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### UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

# Interactions between anticholinesterases in an *in vitro* central nervous system preparation.

By

Iain Ratcliffe Scott

Thesis for the degree of Doctor of Philosophy

December 2008

#### ABSTRACT

### UNIVERSITY OF SOUTHAMPTON FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES SCHOOL OF MEDICINE Doctor of Philosophy

# INTERACTIONS BETWEEN ANTICHOLINESTERASES IN AN *IN VITRO* CENTRAL NERVOUS SYSTEM PREPARATION.

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Organophosphate compounds have been widely developed as pesticides (e.g. paraoxon) and also as chemical warfare agents (nerve agents, e.g. sarin). These compounds rapidly inhibit the enzyme acetylcholinesterase (AChE), causing overstimulation within the cholinergic nervous system. If left untreated, this can be fatal. Current medical countermeasures to nerve agent poisoning consist of pretreatment with pyridostigmine and an emergency therapy comprising atropine, diazepam and pralidoxime. As well as a replacement pretreatment for pyridostigmine, physostigmine has been proposed as a component of a next generation of therapy to nerve agent poisoning, along with scopolamine and HI6. In animal studies, this therapy has been shown to lessen the level of incapacitation and increase survivability post poisoning with nerve agent. The exact mechanism of action of physostigmine in this combination is as yet unclear.

The primary aim of this study was to test the hypothesis that the beneficial effect of physostigmine in the proposed therapy is due to reversible inhibition of AChE, thereby protecting it from irreversible inhibition by nerve agent. To test this, extracellular field potentials were recorded from the molecular layer of the dentate gyrus in an *in vitro* slice model developed from the guinea pig. This response was shown to be modulated by the application of physostigmine and the nerve agent sarin and interactions between the two inhibitors were characterised. The results provided evidence for protection of ChE by physostigmine. The functional response (field potential) was related to cholinesterase activity measured in slices exposed to sarin.

This is the first evidence of the mechanism of action of physostigmine protection against nerve agent in the CNS. Not only will these results support the use of physostigmine as a pre-treatment, it also supports its use as a possible immediate therapy.

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#### Author's declaration

I, Iain Scott, declare that the thesis entitled "Interactions between anticholinesterases in an *in vitro* central nervous system preparation" and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission.

Signed: ..... Date: ....

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I would like to dedicate this thesis to the memory of my father, Allan Beattie Scott. Without the sacrifices he and my family made, I would not have been given the opportunities to further my education.

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#### Abbreviations

<sup>3</sup> [H]TCP	<sup>3</sup> [H]-thienyl-cyclohexylpiperidine	
4-DAMP	4-diphenylacetoxy-N-methyl-piperidine methiodide	
5-HT	5-hydroxy tryptamine (serotonin)	
α-BGT	α-Bungarotoxin	
ACh	Acetylcholine	
AChE	Acetylcholinesterase	
ACSF	Artificial cerebro-spinal fluid	
AP5	D-2-Amino-5-phosphonopentanoic acid	
ATP	Adenosine triphosphate	
BMI	Bicuculline methyliodide	
BuChE	Butyrylcholinesterase	
cAMP	Cyclic adenosine monophosphate	
СВ	Cannabinoid	
ChAT	Choline acetyltransferase	
CN	Cyano	
CNQX	6-Cyano-7-nitroquinoxaline-2, 3-dione	
CNS	Central nervous system	
CREB	Ca <sup>2+</sup> /cAMP response element binding protein	
DAG	Diacylglycerol	
DFP	Di-isopropylflurophosphate	
DHβE	Dihydro-β-ethroidine	
DSE	Depolarisation-induced suppression of excitation	
DSI	Depolarisation-induced suppression of inhibition	
Dstl	Defence Science and Technology Laboratory	
EAA	Excitatory amino acid	
EDTA	Ethylenediaminetetraacetic acid	
EPSP	Excitatory post-synaptic potential	
fEPSP	Field excitatory post-synaptic potential	
G	German	
g	Gram	
GA	Tabun	
GABA	γ–Aminobutyric acid	
GB	Sarin	
GD	Soman	
GDEE	l-glutamic acid diethyl ester	
GF	Cyclosarin	

