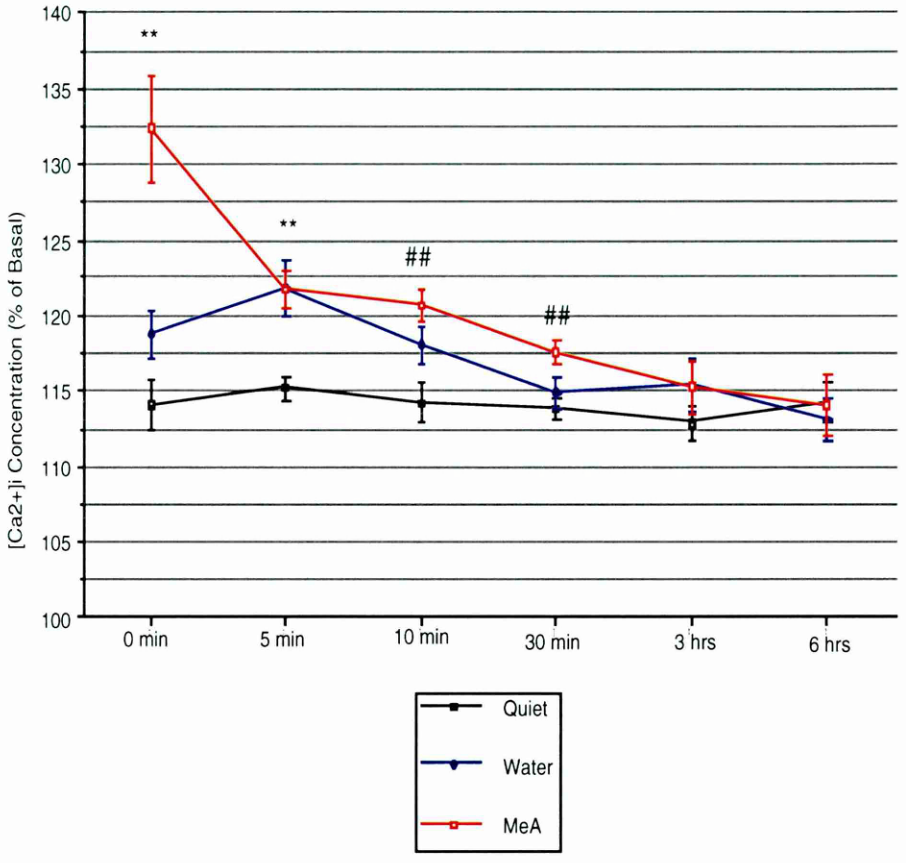


Figure 18 KCl-stimulated $[Ca^{2+}]_i$ changes in synaptoneurosomes prepared from the left IMHV of chicks tested at various times after training on the passive avoidance task. Synaptoneurosomes were stimulated with 70mM KCl. Results are means ± s.e.m. of percentage change in $[Ca^{2+}]_i$ compared with basal $[Ca^{2+}]_i$. n=5-11. ** = $P < 0.05$, ## = $P < 0.025$ (ANOVA). Q = untrained birds, W = water trained birds W = Methylanthranilate trained birds. The x-axis is a non-linear scale

Figure 19 KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptosomes prepared from the right IMHV of Q-birds and W- and M- trained birds tested at specific times after training.

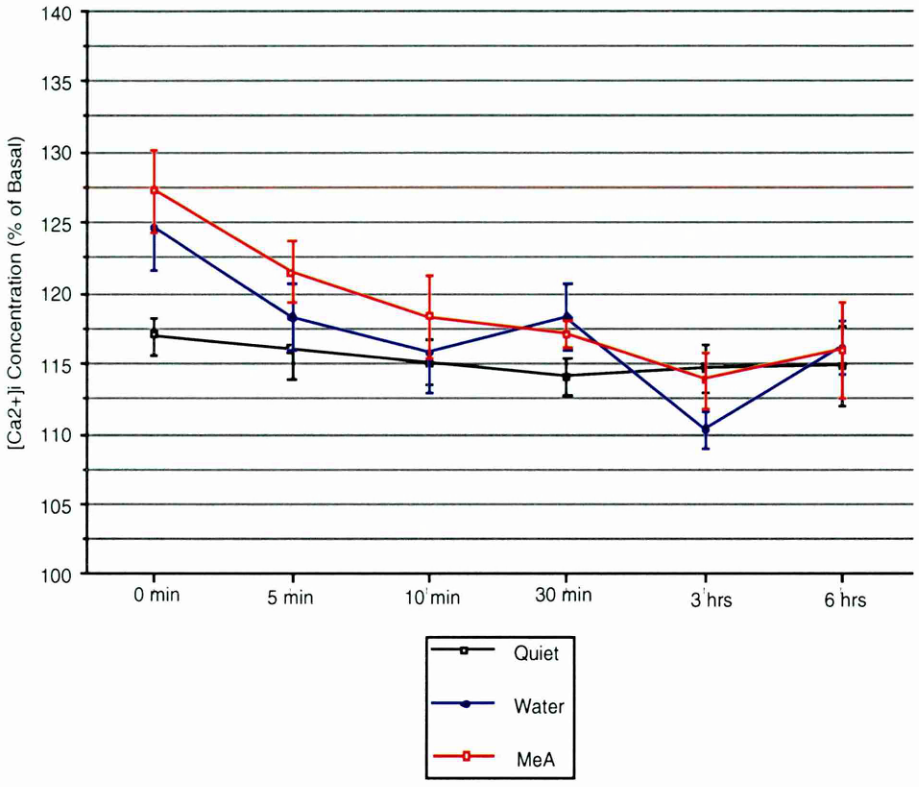


Figure 19 KCl-stimulated $[Ca^{2+}]_i$ changes in synaptoneurosomes prepared from the right IMHV of chicks tested at various times after training on the passive avoidance task. Synaptoneurosomes were stimulated with 70mM KCl. Results are means ± s.e.m. of percentage change in $[Ca^{2+}]_i$ compared with basal $[Ca^{2+}]_i$. n=5-11. Q = untrained birds, W = water trained birds W = Methylanthranilate trained birds. The x-axis is a non-linear scale

Figure 20 KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptosomes prepared from the left LPO of Q-birds and W- and M- trained birds tested at specific times after training.

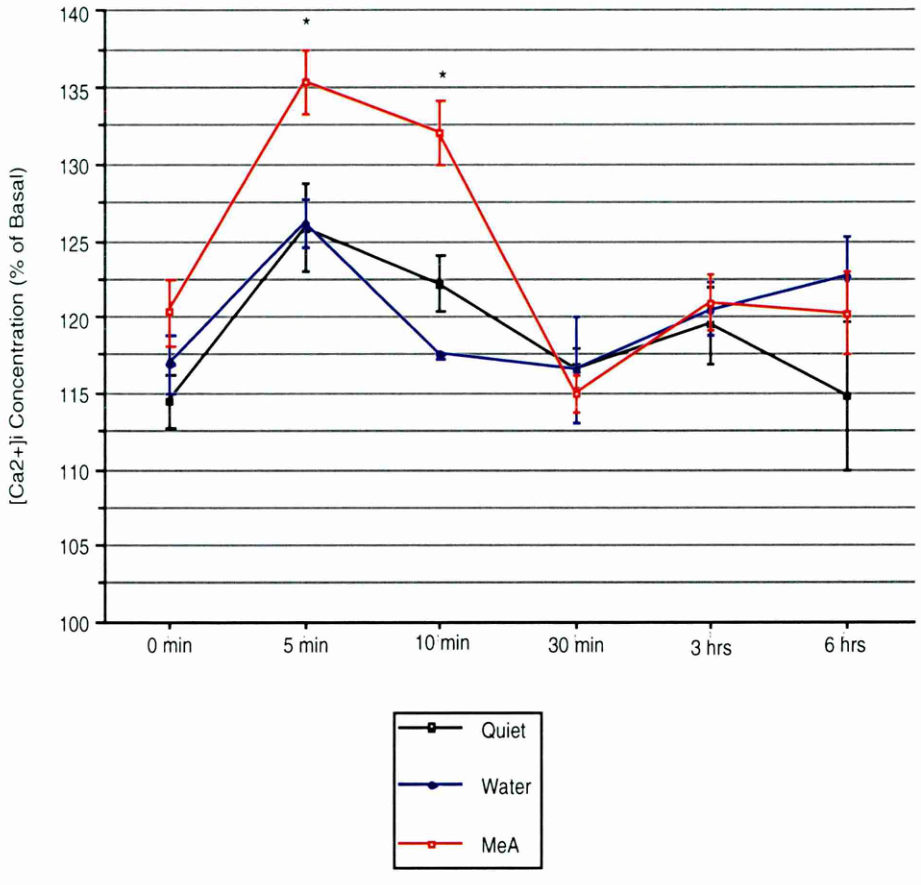


Figure 20 KCl-stimulated $[Ca^{2+}]_i$ changes in synaptoneurosomes prepared from the left LPO of chicks tested at various times after training on the passive avoidance task. Synaptoneurosomes were stimulated with 70mM KCl. Results are means ± s.e.m. of percentage change in $[Ca^{2+}]_i$ compared with basal $[Ca^{2+}]_i$. n=5-11. * = $P < 0.05$ (ANOVA). Q = untrained birds, W = water trained birds W = Methylanthranilate trained birds. The x-axis is a non-linear scale

Figure 21 KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptosomes prepared from the right LPO of Q-birds and W- and M- trained birds tested at specific times after training.

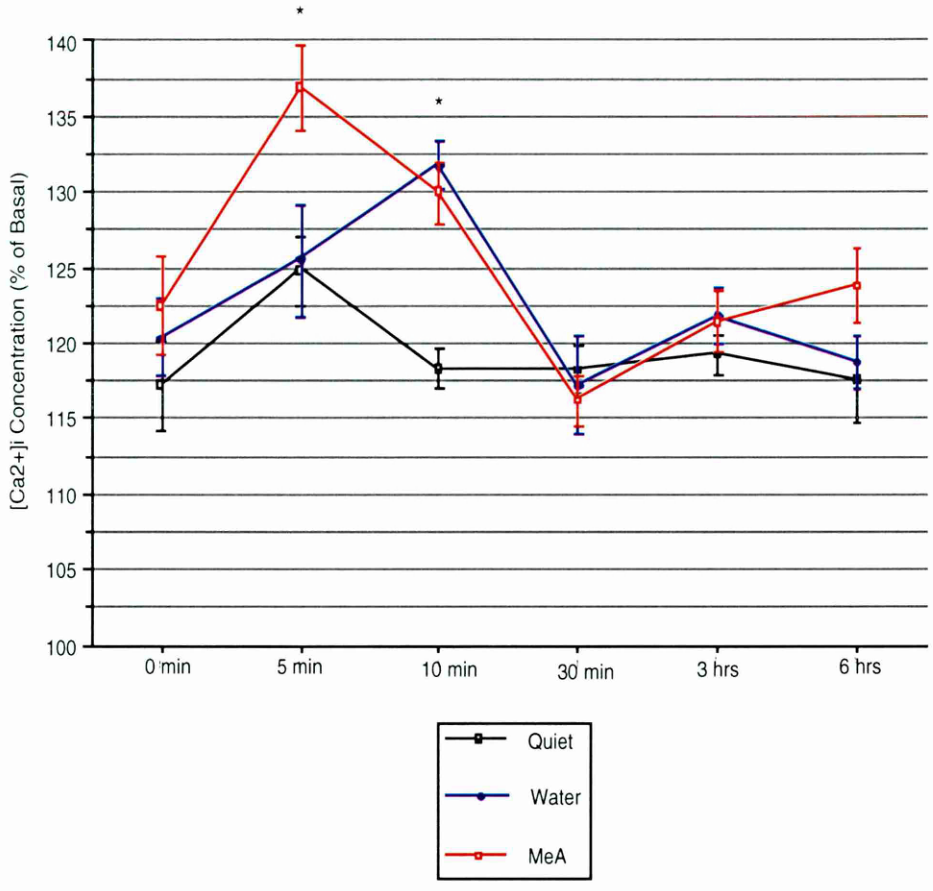


Fig. 21 KCl-stimulated $[Ca^{2+}]_i$ changes in synaptoneurosomes prepared from the right LPO of chicks tested at various times after training on the passive avoidance task. Synaptoneurosomes were stimulated with 70mM KCl. Results are means ± s.e.m. of percentage change in $[Ca^{2+}]_i$ compared with basal $[Ca^{2+}]_i$. n=5-11. * = $P < 0.05$ (ANOVA). Q = untrained birds, W = water trained birds W = Methylanthranilate trained birds. The x-axis is a non-linear scale

4.3. Discussion

The *in vitro* method, used in these experiments of comparing KCl-stimulated calcium influx in untrained and trained chicks, is a way of demonstrating increased VSCC activity in synaptoneurosomes prepared from the IMHV and LPO of the chick brain. This technique is based on the assumption that if training involves *in vivo* Ca^{2+} influx following physiological activation of VSCC, then removal of that tissue at a specific time would render the ion channels in a constant state of activation for a period of time (the channels in the synaptoneurosomes are in the same state as they were *in vivo* prior to removal). These channels would be more sensitive to stimulation by depolarising agents such as KCl compared to ion channels in an inactivated state. Thus, by measuring KCl-stimulated increases in $[Ca^{2+}]_i$ in synaptoneurosomes from chicks tested at different times after training, I was able to determine a time-course of calcium influx in the IMHV and LPO following passive avoidance training. This rationale behind such techniques has been used previously in our laboratory, where for example, Clements and Rose (1993) in some very preliminary work found increased $^{45}Ca^{2+}$ flux in prisms prepared from the IMHV of chicks tested on the PAL task. In addition, Daisley and Rose (1994) found elevated K^+-stimulated glutamate release in prisms from the IMHV of chicks tested on the PAL task. More recently, increased NMDA stimulated calcium influx has been measured in synaptoneurosomes from the IMHV of chicks following PAL training (unpublished observation by Salinska, EJ., Bourne, R,C. and Rose, S.P.R.; Salinska *et al.,* 1999)

KCl-stimulated increases in $[Ca^{2+}]_i$ was greatest in the left IMHV of M-birds immediately after training, an effect that was sustained in these birds up to 30 minutes post-training. Statistical analysis of $[Ca^{2+}]_i$ in M-birds, tested 3 and 6 hours post-training, were not significantly different from Q- and W-birds following KCl-stimulation. The transient increase in Ca^{2+} influx in M-birds soon after passive avoidance training may indicate an important function of the early Ca^{2+} signalling in the biochemical cascade, following learning and memory formation in the IMHV. These data correlates with evidence from previous experiments, where bilateral intracerebral (i.c.) injection of the N-type VSCC antagonist, ω-conotoxin GVIA, at around the time of training induced amnesia in chicks

tested 30 minutes or 3 hours after the training. This was interpreted as an indication that Ca^{2+} influx through these channels must take place up to, at least 30 minutes post-training, if retention for the task is to occur (Clements *et al.,* 1995). Parallel experiments using the L-type VSCC blockers, nifedipine, nimodipine and amlodipine at various doses failed to induce amnesia in chicks tested at different times after training.

In contrast, KCl-stimulated increases in $[Ca^{2+}]_i$ in the right IMHV was similar in all three treatment groups. It should be noted, however, that there was a small, but non significant, increase in M- and W-birds immediately after training. This hemispheric difference in chicks following passive avoidance training has been shown previously in a variety of biochemical assays. For example, within 30 minutes of training a chick on the passive avoidance task glutamate release (Daisley and Rose, 1998), upregulation of post-synaptic NMDA receptor activity (Stewart *et al*, 1992), and increased arachidonic acid release (Clements and Rose, 1996) were detected in the left but not right IMHV. Thus, the changes in calcium influx detected in the left, but not right IMHV is consistent with the previous findings mentioned above. Hemispheric differences for different forms of learning is evident in many vertebrate species (Dudai, 1994). Andrew (1992) suggests that, in chicks, the right hemisphere is particularly involved in spatial relations, such as relationships to distal topographical features. This hemisphere is believed to be involved in analysing the wide range of properties of a stimulus, thus indicating a role in global attention. Therefore, the right hemisphere, when assessing a novel stimulus, may 'use' detailed records of previous comparable experiences to make a decision, and store this information for future use (i.e. the effects may be interpreted as a short-lived and generalised response to the training experience). The left hemisphere, on the other hand, is involved in deciding what response (if any) to give to a stimulus. The chick uses appropriate cues, based on past experiences, remembering the affects of its response (i.e. giving a more specific response to a stimulus). Lesion experiments appear to indicate that memory following passive avoidance learning, is not static, but displays dynamic properties. The memory 'trace' for the passive avoidance task may subsequently 'move' from the left IMHV to the right IMHV then, at some later time, transfers to the left and right LPO(Rose, 1995).

Evidence for the possible involvement of Ca^{2+} in the early stages of memory formation in the chick have been described by Gibbs and Ng (1979a), where i.c. administration of isotonic KCl to chicks between 10 minutes pre-training and 2.5 minutes post-training, resulted in amnesia in birds when tested on a one-trial avoidance task 3 hours post-training. Furthermore, i.c. administration of isotonic KCl, 5 minutes pre-training, resulted in amnesia in chicks on tests at a variety of times between 10 minutes and 24 hours post-training. The authors also found that KCl induced amnesia in chicks trained on a visual discrimination task at 2 hours post-training. The early transient increase in KCl-evoked $[Ca^{2+}]_i$, particularly in the left IMHV, correlates with the designated STM phase of the Gibbs and Ng 3-stage model, where STM appears immediately after training and lasts up to 10 minutes post-training. It has been suggested that STM may be associated with an initial phase of neuronal hyperpolarization, which lasts for several minutes, due to changes in potassium conductance, following neural input (Gibbs and Ng., 1979b). Such an early phase of neural hyperpolarization measured in small diameter, unmyelinated, leech neurons following an impulse were found to be dependent on the presence of extracellular Ca^{2+} (Jansen and Nicholls; 1973). In addition, depolarising agents such as monosodium glutamate and isotonic KCl have been shown to prevent STM formation (Gibbs and Ng, 1979a). Conversely, intracerebral administration of increasing concentrations of calcium chloride, 5 minutes pre-training, into the chick forebrain increases the plateau phase of the designated short-term memory stage for the discrimination task, on birds tested at various times (Gibbs *et al*; 1979b). Subsequent experiments using lanthanum chloride an inhibitor of transmembrane Ca^{2+} flux, showed that administration, 5 minute pre-training, but not 10, 15 or 30 minute post-training, induced amnesia in chicks trained on a visual discrimination task, when tested between 10 and 180 minutes post-training, thus indicating that procedures that increase calcium levels in the chick forebrain also increase the duration of STM. Gibbs and Ng (1979a) therefore, proposed that calcium entry following repetitive spike discharge, associated with gustatory input such as avoidance discrimination learning, may lead to an alteration of the degree of hyperpolarization depending on the extent of potassium conductance; which could then lead to the modulation of several secondary neuronal processes such as the activation threshold, transmitter release and neuronal-glial activation.

In addition to the early transient changes in the IMHV, this area is also involved in long-term memory formation following passive avoidance training. Morphological studies have shown that 24 hours post-training synaptic changes such as increased synaptic bouton volume and synaptic vesicle number were lateralized in the left IMHV (Stewart, 1991). The known biochemical changes associated with passive avoidance learning have already been described in chapter 1 and will not be discussed further here.

The LPO is another area of the chick forebrain that has been shown to be active following passive avoidance learning (Kossut and Rose, 1984), visual discrimination learning and imprinting (Horn and McCabe, 1990; McCabe and Horn, 1991). The earliest changes in KCl-stimulated increases in $[Ca^{2+}]_i$ were detected 5 minutes post-training in both the left and right LPO of M-birds compared to quiet, control, birds. The increase in KCl-stimulated $[Ca^{2+}]_i$ was still evident in chicks tested 10 minutes post-training. However, by 30 minutes post-training there was no difference between any of the three groups. There was no further increase in KCl-stimulated $[Ca^{2+}]_i$ in the left LPO at other times tested, however there was a small but not significant increase in the right LPO of M-birds tested 6 hours after training, compared to quiet-birds.

The significant but transient increases in KCl-stimulated $[Ca^{2+}]_i$ in the LPO of M-birds within 5 minutes of training could also indicate a possible role for this area in STM processing. In addition, these changes may also be suggestive of the early transition from STM to LTM. Furthermore, the increases, although not significant, (post-hoc THSD; $p=0.05$), in KCl-stimulated $[Ca^{2+}]_i$ measured in the right LPO of M-birds tested 6 hours post-training may be indicative of biochemical changes in this area associated with LTM formation. Previous work from our laboratory has shown a role for the LPO in LTM formation, where electrophysiological measurements in chicks at different times after training only showed increased neural burst firing in chicks tested at 4 - 7 hours, but not 1-4 or 7 - 10 hours, after training on the passive avoidance task (Gigg *et al*; 1994). Morphological studies describing changes associated with LTM in the LPO have been briefly described in chapter 1.

As mentioned previously, the left hemisphere is believed to be the primary region involved in memory processing following passive avoidance learning in the chick (Rose, 1991), and the majority of changes in the IMHV following training were found to be localised here. The left hemisphere is also believed to be the site of memory storage for visual discrimination learning in chicks (Gaston and Gaston, 1984). Hemispheric lateralization have also been measured in other animals. For example, the left hemisphere plays a dominant role in song-learning in the canary (Nottenbohm *et al.,* 1990). The degree of lateralization in the LPO is smaller than that observed in the IMHV (Serrano *et al*, 1992). This difference in the degree of lateralisation is also evident on comparisons of percentage increase in KCl-stimulated $[Ca^{2+}]_i$ between the IMHV and LPO. In the IMHV, of M-birds, there was a significant increase in percentage stimulated $[Ca^{2+}]_i$ in the left but not right IMHV immediately after training that was sustained to a greater level than in Q-birds, up to 30 minutes post-training (figure 18). There was no significant difference in the right IMHV between any of the three groups of birds (figure 19). In comparison, changes in the LPO were more symmetrical, where both hemispheres of M-birds, exhibited significant increases in KCl-stimulated $[Ca^{2+}]_i$ 5 minutes post-training, which remained significantly greater then Q-birds up until 10 minutes post-training (figure 20, 21), though as mentioned previously there was a slight, but not significant, increase in the right but not left LPO of M-birds tested at 6 hours post-training.

Changes in KCl-stimulated increases in $[Ca^{2+}]_i$ were also detected in the IMHV and LPO of W-birds at specific times after training. For example, KCl-stimulated increases in $[Ca^{2+}]_i$ comparable to M-birds, was detected in the left IMHV of W-trained birds, particularly in those birds tested at 5 and 10 minutes post-training. Since pecking at either the water- or MeA-coated bead results in some learning experience, as detected by the lasting changes in the behaviour of the bird, where water-trained birds continue to peck at the bead (appetitive learning) and the MeA-trained birds avoid subsequent presentations of the bead (aversive learning), it is thus not surprising that some minor transient changes were also detected in the W-trained birds. The biochemical changes in W-trained birds that I observed agree with previous findings in our laboratory where water-training was found

to affect a variety of biochemical measures in the chick (Anokhin *et al.,* 1991b; Barber *et al.,* 1989; Barber *et al.,* 1991; Daisley and Rose, 1998)

To determine if the act of pecking a water coated bead is related to the observed elevation in KCl-stimulated $[Ca^{2+}]_i$, a series of experiments involving training birds to peck at a dry bead (D) were carried out. The experiments showed that KCl-stimulated increases in $[Ca^{2+}]_i$ in D-birds tested immediately or 5 minutes post-training was similar to Q-birds. This indicates that pecking at the dry-bead alone does not result in any observable increases in Ca^{2+} concentration in the IMHV. Conversely it also indicates that water training alone will result in some learning associated increase in Ca^{2+} concentration in the IMHV and LPO with a time-course different to M-birds.