HCI	Hydrochloric acid	
ICAM-1	Intercellular adhesion molecule-1	
IP <sub>3</sub>	Inositol 1,4,5-triphosphate	
КОН	Potassium hydroxide	
LTP	Long-term Potentiation	
mAChR	muscarinic acetylcholine receptor	
MAP2	Microtubule associated protein-2	
mIPSC	Miniature inhibitory post-synaptic current	
MLA	Methyllycaconititine	
MPSC	Miniature post-synaptic current	
mRNA	Messenger ribonucleic acid	
NAcc	Nucleus accumbens	
nAChR	Nicotinic acetylcholine receptor	
NaOH	Sodium hydroxide	
NAPS	Nerve Agent Pre-treatment Set	
NGFr	Nerve growth factor receptor	
NMDA	N-Methyl-D-aspartic acid	
NMJ	Neuromuscular junction	
NTE	Neuropathy Target Esterase	
OP	Organophosphate	
OPIDPN	Organophosphate induced delayed polyneuropathy	
P2S	Pralidoxime methanesulfonate	
PBS	Phosphate buffered saline	
PCR	Polymerase chain reaction	
RNA	Ribonucleic acid	
S.E.M	Standard error of the mean	
SN	Substantia nigra	
SNc	Substantia nigra pars compacta	
SNr	Substantia nigra reticulata	
SON	Supraoptic nucleus	
TOCP	Tri-orthocrysylphosphate	
ттх	Tetrodotoxin	
VCAM	Vascular cell adhesion molecule	
VGCC	Voltage gated calcium channel	
VGKC	Voltage gated potassium channel	
VGSC	Voltage gated sodium channel	

# 1. INTRODUCTION

#### 1.1. Background

The term organophosphate is used to describe many different types of esters of phosphoric acids. These compounds have found use in a diverse range of applications including flame retardants, lubricants, and medical therapeutics. Some of these compounds are highly toxic to invertebrates and have been developed as insecticides and pesticides. A small class of these compounds have also been developed as chemical warfare agents, known as nerve agents. However, it is as agricultural insecticides and pesticides that organophosphates (OP) have gained widespread use around the world. Their almost ubiquitous presence in modern agricultural practices also leads to a higher frequency of human exposure. It is estimated that in 1985 there were approximately 3 million reported cases of severe acute pesticide poisoning worldwide (World Health Organization, 1990). This figure may be closer to 25 million cases if self-reported data of all levels of poisoning severity was used (Jeyaratnam, 1990). The exact proportion involving OP compounds in both estimates is unknown. Whilst relatively rare in developed countries, human OP pesticide poisoning is an ever present problem in developing countries especially in rural Asia. In this region it is not only exposure to OPs from agricultural practices that is a clinical problem. OP compounds are regularly implicated in cases of self harm with approximately 200 thousand case fatalities each year. This figure greatly exceeds those cases resulting from environmental exposure (Eddleston et al., 2008;Eddleston & Phillips, 2004). Nerve agents have been deployed during military conflicts. There is evidence that tabun filled munitions were deployed during the Iran-Iraq war (1980-1988) (Newmark, 2004). Nerve agents have also been manufactured and released in terrorist attacks against civilian populations (Matsumoto in 1994 and the Tokyo subway in 1995) (Centre for Nonproliferation, 2001).

Despite the presence of the Chemical Weapons Convention which has been ratified by 165 member states, the use of either agricultural OPs or nerve agents by terrorist groups remains a threat to today's society. Fully understanding how OPs affect the human body will allow the development of more effective treatment regimes which will benefit those either exposed occupationally, through self harm, or via a deliberate release.

#### 1.2. The cholinergic system

Acetylcholine (ACh) is a neurotransmitter released from synapses at many sites throughout the central and peripheral nervous systems (CNS and PNS respectively) in vertebrates as well as at excitatory synapses in the invertebrate (i.e. nematodes such as

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*Caenorhabditis elegans*) nervous system (Rand, 2007). It is the main neurotransmitter released in the parasympathetic nervous system and is also released from pre-ganglionic synapses in both the sympathetic and parasympathetic nervous systems. It is also released at the junction between smooth/skeletal muscle and the innervating nerve (the neuromuscular junction (NMJ)). With the exception of sympathetic innervation of glands, ACh is absent, on the whole, from post-ganglionic sympathetic synapses. Although ACh synapses are abundant throughout the body, the majority of our knowledge of cholinergic mechanisms has been gathered through investigation of peripheral cholinergic synapses such as the NMJ.

ACh is synthesised in a reaction between Acetyl-CoA and choline. This is catalysed by the enzyme choline acetyltransferase (ChAT) which is exclusive to cholinergic neurones. The newly synthesised ACh is then packaged into vesicles along with adenosine triphosphate (ATP), proteoglycans, and the ions  $H^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$  prior to release. As the neurone is depolarised by an action potential, the vesicles dock with the presynaptic membrane. The contents of the vesicles are then released by exocytosis into the synaptic cleft. Further release of ACh is terminated by activation of autoreceptors on the presynaptic membrane. Another neurotransmission limiting step is the hydrolysis of the ATP released in conjunction with the ACh. The hydrolysed ATP yields adenosine, which then binds to the  $P_1$  purinoreceptor, which inhibits the activity of G-protein-linked  $Ca^{2+}$  channels. At the NMJ, the action of the  $P_1$  receptor is performed instead by the  $P_2$  receptor, which has a higher affinity for ATP than adenosine (Naguib *et al.*, 2002).