The results presented here suggests that calcium influx in the IMHV and LPO occurs in the early stages of the biochemical cascade; activated following passive avoidance learning in the chick. In conclusion, assuming that calcium may be one of a group of prerequisite activators for several biochemical pathways, it should then be reasonable to assume that calcium influx following learning and memory would occur at the earlier stages of the biochemical cascade that have been described following passive avoidance learning (Rose, 1991). These data indicate that increased calcium influx probably occurs very soon after training in the left IMHV and both hemispheres of LPO. It should be noted that in chapter 1 I discussed earlier findings that the induction of the electrophysiological model of synaptic plasticity, LTP, has also been shown to be dependent on calcium influx. Thus it is reasonable to suggest that pre- and postsynaptic calcium may have an important role in activating some parts of the biochemical cascade necessary for neural plasticity. The early influx of calcium, as observed in the IMHV and the LPO, may be a precursor for the activation of several calcium-dependent enzymes and vesicular proteins leading to changes such as neurotransmitter release and activation of adjacent neurons. The precise mechanism of transmitter release is unknown, however, calcium influx probably triggers transmitter release by binding to, and subsequently activating, vesicular proteins such as synaptotagmin (Augustine *et al.,* 1994). Recently Daisley and Rose (1998) measured calcium-dependent glutamate release in the left IMHV of M-birds tested 30 minutes post-

training. Thus, assuming that calcium influx takes place 'upstream' of glutamate release then these data is in agreement with my findings of the early influx of calcium in synaptoneurosomes prepared from the left IMHV of M-birds. Furthermore, the early time-course of KCl-stimulated increases in $[Ca^{2+}]_i$ in the IMHV and LPO also agrees with the time-course of activation of calcium-dependent enzymes such as cAMPKII in chick brain following passive avoidance training described in chapter 1 (Zhao *et al.,* 1996; Serrano *et al.,* 1994). The multifunctional enzyme cAMPKII has been shown to regulate gene expression in hippocampal neurons, via two distinct calcium signalling pathways following stimulation of either NMDA-receptors or L-type VSCCs (Bading *et al.,* 1993). Thus, it could be hypothesised that the early influx and prolonged / sustained calcium influx in the left IMHV of M-birds may eventually 'trigger' cAMPKII activation which could then activate the transcription protein, cAMP responsive element binding protein (CREB). Activation of CREB may then result in glycoprotein synthesis leading to strengthening / stabilisation of synaptic connections leading to LTM formation. Earlier studies had already shown that other transcription factors were activated in the intermediate stages of the biochemical cascade in the chick brain following passive avoidance training (Anokhin and Rose, 1991a). In addition, it has recently been hypothesised that the synergistic stimulation of adenyl cyclase by calcium and neurotransmitters or PKA may produce exceptionally strong or prolonged cAMP signals required for stimulation of transcription (for a review see Xia *et al.,* 1995).

It should be mentioned briefly that increases in $[Ca^{2+}]_i$ following activation of intracellular calcium stores have also been shown to participate in calcium dependent neuronal plasticity. For example, Ohnuki and Nomura (1996) found that pretraining administration of dantrolene (1-[(5-[p-Nitrophenyl]furfurylidene)-amino]hydantoin) an inhibitor of intracellular calcium mobilisation, impaired avoidance performance and spatial memory in mice. It is most likely that release of calcium from intracellular stores and calcium influx via VSCC are closely linked in modulating a variety of neuronal events associated with learning and memory formation such as membrane excitability, transmitter release and synaptic plasticity (Kostyuk and Verhratsky, 1994).

The observation that KCl-stimulated increases in $[Ca^{2+}]_i$ occurs in the IMHV and LPO, lends further support to the idea that both these regions are involved in information processing following passive avoidance learning. The findings that increases in KCl-stimulated $[Ca^{2+}]_i$ occurred immediately after training, in the left IMHV, and 5 minutes post-training in both LPO's, tentatively agrees with the suggestion by Rose (1991) that the initial changes occur in the left IMHV; which is then transferred to the right IMHV followed by 'movement' of the memory trace to both areas of the LPO. It should be noted, however, that no changes were detected in the right IMHV at any time, which does not agree with the idea of the 'trace' being transferred from the left to the right IMHV. However, my data do agree with previous findings that changes following avoidance learning occurs predominantly in the left IMHV (Rose, 1991). Finally, W-trained birds also showed some interesting increases in KCl-stimulated $[Ca^{2+}]_i$ at certain times after training, and that these changes were lateralized to some degree. These changes may lend further support to the idea that water-training itself will result in some form of learning that may also produce biochemical changes associated with memory formation.

Chapter 5

Voltage-Sensitive Calcium Channels in Crude Synaptic Membranes from the IMHV

5. Introduction

As discussed in chapter 1, there are several subtypes of voltage sensitive calcium channels (VSCC) with differing electrophysiological and pharmacological properties. These channels are impermeable to calcium ions when the cell is hyperpolarised, and opens when the cells are depolarised. Calcium influx into neurons following depolarisation may occur through any one of these subtypes as long as they are present in the cell.

VSCC can be broadly categorised into high voltage activated (HVA) and low voltage activated (LVA) channels. The different ion channels are involved in distinct stages, and to varying degrees, in the depolarisation-induced calcium influx. In chapter 1 I discussed previous findings that KCl-stimulated calcium influx can be divided into two phases. The initial phase which is termed the rapidly inactivating phase, and the secondary stage is called the non-inactivating phase. Following KCl-stimulation, HVA channels are believed to be activated in the rapidly inactivating phase, whereas LVA channels are activated in the secondary non-inactivating phase (Nacshen and Blaustein, 1980; Adam-Vizi and Ashley, 1987; Tibbs *et al.,* 1989). Bowman *et al.* (1993) have shown that the HVA, N-type VSCC, has a dominant role in the plateau phase of the observed KCl-induced increase in $[Ca^{2+}]_i$ in synaptosomes prepared from chick forebrain. Specific VSCC blockers, particularly blockers of N, P and L-type channels have been shown to inhibit, to different degrees, KCl-stimulated increases in $[Ca^{2+}]_i$ in chicken synaptosomal preparations (Lundy *et al.,* 1994; Maubecin *et al.,* 1995).

5.2. Aim of experiments

The experiments described in this chapter were carried out to determine the characteristics of the different VSCC in synaptoneurosomes, and brain slices prepared from the IMHV and LPO of chick brain. The first experiments described in figure 22 - 25, were to determine the effects of different VSCC blockers, on the plateau phase, of KCl-stimulated increases in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the IMHV of untrained day-old chicks. For the dose-response experiments the left and right IMHV were combined, since there is currently

no evidence in the literature for large hemispheric differences in VSCC levels in the brain of untrained birds. The I.C.$_{50}$ values for the different antagonists was calculated by eye. from the respective graphs.

The second set of experiments were carried out to determine the inhibitory effects of adding different antagonists (N, P/Q and L) together on synaptoneurosomes prepared from combined left and right IMHV of untrained day-old chicks (figure 26). The rationale for this was to deduce whether these antagonists exerted their effects on similar or distinct sites on the synaptoneurosomes.

The aim of the experiments, described in figures 27, 28 and 29, was to determine the degree of inhibition of N, P/Q and L-type channels in synaptoneurosomes prepared from the IMHV of chicks tested immediately after training. All data were calculated as a percentage of increases in $[Ca^{2+}]_i$ following KCl stimulation in the absence of antagonist.

Autoradiographic analysis was carried out to determine the degree of binding of L-type channels with [^{3}H]-PN-200-110 in the IMHV and LPO of chicks tested at different times after training (figure 30 and 31 respectively).

5.3. Results

5.3.1. Dose-response curve for nimodipine

The dose-response experiments shows the trend that increasing concentration of nimodipine causes greater inhibition of KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the IMHV of the chick (figure 22). The I.C.$_{50}$ for nimodipine, in the IMHV, was 3.5μM. Maximal inhibition (60±7%) was measured with 60μM of nimodipine.

5.3.2. Dose-response curve for ω-CTX GVIA

The relationship between increasing concentration of the N-type blocker ω-CTX GVIA and inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation in the IMHV is shown in figure 23. Maximal concentration of antagonist (1μM) inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation by 48±7%. In comparison, previous studies in synaptosomes prepared from the chick forebrain showed that 1μM of ω-CTX GVIA elicited approximately 90% inhibition of KCl-stimulated increases in $[Ca^{2+}]_i$ (Bowman *et al.,* 1993; Lundy *et al.,* 1994; Maubecin *et al.,* 1995). The data shows that the effect of the antagonist may be maximal at 1μM, without eliciting total inhibition; however, greater concentrations of antagonist are required for confirmation.

5.3.3. Dose-response curve for ω-CTX MVIIC

The correlation between increasing concentration of ω-CTX MVIIC (non-specific blocker of N/P and Q-channels) and KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the IMHV of untrained chicks is shown figure 24. The antagonist at concentrations of 1, 10 and 100nM had very little inhibitory effect on KCl-stimulated increase in $[Ca^{2+}]_i$ (17±6, 15±7 and 16±6% respectively). On the other hand, 1μM of ω-CTX MVIIC dramatically reduced KCl-stimulated increases in $[Ca^{2+}]_i$ by 50±8% compared to control. Further experiments with greater concentrations of the antagonists might elucidate the effects of the blocker on KCl-stimulated increase in $[Ca^{2+}]_i$.

5.3.4. Dose-response curve for ω-Aga IVA

The effect of the P-type antagonist, ω-Aga IVA, on KCl-stimulated increases in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the IMHV of untrained chick is shown in figure 25. ω-Aga IVA concentrations of 30nM and 100nM did not inhibit KCl-stimulated increases in $[Ca^{2+}]_i$. At 300nM, the antagonist elicited 28±9% inhibition compared to controls. Higher concentrations of this toxin might produce greater inhibition than that observed in these experiments.

5.3.5. Combined effects of ω-CTX GVIA and ω -CTX MVIIC or ω-CTX GVIA and nimodipine on KCl-stimulated increases in $[Ca^{2+}]_i$.

The addition of the N- (ω-CTX GVIA; 1μM), P/N or Q- (ω-CTX MVIIC; 1μM) and L-type(nimodipine; 10μM) VSCC antagonist, inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation by 47±7, 58±7 and 52±13% respectively (figure 26). One-way ANOVA showed significantly greater inhibition when ω-CTX GVIA and ω-CTX MVIIC were added together compared to the effects of the drugs individually (78±5%; $F_{2,15}=7$; p=0.007). In addition, one-way ANOVA, shows greater, bordering on significant, inhibition when ω-CTX GVIA and nimodipine were added together compared to the inhibitory effects of the drugs individually (75±7%; $F_{2,15}=3$; p=0.07).

5.3.6. Effect of 1μM ω-CTX GVIA, ω-CTX MVIIC and 10μM nimodipine on inhibiting KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the IMHV of chicks tested immediately after training.

Addition of 1μM of ω-CTX GVIA (figure 27) or ω -CTX MVIIC (figure 28) evoked greater inhibition in KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the left IMHV of M-birds (69±4% and 73±4% inhibition respectively), compared to Q-(34±8% and 40±9% inhibition respectively), and W-birds (44±12% and 21±15% inhibition respectively). One-way ANOVA for the effect of ω-CTX GVIA shows significant inhibition only in the left IMHV ($F_{2,11}=6$; p=0.02). Post-hoc analysis between the 3 treatment groups showed that ω-CTX GVIA significantly inhibited calcium influx in M-birds compared to Q-birds (THSD; P=0.02). Furthermore, one-way ANOVA for ω-CTX MVIIC also showed a significant effect only in the left IMHV ($F_{2,12}=7$; p=0.01). Post-hoc analysis showed that ω-CTX MVIIC significantly inhibited KCl-stimulated elevation in $[Ca^{2+}]_i$ in M-birds compared to W-birds (THSD; P=0.01). There was greater inhibition in M-birds compared to Q-birds but the difference was not statistically significant (THSD; p=0.08). The degree of inhibition for ω-CTX GVIA and ω-CTX MVIIC in the left IMHV of M-birds is 35 and 33% greater then that observed in Q-birds.

Addition of 10μM nimodipine (figure 29) shows a significant effect of the antagonist in the left IMHV. The graph shows that nimodipine produced greater inhibition of calcium influx in the M-birds (77±11% inhibition) compared to Q- (43±9% inhibition) and W-birds (48±17% inhibition; $F_{2,11}$=4; p=0.04). Post-hoc test, however, did not show any significant difference between any of the three individual data sets, though comparisons between M- and Q-birds shows a trend, indicating greater inhibition in M-birds (THSD; P=0.09). The degree of inhibition for nimodipine in the left IMHV of M-birds is 34% greater than that observed in Q-birds

5.3.7. The effect of training on the binding of [^{3}H]-PN-200-110 in the IMHV and LPO

Statistical analysis to determine the interaction between training and time, with the degree of binding of the L-type VSCC blocker [^{3}H]-PN-200-110 did not show any significant difference between the left and right IMHV, in any of the groups of birds tested. The data in figure 30 does show that [^{3}H]-PN-200-110 binding increases in the left ($F_{2,55}$=4; p=0.02) and right IMHV (F2,52=7; p=0.002) in all 3 groups tested at 6 and 24 hours post-training compared to birds tested 30 minutes post-training. The data shows a trend; where there appears to be greater binding in Q-birds in the 6 and 24 hours post-training group compared to Q-birds in the 30 minutes post-training group. However, one-way ANOVA did not show any significant difference in the degree of binding in the left ($F_{2,18}$=3; p=0.1) or right IMHV ($F_{2,16}$=3; p=0.09).

The data in figure 31 shows the binding of [^{3}H]-PN-200-110 in the LPO of birds tested at either 30 minutes, 6 or 24 hours post-training. Statistical analysis did not show any significant time and training interaction in the left or right LPO. One-way ANOVA showed a significant treatment effect in the left ($F_{2,18}$=4; p=0.03) LPO of birds tested 30 minutes post-training. Though not significant ($F_{2,21}$=3; p=0.05), there may also be some treatment effects in the right LPO in birds tested 30 minutes after training. Post-hoc analysis showed significantly greater binding in the left LPO of W-birds (THSD; p=0.04) compared to Q- and M-birds. An example of the autoradiogram showing enhanced [^{3}H]-PN-200-110 binding in the left LPO of W-trained birds is shown in figure 32. There was no statistically

significant difference in the degree of [^{3}H]-PN-200-110 binding in the left or right LPO of any birds tested 6 or 24 hours post-training. It should be noted, however, that, there is a trend showing greater binding of the antagonists in left and right LPO of W- and M-birds, tested 6 hours post-training compared to Q-birds. Comparison of the left and right LPO of Q-birds in the 3 time-points (30 minutes, 3 and 6 hours post-training) to determine developmental related increases in antagonist binding did not show significant differences in either hemisphere. Though it should be noted that there was a trend showing greater binding in Q-birds from the 24 hours group compared to Q-birds taken from the 30 minutes and 6 hours group ($F_{2,18}$=3; p=0.06).

Figure 22 Effect of nimodipine on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks

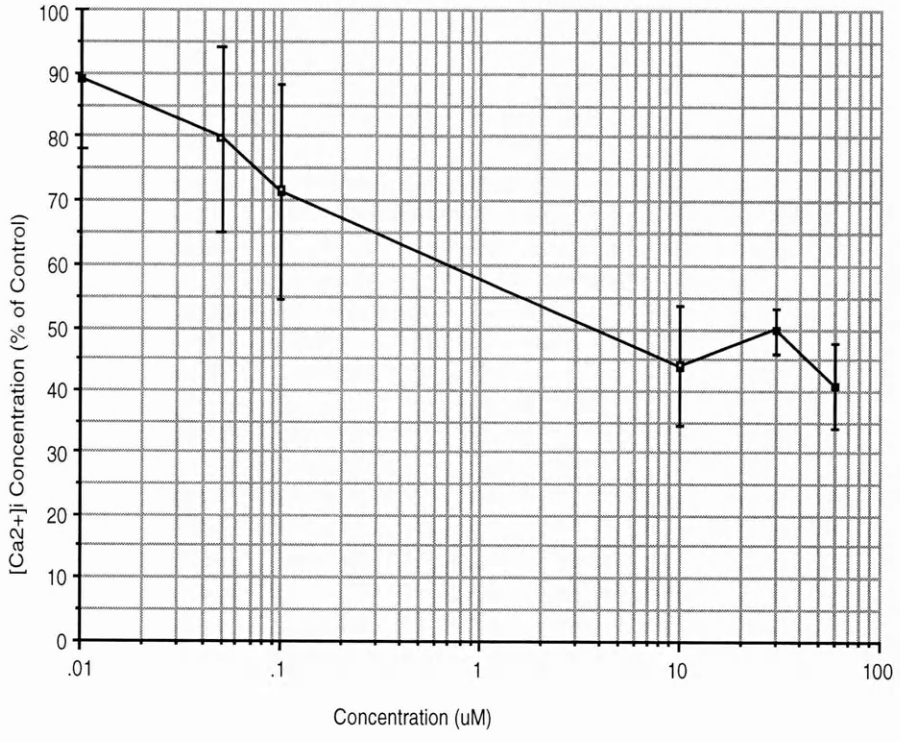


Figure. 22 Inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes exposed to increasing concentration of nimodipine. Each data point represents calcium concentration (% of control) of KCl-stimulated $[Ca^{2+}]_i$ in synaptoneurosomes incubated for 2.5 minutes with antagonist prior to depolarisation with 70mM (final concentration) KCl compared to unantagonised, control, synaptoneurosomes. Each data point is the mean $\pm$ s.e.m. from synaptoneurosomes prepared from 5 - 8 chicks.

Figure 23 Effect of ω-CTX GVIA on KCl-stimulated increases in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks

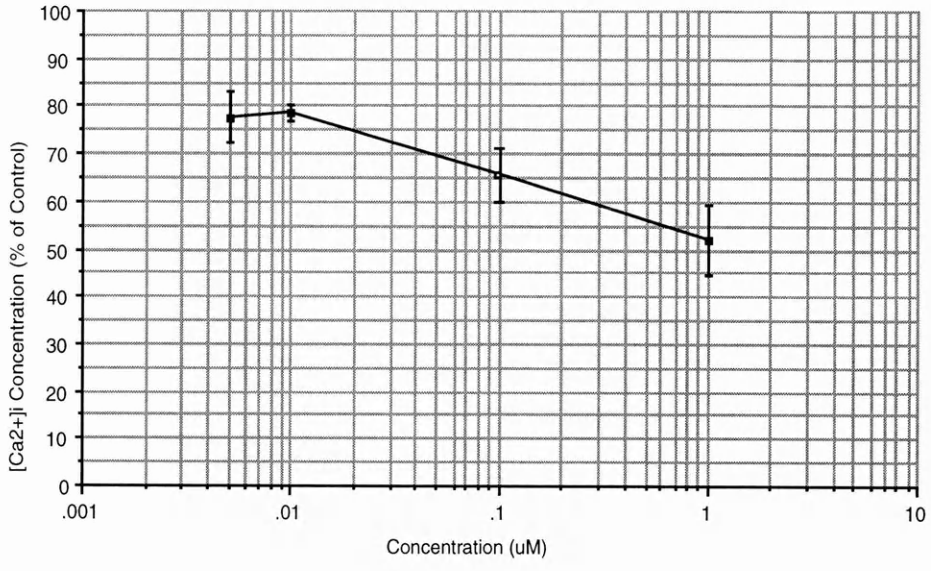


Figure 23 Inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes exposed to increasing concentration of ω-CTX GVIA. Each data point represents calcium concentration (% of control) of KCl-stimulated $[Ca^{2+}]_i$ in synaptoneurosomes incubated for 2.5 minutes with antagonist prior to depolarisation with 70mM (final concentration) KCl compared to unantagonised, control, synaptoneurosomes. Each data point is the mean ± s.e.m. from synaptoneurosomes prepared from 5 - 8 chicks.

Figure 24 Effect of ω-CTX MVIIC on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks

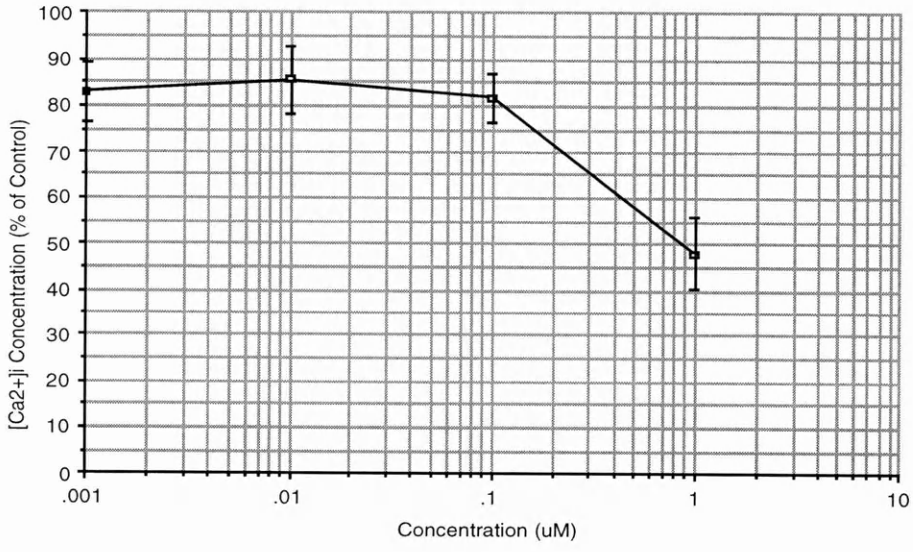


Figure 24 Inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes exposed to increasing concentration of ω-CTX MVIIC. Each data point represents calcium concentration (% of control) of KCl-stimulated $[Ca^{2+}]_i$ in synaptoneurosomes incubated for 2.5 minutes with antagonist prior to depolarisation with 70mM (final concentration) KCl compared to unantagonised, control, synaptoneurosomes. Each data point is the mean ± s.e.m. from synaptoneurosomes prepared from 6 chicks.

Figure 25 Effect of ω-Aga IVA on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks

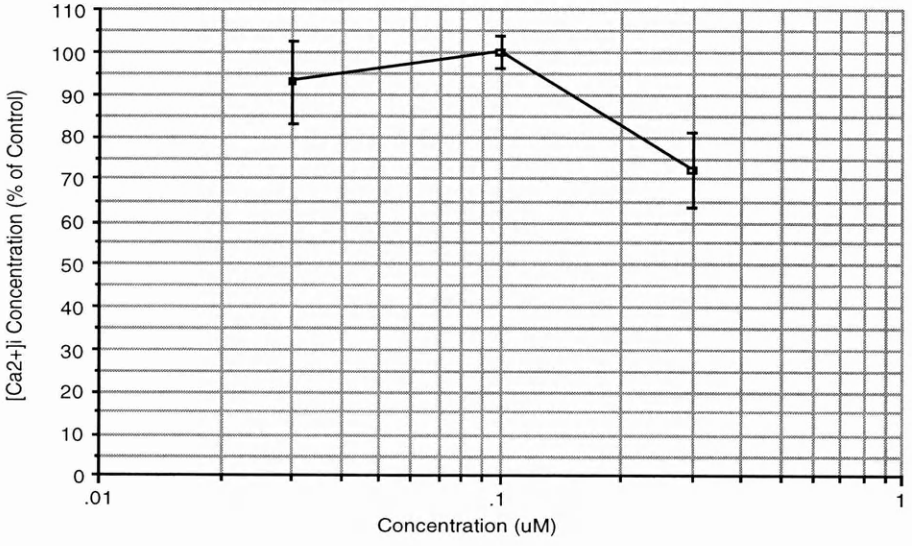


Figure 25 Inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes exposed to increasing concentration of ω-agatoxin IVA. Each data point represents Calcium concentration (% of control) of KCl-stimulated $[Ca^{2+}]_i$ in synaptoneurosomes incubated for 2.5 minutes with antagonist prior to depolarisation with 70mM(final concentration) KCl compared to unantagonised, control, synaptoneurosomes. Each data point is the mean ± s.e.m. from synaptoneurosomes prepared from 4 chicks.

Figure 26 Combined effect of ω-CTX GVIA and ω-CTX MVIIC or ω-CTX GVIA and nimodipine on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks

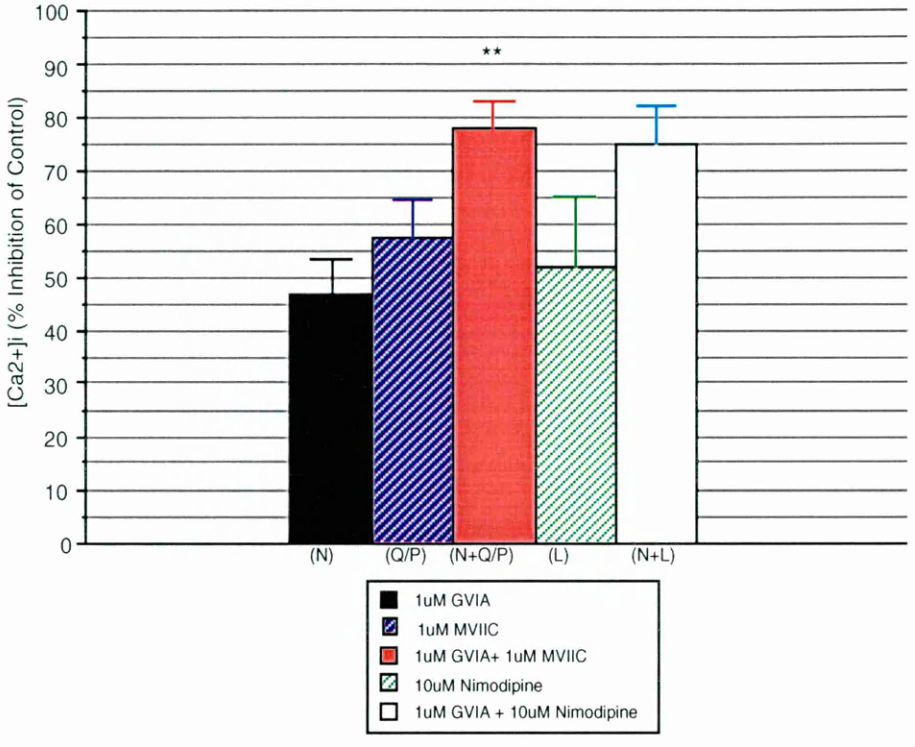


Figure 26 Inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes exposed to increasing concentration of antagonist alone or in combination. Each data point represents % inhibition of synaptoneurosomes incubated for 2.5 minutes with antagonist prior to depolarisation with 70mM (final concentration) KCl compared to unantagonised, control synaptoneurosomes. Data expressed as mean ± s.e.m. prepared from 5 - 8 chicks. ** = $p<0.025$

Figure 27 Comparison of the effect of ω-CTX GVIA on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks and chicks tested immediately after training

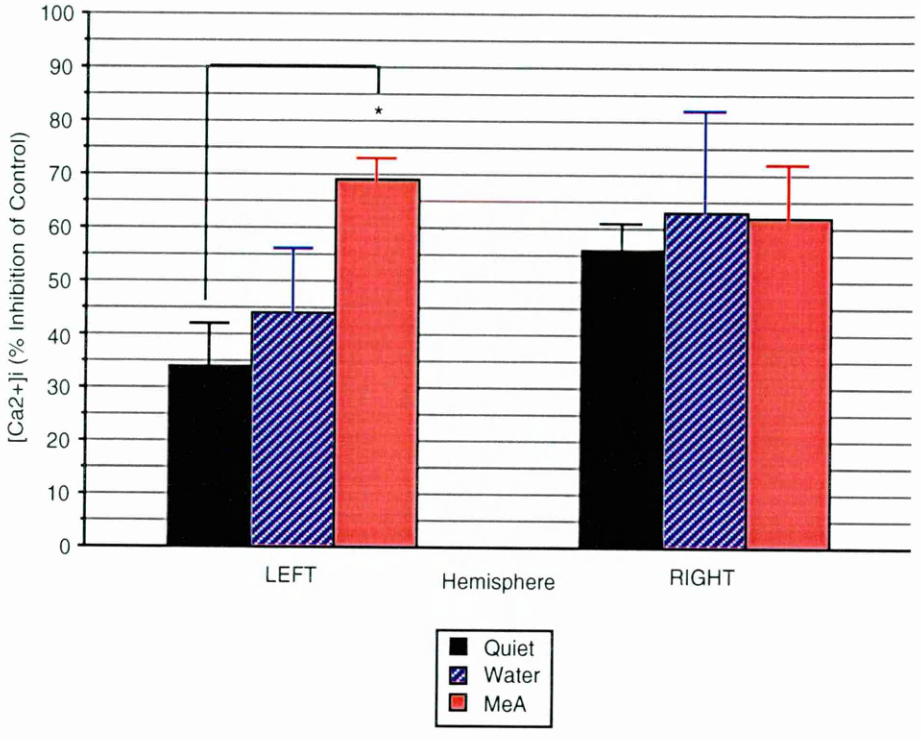


Figure 27 Inhibition of KCl-stimulated $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the left or right IMHV of Q-, W- or M-trained Birds. Synaptoneurosomes were incubated with 1μM of ω-CTX GVIA for 2.5 minutes prior to depolarisation with 70mM(final concentration) KCl compared to uninhibited synaptoneurosomes. Each data point represents mean ± s.e.m. from synaptoneurosomes prepared from 4 - 6 chicks. * = $p<0.05$.

Figure 28 Comparison of the effect of ω-CTX MVIIC on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks and chicks tested immediately after training

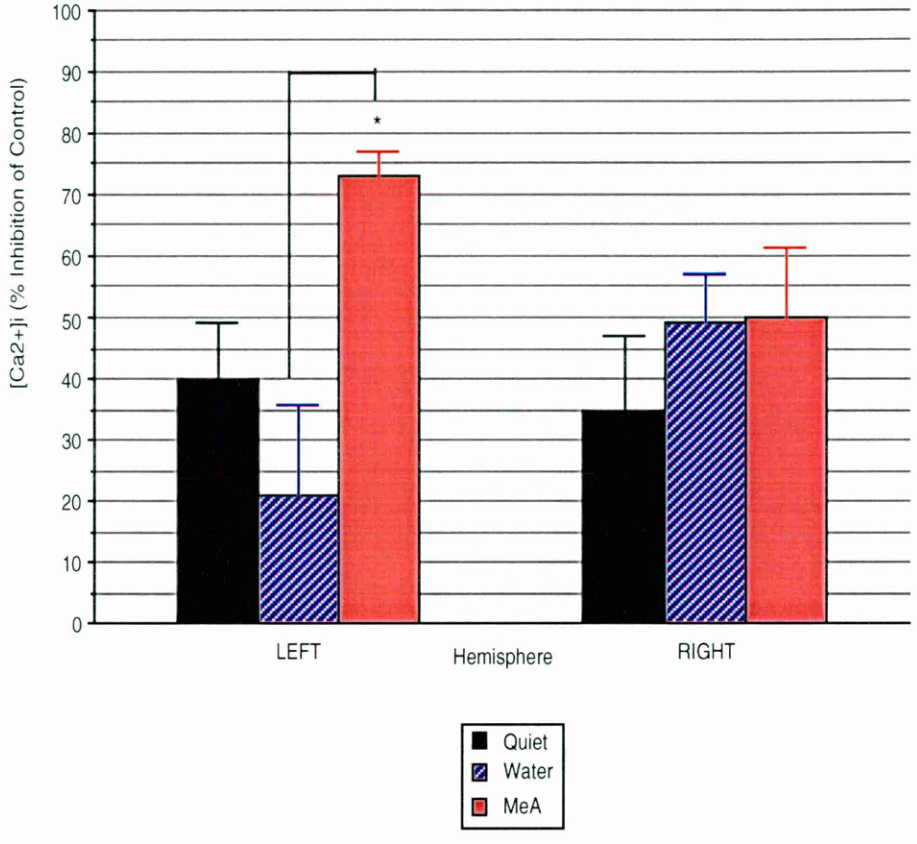


Figure 28 Inhibition of KCl-stimulated $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the left or right IMHV of Q-, W- or M-trained Birds. Synaptoneurosomes were incubated with 1μM of ω-CTX MVIIC for 2.5 minutes prior to depolarisation with 70mM(final concentration) KCl compared to uninhibited synaptoneurosomes. Each data point represents mean ± s.e.m. from synaptoneurosomes prepared from 4 - 6 chicks. * = $P<0.025$.

Figure 29 Comparison of the effect of nimodipine on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chicks and chicks tested immediately after training

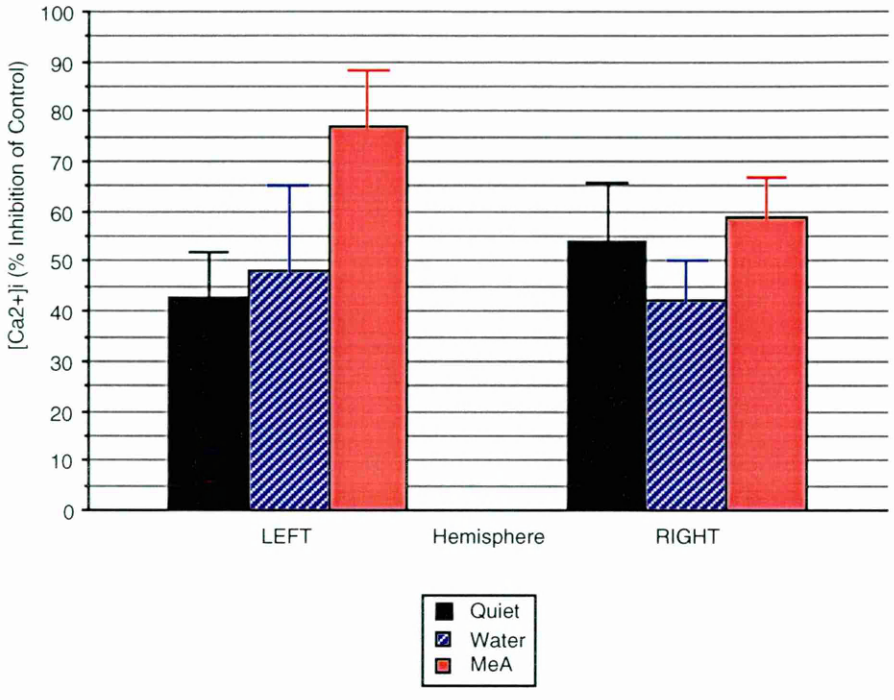


Figure 29 Inhibition of KCl-stimulated [Ca $^{2+}$] i in synaptoneurosomes prepared from the left or right IMHV of Q-, W- or M-trained Birds. Synaptoneurosomes were incubated with 10µM of nimodipine for 2.5 minutes prior to depolarisation with 70mM (final concentration) KCl compared to uninhibited synaptoneurosomes. Each data point represents mean ± s.e.m. from synaptoneurosomes prepared from 4 - 7 chicks.

Figure 30 Binding of [^{3}H]-PN-200-110 in the left and right IMHV

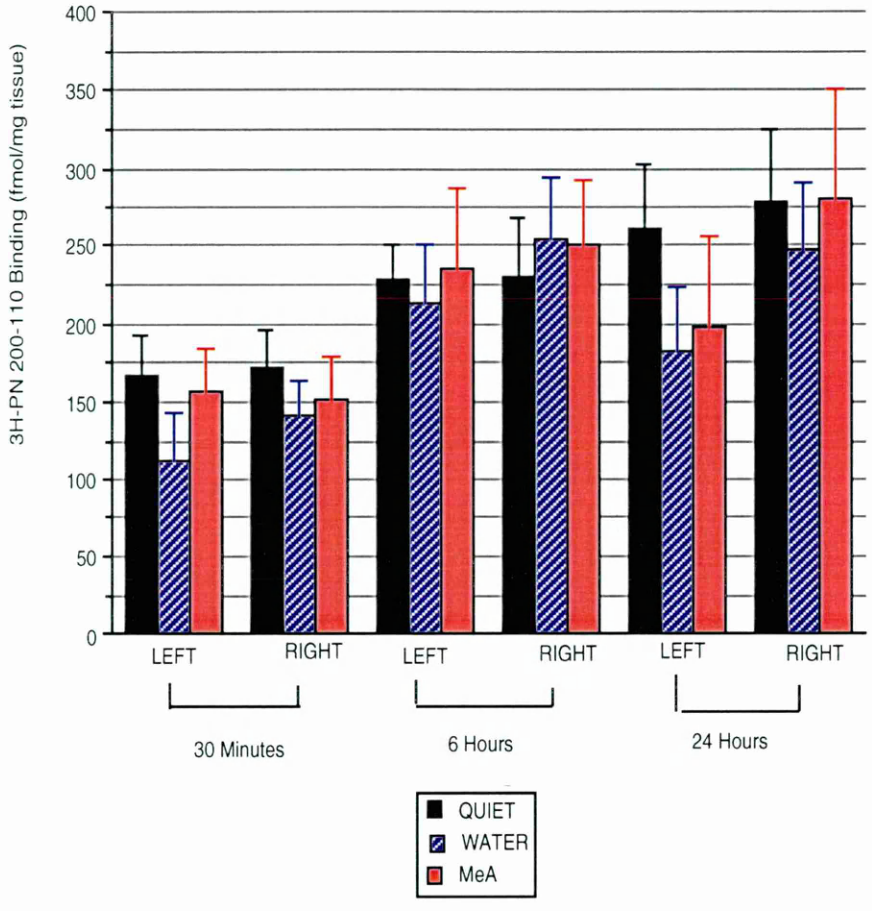


Figure 30 Binding of [^{3}H]-PN-200-110 in the IMHV of chicks tested at 30 minutes, 6 hours or 24 hours post-training. Each column represents the amount of binding in the left and right IMHV. Data are expressed as mean $\pm$ s.e.m. prepared from 4 - 12 chicks.

Figure 31 Binding of [^{3}H]-PN-200-110 in the left and right LPO

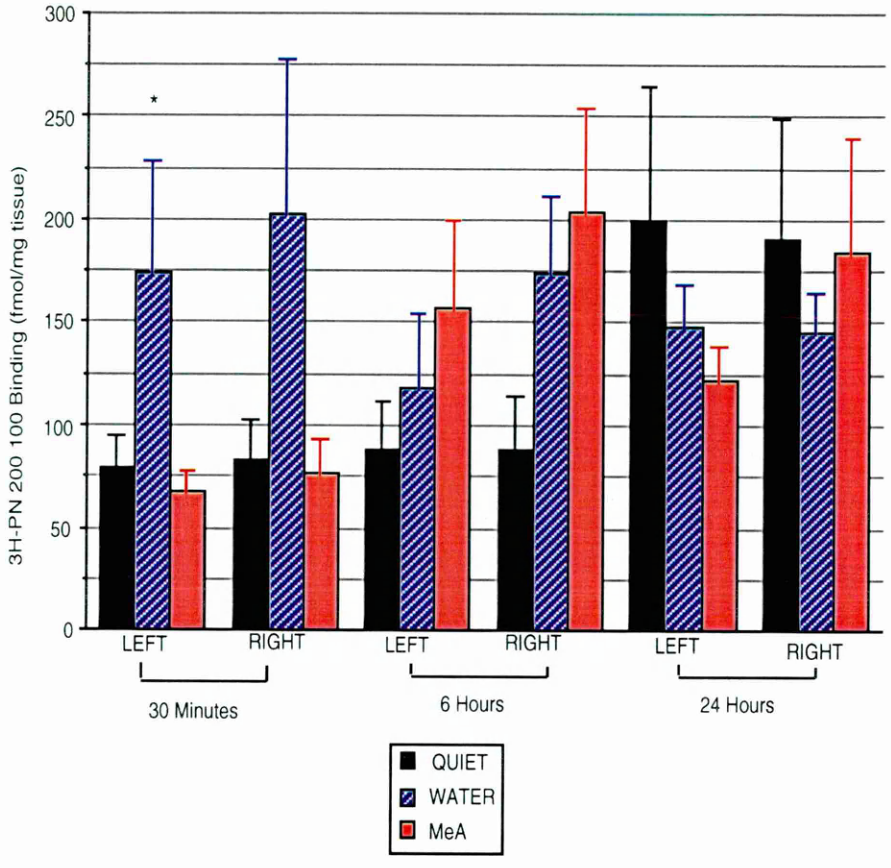
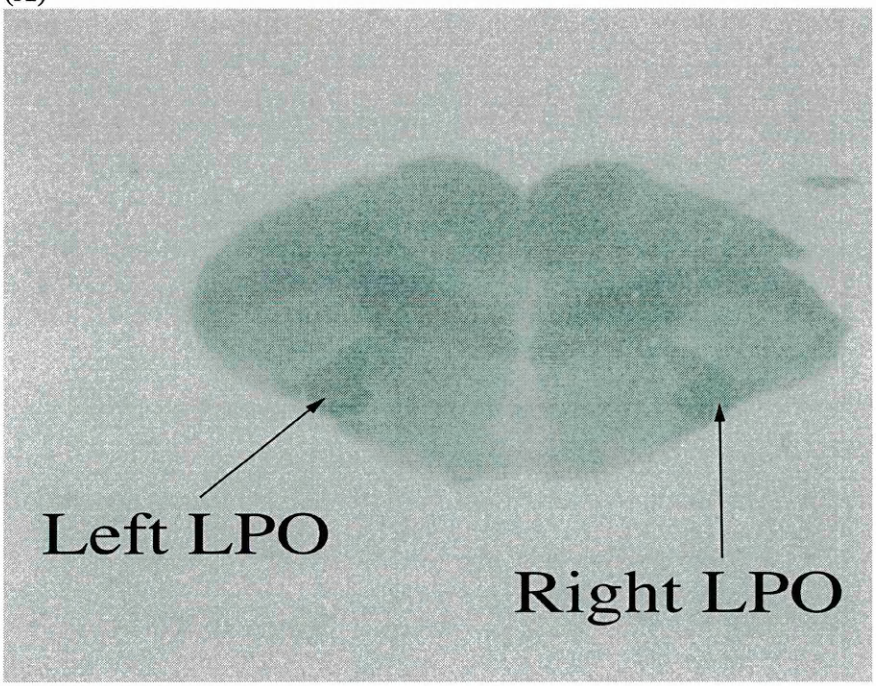


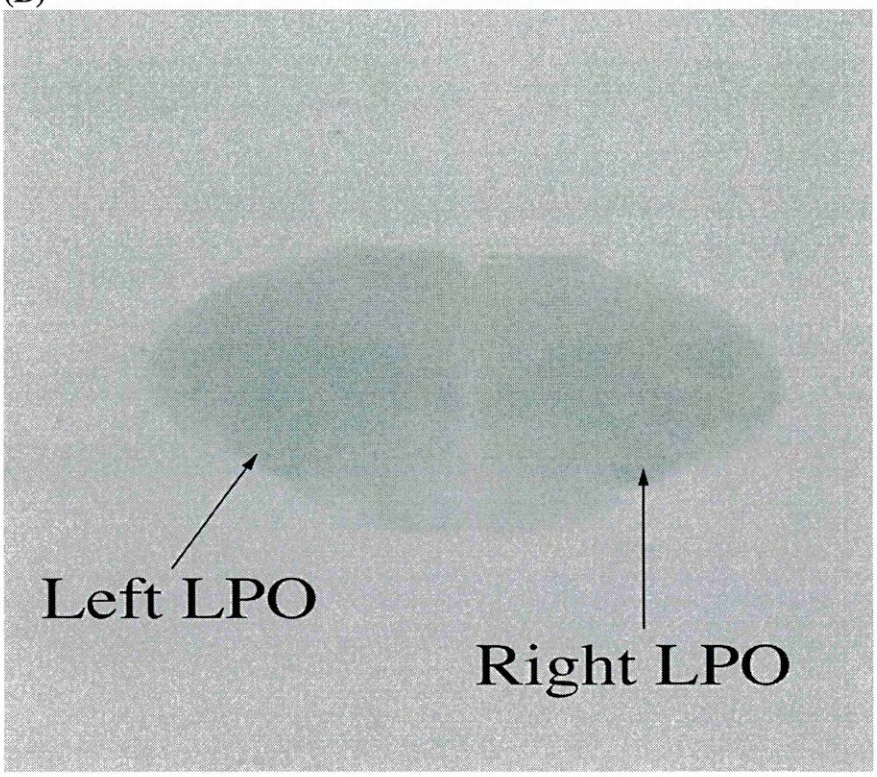
Figure 31 Binding of [^{3}H]-PN-200-110 in the LPO of chicks tested at 30 minutes, 6 hours or 24 hours post-training. Each column represents the amount of binding in the left and right IMHV. Data are expressed as mean $\pm$ s.e.m. n= 4 - 12 chicks. * = $p<0.05$

Figure 32 Autoradiogram of specific (A) and non-specific (B) binding of [^{3}H]-PN-200-110 in the LPO of W-trained birds tested 30 minutes after training.

(A)



(B)



5.4. Discussion

As discussed in chapter 1, calcium is a ubiquitous signalling molecule that is involved in a variety of important biochemical pathways in the cell. There is ample evidence indicating that calcium is involved in the biochemical cascade following learning and memory formation (Rose, 1995). Cells, like neurons, are very sensitive to changes in intracellular calcium concentration. Hence, there are a complex set of systems within cells, that maintain $[Ca^{2+}]_i$ at about 180nM (Disterhoft *et al.,* 1993). Influx of calcium into cells can occur via a number of routes; for example, calcium influx into excitable cells, such as neurons can occur via VSCC. In this chapter I have carried out a series of experiments to determine the presence of different subtypes of VSCC in the IMHV of day-old chicks. Specific types of VSCC blockers were used to determine, if any of the drugs inhibited KCl-stimulated elevation in $[Ca^{2+}]$i compared to controls. A series of dose-response experiments were performed; using the L-type channel blocker (nimodipine), N-type (ω-CTX GVIA), P-type (ω-Aga IVA), and N/P- or Q-type channel blockers (ω-CTX MVIIC). The rationale for using these antagonists was based on previous findings, that these antagonists inhibited calcium influx in synaptosomes prepared from the forebrain of chicks of various ages (Pocock *et al.,* 1992; Bowman *et al,* 1993; Lundy *et al,* 1993; Grantham *et al.,* 1994; Maubecin *et al.,* 1994). I was interested in the effect of these antagonists in a specific area of the day-old chick brain, namely the IMHV, thus giving a region and age-specific effect of these VSCC antagonists.

The first dose-response experiment shown in figure 22 is for the specific L-type blocker, nimodipine. I detected a concentration dependent inhibition of KCl-stimulated elevation in $[Ca^{2+}]_i$ with nimodipine, where the IC_{50} was measured at 3.5μM concentration of the drug. The data shows a 'plateauing off' at nimodipine concentration ranging between 10 and 60μM. Maximal inhibition (approximately 60%) of KCl-stimulated $[Ca^{2+}]$i elevation compared to controls was observed with 60μM of the drug. My results differ from previous studies into the effect of DHP-antagonists on KCl-stimulated calcium influx in chick brain; though it should be noted that the other groups used different DHP-antagonists (e.g. Lundy *et al.,* 1994; Maubecin *et al.,* 1995). I assumed that the effect of nimodipine would be

similar to other DHPs since studies have already shown that DHP-antagonists had similar effects on L-type channels (Dolphin, 1995). Previous studies have shown very little effect of different DHP-antagonists on KCl-stimulated $[Ca^{2+}]i$ increases in synaptosomes from chicken brains (e.g. Bowman *et al.,* 1993) which, are known to be abundant in another sub-type of VSCC, the N-type channels. For example, Bowman *et al.* (1993), when studying the effect of 10μM nifedipine or verapamil on the plateau phase of KCl-stimulated calcium influx in synaptoneurosomes only observed 9±2% and 2±2% inhibition respectively compared to controls. However, Bowman and co-workers observed that the atypical L-type channel blockers, flunarizine and fluspirilene, both produced concentration-dependent inhibition of the plateau phase of $[Ca^{2+}]_i$ elevation in chicken synaptosomes. Furthermore, both agents at 10μM concentration produced greater then 80% inhibition of calcium influx in chick synaptosome. Both flunarizine and fluspirilene have been shown to exhibit mixed pharmacology in various tissue preparations, so it may be possible that these atypical L-type blockers may be inhibiting other VSCC present in chick synaptosomes (for discussion see Bowman *et al.,* 1993). Several reports have been produced from various laboratories that some DHP agonist and antagonists have the ability to bind to N-type channels. For example, Jones and Jacobs (1990) observed that high concentrations of nifedipine (10μM) produced a stronger blocking action on evoked calcium current in the frog sympathetic neuron than 1μM of nifedepine, which itself produced almost total block. Furthermore, more recent experiments, have also shown that nimodipine and nitrendipine caused an almost complete block of high K^+-induced $[Ca^{2+}]_i$ transient in dibutryl cAMP-differentiated neuroblastoma X glioma hybrid (Li *et al.,* 1997). Similarly some DHPs, phenylalkylamines and benzothiazepines were shown to block neuronal N, P/Q- and R-type VSCCs (Diochot *et al.,* 1995). Thus, taken together, one interpretation of my findings with nimodipine would suggest that this 'classical' L-type VSCC blocker is also capable inhibiting N-type and possible some other types of VSCC in the synaptoneurosomes from the chick IMHV.