The newly released ACh diffuses across the synaptic cleft, a distance of approximately 20 nm to the post-synaptic membrane. Depending upon the location of the synapse, the postsynaptic membrane will be populated with either muscarinic or nicotinic acetylcholine receptors (mAChR and nAChR respectively). Binding of ACh to these structures causes a depolarisation of the postsynaptic cell thus either propagating the action potential or affecting a target organ/cell to effect a response. The structure and function of the two types of cholinergic receptor will be dealt with in more detail in a later section.

Once ACh has bound to the receptor it rapidly dissociates and is free to circulate again within the synaptic cleft. There is no limit to how many receptors a molecule of ACh can theoretically activate in succession; therefore a rate-limiting step is needed to terminate the transmitted signal. This is performed by the enzyme acetylcholinesterase (AChE) which hydrolyses the ACh into its constituent parts (Massoulie *et al.*, 1993). The recently cleaved choline is then recycled into the nerve terminal of the pre-synaptic cell via an active uptake system. Further release of ACh is also reduced by negative feedback on the presynaptic neurone by autoreceptors.



#### Figure 1.1. Cholinergic agonists.

Acetylcholine and carbachol are non-specific agonists and have agonist properties at both muscarinic and nicotinic receptors. Nicotine will only activate nicotinic receptors.

#### 1.2.1. Muscarinic receptors

To date, five subtypes of the mAChR have been identified. These are termed M1 to M5 (Caulfield & Birdsall, 1998;Eglen *et al.*, 2001;Eglen *et al.*, 1996). The mAChR is a metabotropic receptor, which unlike the ionotropic nicotinic receptor does not directly form channels to allow ion movement to modulate the cell system in response to the binding of an agonist. Instead, the muscarinic receptor is bound to a guanosine nucleotide binding protein (G protein) which acts as a trigger for other second messenger molecules to facilitate or inhibit various cellular processes (Caulfield, 1993).



#### Figure 1.2. Muscarinic antagonists.

Both atropine and hyoscine show no selectivity for the different subtypes of mAChR. Telenzepine and methoctramine shown selectivity for the M1 and M2 subtypes respectively.

Five distinct but related genes code for the muscarinic acetylcholine receptor family and the amino acid sequence is very highly conserved throughout the mammalian family tree. Each mAChR consists of seven transmembrane spanning domains with three intracellular loops. It is to the third intracellular loop, the G protein binds when the receptor is activated by an agonist. The muscarinic acetylcholine receptors are normally associated with either the  $G_{q/11}$  protein (M1, M3, and M5) or to the  $G_{i/o}$  protein (M2 and M4). Activation of the  $G_{q/11}$  coupled receptors stimulates the enzyme phospholipase C that in turn stimulates the production of two intracellular second messengers, inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). Activation of IP<sub>3</sub> and DAG then initiates various intracellular processes via the release of calcium from intracellular stores and the phosphorylation of many differing enzymes. In contrast, activation of the  $G_{i/0}$  protein inhibits the enzyme adenylate cyclase thereby reducing the amount of cAMP available within the cell. There is also evidence that this G protein can directly interact with potassium channels causing their activation without the need for a second messenger. Through activation of these channels the cell becomes hyperpolarised reducing the likelihood of synaptic activity occurring (Delmas & Brown, 2005). Examples of agonists and mAChR selective antagonists are shown in Figure 1.1 and 1.2.

Muscarinic acetylcholine receptors can be found throughout the CNS, with all five subtypes being successfully identified. M1 mAChRs have been shown to be located in high densities in the cortex, hippocampus, and striatum (Piggott et al., 2002). M2 mAChRs have been reported to be present in high densities in the thalamus and areas of the brainstem (Perry et al., 1989; Piggott et al., 2002). They can also be found in the cortex, hippocampus (Levey et al., 1995) and striatum (Rodriguez-Puertas et al., 1997). The M3 mAChR subtype has been reported in the hippocampus and cortex (Buckley et al., 1988), but is expressed at much lower levels than the M1 and M2 subtypes (Levey et al., 1994). M4 mAChRs are expressed within the hippocampus (Levey et al., 1995), cortex (Buckley et al., 1988) and the striatum (Harrison et al., 1996). Finally, M5 mAChRs have been identified on dopaminergic neurons within the ventral tegmental area (Forster et al., 2002), substantia nigra, and also in the hippocampus (Vilaro et al., 1990). As well as their anatomical locations, the various mAChR subtypes also show differences in their location within the synapse. The M1 mAChRs are located on postsynaptic membranes (Levey et al., 1991), and the M2 subtype is found predominantly on presynaptic membranes where it acts as an autoreceptor (McKinney et al., 1993). The M3 and M4 subtypes can be found on both pre- and postsynaptic membranes (Rouse et al., 1998).