The difference between my data and that of Bowman *et al.* (1993) may also be, partly, due to the fact that I incubated my samples with the antagonist prior to adding 70mM KCl, whereas Bowman and co-workers added the antagonist 3 minutes after the addition of 20mM KCl when the $[Ca^{2+}]_i$ elevation had reached the plateau phase of the biphasic KCl-

stimulated calcium influx cascade. Therefore, it may be possible that the antagonist used in my experiments inhibited the whole KCl-stimulated calcium influx cascade compared to the plateau phase inhibition measured by Bowman and co-workers. The fact that Bowman and his co-workers observed very slight inhibition with L-type blockers may be indicative of a functional role of this channel in the initial rapidly inactivating phase of the biphasic KCl-stimulated calcium influx sequence. In addition it has been suggested that HVA calcium channels such as the L-type channels are activated prior to the initial rapidly inactivating phase of the biphasic KCl-stimulated calcium influx sequence (Suszkiew, 1986). Thus the inhibition I observed with nimodipine may be demonstrating an affect for the initial calcium influx phase. However, Maubecin *et al.* (1995) did not detect any inhibition with another DHP L-type blocker, nifedipine (10μM), when measuring the initial non-inactivating phase of calcium influx. It should be noted that this group used pure synaptosomal preparations, and, since L-type channels are believed to be predominantly located in postsynaptic terminals, this may explain the lack of inhibition observed by this group.

Support for the existence of L-type channels in the chick brain has come from neurodevelopmental studies; where it has been shown that there was a steady increase in ^{3}H-nitrendipine binding from embryonic day 3, reaching adult levels at around the time of hatching, at embryonic day 19 (Marangos *et al.,* 1984). It should be noted, however, that discrepancies have been observed between binding and uptake studies, particularly with ω-CTX GVIA (Venema *et al.,* 1992). Pocock *et al*. (1992) also detected minor inhibition of 7.5±10.3% of KCl-stimulated $^{45}Ca^{2+}$ influx compared to control when pure synaptosomes were exposed to 1μM nifedipine.

The second dose-response curve (figure 23) shows the inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation with increasing concentration of the specific N-type channel blocker, ω-CTX GVIA. N-type channels are abundant in the chicken brain (Azimi-Zonooz and Litzinger, 1992). Previous studies have shown that KCl-stimulated calcium influx in chick synaptosomes is readily inhibited by ω-CTX GVIA (Suzkiew *et al.,* 1987, Bowman *et al.,* 1993; Lundy *et al.,* 1994; Maubecin *et al.,* 1995; Pocock *et al.,* 1992; Grantham *et al.,* 1994). These groups had shown that ω-CTX GVIA totally inhibited KCl-stimulated calcium

influx at concentrations ranging from 0.3μM to 1μM. As shown in figure 23, 1μM of ω-CTX GVIA only produced 48±7% inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from IMHV compared to controls. There is a large variation in the measured IC_{50} value for ω-CTX GVIA between previous studies, where values ranged from 2nM to 250nM, whereas in my experiment IC_{50} was around 1μM, the maximal concentration of drug tested. There may be several reasons for the discrepancy between my data and the others. For example, I used a specific area of the chick forebrain, namely the IMHV, whereas the other groups used either the whole forebrain or whole brain samples. Furthermore, the fact that I had used synaptoneurosomal samples whereas most of the other groups used synaptosomes would also indicate that I was also measuring post-synaptic calcium influx which may not be as sensitive to ω-CTX GVIA inhibition, since, N-type channels are believed to be predominantly located at pre-synaptic terminals.

It has been observed, that extracellular calcium inhibits the binding of ω-CTX GVIA by lowering the affinity of the peptide to its binding site on the N-type channel (Feigenbaum *et al.,* 1988; Wagner *et al.,* 1988;K_i for calcium 0.45mM). This finding may partially explain some of the discrepancy in inhibition between the different groups, where the concentration of calcium chloride ($CaCl_2$) in the incubation medium varied from 0.5mM to 1.5mM. I used 2mM (1.6mM final concentration) of $CaCl_2$ which may be high enough to significantly decrease antagonist binding in my synaptoneurosome preparation. Finally, Azimi-Zoonoz and Litzinger (1987), in developmental studies, have shown increased binding of ^{125}I-ω-CTX GVIA in embryonic chick day 9 to post-hatch day 10. I used day-old chicks whereas, other groups had used older chicks. For example, Grantham *et al.* (1994), who detected 90% inhibition of KCl-stimulated calcium influx with 1μM of ω-CTX GVIA, used older chicks (7 - 14 days old), where the properties of VSCC in synaptosomes may be different in terms of affinity and / or number of sites for antagonist binding. Finally, ω-CTX MVIID, a VSCC blocker with a high index of discrimination against the N-type channels was shown to inhibit 60% of KCl-stimulated calcium influx in chick and rat brain (Maubecin *et al.,* 1994). These authors suggested that, in view of this finding, ω-CTX GVIA, in avian brains, may be acting on VSCC's with pharmacological characteristics, different from N-type channels in mammalian neural tissue. Also, Katz *et al.* (1995) found that ω-CTX GVIA and the

synthetic funnel web toxin (FTX; P-type channel blocker) inhibited transmitter release in frog neuromuscular junction, a function originally thought to be predominantly N-channel dependent. Thus, Katz and co-workers proposed that in this preparation calcium channel subtypes involved in mediating transmitter release may not necessarily be the classical N-type channel described in mammals. This is in accordance with the hypothesis put forward by Adams and Olivera (1994), that ω-CTX's may have a broader specificity in vertebrates such as avians and amphibians than in mammals. In relation to my experiments, however, the lower potency of ω-CTX GVIA may be indicative of the existence of a small population of a specific subtype of N-type channels in synaptic terminals of the IMHV.

The calcium channel blocker, ω-CTX MVIIC, which has been previously reported to block both N and P currents in mammalian brains (Hillyard *et al.,* 1992), and also Q-currents in cerebellar granule neurons (Zhang *et al.,* 1993) in rats, also caused a dose-dependent block of KCl-stimulated $[Ca^{2+}]_i$ increase in the IMHV of day-old chicks (figure 24). Previous studies using ω-CTX MVIIC had shown that this blocker was more potent, than ω–CTX GVIA, in blocking KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptosomes from the chick forebrain, where the IC_{50} was 2.3nM. Total inhibition (70 - 100 % of control) was achieved with 40nM of the toxin (Lundy *et al.,* 1994) compared to an IC_{50} of 14nM, for ω-CTX GVIA, with complete inhibition at 1μM. These authors disputed the argument that ω-CTX MVIIC blocks only N-type channels since previous studies had shown that ω-CTX MVIIC was more potent in blocking KCl-stimulated calcium influx than ω-CTX GVIA in mammalian neurons (Hillyard *et al.,* 1992). This suggests that ω-CTX MVIIC may have the ability to block more then one subtype of VSCC.

The notion that ω-CTX MVIIC may be blocking both N- and P- type channels may explain the profile of the dose-response curve for this antagonist in the IMHV, where low concentrations of the drug had little or no affect on KCl-stimulated increases in $[Ca^{2+}]_i$. On the other hand, at higher concentrations, there was significant inhibition. For example, 1, 10 and 100nM concentrations of the antagonist only blocked stimulated calcium influx by 17 ± 6, 15 ± 7 and 18 ± 5% respectively compared to control. However, much greater inhibition was observed with 1μM of ω-CTX MVIIC (52 ± 8% inhibition compared to control). This

may indicate a concentration-dependent effect of the drug on channel specificity. For example, at lower concentrations ω-CTX MVIIC binds to a small number of P-type channels, and at higher concentration the antagonist is also capable of binding to N-type or to Q-type channels. Alternatively, as proposed by Lundy *et al.* (1994), there may be a novel N/P channel present in chick synaptoneurosomes and that this antagonist may be binding to both N- and P-sensitive domains on the 'NP' channel. Lundy and his co-workers detected a greater inhibition of stimulated calcium influx with ω-CTX MVIIC when compared to ω-CTX GVIA, and they suggested that ω-CTX MVIIC has the ability to bind to P-domains which then has the ability to 'shut-down' the N-domains. Thus, inhibition with low concentrations of the drug could represent binding of the drug to the P-domain while at higher concentration the P-domains may be activated sufficiently to initiate the inhibition of the N-domains.

In contrast, Grantham *et al.* (1994) found that ω-CTX MVIIC was less potent than ω-CTX GVIA in synaptosomes prepared from the forebrain of 7 - 14 day-old chicks, where threshold inhibition of 20% with 10nM of ω-CTX MVIIC and 65% inhibition with 1μM of the drug was observed. In comparison, my data shows increased sensitivity of VSCC to ω-CTX MVIIC at a threshold concentration (1nM) in the IMHV of day-old chicks. However, 1μM of ω-CTX MVIIC produced 52% inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation. This difference in inhibition with ω-CTX MVIIC may be due to inhibition of different stages of the biphasic calcium influx following K^+-stimulation since Grantham and co-workers added the inhibitor after KCl stimulation, while I pre-incubated my samples with antagonist prior to depolarisation.

In light of recent findings, that the family of ω-CTX's displayed preferential binding with inactivated N-type channels (Stocker *et al.,* 1997), which may also be the case for the other calcium channels; this may explain my findings that lower concentrations of ω-CTX MVIIC was required for threshold inhibition of KCl-stimulated $[Ca^{2+}]_i$ compared to that found by Grantham and co-workers. In addition, the greater inhibition Grantham and co-workers report with 1μM of the drug may indicate increased affinity of the toxin to VSCC after threshold inhibition is reached. A further explanation for the difference in threshold

and maximal inhibition between my data and that of Grantham and his co-workers may be the difference in ages of chicks used, where Grantham *et al.* (1994) used older chicks, 7 - 14 days old, compared to day-old chicks in my experiments. Their preparation may have a greater number of channels, sensitive to ω-CTX MVIIC, since it has been observed that there is a relatively large increase in calcium channels, such as the N-type channels, during neurodevelopment (Azimi-Zonooz and Litzinger, 1992). Thus, greater number of channels may require larger concentrations of toxins for threshold inhibition. However, if this was so then I would also expect total block with a lower concentration of ω-CTX MVIIC in my preparation. As mentioned previously another factor that could effect the apparent potency of an antagonist could be the type of preparation used. Finally, the existence of pre- and postsynaptic calcium influx, as seen in synaptoneurosomes, could 'dilute' the inhibitory effect of an antagonist that may predominantly exert its effects on presynaptic terminals.

The P-type blocker ω- Agatoxin IVA was shown to have little affect at the concentration used, in blocking KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the IMHV of day-old chicks (figure 25). The results show that 30nM of the drug inhibited 9±10% of KCl-stimulated elevation in $[Ca^{2+}]_i$. However, 100nM of the toxin did not inhibit KCl-stimulated $[Ca^{2+}]_i$, while 300nM produced 28±9% inhibition of KCl-stimulated elevation in $[Ca^{2+}]_i$ compared to control. These results are similar to the inhibition observed by Bowman *et al.* (1993), who found that 300nM of the toxin inhibited the plateau phase of KCl-stimulated increase in $[Ca^{2+}]_i$ by 28% compared to control in chicken synaptosomes. In addition, this group only observed 39% inhibition of stimulated calcium influx in synaptosomes exposed to 1μM of the toxin. In contrast, Lundy *et al.* (1994) managed to obtain 65% inhibition of control with 3μM of the toxin. Maubecin *et al.* (1995) using 100nM of ω-Aga IVA observed 10% inhibition of calcium influx, which was similar to the inhibition I obtained. These authors observed only 40% inhibition with 5μM of the toxin. Thus the data, including mine, appear to indicate, that there is a relatively small population of ω-Aga IVA sensitive P-type channels in the chick brain.

Other antagonists of the ω-Agatoxin family, ω-Aga IA, IIA and IIIA were found to differentially inhibit KCl-stimulated calcium influx in chicken synaptosomes (Pocock *et al.*,

1992). ω-Aga IIA and IIIA, but not IA inhibited calcium influx. A dose-response curve demonstrated that ω-Aga IIA had an IC_{50} of 2nM in synaptosomes prepared from the chick forebrain, and a maximum concentration, of 100nM, of the toxin caused maximal inhibition (65% compared to control). Pocock *et al.* (1992) has suggested that the difference in potency of the family of agatoxins, for blocking VSCC, may help to distinguish sub-classes of these calcium channels, not already delineated by currently available pharmacological agents. Furthermore, these agents may also have the capacity to distinguish between central and peripheral neurons, or between calcium channels on cell bodies and at synapses.

Displacement studies have shown that ω-aga IIA, but not IA, inhibits binding of ^{125}I-ω-CTX GVIA in chick neuronal membranes (Adams *et al.,* 1990). The evidence that the incubation time required for maximal block of calcium influx in chick synaptosomes with ω-Aga IIA was shorter compared to ω-CTX GVIA indicates that in this preparation, ω-Aga IIA has a greater affinity for N-type channels than ω-CTX GVIA channels (Pocock *et al.,* 1992). To summarise, it would appear that the family of agatoxins have differential binding capacities in synaptosomes prepared from different species. Additionally, within species intra-differential binding has been found, further suggesting that differences exist within the family of agatoxins for different VSCC's. It should be noted that Lundy (unpublished observations) found that the inhibitory potency and total percentage block for ω-Aga IVA in a rat synaptosomal preparation decreases when the concentration of KCl was increased to 60mM. However, comparisons with previous experiments show that different groups observed similarly low percentage block with ω-Aga IVA whether 20mM, 40mM KCl or as in my experiments 70mM KCl were used. This, may, however, partially explain the differences in block with the other antagonists used, since, as far as I am aware there is no quantitative or qualitative study into the effects of different concentrations of KCl on the binding of different antagonists on synaptosomal preparation.

Another factor that may be responsible for the variable effects of the different VSCC antagonists between groups may due to the preparation of tissue and the area of brain tissue used. For example, neurons in different area of the brain may have different fractions of neurons with different populations of VSCC's. Similarly, Bowman *et al.* (1995) suggested

that the lack of antagonism with ω-CTX GVIA in the rat brain may not be due to the smaller number of N-type channels in each synaptosomes but that there are less synaptosomes with N-type channels in the rat compared to the chick.

Figure 26 shows a comparison of the data collected from experiments investigating the effects of different antagonists on KCl-stimulated $[Ca^{2+}]_i$ elevation, when added in combination or individually. The aim of these experiments was to determine if ω-CTX GVIA, ω-CTX MVIIC and nimodipine were exerting their block on separate or similar sites in the synaptoneurosomal preparation from the IMHV of untrained day-old chicks. A comparison of the effects of ω-CTX GVIA and ω-CTX MVIIC separately and when added together, shows that the separate addition of ω-CTX GVIA or ω-CTX MVIIC inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation by 47±7% and 58±7% respectively compared to control. Addition of the antagonists together, however, resulted in 78±5% inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation, compared to control. The near additive effect of the two antagonists indicates that ω-CTX GVIA and ω-CTX MVIIC exert most of their affects on separate VSCC. However, it should be noted, that since ω-CTX MVIIC has been shown to have some affinity for N-type channels (Dolphin, 1995), it is possible that the lack of a total additive inhibitory effect of the two antagonist may be due to some interaction between ω-CTX MVIIC and N-type channels in synaptoneurosomes from the IMHV. As discussed previously, ω-CTX MVIIC has been reported to bind to different subtypes of VSCC with different potencies depending on species and tissues studied. For example, in cloned mammalian N-type channels, expressed in *Xenopus* oocytes, Grantham *et al.* (1994) found that ω-CTX MVIIC had a greater inhibitory capacity than ω-CTX GVIA, and that the inhibition was irreversible, thus indicating that cloned mammalian N-type channels are more sensitive to ω-CTX MVIIC then to ω-CTX GVIA. However, in the chicken synaptosomes, Grantham and co-workers, found that ω-CTX GVIA was more potent in blocking KCl-stimulated calcium influx than ω-CTX MVIIC. I mentioned previously that chick synaptosomes prepared from the forebrain have a high proportion of N-type channels. Thus, the study by Grantham and co-workers suggests, that in chick synaptosomes ω-CTX MVIIC either, has affinity for N-type channels, or it may bind to a different subtype of VSCC in this preparation. Lundy *et al.* (1994) had already proposed that ω-CTX MVIIC

may be blocking a novel 'N/P'-channel in the chick brain demonstrating the greater potency of ω-CTX MVIIC compared to ω-CTX GVIA in this preparation. According to my data ω-CTX MVIIC has the ability to bind to a novel 'N/P'- or Q-type channels and possibly N-type channels, whereas ω-CTX GVIA most probably binds to the N-type channel alone.

The second combination experiment; to determine the effects of adding ω-CTX GVIA and nimodipine together, shows that nimodipine alone produced 52±13% inhibition compared to control, and that the combination of ω-CTX GVIA and nimodipine produced 75±7% inhibition. The near additive inhibitory effect of the two antagonists suggests that at these concentrations, ω-CTX GVIA and nimodipine block KCl-stimulated $[Ca^{2+}]_i$ elevation at distinct epitopes on chick synaptoneurosomes prepared from IMHV of day-old chicks. L-type channels are usually found on post-synaptic terminals, such as the soma and proximal dendrites of the brain (Hell, *et al.,* 1993). The additive inhibitory effect of these drugs may, therefore, suggest that nimodipine exerts its effects on post-synaptic terminals, whereas ω-CTX GVIA blocks pre-synaptic terminals. This is in accordance with my EM, pictures showing the existence of post-synaptic densities in my preparation. It should be noted, however, that since, the combination of ω-CTX GVIA and nimodipine was not perfectly additive, it may also indicate that L-type channels in the IMHV may possess the α_{1d} subunit which is sensitive to reversible block by ω-CTX GVIA (Fox *et al.,* 1987; Pearson *et al.,* 1993).

The fact that the combined addition of the antagonists did not totally abolish KCl-stimulated increase in $[Ca^{2+}]_i$ further suggests that there may be other subtypes of VSCC present in synaptoneurosomes prepared from the chick IMHV. Previous pharmacological studies have highlighted the possible existence of novel subtypes of VSCC in chick synaptosomes, which have yet to be defined, and characterised. Bowman *et al.*(1993), using submaximal concentrations of, ω-CTX GVIA (30nM), and ω-Aga IVA (300nM), did not observe any additive inhibitory effects, and concluded that these drugs have a common site of action on avian presynaptic terminals, at least during the plateau phase of KCl-stimulated calcium influx. Maubecin *et al.* (1995) found further evidence to support the possible existence of another novel VSCC, where FTX, a polyamine inhibitor of P-type channels, and ω-CTX

GVIA, when added together, did not have an additive affect; thus, indicating a common site of action for these drugs. Since FTX has not been shown to inhibit mammalian N-type channels (Manfridi *et al.,* 1993), it has been proposed that, at least two populations of VSCC exist in chick brain, one sensitive to ω-CTX GVIA and another to FTX (Lundy *et al.,* 1994; Maubecin *et al.,* 1995).

Lundy and co-workers (1994) have shown that FTX was able to inhibit 80% of KCl-stimulated calcium influx indicating considerable N-type activity, a theory further supported by Manfridi *et al.* (1993). This research showed that synthetic FTX was able to displace ^{125}I-ω-CTX GVIA binding in rat synaptosomes. In addition, since ω-Aga IVA did not compete with ω-CTX MVIIC, a potent 'N/P' channel blocker in the chicken brain (Hillyard *et al.,* 1992), it was concluded that synthetic FTX blocks calcium influx in chicken brain, partly through an affect on P-type channels and partly through an affect on N-type channels. Lundy and co-workers (1994) have suggested that in the chicken brain there is a novel type of VSCC, since combination of P-type channel blockers (synthetic FTX, spermine and ω-Aga IVA) with N-channel blocker, ω-CTX GVIA did not inhibit in an additive manner, but did result in complete block of KCl-stimulated calcium influx in chicken brain. They suggest that this novel 'N/P' channel may consist of two distinct domains; one sensitive to N-type block and the other to P-type block and that the two domains may be functionally linked together, in such a way that inhibition of one site "shuts' down the other domain. Thus ω-CTX GVIA blocked all the KCl-stimulated increase in $[Ca^{2+}]_i$ including the 80% of channel sensitive to either the ω-Aga IVA or the polyamine antagonists.

My data shows that both ω-CTX GVIA and ω-CTX MVIIC exhibit similar degrees of inhibition (48 and 52% respectively) at 1μM. However, the general profile of the dose-responses are quite different for the two antagonists. For example, the degree of inhibition with ω-CTX MVIIC was very small (approximately 20%) at 1, 10 and 100nM, but considerably larger with 1μM (47%). On the other hand, ω-CTX GVIA showed a gradual increase in the level of inhibition with increasing concentration of antagonist. Since lower concentrations of ω-CTX MVIIC have very little inhibition of stimulated calcium influx and since the effects of 1μM of both of these drugs were almost additive, indicating

different binding sites, I suggest that this may be further support to the existence of the hypothetical 'NP'-channel. Furthermore the dose-response curve for ω-CTX MVIIC, as discussed earlier, may be explained by the existence of a novel 'NP'-channel, whereby at low concentrations the peptide binds to the P-domain of the channel, but at higher concentrations, the peptide binds to the N-domain. Alternatively, threshold inhibition of the P-domain may result in some form of negative feed-back signal resulting in inhibition of the N-domains.

The data in figures 27, 28 and 29 shows the effects of ω-CTX GVIA, ω-CTX MVIIC or nimodipine respectively in synaptoneuromes prepared from the IMHV of chicks tested immediately after training. The aim of these experiments was to determine if different VSCC's were activated immediately after training. The rationale for these experiments was based on previous findings that some toxins binds more strongly to the inactivated ion channel then to the resting ion channel, i.e. exhibit voltage-dependence of block (Hondeghem and Katzung, 1997). Furthermore, Stocker *et al.* (1997) has recently found that the family of ω-CTX's antagonists including, ω-CTX GVIA and ω-CTX MVIIC, preferentially inhibited inactivated N-type channels in the *Xenopus* oocyte. Stocker and co-workers found that the degree of N-type calcium channel inactivation increased when the membrane potential in the oocyte was made less negative. For example, at the holding potential of -30mV the amount of channel inactivation was greater then that observed at -90mV. Therefore, it was argued that if the neurons in the IMHV were at a less negative potential, as a result of some neuronal activity, then the VSCC in these tissues may have greater affinity for the respective antagonists. DHP antagonists have also been shown to exhibit voltage dependence of inhibition. For example, L-type blockers were observed to bind with a higher affinity to an inactivated state of the calcium channel (Spedding *et al.,* 1989). Electrophysiological experiments have also shown that the inhibition of calcium channels by nifedipine in cultured hippocampal neurons is dependent on the holding potential (Meyers and Barker, 1989).The inhibitory effect of nifedipine was also shown to be lower following membrane hyperpolarization (Gahwiler and Brown, 1987). The data for all the antagonists shows that greatest inhibition occurred in synaptoneurosomes from the left but not right IMHV of M-birds compared to Q- and W-birds. Interestingly ω-CTX

GVIA, ω-CTX MVIIC and nimodipine all exhibited similarly high percentages of inhibition, 69, 73 and 77% respectively. A reason for a total inhibitory value greater then 100% could be indicative of some cross-reactivity between the antagonist with other VSCC subtypes in the synaptoneurosomes. Alternatively, there may be some form of down-regulatory signalling between different VSCC's following *in vivo* changes in M-birds resulting in a greater then normal level of inhibition following addition of certain antagonists *in vitro*. Furthermore, a review of the literature has shown that several antagonists for different subtypes of VSCC produced high degrees of inhibition in chick synaptosomes ranging from 65% to 80%, which when added together, would give inhibition of calcium influx greater then 100% inhibition. (e.g. Bowman *et al.,* 1993; Grantham *et al.,* 1994; Maubecin *et al.,* 1995).