It is not surprising given the almost ubiquitous nature of the mAChRs that they have been shown to have an important role in many functions. In particular the high densities of mAChRs (predominately M1) in the cortex and hippocampus have been shown to play a major role in higher cognitive processes such as learning and memory (Levey, 1996;Hasselmo, 2006). Other functions including attention (Herrero *et al.*, 2008) and sleep (Goutagny *et al.*, 2005;Coleman *et al.*, 2004) have been demonstrated to rely on the involvement of mAChR. Muscarinic receptors within the CNS are also involved in non-

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executive functions. An example of this is the M5 mAChR which is required to maintain the patency of cerebral vasculature (Hamel, 2004).

Having such a wide ranging involvement in so many functions, it is not surprising that mAChR are implicated in many disease states which affect the CNS. The most dramatic and well studied of these is Alzheimer's disease. This condition is typified by a progressive loss of cognitive ability and the formation of characteristic amyloid β plaques in neuronal tissue (Terry, 2006;Blennow *et al.*, 2006). Apart from the formation of these plaques, the other major component of Alzheimer's disease is the loss of cholinergic function including a marked decline in the synthesis of ACh and also deterioration of major cholinergic projections to the hippocampus and cortex (Geula *et al.*, 2008). It is thought that whilst the expression of the M1 mAChR is largely unaffected, the number of these receptors which are functionally coupled to G proteins is greatly decreased (Tsang *et al.*, 2006). This means that although the receptors are present, their activation will not result in excitation of the postsynaptic cell membrane. Changes in the expression of M2 mAChR in the cortex and hippocampus have also been reported (Lai *et al.*, 2001).

Alteration of mAChR expression and function has also been implicated in the pathophysiology of schizophrenia. Reduction in the density of mAChRs (mostly M1) in the hippocampus (Crook *et al.*, 2000), prefrontal cortex (Dean *et al.*, 2002) and caudate putamen (Dean *et al.*, 1996) has been shown in the brains of schizophrenic patients. In this condition, genetic changes in the coding for mAChR have been reported. A single nucleotide polymorphism in the structure of the gene coding for the M1 mAChR has been identified in the brains of some patients with schizophrenia (Liao *et al.*, 2003).

Other conditions which have shown an involvement of mAChRs include Parkinson's disease (striatal M4 dysfunction (Ding *et al.*, 2006), progressive supranuclear palsy (decrease in M2 and M4 mAChR expression in thalamic nuclei (Warren *et al.*, 2007)), and drug dependence (M5 mAChR (Basile *et al.*, 2002;Fink-Jensen *et al.*, 2003)).

#### 1.2.2. Nicotinic receptors

The nicotinic acetylcholine receptor (nAChR) is a ligand gated cation permeable channel, which belongs to the superfamily of receptors, which includes the GABA<sub>A</sub>, glycine, and 5-HT<sub>3A</sub> receptors (Dani, 2001). Each nicotinic receptor is made up of five membrane spanning subunits. To date, five different subtypes of receptor subunit have been recognised (Table 1.2). These are termed  $\alpha$  ( $\alpha$ 1 -  $\alpha$ 10),  $\beta$  ( $\beta$ 1 -  $\beta$ 4),  $\epsilon$ ,  $\delta$  and  $\gamma$ . Nicotinic acetylcholine receptors can exist as homomeric structures (where all of the subunits are of the same type e.g.  $\alpha$ 7), or heteromeric structures where a mixture of subunit types are

combined to form the receptor. In the membrane the five subunits are assembled such that they form a ring structure with a central pore forming the ion channel.

The nAChR can be further subdivided into muscle-type and neuronal receptors. Muscletype nAChR are composed of  $(\alpha 1)_2\beta 1\gamma\delta$ .subunits (found in developing muscle and also in extrajunctional locations). In mature muscle they are composed of  $(\alpha 1)_2\beta 1\epsilon\delta$  subunits. Neuronal nAChRs in contrast are only ever composed from either the  $\alpha$  and  $\beta$  subunits either as homomers (all five subunits are the same ( $\alpha 7$ ,  $\alpha 8$  and  $\alpha 9$ )), or heteromers (composed of 2  $\alpha$  and 3  $\beta$  subunits). The subtype composition of the receptor engenders many different characteristics such as agonist affinity, ion permeability and desensitisation rates. These differences, especially the agonist affinity and rate of desensitisation, allow for a level of fine adjustment in the modulation of the output (usually the release of other neurotransmitters) of neurones expressing these receptors.