However, comparisons of the increase in inhibition in the left IMHV of M-birds relative to Q-birds shows that there was a 35, 33 and 34% increase in the degree of inhibition with ω-CTX GVIA, ω-CTX MVIIC and nimodipine respectively. Thus, the data may be interpreted to suggest that at around the time of training the membrane potential of neurons in the left IMHV of M-birds is becoming less negative, probably as a result of small learning related electrochemical changes. This may then increase the sensitivity of the N-, N/P or Q and L-type channels to be blocked by the respective antagonists. It should be noted that I have interpreted these data with caution for several reasons:

(1) As far as I am aware there is no known data on the differences in the affinity of VSCC antagonists for the channels in the resting and open states.
(2) Inhibition of VSCCs with the respective antagonists can differ at different depolarisation potentials. For example, blockade of N-type channels by ω-CTX GVIA is not as readily removed by strong depolarisation as the inhibition of P-type channels by ω-Aga IVA (Mintz *et al.,* 1992a; Randall and Tsien, 1995). Furthermore, Stocker *et al.* (1997) had only shown the effects of ω-CTX's on N-type channels. Different VSCC may have different properties to N-type channels for their respective blockers at different potentials.

An additional analysis of the effects of the different VSCCs antagonists on absolute basal $[Ca^{2+}]_i$ did not show significant inhibition by nimodpine, ω-CTX GVIA, ω-CTX MVIIC or ω-Aga IVA (table 8; A, B, C and D respectively) on unstimulated VSCC in the synaptoneurosomal preparations. The table shows that in some samples the absolute value decreased following the addition of antagonist, however, in other samples the absolute $[Ca^{2+}]_i$ value increased. Generally, however, the absolute values decreased following the addition of antagonists. This shows that some block may occur, prior to depolarisation, in the resting synaptoneurosome. Finally, I analysed the absolute values of basal $[Ca^{2+}]_i$, following the addition of ω-CTX GVIA, ω-CTX MVIIC and nimodpine, in synaptoneurosomes prepared from chicks tested immediately after training (table 8; F, G and H respectively). I had expected a trend showing decreases in $[Ca^{2+}]_i$ in the left IMHV of W- and M-birds since as already hypothesised, the VSCCs would be in a more depolarised potential following PAL. The data did show some decrease, though not significant and consistent, in absolute $[Ca^{2+}]_i$, indicating that training may, slightly, increase the tonic binding of these antagonist to the respective channels. The rather inconsistent findings is most probably due to the relatively large variability in absolute $[Ca^{2+}]_i$ measurements between samples.

The data shown in figure 30 and 31 are results from experiments carried out to determine the degree of binding of the dihydropyridine L-type channel antagonist [^{3}H]-PN-200-110 in the IMHV and LPO respectively, of chicks tested at either 30 minutes, 6 or 24 hours after training. The rationale behind these experiments was based on the idea that a consequence of VSCC activation may be an increase in the channel population in neurons. Thus, if there was an increase in the channel population, it may be possible to quantify the increase, using radiolabelled antagonists. The data in figure 30 shows that there was no significant differences in the degree of [^{3}H]-PN-200-110 binding in the left or right IMHV of Q-, W- or M-birds at any of the times tested. These data suggests that in birds tested 30 minutes, 6 or 24 hours after training L-type VSCC may not be activated in the IMHV. This binding study shows some correlation to my findings described in chapter 4 (figure 18), where increased KCl-stimulated $[Ca^{2+}]_i$ elevation was significant in the left IMHV of M-birds only when they were tested up to 30minutes post-training. The discrepancy between KCl-stimulated

$[Ca^{2+}]_i$ elevation and ^{3}H-PN 200 110 binding in the left IMHV of M-birds tested 30 minutes after training could be due to increased L-type channel susceptibility to activation and not increased channel population. Alternatively, subtypes of VSCC other than the L-type channels may be activated in the IMHV of M-birds which were not tested in the autoradiography studies. It is already known that subtypes of VSCC are differentially distributed and activated in neurons (Dolphin, 1995).

The data in figure 31 shows some interaction between training and [^{3}H]-PN-200-110 binding in the LPO of birds tested at particular times after training. Significant binding of the antagonist in the LPO of W-birds tested 30 minutes post-training compared to Q- and M-birds could be further evidence of the differential activation of VSCC, depending on the type of test used; where, for example, the appetitive water training leads to different biochemical changes compared to MeA training. I have shown a representative autoradiogram showing the level of binding of [^{3}H]-PN-200-110 in the LPO of W-birds tested 30 minutes after training (figure 32). There is a trend showing greater [^{3}H]-PN-200-110 binding in the left and right LPO of M birds tested at 6 hours after training compared to control. The possible increase in the population of L-type channels in M-birds could be related to the earlier observations by Gigg *et al.*(1994) who found increased neural burst firing in the LPO of birds tested 4 - 7 hours after training. In addition, the data in chapter 4 (figure 21) shows, although not significant, an increase in KCl-stimulated $[Ca^{2+}]_i$ elevation in the right LPO of M-birds compared to Q- and W-birds. It is possible that the increased presynaptic neural firing causes hebbian strengthening between pre- and postsynaptic terminals, leading to an increase in the number of postsynaptic L-type channels. It should be noted that correlating the binding data with the KCl-stimulated $[Ca^{2+}]_i$ elevation data should be carried out with caution since, previous works have shown discrepancies between the two techniques (Maubecin *et al.,* 1995).

Interpretation of the binding data may be complicated by possible developmental changes taking place in the IMHV and LPO of the young chick. A comparison of [^{3}H]-PN-200-110 binding in the IMHV of Q-birds used from each of time points, where the ones used in the 30 minutes group would be younger then ones used in the 24 hour group, shows a trend of

increased binding in birds taken from the 6 hour and 24 hour groups compared to the younger birds from the 30 minute group. In the LPO the possible developmental increases in [^{3}H]-PN-200-110 binding was only observed in Q-birds taken from the 24 hour group. Taken together, these data shows that different parts of the chick brain may exhibit different rates of calcium channel development. Earlier binding studies using ^{125}I-ω-CTX GVIA have already shown small increases in the population of N-type channels in synaptosomes prepared from chicks of different ages (Azimi-Zonooz and Litzinger, 1992). However, L-type channel studies using ^{3}H-nitrendipine binding in the whole brain preparation was shown to level off at around the time of hatching (Marangos *et al.,* 1984). Developmental changes taking place in the IMHV and LPO may have been 'diluted out' by other areas of the whole brain preparation used by Marangos and co-workers.

Chapter 6

GABAergic Modulation of Voltage-Sensitive Calcium Channels in Crude Synaptic Membranes Prepared from the IMHV

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6. Introduction

As described in chapter 1, GABAergic systems have been shown to be involved in biochemical and physiological changes associated with learning and memory. Work in our laboratory has already shown that $GABA_A$ agonists and antagonists can influence memory formation in chicks trained on the passive avoidance task (Clements and Bourne, 1996). Furthermore, Daisley and Rose (1998) have shown increased GABA release in the left and right IMHV, 1 hour and 6.5 hours post-training, respectively.

Electrophysiological and pharmacological evidence indicates that GABA receptors can modulate VSCC (e.g. Stirling *et al.,* 1989; Wall and Dale, 1994; Huston *et al.,* 1995). This chapter explores possible interactions between $GABA_A$ and $GABA_B$ receptors with VSCC, in synaptoneurosomes prepared from the IMHV of day-old chicks.

6.2. Aim of experiments

This chapter has two aims. Firstly, to determine the effects of $GABA_A$ and $GABA_B$ agonist and antagonists, on KCl-stimulated increases in $[Ca^{2+}]_i$, in synaptoneurosomes prepared from the IMHV of untrained day-old chicks. Secondly, to determine if training day-old chicks affects the modulation of the GABAergic receptors on VSCC in synaptoneurosomes prepared from the IMHV.

6.3. Results

6.3.1. Dose-response curve for GABA

GABA inhibits KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes from the IMHV of untrained chicks, in a dose-dependent manner. GABA has an IC_{50} value of 50nM. Figure 33 shows that maximal inhibition ($63\pm7\%$ inhibition) was produced with 100nM of GABA. The degree of inhibition reaches a plateau after this concentration whereby 1μM, 10μM and

100μM of GABA produced 53±12%, 53±10% and 45±8% inhibition respectively. Statistical analysis gave a value of ($F_{5,16}$=3; p=0.03).

6.3.2 Dose-response curve for $GABA_A$ agonist muscimol

The $GABA_A$ receptor agonist, muscimol (figure 34), produced a dose-dependent inhibition of KCl-stimulated increase in $[Ca^{2+}]_i$ with an IC_{50} = 8nM. Very little inhibition (16±12% inhibition) was produced with 1nM of muscimol. On the other hand, 10nM and 50nM of muscimol produced 53±14% and 45±6% inhibition, respectively. Almost total inhibition (82±12% of control) was produced with 10μM of muscimol. Statistical analysis gave a value of ($F_{3,11}$=2; p=0.08).

6.3.3. Dose-response curve for $GABA_A$ antagonist bicuculline

There was no inhibitory effect of bicuculline (figure 35) on KCl-stimulated increase in $[Ca^{2+}]_i$. Incubation of synaptoneurosomes with 50μM bicuculline augmented KCl-stimulated$[Ca^{2+}]_i$ elevation compared to the control (54±1% increase). Statistical analysis gave a value of ($F_{5,12}$=5; p=0.08).

6.3.4. Dose-response curve for $GABA_B$ agonist baclofen

Baclofen (figure 36) produced a dose-dependent inhibition of the KCl-stimulated increase in $[Ca^{2+}]_i$ with an IC_{50} = 3.5μM. There was very little inhibition produced by 10nM and 100nM of baclofen (22±6% and 14±1% respectively), whereas, 10μM baclofen produced 72±14% inhibition. Statistical analysis gave a value of ($F_{3,7}$=2; p=0.18).

6.3.5. Dose-response curve for $GABA_B$ antagonist saclofen

At all concentrations used, saclofen inhibited KCl-stimulated $[Ca^{2+}]_i$ elevations in synaptoneurosomes, and the IC_{50} was 200nM. The graph in figure 37 shows a relatively

shallow curve. There was no difference in inhibition at antagonist concentrations of 10nM or 100nM (44±11% and 45+10% respectively). Greater inhibition, 68±10%, 61±14% and 74±11%, was produced by 1μM, 10μM and 30μM respectively. Statistical analysis gave a value of ($F_{5,19}$=1; p=0.3).

6.3.6. Combined effect of baclofen and saclofen on KCl-stimulated elevation in $[Ca^{2+}]_i$ in untrained chicks.

At concentration of 1μM baclofen and 100nM saclofen, 45±8 and 56±11% inhibition was produced respectively. In comparison, the combination of 1uM baclofen and 100nM saclofen produced 75±7% inhibition (figure 38). There was no significant differences in inhibition between the three groups (p=0.08). This results suggest, that at submaximal concentrations, 1μM baclofen and 100nM saclofen inhibit KCl-stimulated $[Ca^{2+}]_i$ elevation by binding to the same site in synaptoneurosomal preparations from the chick IMHV.

6.3.7. Comparing the effects of GABA, baclofen and muscimol, on KCl-stimulated increase in $[Ca^{2+}]_i$ in crude synaptic membranes prepared from the IMHV of: untrained chicks; and, chicks tested at 5 minutes and 30 minutes post-training.

Experiments were performed to determine the inhibitory effects of submaximal concentrations of GABA, baclofen and muscimol, on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from untrained and trained chicks. There was no difference in the degree of inhibition between untrained chicks and chicks tested 5 minutes post-training (figure 39). There was, however, much greater baclofen induced inhibition in chicks tested 30 minutes post-training (79±10% inhibition), than there was in untrained chicks (47±7%) or in chicks tested 5 minutes post-training (40±7%). It should be noted, however, that the only significant difference was between M-birds tested at 30 minutes after training and M-birds tested 5 minutes after training (THSD; p=0.01); while the difference between M-birds tested at 30 minutes after training and Q-birds bordered significance (THSD; P=0.05). This

result suggests that $GABA_B$ receptors have a greater potency in inhibiting VSCC in the IMHV of chicks tested 30 minutes after training.

Figure 33 Effect of GABA on KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the IMHV of untrained chicks.

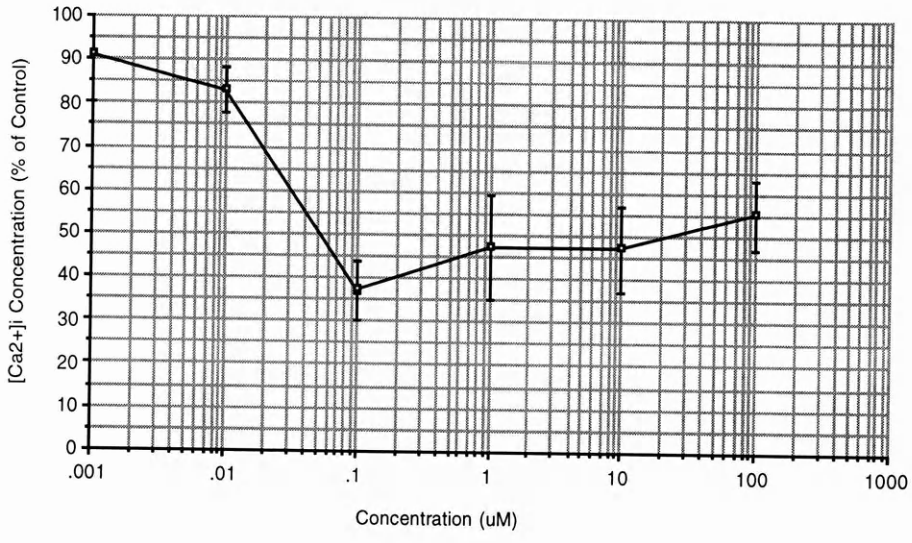

Figure 33 Effect of the increasing concentration of GABA on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the left and right IMHV of untrained day-old chicks. Each data point represents calcium concentration (% of control) following KCl-stimulation. Synaptoneurosomes were incubated for 2.5 minutes with GABA prior to depolarisation with KCl (final concentration: 70 mM). Each data point is the mean ± s.e.m. n=3-7.

Figure 34 Effect of the $GABA_A$ receptor agonist muscimol on KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the IMHV of untrained chicks.

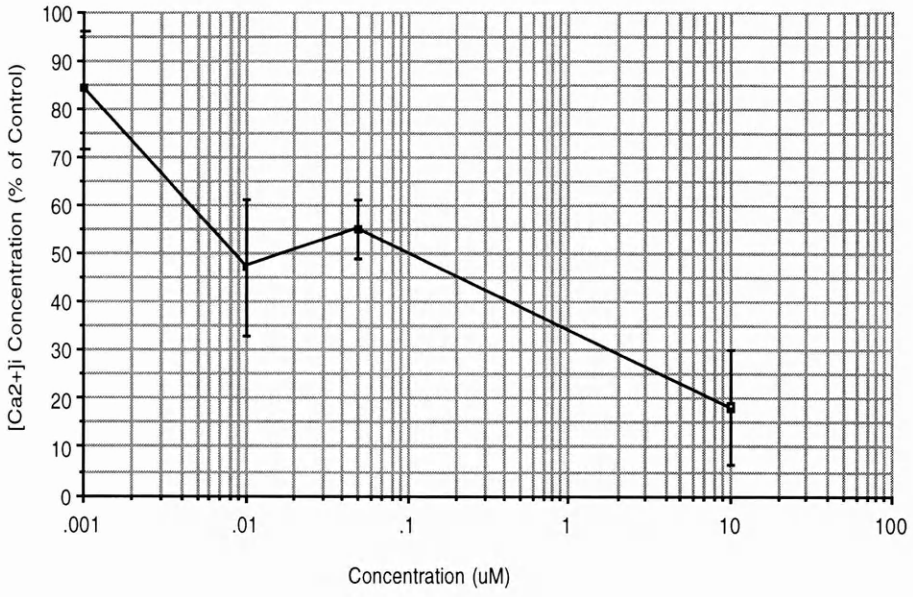

Figure 34 Effect of increasing concentration of the $GABA_A$ receptor agonist muscimol on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the left and right IMHV of untrained day-old chicks. Each data point represents calcium concentration (% of control) following KCl-stimulation. Synaptoneurosomes were incubated for 2.5 minutes with muscimol prior to depolarisation with KCl (final concentration: 70 mM). Each data point is the mean $\pm$ s.e.m. n=4-7.

Figure 35 Effect of the $GABA_A$ receptor antagonist bicuculline on KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from IMHV of untrained chicks.

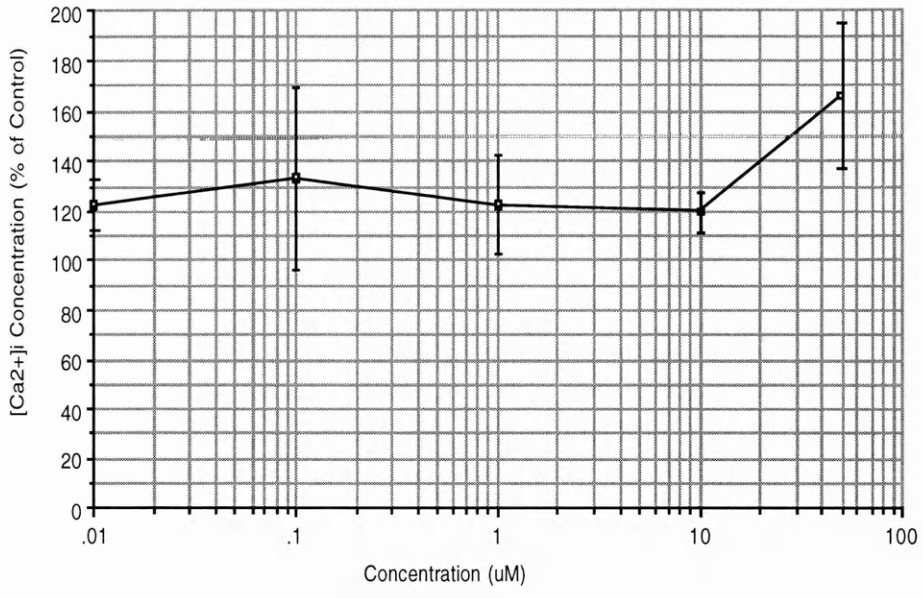

Figure 35 Effect of increasing concentration of the $GABA_A$ receptor antagonist bicuculline on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the left and right IMHV of untrained day-old chicks. Each data point represents calcium concentration (% of control) following KCl-stimulation. Synaptoneurosomes were incubated for 2.5 minutes with muscimol prior to depolarisation with KCl (final concentration: 70 mM). Each data point is the mean $\pm$ s.e.m. n=4 - 6.

Figure 36 Effect of the $GABA_B$ receptor agonist baclofen on KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from the IMHV of untrained chicks.

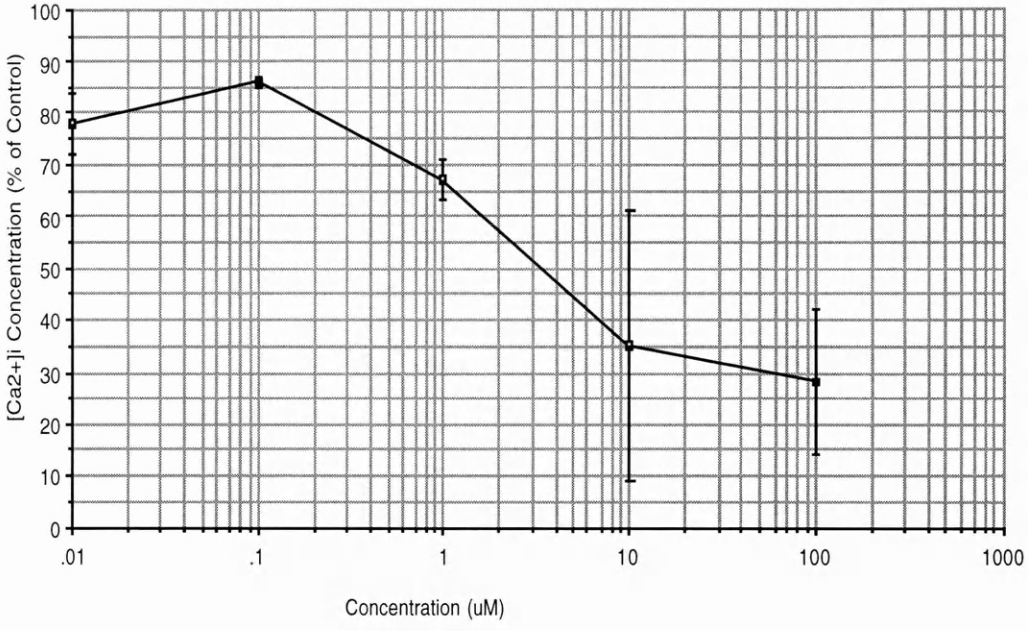

Figure 36 Effect of increasing concentration of the $GABA_B$ receptor agonist baclofen on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the left and right IMHV of untrained day-old chicks. Each data point represents calcium concentration (% of control) following KCl-stimulation. Synaptoneurosomes were incubated for 2.5 minutes with muscimol prior to depolarisation with KCl (final concentration: 70 mM). Each data point is the mean ± s.e.m. n=3 - 5.

Figure 37 Effect of the $GABA_B$ receptor antagonist saclofen on KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from IMHV of untrained chicks.

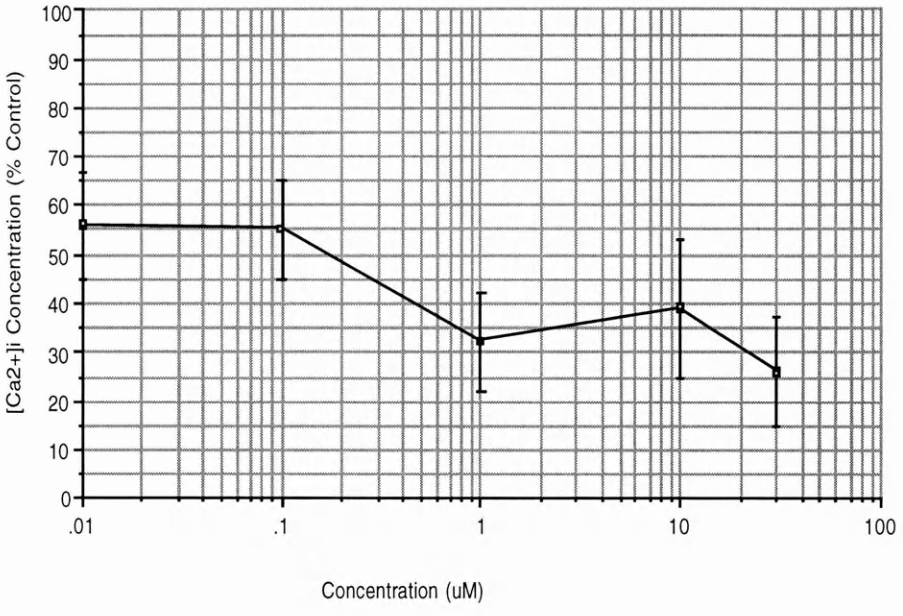

Figure 37 Effect of increasing concentration of the $GABA_B$ receptor antagonist saclofen on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from the left and right IMHV of untrained day-old chicks. Each data point represents calcium concentration (% of control) following KCl-stimulation. Synaptoneurosomes were incubated for 2.5 minutes with muscimol prior to depolarisation with KCl (final concentration: 70 mM). Each data point is represented as the mean $\pm$ s.e.m. n=4 - 8.