Neuronal nicotinic acetylcholine receptors are prevalent throughout the brain and spinal cord (Woolf, 1991). The mRNA of the various subunits has been detected in most areas of the brain, with higher concentrations being found in the cerebral cortex, periaqueductal grey matter, basal ganglia, thalamus, hippocampus and the cerebellum. The  $\alpha$ 7 nAChR is the most widely distributed of the homomeric receptors, with the mRNA of this subunit being found in the hippocampus, auditory cortex, optical cortex, and mesocorticolimbic structures. The  $\alpha$ 7 homomeric receptor rapidly activates allowing the flow of cations especially Ca<sup>2+</sup>, but then equally rapidly desensitises. The rank order of agonist efficacy at the  $\alpha$ 7 receptor is nicotine>cytisine>DMPP>ACh (Lucas-Meunier *et al.*, 2003). The  $\alpha$ 7 receptor is also highly sensitive to choline (which appears to act as an agonist and also causes desensitisation) whereas the heteromeric receptors are not (Pereira *et al.*, 2002). This then means that this receptor may be activated not only by the release of ACh, but also by the major product of its hydrolysis. This characteristic of the  $\alpha$ 7 receptor allows for fine-tuning of neurotransmitter release modulated by the release of ACh.

The  $\alpha 4\beta 2$  nAChR is the most abundant of the heteromeric nAChR within the mammalian CNS with high densities of mRNA of the  $\alpha 4$  and  $\beta 2$  subunits being found in the cerebral cortex and the hippocampus. In contrast to the  $\alpha 7$  receptor, the  $\alpha 4\beta 2$  receptor has a much slower rate of desensitisation, and a differing affinity for cholinergic agonists (ACh and nicotine>DMPP>cytisine) (Lucas-Meunier *et al.*, 2003).

The function of the nicotinic nAChR within the CNS is mainly one of a modulator of other neurotransmitter systems. From receptor localisation studies it has been shown that the

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#### Figure 1.3. Nicotinic antagonists.

Methyllycaconitine is selective for  $\alpha$ -bungarotoxin sensitive neuronal nAChR (mainly homomers of  $\alpha$ 7-10 subunits). Mecamylamine is selective for neuronal nAChR that are insensitive to  $\alpha$ -bungarotoxin including those composed of multiple  $\alpha$  and  $\beta$  subunits (e.g.  $\alpha$ 4 $\beta$ 2).

majority of nAChRs are to be found on presynaptic membranes where they modulate the release of other neurotransmitters such as dopamine (Wonnacott, 1997). There is however evidence for a direct fast transmission involving nAChRs in the GABAergic interneurones of the rat hippocampus and the human cerebral cortex(Albuquerque *et al.*, 2000). The evidence implies that the various nAChRs have a critical role in maintaining a balance between excitation and inhibition within the CNS. As such, the nAChR has been implicated in many cognitive processes such as learning, attention and memory consolidation, as well as control of locomotion, noiception, and temperature regulation. With the great complexity of subunits and configurations of the nAChR it is not surprising that any fault will have implicated in various pathophysiological conditions such as nocturnal epilepsy (a deficiency in  $\alpha$ 2 and  $\alpha$ 4 subunits), febrile convulsions (Chou *et al.*,

2003), Tourette's syndrome (Gotti *et al.*, 1997), and schizophrenia (Freedman *et al.*, 2000).

#### 1.2.3. Acetylcholinesterase and butyrylcholinesterase

AChE is a type 3 hydrolase enzyme which hydrolyses ACh into its constituent parts, acetate and choline (Figure 1.4). The cholinesterase family of enzymes belongs to the much larger hydrolase group of enzymes, which also includes other serine esterases and protease enzymes such as trypsin. The different cholinesterase enzymes are named after the substrate for which the enzyme shows the highest affinity. Thus AChE (EC 3.1.1.7) has a high affinity for ACh and butyrylcholinesterase (EC 3.1.1.8) has a higher affinity for butyrylcholine than ACh. However, whilst AChE has a high affinity for ACh, it is also able to hydrolyse other esters. Whilst the role of AChE is relatively well defined with respect to the hydrolysis of ACh, the physiological role of BuChE is not so clear cut. Whilst it is known that BuChE can hydrolyse ACh, it can also metabolise other compounds such as heroin, physostigmine and succinylcholine. It has been shown however that in some individuals, BuChE can be found in extremely low concentrations (or in some cases is totally absent) when compared to the normal population, with no apparent physiological detriment. Therefore it has been hypothesised that BuChE, whilst sharing a common ancestry with AChE, has evolved into a scavenger but has retained the cholinesterase catalytic activity that it shares with AChE (Massoulie et al., 1993).