Figure 38 Combined effect of 100μM baclofen and 100nM saclofen on KCl-stimulated increase in $[Ca^{2+}]_i$ from synaptoneurosomes prepared from the IMHV of untrained chick.

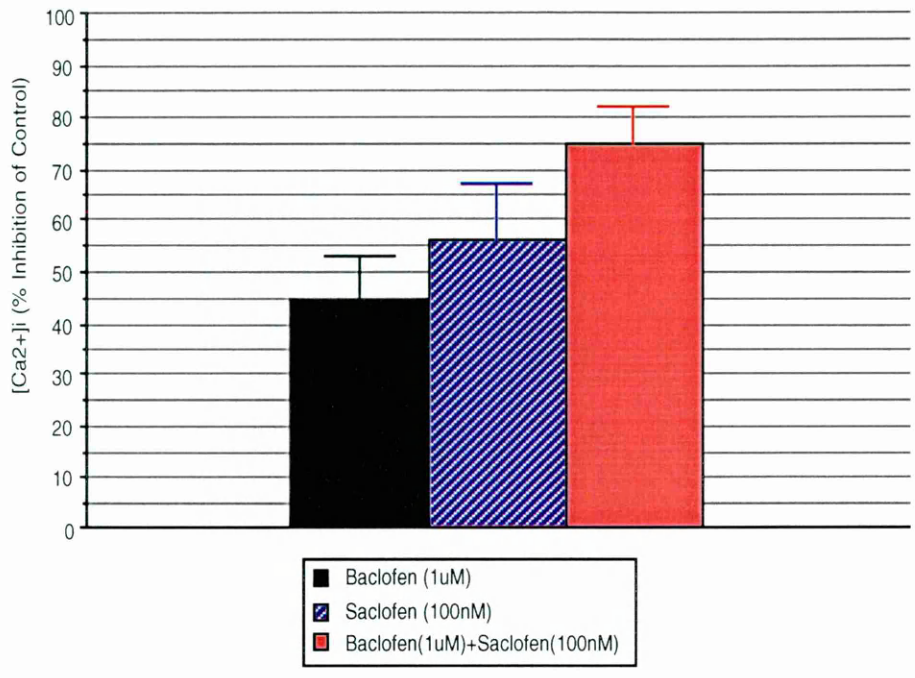

Figure 38 Comparison of the $GABA_B$ agonist baclofen and the $GABA_B$ antagonist saclofen on KCl-stimulated $[Ca^{2+}]_i$ elevation. Synaptoneurosomes were incubated with drugs 2.5 minutes prior to depolarisation with KCl (final concentration: 70 mM). Results are represented as mean ± s.e.m. n=4 - 5.

Figure 39 Comparison of the effect of GABA, muscimol and baclofen on KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes from the IMHV of untrained chicks, chicks tested 5 minutes and 30 minutes post-training.

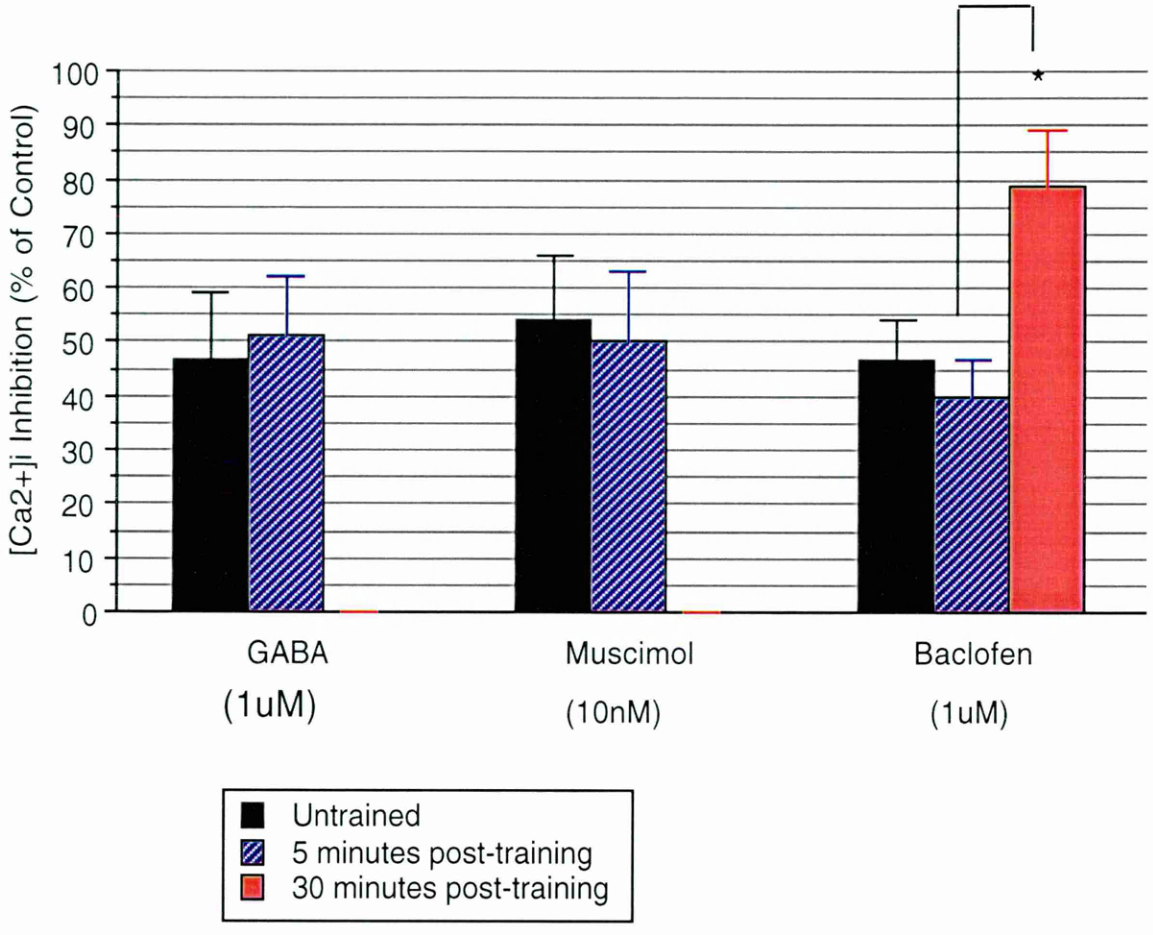

Figure 39 Comparison of the effect of GABA, muscimol and baclofen on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from untrained chicks, chicks tested 5 minutes and 30 minutes post-training. Synaptoneurosomes were incubated with GABA, muscimol or baclofen for 2.5 minutes prior to depolarisation with KCl (final concentration, 70mM). Results are represented as mean $\pm$ s.e.m. n=4 - 7. * = $P<0.05$

6.4. Discussion

Previous findings have shown that the inhibitory neurotransmitter GABA is involved in modulating memory formation. For example, intraseptal injections of muscimol ($GABA_A$ agonist) impairs both radial arm maze (Chrobak *et al.,* 1989) and Morris water maze (Brioni *et al.,* 1990) learning in rats. More recently it was observed that bilateral injections of muscimol into the amygdala complex, a region with a large population of GABAergic neurons, prior to testing 24 hours after training rendered rats amnesiac for an inhibitory avoidance task (Coleman-Meches and McGaugh, 1995). Furthermore, the differences were lateralized, where unilateral administration of muscimol into the right but not left amygdala complex rendered rats amnesiac. $GABA_B$ receptors were also found to have an affect on memory formation. In mice, administration of the specific $GABA_B$ agonist baclofen immediately after training impaired, retention in animals tested on either a one-trial inhibitory avoidance task, or classical conditioning task (Castellano *et al.,* 1989). Furthermore, in a similar manner to muscimol, post-training intra-amygdala administration of baclofen, impaired retention in rats tested in an inhibitory avoidance response.

Developmental studies in the chick have shown that total ^{3}H-muscimol binding increases rapidly *in ovo*, reaching a peak at around day one post-hatch, and slowly declining to approximately 50% of the maximal level by day 21 post-hatch (Stewart and Bourne, 1987). As mentioned previously, the $GABA_A$ system has been shown to modulate the early phase of memory formation in day-old chicks tested on the passive avoidance task (Clements and Bourne, 1996). As a result of this, and previous findings that the GABAergic system modulates memory formation in a wide variety of animal models, I decided to determine if the GABAergic systems ($GABA_A$ and $GABA_B$) exert any of their memory modulatory affects via voltage sensitive calcium channels.

Firstly, I attempted to determine the dose-response curves for a variety of GABAergic agents in synaptoneurosomes prepared from the IMHV of untrained day-old chicks. The rationale behind these experiments was to establish if these GABAergic agents modulated VSCC in synaptic terminals, and also to assess the potency of modulation between different

GABAergic agents. Figure 33 shows a dose-dependent inhibition of GABA on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes. Plateau was observed with 100nM of GABA. Subsequent additions with larger concentrations of GABA (1μM, 10μM and 100μM) did not evoke greater inhibition. The relatively large degree of inhibition of the VSCCs with a relatively small concentration of GABA (100nM) is most probably the result of $GABA_A$ and $GABA_B$ receptor modulation of VSCCs, following the activation of both GABAergic systems. In support of my data, previous electrophysiological work has also shown that GABA inhibits calcium channel currents. Robertson and Taylor (1986) reported that GABA reduced the peak amplitude of the sustained calcium current in isolated dorsal root ganglion cells of the cat, under voltage clamp conditions.

The dose-response curve for the $GABA_A$ agonist muscimol, also shows a dose-dependent inhibition of KCl-stimulated increase in $[Ca^{2+}]_i$ (figure 34). This results indicates that $GABA_A$ receptors are present in synaptoneurosomal preparations and have the ability to inhibit presynaptic VSCC. It may be, however, that, since I used a crude membrane preparation containing synapto neurosomes, the effect could also be due to inhibition on the postsynaptic densities. It is interesting that $GABA_A$ receptors have the capacity to inhibit KCl-stimulated $[Ca^{2+}]_i$ elevation with a greater potency than the $GABA_B$ agonist baclofen, where the IC_{50} for muscimol was 8nM and for baclofen was 3.5μM. I initially assumed that muscimol would not have any effect on VSCC, because most previous studies have shown that the metabotropic $GABA_B$ receptors modulate VSCC (Zamponi and Snutch, 1998). However, some earlier work using synaptosomes prepared from rat cortical tissue, did show that muscimol inhibited KCl-stimulated increase in $[Ca^{2+}]_i$ (Stirling *et al.,* 1989), although the degree of inhibition was a lot less. Stirling and co-workers found that 300μM of muscimol inhibited 32% of KCl-stimulated $[Ca^{2+}]_i$ elevation. In comparison, I found almost total inhibition with 100μM of muscimol (figure 34). The relatively large difference in potency between my data and that of Stirling and co-workers may be due to species differences. For example, there may be a greater number of GABAergic neurons in the chick IMHV than there is in rat cortical tissue; hence the greater potency of muscimol on inhibiting KCl-stimulated $[Ca^{2+}]_i$ elevation in the chick preparation. In addition, it has previously been shown that muscimol has some activity at $GABA_B$ receptors (Bowery *et al.,* 1983). The

greater potency of muscimol compared to baclofen could thus be a consequence of cross-reactivity of the $GABA_A$ agonist with $GABA_B$ receptors. Finally, $GABA_A$ stimulation may be having a more indirect and global effect on VSCC. The GABA receptors might be undergoing developmental changes in the developing chick brain. These receptors may therefore express different pharmacological properties than GABA receptors in fully matured neurons. Activation of $GABA_A$ receptors leads to chloride influx resulting in neural hyperpolarization which may inactivate a larger population of VSCC. In comparison, $GABA_B$ receptor activation has previously been shown to inhibit specific VSCC, particularly N-type (Wu and Saggau, 1995) and P-type channels (Dittman and Regehr, 1996), thus giving a smaller degree of VSCC inhibition.

The dose-response curve for the $GABA_A$ antagonist, bicuculline, does not show any inhibition of KCl-stimulated increase in $[Ca^{2+}]_i$ (figure 35). In fact, the antagonist produced slight increases in KCl-stimulated $[Ca^{2+}]_i$ at concentrations of 10nM, 100nM, 1μM and 10μM. At the highest dose of 50μM, however, bicuculline produced a relatively large (54%) increase in KCl-stimulated $[Ca^{2+}]_i$ elevation. In summary bicuculline did not inhibit VSCCs in synaptoneurosomes, indicating that, in the developing chick brain, bicuculline does behave as a $GABA_A$ antagonist. This would be in agreement with the literature (e.g. Rang and Dale, 1991). As far as I am aware there is no direct *in vitro* experimental data showing the effects of bicuculline on muscimol-mediated modulation of K^+-induced increase in $[Ca^{2+}]_i$, however, indirect evidence from *in vivo* experiments have shown that bicuculline increased memory retention in day-old chicks trained on a weak PAL task while muscimol decreased memory retention in chicks tested on the weak PAL task (Clements and Bourne, 1996). In contrast the results with the $GABA_B$ antagonist saclofen (figure 37), suggest that it may have some GABAergic agonistic effect on synaptoneurosomal preparation.

The $GABA_B$ agonist baclofen produced a dose-dependent inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation with an IC_{50} of 3.5μM (figure 36). A similar effect was previously measured in rat cortical neurons where the IC_{50} for baclofen inhibiting KCl-stimulated $[Ca^{2+}]_i$ elevation was also calculated to be 3.5μM (Stirling *et al.,* 1989). Stirling and co-workers found that GABA and baclofen produced similar dose-dependent inhibition of Kcl-

stimulated $[Ca^{2+}]_i$ elevation. I, however, found quite large differences in the dose-response curves of GABA and baclofen. For example, GABA had two IC_{50} values, 50nM and 20μM; while baclofen inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation in a classical dose-dependent manner with an IC_{50} of 3.5μM.

It has been known for some time that presynaptic $GABA_B$ receptors have the ability to modulate voltage dependent calcium currents (Tsien *et al.,* 1988). Since neurotransmitter release is dependent on calcium influx, via a variety of VSCC subtypes (Olivera *et al.,* 1994), this implies that $GABA_B$ receptors have an important function in modulating transmitter release, and therefore synaptic plasticity. Experiments have already shown that KCl-stimulated release of 5-hydroxytryptamine (5-HT) and noradrenaline from cortical slices were inhibited in the presence of baclofen (Bowery *et al.,* 1980; Schlicker *et al.,* 1984; Gray and Green, 1987). A lot of work has already been carried out to determine the mechanism of VSCC inhibition following $GABA_B$-activation. For example, the $GABA_B$ agonist baclofen reversibly reduced the amplitude of calcium currents in isolated *Xenopus* spinal neurons (Wall and Dale, 1994). Furthermore, the specific $GABA_B$ antagonist CGP35348 blocked the affect of baclofen. In addition, the application of the specific N-type blocker, ω-CTX GVIA, also attenuated the effect of baclofen in isolated *Xenopus* spinal neurons, which lead to the conclusion that $GABA_B$ receptors have the ability to modulate synaptic transmission by reducing the amplitude of ω-CTX GVIA-sensitive calcium currents.

My experiments demonstrate that baclofen has the ability to inhibit VSCC activity in the chick IMHV. It should be noted, however, that Gahwiler and Brown (1985) have reported that, in hippocampal neuronal cultures, $GABA_B$ activation leads to increased potassium conductance with no direct effect on calcium conductance. They argued that the effect of increased potassium conductance following $GABA_B$-receptor activation resulted in the activation of potassium-channels allowing the efflux of potassium ions causing the membrane potential to reach a hyperpolarized potential. As a result, the increased hyperpolarized potential would lead to the inactivation of VSCCs, which are activated at a more depolarised potential (i.e. voltage-dependence of VSCC activation). In light of more recent work, however, it would appear that the majority of the inhibitory effect of $GABA_B$

receptors on presynaptic calcium influx occurs via the inhibition of VSCC's. For example, Huston *et al.* (1995) found that baclofen inhibited KCl-stimulated (VSCC activating) glutamate release in cerebellar granule neurons, whereas non-VSCC-dependent stimulated glutamate release was not affected by baclofen. Huston and co-workers also found that different subtypes of VSCC were modulated to different degrees by $GABA_B$ receptors, where the ω-CTX GVIA sensitive N-type channels were the primary targets of $GABA_B$ modulation. However, blockade of N-type channels also showed that $GABA_B$ receptors were able to modulate ω-Aga IVA-sensitive P/Q-type channels, though with a lower affinity.

Wu and Saggau (1995) simultaneously measured presynaptic calcium transients and field excitatory postsynaptic potential [f(EPSP)] in the hippocampal area CA1 of the guinea-pig. They found that baclofen rapidly reduced $[Ca^{2+}]_i$ which subsequently lead to decreased f(EPSP) in a manner similar to that previously observed with the addition of VSCC antagonists (Wu and Saggau, 1994). The effects of baclofen on $[Ca^{2+}]_i$ and f[EPSP] were blocked by CGP35348. Prior application of ω-CTX GVIA, but not ω-AGA IVA (a P-type blocker), attenuated the effects of baclofen on calcium current, indicating that $GABA_B$ receptors inhibit N-type channels but not P-type channels in the guinea-pig hippocampus. Further experiments also confirmed that baclofen inhibition of $[Ca^{2+}]_i$ is not a secondary consequence of K^+-channel modulation, because baclofen differentially inhibited the three components of $[Ca^{2+}]_i$ where, baclofen inhibited 49% of N-type currents and 40% of the ω-CTX GVIA and ω-AGA IVA -insensitive current. In thalamacortical neurons, different concentrations of baclofen were shown to have different affects on HVA calcium currents. Low concentrations of baclofen (0.5 - 10μM) inhibited 13.5% of the current, whereas 50μM baclofen inhibited 41% of the current. Moreover, low doses of baclofen did not inhibit N-type channels but high doses did. Addition of CGP35348 attenuated the affects of high but not low concentrations of baclofen. Taken together these data show that modulation of VSCC receptors involves two subtypes of $GABA_B$ receptors. One subtype which has a high affinity to baclofen acts on the residual HVA current, and a low affinity $GABA_B$ subtype which acts on N-type currents. Bonanno and Raitery (1993a/b) have also described the existence of two types of $GABA_B$ receptors on glutamate terminals. One type is insensitive to CGP35348 and phaclofen (a weak selective $GABA_B$ antagonist), the other ($GABA_B$

autoreceptor) type which is CGP35348-insensitive but phaclofen-sensitive. Other groups have also demonstrated the existence of different subtypes of $GABA_B$ receptors. For example, Calabressi *et al.* (1991), using neocortical slices have shown the existence of two types of presynaptic $GABA_B$ receptors. Dutar and Nicoll (1988) and Thompson and Gahwiler (1992) have both shown the existence of pre and postsynaptic $GABA_B$ receptors with distinct pharmacology in the hippocampus. Others had additionally found evidence for the existence of $GABA_B$ receptors with distinct pharmacology's in the cortex (Diesz *et al.,* 1993), and in thalamus (Emri *et al.,* 1994). Expression of different $GABA_B$ receptors have also been found in the same neuron, where for example, cultured cerebellar granule neurons expressed high and low affinity $GABA_B$ receptors which were blocked by CGP35348 (Wojcik *et al.,* 1990). Dutar and Nicoll (1988) suggested that there was heterogeneity of postsynaptic $GABA_B$ receptors in the hippocampal pyramidal neurons where one subtype was coupled to decreases in calcium conductance and the other, was responsible for late IPSP. Finally, Toselli and Taglietti (1993) using rat hippocampal neurons found that baclofen inhibited HVA calcium currents by two distinct modes. One mode was voltage-dependent and induced the slow-down of the current; the other mode was voltage independent and was not associated with significant changes in the kinetics of the calcium current. The existence of different subtypes of $GABA_B$ receptors with different affinities for baclofen and different modulatory affects on VSCC may explain the lack of inhibition with 10nM and 100nM baclofen. The amount of inhibition increased significantly, however, when synaptoneurosomes were exposed to 1μM, 10μM and 100μM baclofen. In addition, my synaptoneurosomal preparations (which contained both pre- and postsynaptic terminals) may be indicating the presence of $GABA_B$ and $GABA_A$ subtypes on the different terminals ,or, as suggested by Thompson and Gahwiler (1992), the existence of different subtypes of pre- and postsynaptic $GABA_B$ receptors. Thus, this 'step-wise' dose-dependent inhibition suggests the possible existence of different $GABA_B$ receptor subtypes with differential sensitivity to baclofen.

The $GABA_B$ antagonist saclofen (figure 37) produced dose-dependent inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation with greater potency then the $GABA_B$ agonist baclofen, where the IC_{50} for saclofen and baclofen were 200nM and 3.5μM respectively. In light of the fact

that activation of GABAergic receptors leads to inhibition of VSCC, and therefore inhibition of calcium influx, the dose-response curve for saclofen was unexpected. This initial finding indicates that, in chick synaptoneurosomal preparations, saclofen behaves like a $GABA_B$ agonist. Together with this, the potency of saclofen-evoked inhibition of VSCC is greater than baclofen. Earlier experiments using rat cortical synaptosomes found that 3 putative $GABA_B$ antagonists; δ-aminovaleric acid; phaclofen; and β-phenyl GABA all significantly inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation by 56, 64 and 31% respectively (Stirling *et al.,* 1989). It was argued that the drugs were only acting on the $GABA_B$ receptors, and no other receptor site, because earlier work had shown that these drugs bound to $GABA_B$ receptors in cortical membranes from rats (Bowery *et al.,* 1983). Aditionally, β-phenyl GABA, phaclofen and δ-aminovaleric acid had shown $GABA_B$ antagonistic properties in a variety of peripheral tissues (Ong *et al.,* 1987; Sawynok, 1986). Furthermore, these drugs have also shown $GABA_B$ antagonistic actions in central systems, where for example, phaclofen blocked postsynaptic $GABA_B$ receptors, which are usually involved in modulating potassium conductance in hippocampal slices (Dutar and Nicoll, 1988). Also, δ-aminovaleric acid has been shown to attenuate baclofen induced reduction of population spikes in hippocampal slices (Nakahiro *et al.,* 1985). My results with saclofen along with those of Stirling and co-workers (with their putative $GABA_B$ antagonists), appear to indicate that presynaptic synaptosomal and synaptoneurosomal $GABA_B$ receptors may have distinct pharmacological properties in comparison to peripheral and some other central $GABA_B$ receptors (as seen with the use of other known $GABA_B$ antagonists). It should be noted, however, that other $GABA_B$ antagonists (such as CGP35348) did attenuate the affects of baclofen on $GABA_B$ receptors in the dorsal lateral cingulate and hippocampal slices (Wu and Saggau, 1995; Guyon and Leresche, 1995).