The cholinesterase enzymes exist either as a sole catalytic unit, or as a complex between a catalytic unit and a structural subunit allowing further modification of the enzyme characteristics. The attachment of the structural subunit also allows the enzyme to attach to the cell membrane. AChE and BuChE can exist in one of two basic forms, termed globular (G) and asymmetric (A). The globular form of the enzyme exists as a monomer (G<sub>1</sub>), dimer (G<sub>2</sub>), or tetramer (G<sub>4</sub>). The multimeric forms of the enzyme are held together by disulphide bonds. The isoforms of the enzyme can be subdivided still further, into those which are soluble, those which contain an amphiphilic region (ethanolamine-glycan-phosphoinositol attached via an amide bond to the C terminus of the structure of the catalytic unit) allowing binding with the plasma membrane, and those which have a fully hydrophobic chain attached allowing deep attachment within the lipid bilayer of the plasma membrane. In contrast, the asymmetric form can only exist as a homomer (A<sub>4</sub>, A<sub>8</sub>, and A<sub>12</sub>). Structurally, this form of AChE is found as four catalytic subunits which are bound together by an attached collagen tail. Both forms of AChE can be found either tethered to the plasma membrane or as soluble entities within the cytosol (Massoulie *et al.*, 1993).

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Cholinesterase enzymes have two active sites per molecule, which are located in gorges deep within the folds of the protein structure. Each of the gorges contains two regions termed the anionic and esteratic sites. The anionic site comprises of a glutamate residue and the esteratic site contains a histamine imidazole ring and serine hydroxyl group. These are both necessary for the successful hydrolysis of ACh and similar substrates. It has been postulated that the process of catalysis of the substrate is first initiated by nucleophilic attack from the serine hydroxyl residue on the acyl group of the ester substrate, resulting in acylation of the serine residue. Transfer of electrons from the aspartate (or glutamate) residue to the serine, thus strengthens the attack. Reversal of the electron flow following acylation of the serine, back to the aspartate (or glutamate) residue facilitates hydrolysis of the substrate into its component parts and subsequent reactivation of the enzyme(Whittaker, 1993). The histidine ring is believed to govern this process by relaying the electrons between the other two catalytic residues. The acidic anionic group also acts as guide to the correct insertion of the substrate into the esteratic site. An example of this can be shown using ACh as the substrate. The positively charged guaternary nitrogen ion within the ACh molecule is electronically attracted to the carboxyl group of the anionic site. This in turns locates the ester portion of the molecule over the esteratic site of the enzyme, thereby allowing efficient cleavage of the substrate into its constituent components (Whittaker, 1993). This is an extremely rapid and efficient process, which has been estimated to have a turnover rate of 5000 molecules of ACh per molecule/enzyme s<sup>-1</sup>. This equates roughly to a time course of 0.2 milliseconds per hydrolysis event.

Both AChE and BuChE can be found throughout the mammalian body system. All mammalian muscle contains cholinesterase, with AChE appearing as the dominant subtype (Massoulie *et al.*, 1993). BuChE is also present at low levels, but in certain muscle types such as cardiac muscle, the level of BuChE is greatly elevated such that it is found in approximately the same concentration as AChE. Within the CNS, AChE predominates, both in areas that have cholinergic properties and also those that have no known cholinergic properties, such as the locus coeruleus and the dopaminergic neurones of the substantia nigra. BuChE has been demonstrated to be confined mostly to glial cells (Giacobini, 2004) whereas AChE can be found almost ubiquitously throughout the brain in the  $G_1$  and  $G_4$  isoforms (Marquis & Fishman, 1985).

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The active site of AChE comprises of two separate areas, the esteratic (histidine (His) and serine (Ser) residue based) and anionic sites (glutamate (Glu) residue based) (A). ACh is attracted to the anionic site of the enzyme (B), and the process of hydrolysis begins. During hydrolysis, the serine residue becomes acetylated and a molecule of choline is released (C). Following spontaneous hydrolysis of the acetylated serine residue, a molecule of acetate is liberated and the enzyme is free to receive another molecule of ACh (D).

#### 1.2.4. Volume transmission of ACh within the CNS

Whilst the action of ACh in the peripheral nervous system is largely as a direct neurotransmitter (e.g. as seen at the neuromuscular junction), the same cannot be said for the action of ACh within the central nervous system. There is gathering evidence that ACh not only acts as a classical neurotransmitter , but also may act by volume transmission (Descarries *et al.*, 1997).

The concept of volume or non-synaptic transmission was first proposed for serotonin and noradrenaline neurones within the rat cortex. In this model, there is an ever-present basal level of the neurotransmitter within the extracellular space. Further release of the neurotransmitter in response to stimulation results in a transient increase before returning to the basal level following elimination of the neurotransmitter. The superimposed spike will then act as a signal to the cholinergic receptors on the neuronal membranes. The basal level is tightly maintained by uptake mechanisms or catalytic enzymes that remove the neurotransmitter molecule, preventing further stimulation of the receptors. In volume transmission, these processes do not remove all of the neurotransmitter thus allowing a constant basal level to be maintained. The evidence for this model has come from

ultrastructural analysis which has shown that the axon nerve terminals (both boutons and varicosities) found on neurones containing these neurotransmitters showed little of the structure commonly associated with synapse formation (such as inclusion of a post-synaptic membrane) (Descarries *et al.*, 1997).

Investigation of central cholinergic axons has revealed a similar picture for cholinergic varicosities. In the cortex, only 10-20% of observed varicosities have synaptic properties, whilst in the hippocampus and the neostriatum the level of synaptic differentiation is lower at around 7-9%. Therefore it is proposed that ACh acts by volume transmission at least in areas of the brain which has a dense cholinergic cell population, as the number of synaptic structures is far lower than the total number of potential release sites. This would allow for axo-axonic transmission (transmission between adjoining axons) to occur in the absence of direct synaptic interfaces (Descarries et al., 1997;Descarries, 1998;Contant et al., 1996). Similarly, the distribution of M2 mAChR on striatal interneurones has been shown to be largely asynaptic. This suggests that cholinergic transmission mediated by these receptors must take place by a volume transmission-like process (Bernard et al., 1998). It is theorised that the large number of subtypes of the ACh receptors, especially the nAChRs with their varying properties, will act as filters to the different levels of ACh present and provide the necessary graded transmission of the cholinergic signal. Further evidence for a basal level of ACh comes from the results of microdialysis studies which have shown that ACh can be detected even in the absence of AChE inhibitors such as physostigmine (Testylier & Dykes, 1996).

#### 1.2.5. Implications for poisoning

As can be seen from this brief review, the role of cholinergic receptors within the central nervous system are numerous and extremely diverse. Any alteration to the functioning of these systems by poisoning with organophosphates will have numerous implications of varying severity. Overstimulation of classical cholinergic synapses may result in alterations to the release of other neurotransmitters within areas of the brain that are directly modulated by cholinergic receptors.

The volume transmission model of ACh transmission within the CNS has many farreaching implications following organophosphate poisoning. Poisoning would cause an increase in the basal level of ACh by inhibiting AChE leading to one of two possible outcomes. Firstly, the inhibition of AChE would cause a rapid increase in the basal level of ACh that would overstimulate the various cholinergic receptors regardless of their sensitivity for ACh thereby affecting the release of other neurotransmitters. Secondly, the inhibition of AChE and subsequent elevation of basal ACh levels may result in the stimulated release signal being lost amongst the noise of the increased background. If the case for volume transmission for ACh within the CNS is proven to be correct, this will have implications for the design of future clinical management of organophosphate poisoned casualties. The need for a centrally active muscarinic antagonist such as atropine remains, but there may also be the need to antagonise the actions of the central nAChR as well. This may decrease the involvement of other neurotransmitter systems that will have been affected by this disturbance within the cholinergic system.

It is not just the direct effects of inhibiting AChE that will affect cholinergic neurotransmission. Elevated levels of neuronal activity such as that seen during seizures cause a decrease in the level of extracellular calcium. Calcium is an allosteric modulator of the nAChR and is required for the normal functioning of this receptor subtype. Any reduction of the levels of available calcium will dramatically decrease the opening probability of the nAChR leading to a failure in signal transmission (Dani, 2001).

#### 1.3. Cholinergic pathways in the brain

Many areas of the mammalian brain receive a rich cholinergic innervation that has been shown to be involved in many cortical functions involving cognition and arousal (Everitt & Robbins, 1997). Deficits in the cholinergic system have been seen in the brains of sufferers of neurodegenerative conditions such as Alzheimer's dementia (Mesulam, 2004b) and Parkinson's disease (Bosboom *et al.*, 2004). Changes in the central cholinergic system are also a large part of the degenerative aging processes (Court *et al.*, 1993). The rodent brain has been well characterised (Mesulam *et al.*, 1983b), with similar studies having been conducted on the brains of primates and humans (Mesulam *et al.*, 1992b;Mesulam & Geula, 1988;Mesulam, 2004a;Mesulam *et al.*, 1992a;Mesulam, 1990;Selden *et al.*, 1998). Whilst there are many similarities between the cholinergic innervation of the primate and rodent brain, there are several distinct differences especially in the innervation of the cerebral cortex which can make direct comparison difficult (Mesulam, 1990).