To determine if saclofen was exerting its inhibitory affect on VSCC via the activation of the same site as baclofen I did a combination experiment. The rationale was as follows: if the two drugs were causing their respective inhibitory effects via the same site on the presynaptic terminal, then, addition of the two compounds at the same time prior to depolarisation with KCl (70mM) would not be additive when compared to the inhibitory effects of the drugs separately. However, if the drugs were acting on separate sites on the synaptoneurosome,

then this would indicate inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation at two distinct sites of the synaptoneurosome. The results (in figure 38) show a comparison between the effects of submaximal concentrations of the drugs on KCl-stimulated $[Ca^{2+}]_i$ elevation, when added separately, and when combined. The results suggests that baclofen and saclofen exert (at least the majority of) their inhibitory effects by activating the same site, which is most probably the $GABA_B$ receptor. An explanation for the agonistic like action of saclofen in synaptoneurosomes from the IMHV may be due to developmental changes in $GABA_B$ receptors in the neurons of the young chick. There is evidence that GABA receptors may express atypical behaviour in young developing neurons. For example, GABA has been shown to have transient excitatory affects in immature rat neuroblasts and neurons (for review see Ben-Ari *et al.,* 1997). Another reason for the "agonist-like" behaviour of saclofen in the synaptoneurosomes could be due to the existence of $GABA_B$ receptor subtypes that behave differently to saclofen from the classical $GABA_B$ receptors. Although there is nothing in the literature showing a direct $GABA_B$ agonist action of saclofen, Matthews *et al.* (1994) found evidence that saclofen did not block the affects of GABA in isolated goldfish bipolar neurons. They went on to propose that this was evidence for the existence of a variety of $GABA_B$ receptor subtypes, that can be determined by their different degrees of sensitivity to $GABA_B$ agonist and antagonist. Furthermore, Bonanno and Raiteri (1993) also found evidence for the existence of different subtypes of $GABA_B$ receptors in the spinal cords and cerebral cortex of the rat. It should be noted that since the studies quoted above do not directly explain the effects of saclofen on $GABA_B$ receptors more detailed analysis will have to be carried out in the chick synaptoneurosomal system.

Behavioural studies have already shown that GABAergic systems are involved in the early stages of memory formation following passive avoidance training in the day-old chick (Clements and Bourne, 1996). Daisley and Rose (1998) found lateralized differences in calcium-dependent GABA release in chicks following passive avoidance training. They found a significantly greater release of GABA (1 hour post-training) in the left IMHV of M-birds compared to W-birds. At 6.5 hours post-training, however, increased calcium-dependent GABA release was detected in the right IMHV of M-birds compared to W-birds. Increases in calcium-dependent GABA release at 6.5 hours post-training indicates a role for

the inhibitory neurotransmitter in longer-term memory processes. Furthermore Martijena and Arce (1994) found increased ^{3}H-flunitrazepam binding in the chick forebrain, indicative of increased $GABA_A$ receptor number, following passive avoidance training. The increase was most evident in birds tested 30 minutes post-training, thus further supporting the hypothesis of an early involvement of the GABAergic system in memory formation following passive avoidance training. In addition to the increased release of GABA in animals trained on various paradigms; increased release of GABA has also been measured in the electrophysiological model of memory and synaptic plasticity (LTP). For example, Ghisjen *et al.* (1992) showed increased release of GABA following LTP. The observations that GABA plays a part in the biochemical correlates of memory formation lead to the final experiments in this chapter. The aim of the final experiments, the results of which are shown in figure 39, was to determine if training effected GABAergic modulation of VSCC in the left and right IMHV of day-old chicks. In the limited time available I studied the inhibitory effects of submaximal concentration of GABA, muscimol and baclofen in synaptoneurosomes prepared from chicks tested 5 minutes post-training. In addition, I was able to measure the inhibitory effects of baclofen on synaptoneurosomes from chicks tested 30 minutes post-training.

Comparisons of the effect of GABA, muscimol and baclofen, on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosomes prepared from untrained chicks, and chicks tested five minutes post-training, shows that that there was no difference in the degree of inhibition in the synaptoneurosomes. The similarity in potencies of $GABA_A$ and $GABA_B$ receptors on VSCC inhibition between untrained chicks, and chicks tested 5 minutes post-training, perhaps suggests that neither receptor subtype has any modulatory role on VSCC at this time. This observation agrees with earlier findings in chapter 4 (figure 18), where the VSCC's were found to be readily activated (as measured by the higher degree of KCl-stimulated increase in $[Ca^{2+}]_i$ in synaptoneurosomes prepared from M-birds tested 5 minutes post-training compared to Q-birds).

Baclofen evoked significantly greater inhibition in synaptoneurosomes prepared from the IMHV of chicks tested 30 minutes post-training, compared to untrained chicks and chicks

tested 5 minutes post-training. This shows that $GABA_B$ receptors may be activated sometime between the time of training and thirty minutes post-training. Consequently *in vitro* addition of baclofen in synaptoneurosomes prepared from trained birds had a greater inhibitory effect on VSCC. Furthermore, the increased potency of $GABA_B$ inhibition of VSCC may be responsible for the decrease in VSCC activation observed in the left IMHV of M-birds (chapter 4, figure 18). These data gives further support to the notion that GABAergic systems are involved in the early time-course of memory formation following passive avoidance learning (Clements and Bourne, 1996).

The results in figure 39 suggest that $GABA_B$ systems have a role to play in memory formation in the day-old chick. As mentioned previously, $GABA_B$ receptors are predominantly situated in presynaptic terminals where they behave as autoreceptors, activation of which results in negative feedback modulation of further transmitter release. In fact, $GABA_B$ autoreceptors have been shown to regulate the induction of LTP (Davies *et al.,* 1991). Daisley and Rose (1998) hypothesised that, the increased GABA release in the IMHV of chicks following passive avoidance training may act on $GABA_B$ autoreceptors, resulting in feedback inhibition of further GABA release and thus remove GABA-mediated inhibition of neural tissue. Since they found increased GABA release within 1 hour post-training in the left IMHV of trained chicks, this correlates with my findings of an increased effect of baclofen on inhibiting VSCC in synaptoneurosomes prepared from the IMHV of chicks tested 30 minutes post-training.

Chapter 7

General Discussion and Future Directions

7. General Discussion and Future Directions

This aims of this thesis can be divided into three parts:

(1) Determine the time-course of calcium influx, via voltage sensitive calcium channels (VSCC), in the intermediate medial hyperstriatum ventrale (IMHV), and lobus parolfactorius (LPO) of day-old chicks trained on the passive avoidance task.

(2) Identify the type or types of VSCC in the IMHV of day-old chicks, and determine if different types of VSCC are activated at different times after training on the passive avoidance task.

(3) Study the modulation of VSCC by agonists and antagonists of GABA receptors in the IMHV, and subsequently determine if the degree of modulation varies between untrained and trained chicks.

The first part of chapter 3 describes some preliminary work from the 1960's and 1970's into the validity of using crude membrane preparations, containing a mixture of synaptosomes and synaptoneurosomes, for measurements of calcium influx in the chick brain. Since synaptosomes and synaptoneurosomes have the same physiological properties, evidence in the literature showing he usefulness of synaptosomes in the *in vitro* studies of synaptic function was taken to indicate that of synaptoneurosomes were useful in studying synaptic function. The advantage of having synaptoneurosomes is that it enables the experimenter to study pre and postsynaptic function.

Blaustein and Goldring (1975) among others, have shown that synaptosomes retain most of the presynaptic 'machinery' for studying presynaptic mechanisms. Thus synaptosomes still contain the original synaptic cytosol, including calcium binding sites and the machinery necessary for neurotransmission. In addition to this, synaptosomes have been shown to be able to maintain a membrane potential as a result of an active Na^+/K^+-ATPase; they have high affinity binding sites for receptor agonists; and functional, selective, ionic channels (for review see Bradford, 1986). Experiments undertaken to evaluate free calcium concentration ($[Ca^{2+}]_i$) in synaptosomes, using fura-2/AM and quin-2 analysis, has shown that $[Ca^{2+}]_i$

measurements were critically dependent on several factors (Verhage *et al.*, 1988). These were:

(1) Synaptosomal preparation

(2) Type of dye used

(3) Dye/protein ratio and the time and temperature during loading

(4) Calibration procedure

(5) Prolonged incubations at 37°C lead to decreased synaptosomal viability

Verhage and co-workers (1988) concluded that, as long as precautions were taken in preparing synaptosomal samples, interpretation of measured $[Ca^{2+}]_i$ as a representation of physiological values were legitimate. Thus, as a result of the work by Verhage and various other groups such as Blaustein and Goldring (1975), and Bradford (1986), I decided to use the crude membrane preparations (containing synaptosomesand synaptoneurosomes) to measure KCl-stimulated $[Ca^{2+}]_i$ in the IMHV and LPO of chicks following passive avoidance training (as described in chapter 4). A review of recent literature confirms that synaptosomal preparations are valid in determining the identity of VSCC (e.g. Bowman *et al.*, 1993; see chapter 5) and the modulation of these channels (e.g. Stirling *et al.*, 1989; see chapter 6).

Later in chapter 3 I report some preliminary results from experiments using radiolabelled calcium ($^{45}Ca^{2+}$) as a measure of calcium influx in synaptosomal and synaptoneurosomal preparations from the chick brain. Previous work had demonstrated that this method was valid in studying calcium influx (e.g. Blaustein *et al.*, 1975; Lundy *et al.*, 1994). It soon became evident, however, that I was not able to consistently reproduce $^{45}Ca^{2+}$ influx following depolarisation with KCl (70mM). Additionally, when stimulation of $^{45}Ca^{2+}$ influx was successful I was unable to get inhibition by exposing synaptoneurosomes to the potent, irreversible N-type VSCC channel blocker, ω-CTX GVIA. Since N-type channels have been shown to exist in the chick forebrain, though not specifically in the IMHV and LPO, it seemed to me that another approach may be more fruitful. Thus, I started using the

fluorescent free calcium ion indicator, fura-2/AM to measure changes in $[Ca^{2+}]_i$ after KCl-stimulation (70 mM).

The last section of chapter 3 describes some preliminary experiments to determine the optimal concentration of KCl needed to stimulate calcium influx; using fura-2/AM to measure $[Ca^{2+}]_i$ in the crude membrane preparations. The amount of calcium influx was represented as a percentage of the control to standardise the data. I did this because in some experiments the basal $[Ca^{2+}]_i$ values from different preparations of synaptoneurosomes were quite variable.

Chapter 4 describes experiments to determine a time-course of KCl-stimulated changes in $[Ca^{2+}]_i$ in synaptoneurosomes, in the left and right IMHV and LPO of chicks, tested at certain times after training on the passive avoidance task compared to control and water-trained birds. I observed large increases in KCl-stimulated increase $[Ca^{2+}]_i$ in the left IMHV of trained chicks tested immediately, 5 minutes, 10 minutes and 30 minutes post-training. There was no statistical difference at later times between any of the three groups in the left IMHV. ANOVA did not show any time and training interaction in the right IMHV, thus further post-hoc analysis was not carried out. These data shows that following training on the passive avoidance task, there is a large and transient increase in KCl-stimulated calcium influx in the left IMHV, and this is sustained up until 30 minutes post-training, thus indicating activation of the VSCC. This is in accordance with previous findings that pre-training, but not post-training, injections of the N-type VSCC blocker, ω-CTX GVIA rendered chicks amnesiac when tested on the task 30 minutes or 3 hours later (Clements *et al.*, 1995).

One would expect, if memory for an event was to form, changes in neural firing to occur almost immediately after the training event. This would mean an alteration in synaptic communication between neurons of the activated brain region. Recently, Power *et al.* (1997), using the *in vitro* electrophysiological analysis of rabbit hippocampal slices, found significantly enhanced synaptic transmission in the CA1 region in slices prepared 1 hour after training rabbits on the eye-blink conditioning paradigm. However, there was no

enhancement of synaptic transmission in hippocampal slices prepared from trained rabbits 24 hours post-training. This demonstrates that associative learning enhances synaptic transmission between CA3 and CA1 regions immediately after training, but not at later times. One possible mechanism for such an increase in synaptic transmission, put forward by Power and co-workers, was an increase in transmitter release in the hippocampus 1 hour after training, which as mentioned in chapter 1 is usually dependent in calcium influx. Daisley and Rose (1998), using the *in vivo* microdialysis technique, showed increased bilateral calcium-dependent glutamate release in the IMHV of chicks 30 minutes after-training on the passive avoidance task. This increase was sustained up to 1 hour post-training in the left, but not right IMHV. The present result suggests that memory formation following passive avoidance training leads to the early transient calcium influx in the left IMHV. One of the consequences of this is calcium dependent glutamate release, necessary for increased neural communication to 'encode' information learned during the passive avoidance task. The increased calcium-dependent glutamate released in the right IMHV measured by Daisley and Rose needs however, to be resolved with the non significant calcium influx in the IMHV in my experiments. A possible reason for such a discrepancy may be due to differences in the experimental techniques used to measure calcium influx and glutamate release respectively.

In the LPO there was a slight 'delay' in calcium influx. Significant increases in KCl-stimulated calcium influx was measured in M-birds 5 minutes post-training, and sustained in birds tested 10 minutes post-training, but it declined to control levels by 30 minutes post-training in both left and right LPO. The slight difference in activation of calcium influx in the LPO agrees with previous suggestions that the memory trace is transferred from the IMHV to the LPO (Rose, 1995).

Chapter 5 initially describes experiments to determine the presence of the type, or types, of VSCC in synaptoneurosomal preparations from the IMHV of untrained chicks. The left and right hemisphere were analysed together so as to limit the amount of toxins used. To do this I carried out dose-response experiments, where the potency of the increasing concentration of each antagonist at blocking KCl-stimulated $[Ca^{2+}]_i$ elevation was measured. The results

showed the existence of: ω-CTX sensitive N-type channels; nimodipine sensitive L-type channels; ω-agatoxin IVA sensitive P/Q-type channels; and ω-CTX MVIIC sensitive N/P/Q-type channels in the IMHV. Interestingly, the potencies of some of these antagonists showed some variation from previous findings by groups who had also used synaptosomes prepared from the chick brain (Suszkiew *et al.*, 1987; Bowman *et al.*, 1992; Lundy *et al.*, 1994; Pocock *et al.*, 1992; Grantham *et al.*, 1994; Maubecin *et al.*, 1995). For example, the chick brain has been shown to be abundant in N-type channels on the basis of binding studies in the whole brain of developing chicks. There is an increased ω-CTX GVIA binding from embryonic day 9 to post-hatch day 10, with the largest increase in binding at around the time of hatching (Azimi-Zoonoz and Litzinger, 1992). Dose-response experiments carried out by Suszkiew *et al.* (1987), Bowman *et al.* (1992), Lundy *et al.* (1994), Grantham *et al.* (1994), Maubecin *et al.* (1995) showed that 1μM of ω-CTX GVIA almost completely blocked calcium influx in their preparations. Similarly, L-type blockers such as the group of drugs belonging to the family of dihydropyridines, nimodipine or nitrendipine had little or no effect on calcium influx in the chick brain preparations. In contrast to previous work, I found that specific blockers of N-type and L-type channels at the maximal concentration used (1μM and 60μM respectively), evoked approximately 50% and 60% inhibition respectively of KCl-stimulated $[Ca^{2+}]_i$ elevation. Comparisons of my results with the degree of inhibition observed by other laboratories did show differences in the amount of inhibition in the chick neural preparation. The differences in data are probably due to a variety of reasons such as:

(a) Age of chick used

(b) Area of brain studied

(c) Composition of incubation medium

(d) Concentration of KCl (depolarising agent) used

(e) Species differences

(f) Tissue preparation (existence of synaptosomes and synaptoneurosomes in my preparation).

The age of chick used for the experiments may affect the amount of inhibition since the number of calcium channels varies in the developing organism. For example, Azimi-Zoonoz and Litzinger (1987) have shown a steady 40% increase in N-type channels in the chick

brain between day 1 of hatching and 8 days post-hatch. Furthermore, developmental changes in the sensitivity of VSCC to antagonist should not be disregarded since it is possible that during the processes of neurodevelopmental maturation, properties of calcium channels such as affinity and / or numbers of binding sites may vary.

Differences in channel distribution is evident between different areas of the brain. For example, autoradiography analysis of [^{3}H]-PN-200-110 (an L-type VSCC blocker) binding showed relatively large differences in the population of L-type channels in different areas of the chick brain (data not shown).

The composition of the medium in which membrane preparations are suspended, such as the concentration of particular ions, has been shown to affect antagonist binding. For example, Feigenbaum *et al.* (1988) and Wagner *et al.* (1988) demonstrated that high concentrations of extracellular calcium decreased the affinity of ω-CTX GVIA peptide binding to N-type channels. Furthermore, a relatively high intracellular magnesium concentration ($[Mg^{2+}]_i$) is known to decrease calcium currents in a variety of tissues. White and Hartzell (1988), for example, had showed that 1 - 3mM of $[Mg^{2+}]_i$ inhibited calcium currents by 50% in guinea-pig cardiac myocytes. Recently, $[Mg^{2+}]_i$ at 0.5mM concentration was shown to significantly inhibit ω-CTX GVIA sensitive calcium currents in rat cerebellar granule neurons (Pearson and Dolphin, 1993; Pearson *et al.*, 1993). Pearson and co-workers also found some evidence that $[Mg^{2+}]_i$ may also affect ω-Aga IVA sensitive channels, and that only small differences in $[Mg^{2+}]_i$ concentration may cause significant inhibition in channel activity. Thus differences in $[Mg^{2+}]_i$, possibly as a result of the type of tissue preparation used and concentration of Mg^{2+} in the incubation medium, may significantly affect calcium channel function and therefore lead to variation in data between groups.

The concentration of KCl used to depolarise membrane preparations has also been shown to affect calcium influx via VSCC. Here both internal and external $[K^+]$ concentration was shown to affect calcium uptake, so that when $[K^+]_i$ is reduced, the amount of calcium influx in synaptosomes following addition of extracellular potassium concentration ($[K^+]_e$) was lower (Blaustein, 1975). In addition KCl concentration may also affect the potencies of

various VSCC antagonists. For example, Lundy and co-workers (1994) mention some unpublished observations that high concentrations of KCl (> 60 mM) decrease the inhibitory potency, and total block, of ω-agatoxins on VSCC in synaptosomal preparations from the chick forebrain.

As far as I am aware, studies have not been carried out to determine differences in VSCC distribution in chicks of different strains. The literature shows variations in the type of chicks used, thus this may lead to some differences in calcium channel inhibition. However, the differences, if any, should only cause small differences in experiments such as channel inhibition.

Another reason for the variation in the effects of antagonists on KCl-stimulated $[Ca^{2+}]_i$ elevation between different laboratories, may be due to the use of different tissue preparations. A survey of the literature shows the type of tissue used varies from, slice preparations for electrophysiology work, to synaptosomes for biochemical and pharmacological analysis of channels. As mentioned previously differences in synaptosomal preparation have also been shown to be important. For example, Verhage *et al.* (1988) showed that $[Ca^{2+}]_i$ in pure synaptosomal fractions, measured with fura-2/AM, varied depending on the fraction of synaptosome used for the experiments. In my experiments I used crude synaptosomes and synaptoneurosomes, whereas some groups had used purer fractions of synaptosomes (Lundy *et al.*, 1994; Maubecin *et al.*, 1995).

After determining the presence of different VSCC's in the synaptoneurosomes, experiments were carried out to determine if the antagonists were exerting their effects on the same, or different, epitopes in the membrane preparation from the IMHV. Addition of antagonists, at concentrations that produced sub-maximal inhibition of KCl-stimulated elevation in $[Ca^{2+}]_i$, showed that the addition of antagonists together had some additive inhibitory effect when compared to the inhibitory effect of antagonist alone. The graph in figure 26 shows that ω-CTX GVIA and nimodipine bind to separate sites on the synaptoneurosome. It should be noted, however, that the block was not totally additive, indicating some cross-reactivity. Similarly, addition of ω-CTX GVIA and ω-CTX MVIIC demonstrates some additive

inhibition, showing that the drugs act on separate epitopes (i.e. N-type and P/Q-type channels respectively). However, there is also some cross-reactivity between the two antagonist which may be the result of ω-CTX MVIIC binding to a small population of N-type channels. In fact, ω-CTX MVIIC has been shown to inhibit N-type channels in synaptosomes prepared from mammalian brains (Grantham *et al.,* 1994). It would be interesting to measure the combined effects of the P-type blocker ω-agatoxin, which evoked a relatively small degree of inhibition in chick synaptoneurosomes, and ω-CTX MVIIC to further confirm the site of action of ω-CTX MVIIC in the chick brain.

The final part of chapter 5 describes some preliminary experiments to determine if different VSCC's are activated at different times after training. As shown in chapter 4, increased calcium influx via VSCC takes place immediately after training. Subsequently I demonstrated that N-type, L-type, P-type and possibly N/P- or Q-type channels are present in synaptoneurosomes from the IMHV of day-old chicks. Following on from this, I wanted to determine if the different calcium channels were all activated immediately after training. In order to do this I compared the effects of ω-CTX GVIA, ω-CTX MVIIC and nimodipine on calcium fluxes in synaptoneurosomes prepared from the IMHV of untrained birds, and birds tested immediately after training. The preliminary data shows that the three antagonists produced greater inhibition in the left IMHV of M-birds tested immediately after training compared to the control. In comparison, there was no difference in potency of any of the antagonists between the treatment groups in synaptoneurosomes from the right IMHV. These findings can be interpreted as demonstrating the early activation of N-, N/P/Q- and L-type VSCC in the left but not right IMHV of birds immediately after the passive avoidance training experience. The early activation of the N-, L- and N/P/Q-type VSCCs observed in these experiments would indicate that these VSCCs have a primary role in gating calcium necessary for activating a variety of calcium-dependent intracellular signals leading to memory formation following the PAL task. A proposed model describing a hypothesised sequence of events will be described later in section 7.1.

Chapter 6 describes a series of experiments to determine if chick synaptoneurosomal preparations can be used to study VSCC modulation by metabotropic receptors. Several

pieces of work, particularly electrophysiological analysis using rat cultured cells (e.g. Wu and Saggau, 1995), have shown that the activation of $GABA_B$ receptors (pre- and postsynaptic) decreases calcium currents; and that modulation of theses calcium currents can be varied depending on the type of VSCC antagonists present in the preparation (Wu and Saggau, 1995; Cardozo and Bean, 1995). Experiments on rat cultured brain tissue show that ω-CTX GVIA sensitive N-type channels, and ω-Aga IVA sensitive P-type channels, were readily modulated by $GABA_B$ receptors. Since presynaptic $GABA_B$ autoreceptors inhibit calcium-dependent transmitter release it is not suprising that most of the electrophysiological work has shown a $GABA_B$ inhibitory affect on the N- and P-type channels, subtypes of VSCC believed to be involved in calcium-dependent transmitter release. Furthermore, Sitges and Chiu (1995a) found that GABA release from rat brain synaptosomes was inhibited in the presence of ω-Aga IVA (IC_{50}=50nM) , thus further supporting a role for this VSCC in transmitter release.

Further examination of the possible involvement of other VSCC in regulating GABA release showed that ω-CTX MVIIC inhibited GABA release with an IC_{50}=3μM (Sitges and Chiu, 1995b). ω-CTX MVIIC has been shown to inhibit Q-type currents in the nM concentration range (Randall *et al.,* 1993), however, in the μM concentration it also inhibits P-type channels (Hillyard *et al.,* 1992). Thus, the second experiment by Sitges and Chiu confirms previous findings that the predominant channel involved in GABA release is of the T-type class of VSCC. In addition to electrophysiological work using tissue cultures slices, Stirling *et al.* (1989) have shown that pure synaptosomes were also useful in studying VSCC modulation by GABAergic systems. This group used rat synaptosomes prepared from whole brain cerebral cortex. they found that $GABA_A$ agonist (muscimol), $GABA_R$ agonist (baclofen), and $GABA_B$ antagonist (δ-aminovaleric acid, phaclofen and β-phenyl GABA) all inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation. I found qualitative similarities with chick synaptoneurosomes, where GABA and muscimol (a $GABA_A$ agonist) inhibited KCl-stimulated $[Ca^{2+}]_i$ elevation in a dose-dependent fashion. The $GABA_A$ evoked inhibition of KCl-stimulated increases in $[Ca^{2+}]_i$ may be due to the activation of post-synaptic receptors, leading to neural hyperpolarization and subsequent VSCC inhibition. Similarly, the $GABA_B$ agonist (baclofen) and $GABA_B$ antagonist (saclofen) also inhibited KCl-stimulated $[Ca^{2+}]_i$

elevation. This was a suprise, since I had expected baclofen to inhibit KCl-stimulated increase in $[Ca^{2+}]_i$, and saclofen not to have any affect. In addition, Stirling *et al.* (1989) have shown that certain $GABA_B$ antagonists inhibit VSCC, which had been previously shown to bind to $GABA_B$ receptors in cortical membranes prepared from the rat (Bowery *et al.*, 1983).

It could be argued that in the chicken brain these antagonists may have different actions, possibly as a result of developmental changes that may be taking place in the day-old chick . Recent studies in embryonic neural tissues have shown that $GABA_A$ receptor activation can actually lead to depolarisation of neural tissue in rat neonatal tissues, such as the hippocampus and cortex (for a review see Ben-Ari *et al.*, 1997). The mechanism underlying the depolarising actions of $GABA_A$ are believed to be due to greater than normal $[Cl^-]_i$ levels, probably due to developmental changes in $[Cl^-]_i$ homeostasis. Thus, activation of $GABA_A$ receptors depolarises the neuron which subsequently allows it to reach the threshold for generating the sodium (Na^+) action potential. Confocal microscopy also shows that $GABA_A$ activation can lead to increased $[Ca^{2+}]_i$ in the developing rat hypothalamic neuron (Chen *et al*, 1996). This effect was blocked by bicuculline, VSCC antagonists and by voltage clamping cells at a hyperpolarised potential. An initial model proposed for the mechanism of $GABA_A$ activation of VSCC suggested that $GABA_A$ activation leads to chloride efflux from the neuron. This causes depolarisation of the neuron, and leads to the activation of Na^+-channels, consequently Na^+-influx further depolarises neural tissue, and subsequently leads to the activation of VSCC's and calcium influx. In contrast, post-synaptic $GABA_B$ receptors in rats have no known function until one week post-natal (Luhmann and Prince 1991; Fukuda *et al.*, 1993; Gaiarsa *et al.*, 1995). This is probably due to undeveloped connections between G-proteins and VSCC. On the other hand, pre-synaptic $GABA_B$ receptors seem well developed from birth. In view of the fact that GABA receptors have different developmental properties, this could lend further support to the idea of differential effects of GABAergic drugs in the young chick brain, compared to previous work carried out in older animals. It should be noted, however, that the depolarising effects of GABA in immature neurons leading to the activation of VSCC, and thus resulting in increased $[Ca^{2+}]_i$ (Chen *et al.*, 1994) was not observed in my synaptoneurosomal preparation.

In addition, ligand-mediated studies have hinted at the possible existence of distinct $GABA_B$ receptor sites (Bowery *et al.,* 1985). More recently, differential effects have been reported for $GABA_B$ receptor antagonists on baclofen-mediated inhibition of GABA and glutamate release from rat brain cerebral cortical synaptosomes (Bonanno *et al.,* 1993; Bonanno and Raiteri, 1994).Also, different $GABA_B$ antagonists were found to have different effects on baclofen mediated augmentation, and inhibition, of isoproterenol - and forskolin-stimulated cAMP accumulation respectively(Cunningham and Enna, 1996). These results were interpreted as an indication of the existence of at least two pharmacologically and molecularly distinct subclasses of $GABA_B$ receptors in regulating cAMP production in the brain. Thus, it may well be the case that the '$GABA_B$-agonist like' affect of saclofen in synaptoneurosomes from the IMHV of day-old chicks indicates the possible existence of a different subtype of $GABA_B$ receptors with a different response to saclofen.

The second set of experiments was carried out to determine if the inhibitory modulatory effects of baclofen and saclofen were due to the action of both drugs on the same site of the synaptoneurosome. The findings that both drugs were acting on the same epitope on the synaptoneurosomes, evident by the non-additive inhibitory effect, suggests that saclofen 'behaves' like a $GABA_B$ agonist in terms of VSCC inhibition in the IMHV. As mentioned previously this may be indicative of the existence of a novel subtype of $GABA_B$ receptors in the IMHV or, as it has been described previously, the existence of the developing $GABA_B$ receptors in the young chick may exhibit atypical properties.

The final set of experiments (shown in figure 39) is a comparison of GABA, muscimol and baclofen respectively on KCl-stimulated $[Ca^{2+}]_i$ elevation in synaptoneurosome prepared from the IMHV of untrained and trained chicks. The aim of these experiments was to determine if training resulted in changes in the profile of GABA, muscimol and baclofen inhibition of VSCC. The rationale behind these experiments was based on the principle that GABAergic modulation of VSCC would occur following prolonged neural activity, such as during memory formation following passive avoidance training in the chick. The data shows that there is no difference in the degree of inhibition of KCl-stimulated $[Ca^{2+}]_i$ elevation with GABA, muscimol or baclofen in synaptoneurosomes prepared from the IMHV of chicks

tested 5 minutes after training compared to untrained birds. Baclofen, however, did produce significantly greater inhibition in birds tested 30 minutes post-training which could be evidence for *in·vivo* modulation of VSCC by $GABA_B$ autoreceptors. In fact, comparison of the increases in KCl-stimulated $[Ca^{2+}]_i$ in the IMHV of birds tested 30 minutes post-training was lower than in birds tested at earlier times (chapter 4, figure 18) showing a lower susceptibility for VSCC activation. It should be noted that further experiments need to be carried out to determine if GABA and muscimol show similarly greater inhibition in birds tested 30 minutes post-training.

7.1. Conclusion

The results from this thesis suggests that, pre and postsynaptic neuronal activity in the left IMHV immediately after training on the passive avoidance task stimulates the opening of N-, N/P/Q and L-type VSCC channels. The resultant increase in $[Ca^{2+}]_i$ is then capable of activating a variety of biochemical pathways in the neurons. In addition to the left IMHV, early increases in calcium influx also occurs in both hemispheres of the LPO. The early increase in the left but not the right IMHV, and in both hemispheres of the LPO, supports earlier observations by Rose (1991), who suggested that initial changes take place in the left IMHV, then in the right IMHV, and later on in both hemispheres of the LPO. Overlapping increases in the left IMHV and both hemispheres of the LPO also strengthens the idea of memory formation occurring in a parallel fashion, as opposed to a serial cascade of events. The influx of calcium then evokes a number of possible calcium-signalling cascades, such as the activation of the intracellular enzymes CaMKII and adenylate cyclase, which then leads to the activation of transcription factors necessary for protein synthesis. Activation of transcription factors has been detected in the left IMHV of chicks within 30 minutes of training (Anokhin and Rose, 1991). Another consequence of calcium influx is transmitter release leading to neuronal communication as measured by Daisley and Rose (1998). Thirty minutes after training, activation of a possible subtype of, the presynaptic $GABA_B$ receptor may then cause G-protein activation leading to the inhibition of VSCC and so resulting in a to return to basal levels of $[Ca^{2+}]_i$.

The mechanism of changes in the IMHV, LPO and possibly the rest of the chick brain may turn out to be more complicated by the possibility that particular receptor systems, such as the GABAergic system, may have some atypical modes of action.

7.2. Future experiments

The results described in this thesis have raised more questions regarding the role of VSCC in the biochemical cascade in the chick brain following passive avoidance learning. As a result, there is still a large amount of work that needs to be carried out to answer some of these questions. Examples of work that still need to be carried out are listed below :-

(1) A more detailed dose-response curve for the P-type channel blocker ω-Aga IVA in the IMHV.

(2) Determine the pharmacological characteristics of VSCC in the LPO of untrained chicks.

(3) Analyse the distribution of different VSCC in synaptic terminals using fluorescent tagged specific VSCC antagonists. After this, determine if the population of the channel varies following testing at different times after training.

(4) Determine the effect of different VSCC antagonists in the IMHV and LPO of birds tested immediately, 5, 10 and 30 minutes after training .

(5) Determine if nimodipine, ω-CTX GVIA and ω-CTX MVIIC have additive inhibitory affects in the LPO similar to that detected in the IMHV.

(6) Carrying out pre- and post-training $GABA_B$ agonist / antagonist injection in the IMHV and LPO to determine if they affect strong / weak learning for the passive avoidance task.

(7) Carry out a more detailed *in vitro* analysis of GABAergic modulation of VSCC in the left and right IMHV and LPO at different times after training . Furthermore use specific calcium channel blockers such as ω-CTX GVIA, nimodipine and ω-Aga IVA to determine the degree of GABAergic modulation for the different subtypes of VSCC.

(8) In addition, it would be interesting to analyse the degree of glutamatergic modulation of different VSCC in membrane preparations from the IMHV and LPO. If the glutamatergic system was shown to modulate VSCC, then experiments should be carried out to study time dependent changes in glutamatergic modulation.

(9) Electrophysiological measurements of different calcium currents prepared from chick brain slices to compare with the pharmacological data.

(10) Use confocal microscopy to try to 'visualise' calcium influx in the IMHV and LPO of chicks tested at different times after training.

APPENDIX

Table 4: **Table of average absolute $[Ca^{2+}]_i$ values in synaptoneurosomes prepared from the left IMHV of chicks tested at different times after training.**

(A)

Q-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	254.1±20	280.3±18	170.1±17	193.0±23	185.4±10	213.4±32
Stimulated $[Ca^{2+}]_i$	290.1±24	322.6±20	190.5±19	220.3±27	209.3±12	245.4±38
% Increase	114.2±1.7	115.1±0.8	112.00±1.4	114.2±1	112.9±1.3	115.0±1.2
n	5	5	9	8	6	6

(B)

W-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	217.2±9	267.5±10	201.0±14	176.2±14	216.6±25	271.4±25
Stimulated $[Ca^{2+}]_i$	258.3±12	321.8±10	234.5±18	202.0±16	249.0±26	307.5±30
% Increase	118.9±1.6	120.3±2.2	116.7±1.6	114.6±1.8	114.8±1.4	113.3±1.2
n	5	7	11	8	5	5

(C)

M-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	229.4±11	299.2±43	207.3±15	207.8±20	236.4±23	215.7±29
Stimulated $[Ca^{2+}]_i$	305.4±19	370.6±52	248.8±19	244.5±24	271.4±25	254.0±36
% Increase	133.1±3.5	123.9±1	120.0±1.1	117.7±1	114.8±1.7	117.8±3.3
n	9	8	12	7	8	10

Table 5: **Table of average absolute $[Ca^{2+}]_i$ values in synaptoneurosomes prepared from the right IMHV of chicks tested at different times after training**

(A)

Q-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	281.1±27	264.1±20	247.1±58	188.1±12	166.6±18	207.5±31
Stimulated $[Ca^{2+}]_i$	329.2±32	306.0±22	278.2±65	214.3±13	191.5±22	238.5±35
% Increase	117.1±1.2	115.9±2.2	112.6±1.6	114.0±1.3	114.9±1.7	114.9±2.8
n	6	5	9	7	6	6

(B)

W-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	239.1±17	309.9±36	289.3±42	227.9±39	208.4±16	276.7±26
Stimulated $[Ca^{2+}]_i$	296.8±19	369.6±48	323.9±46	269.3±44	229.6±17	322.2±33
% Increase	124.1±2.9	119.3±2.4	112.0±2.8	118.2±2.3	110.2±1.3	116.5±2
n	5	7	8	8	6	4

(C)

M-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	229.5+11	301.5+43	193.5+10	208.8+19	185.7+11	195.7+29
Stimulated $[Ca^{2+}]_i$	288.4+16	372.5+62	217.8+10	244.1+21	211.9+13	227.6+35
% Increase	125.7±2.9	123.6±2.1	112.6±2.9	117.0±0.9	114.1±2	116.3±3.4
n	9	8	11	6	8	10

Table 6: Table of average absolute $[Ca^{2+}]_i$ values in synaptoneurosomes prepared from the left LPO of chicks tested at different times after training

(A)

Q-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	215.3±35	218.9±25	247.7±26	263.3±32	240.1±18	242.5±40
Stimulated $[Ca^{2+}]_i$	246.3±41	275.7±33	306.0±33	305.8±35	285.9±19	275.1±37
% Increase	114.5±1.7	125.8+2.8	123.1+1.9	116.6+1.3	119.4+2.6	114.8+4.8
n	4	6	7	6	4	4

(B)

W-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	271.6±35	190.9±22	255.4±22	343.2±42	277.7±68	157.5±11
Stimulated $[Ca^{2+}]_i$	317.8±43	241.2±29	298.1±25	395.8±38	334.4±83	194.1±12
% Increase	116.9±1.9	126.1±1.6	116.8±1.2	116.5±3.5	120.4±1.8	122.6±2.6
n	4	5	7	4	5	6

(C)

M-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	246.1±33	210.8±19	173.6±19	310.8±40	218.3±21	174.0±9
Stimulated $[Ca^{2+}]_i$	298.6±45	284.9±25	227.5±24	356.4±46	265.6±28	206.4±10
% Increase	120.3±2.1	135.3±2.1	131.6±2.1	114.9±1.2	121.0±1.9	120.2±2.7
n	8	6	8	8	9	8

Table 7: **Table of average absolute $[Ca^{2+}]_i$ values in synaptoneurosomes prepared from the right LPO of chicks tested at different times after training**

(A)

Q-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	305.3±70	208.8±37	227.3±18	266.4±54	277.8±24	187.5±23
Stimulated $[Ca^{2+}]_i$	355.1±78	264.2±52	269.7±22	317.4±67	330.8±29	218.1±22
% Increase	117.3±3	124.8±2.3	118.5±1.3	118.2±1.6	119.3±1.4	117.6±2.9
n	4	6	7	6	5	5

(B)

W-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	321.7±64	207.3±28	273.2±33	340.1±73	395.5±99	164.8±11
Stimulated $[Ca^{2+}]_i$	386.4±78	259.8±37	356.2±49	400.2±88	485.7±122	200.1±12
% Increase	120.4±2.6	125.6±3.7	129.5±2.7	117.2±3.3	121.8±1.8	118.8±1.8
n	4	5	7	4	7	7

(C)

M-birds	0 min	5 min	10 min	30 min	3 hrs	6 hrs
Basal $[Ca^{2+}]_i$	257.2±42	190.2±20	245.3±22	312.4±34	244.3±17	170.5±12
Stimulated $[Ca^{2+}]_i$	314.1±50	260.0±27	314.5±29	362.1±39	297.9±23	217.1±13
% Increase	122.6±3.3	136.9±2.8	128.3±2	116.0±1.5	121.5±2.1	123.8±2.5
n	8	6	8	8	9	8

Table 8: **Table of absolute $[Ca^{2+}]_i$ values from the synaptoneurosomes prepared from the IMHV in the presence of different VSCC antagonists**

(A) Nimodipine

	Control	10nM	50nM	100nM	10μM	30μM	60μM
Pre-Nimodipine $[Ca^{2+}]_i$	255±17	263±34	238±23	260±37	305±54	314±76	236±11
Basal $[Ca^{2+}]_i$	320±32	299±54	266±53	255±86	311±32	144±15	167±22
Stimulated $[Ca^{2+}]_i$	382±43	352±63	309±62	301±109	346±38	157±15	177±23
n	8	8	8	8	8	4	3

(B) ω-CTX GVIA

	Control	5nM	10nM	100nM	1μM
Pre-ω-CTX GVIA $[Ca^{2+}]_i$	241±7	251±21	230±6	222±10	217±5
Basal $[Ca^{2+}]_i$	238±13	180±13	184±9	158±7	171±26
Stimulated $[Ca^{2+}]_i$	301±14	222±22	220±13	186±10	199±32
n	6	4	7	6	7

(C) ω-CTX MVIIC

	Control	1nM	10nM	100nM	1μM
Pre-ω-CTX MVIIC $[Ca^{2+}]_i$	200±24	195±21	197±21	196±20	208±24
Basal $[Ca^{2+}]_i$	233±24	193±14	201±21	193±17	121±22
Stimulated $[Ca^{2+}]_i$	275±30	222±18	233±27	220±21	134±27
n	6	6	6	6	6

(D) ω-Aga IVA

	Control	30nM	100nM	300nM
Pre-ω-Aga IVA $[Ca^{2+}]_i$	327±58	319±48	321±39	307±40
Basal $[Ca^{2+}]_i$	251±40	226±14	253±47	193±38
Stimulated $[Ca^{2+}]_i$	316±54	277±16	321±66	231±49
n	4	4	4	4

(E) 1μM ω-CTX GVIA + 1μM ω-CTX MVIIC / 10μM Nimodipine

	Control	ω-CTX GVIA	Nimodipine	ω-CTX GVIA + Nimodipine
Pre-antagonist $[Ca^{2+}]_i$	250±7	145±2	201±18	274±9
Basal $[Ca^{2+}]_i$	243±28	210±31	188±41	243±14
Stimulated $[Ca^{2+}]_i$	289+±35	239±37	216±51	260±15
n	7	7	7	7
	Control	ω-CTX MVIIC	ω-CTX GVIA + ω-CTX MVIIC	
Pre-antagonist $[Ca^{2+}]_i$	241±16	182±24	182±12	
Basal $[Ca^{2+}]_i$	248±16	218±11	260±17	
Stimulated $[Ca^{2+}]_i$	311±31	239±18	276±16	
n	7	7	7	

(F) Training + 1μM ω-CTX GVIA

	QL (-GVIA)	QL (+GVIA)	QR (-GVIA)	QR (+GVIA)	WL (-GVIA)	WL (+GVIA)
Pre-ω-CTX GVIA	243±29	251±33	228±17	248±15	208±35	236±15
Basal $[Ca^{2+}]_i$	245±28	167±20	200±18	231±15	191±37	114±29
Stimulated $[Ca^{2+}]_i$	300±40	190±27	232±21	251±18	223±47	126±33
n	5	5	6	6	4	4

	WR (-GVIA)	WR (+GVIA)	ML (-GVIA)	ML (+GVIA)	MR (-GVIA)	MR (+GVIA)
Pre-ω-CTX GVIA	214±35	239±22	228±21	223±22	248±43	225±16
Basal $[Ca^{2+}]_i$	221±30	230±26	219±22	180±9	215±24	237±17
Stimulated $[Ca^{2+}]_i$	266±36	254±31	272±28	192±10	271±31	263±20
n	4	4	5	5	4	4

(G) Training + 1μM ω-CTX MVIIC

	QL (-MVIIC)	QL (+MVIIC)	QR (-MVIIC)	QR (+MVIIC)	WL (-MVIIC)	WL (+MVIIC)
Pre-ω-CTX MVIIC	209±16	219±20	217±14	208±19	219±27	229±35
Basal $[Ca^{2+}]_i$	200±21	131±26	216±21	242±25	219±27	183±41
Stimulated $[Ca^{2+}]$	230±26	145±30	248±24	266±28	257±33	208±49
n	5	5	5	5	5	5

	WR (-MVIIC)	WR (+MVIIC)	ML (-MVIIC)	ML (+MVIIC)	MR (-MVIIC)	MR (-MVIIC)
Pre-ω-CTX MVIIC	204±21	218±25	200±22	194±20	183±19	193±18
Basal $[Ca^{2+}]_i$	199±25	117±24	200±27	193±8	173±20	132±26
Stimulated $[Ca^{2+}]$	248±27	130±30	264±38	209±10	216±27	151±33
n	5	5	5	5	5	5

(H) Training + 10μM Nimodipine

	QL (-Nim)	QL (+Nim)	QR (-Nim)	QR (+Nim)	WL (-Nim)	WL (+Nim)
Pre-Nimodipine	167±11	166±12	198±20	214±28	167±14	173±15
Basal $[Ca^{2+}]_i$	178±10	129±17	185±18	213±21	159±21	135±34
Stimulated $[Ca^{2+}]_i$	206±11	142±19	215±21	232±24	188±23	151±39
n	5	5	4	4	4	4

	WR (-Nim)	WR (+Nim)	ML (-Nim)	ML (+Nim)	MR (-Nim)	MR (+Nim)
Pre-Nimodipine	225±35	243±55	185±11	186±10	191±9	195±10
Basal $[Ca^{2+}]_i$	259±74	174±69	179±8	139±21	192±9	221±14
Stimulated $[Ca^{2+}]_i$	305±78	192±75	223±11	151±24	237±12	245±16
n	4	4	6	6	7	7

Table 9: Table of absolute $[Ca^{2+}]_i$ values from synaptoneurosomes prepared from the IMHV in the presence of $GABA_{A/B}$ agonist or antagonist

(A) GABA

Control	1nM	10nM	100nM	1μM	10μM	100μM
Basal $[Ca^{2+}]_i$	163±22	234±46	178±10	196±18	177±12	166±10
Stimulated $[Ca^{2+}]_i$	182±22	258±47	201±10	213±21	194±13	185±11
GABA						
Basal $[Ca^{2+}]_i$	159±31	218±61	200±9	200±26	150±19	215±17
Stimulated $[Ca^{2+}]_i$	167±33	234±61	210±8	210±27	158±19	232±19
n	5	3	3	6	3	5

(B) Muscimol

Control	1nM	10nM	50nM	10μM
Basal $[Ca^{2+}]_i$	225±32	211±22	245±41	223±32
Stimulated $[Ca^{2+}]_i$	256±39	232±23	272±50	241±32
Muscimol				
Basal $[Ca^{2+}]_i$	198±50	198±35	144±34	195±33
Stimulated $[Ca^{2+}]_i$	224±60	212±38	151±37	199±33
n	3	6	3	3

(C) Baclofen

Control	10nM	100nM	1μM	10μM	100μM
Basal $[Ca^{2+}]_i$	286±63	297±96	278±76	303±89	313±113
Stimulated $[Ca^{2+}]_i$	334±73	331±101	312±77	336±98	345±121
Baclofen					
Basal $[Ca^{2+}]_i$	228±52	239±62	183±39	320±51	210±36
Stimulated $[Ca^{2+}]_i$	260±60	262±65	204±46	339±56	216±38
n	4	3	3	3	3

(D) Saclofen

Control	10nM	100nM	1μM	10μM	30μM
Basal $[Ca^{2+}]_i$	224++20	201±10	203±18	209±7	227±19
Stimulated $[Ca^{2+}]_i$	251±22	229±9	221±20	229±7	248±19
Saclofen					
Basal $[Ca^{2+}]_i$	229±26	115±20	174±19	156±31	256±12
Stimulated $[Ca^{2+}]_i$	245±29	123±21	182±20	165±33	261±13
n	6	6	5	6	3

(E) **Bicuculline**

Control	10nM	100nM	1μM	10μM	50μM
Basal $[Ca^{2+}]_i$	232±33	197±17	220±22	229±21	248±19
Stimulated $[Ca^{2+}]_i$	251±30	220±19	240±21	248±21	270±20
Bicuculline					
Basal $[Ca^{2+}]_i$	278±39	196±43	317±51	299±25	370±16
Stimulated $[Ca^{2+}]_i$	306±36	214±48	346±58	331±23	419±19
n	4	3	3	4	4

(F) **Baclofen + Saclofen**

Control	(-) Baclofen	(-) Saclofen	(-) Baclofen+Saclofen
Basal $[Ca^{2+}]_i$	172±13	200±16	282±23
Stimulated $[Ca^{2+}]_i$	199±16	232±19	320±23
	(+) Baclofen	(+) Saclofen	(+) Baclofen+Saclofen
Basal $[Ca^{2+}]_i$	153±15	159±18	245±55
Stimulated $[Ca^{2+}]_i$	168±17	174±20	237±52
n	4	4	5

(G) **Train + GABA**

Control	Untrain	5' P.T
Basal $[Ca^{2+}]_i$	183±8	213±8
Stimulated $[Ca^{2+}]_i$	206±9	235±11
GABA		
Basal $[Ca^{2+}]_i$	101±22	242±24
Stimulated $[Ca^{2+}]_i$	109±25	255±25
n	5	6

(H) **Train + Muscimol**

Control	Untrain	5' P.T
Basal $[Ca^{2+}]_i$	177±13	196±36
Stimulated $[Ca^{2+}]_i$	193±12	243±12
Muscimol		
Basal $[Ca^{2+}]_i$	190±25	106±35
Stimulated $[Ca^{2+}]_i$	200±27	127±47
n	8	6

(I) **Train + Baclofen**

Control	Untrain	5' P.T	30'P.T
Basal $[Ca^{2+}]_i$	187±5	204±16	153±4
Stimulated $[Ca^{2+}]_i$	211±3	247±26	173±4
Baclofen			
Basal $[Ca^{2+}]_i$	103±11	128±15	194±14
Stimulated $[Ca^{2+}]_i$	111±14	146±20	204±14
n	5	7	4

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