Using labelled markers of neuronal cholinergic activity such as choline acetyltransferase (ChAT), it has been shown that the vast majority of the cholinergic innervation of the brain is provided by projection neurones arising from a small number of cholinergic nuclei within the basal forebrain and the brainstem (Mesulam, 1990). These cell body groups are often not very well delineated from adjoining cellular structures and show markers not only for cholinergic neurones but also other transmitters such as GABA (Mufson *et al.*, 2003). An example of this is the medial septal nucleus and it's projections to the hippocampus.

So far eight cholinergic pathways have been defined within the mammalian brain (Mesulam, 1995). To avoid the confusion often found with the naming of structure of the brain, Mesulam et al., (1983) proposed an alternative nomenclature for the naming of each pathway. Each pathway is designated Ch (for cholinergic) followed by the appropriate identifying number (1-8) (Mesulam et al., 1983a). This nomenclature has been adopted for the remainder of this review. Four of these pathways (Ch1-4) originate in the magnocellular nuclei of the basal forebrain, including the medial septal nucleus, the nucleus of the vertical limb, the nucleus of the horizontal limb, and the nucleus basalis of Meynert. These pathways project to the hippocampus and cortical areas, are involved in cognitive processes including sustained attention and memory (Sarter & Bruno, 1997a). Two more pathways (Ch 5 and Ch6) arise from the pedunculopontine and laterodorsal nuclei and provides the major cholinergic innervation of the thalamus (Mesulam et al., 1989), the ventral tegmental area (VTA) and substantia nigra (Woolf, 1991). Cholinergic projections from these nuclei have been implicated in arousal (Parikh & Sarter, 2008;Sarter & Bruno, 1997b), rapid eye movement sleep (Garcia-Rill et al., 2008), control of central respiratory patterns (Saponjic et al., 2003), and maintenance of airway patency during sleep (Rukhadze & Kubin, 2007). The projections to the VTA and substantia nigra have been shown to activate dopaminergic neurons in these areas therefore providing control over locomotion, feeding and reward behaviours (for review refer to (Yeomans, 1995). The final two pathways (Ch7 and Ch8) are contained within the medial habenula and parabigeminal nuclei (Mesulam, 1990;Mesulam et al., 1983b;Wenk, 1997). A schematic diagram of these pathways is shown in Figure 1.5.

Not all of the cholinergic innervation of the brain is supplied by projection neurones via the pathways described above. In the striatum the innervation is almost exclusively intrinsic, being supplied by cholinergic interneurones contained within the structure (Wilson, 2004), although there may be a minor extrinsic component from the Ch6 pathway (Mesulam *et al.*, 1992b).

Many disease states and toxic insults can affect the functioning of cholinergic systems within the brain (McKinney, 2005). Following poisoning with nerve agents, a correlation between the length and severity of seizures and the severity of neuropathology has been reported (Shih *et al.*, 2003). In animals which have received a convulsive dose of the nerve agent soman and survived, there is extensive damage and cell loss in the forebrain and hippocampal formation (Petras, 1994;Baille *et al.*, 2001;Baille *et al.*, 2005;Filliat *et al.*, 1999). The soman-induced damage is largely indistinguishable from that caused by ischemia (Petras, 1994). These structures are mainly aligned with the Ch1-4 pathways. Following the sarin attack on the Tokyo subway system in 1995, exposed survivors

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showed a decrease in the volume of grey matter in the temporal cortices and the hippocampus. A decrease in the volume of the white matter of the temporal stem was also reported (Yamasue *et al.*, 2007). In animals exposed to sarin, histological analysis reveals cell death occurring in the hippocampus, piriform cortex and thalamic nuclei (Grauer *et al.*, 2008).

Similar decreases in cell numbers in the areas of the Ch1-4 pathways have also been reported following physical brain trauma such as fluid percussion injury (Schmidt & Grady, 1995). Closed head trauma has been shown to primarily affect the cholinergic pathways of the forebrain (Salmond *et al.*, 2005). Whilst the cholinergic pathways that innervate the hippocampus and forebrain have been conclusively shown to be involved in cognitive processes, other cholinergic nuclei have also been shown recently to have possible roles in these processes. Lesions of the pedunculopontine nucleus (Ch5) have been shown recently to result in disruption to operant tasks (lever pressing) and also a possible impairment in sustained attention (Rostron *et al.*, 2008). How the function of this structure is affected by anticholinesterase exposure is as yet unclear.



## Figure 1.5. Schematic of the principal central cholinergic pathways within the human brain.

(A) Pathways Ch1 (arising in the septal nucleus) and Ch2 (arising in the vertical limb of the diagonal band).
(B) Pathways Ch3 (arising in the horizontal limb of the diagonal band) and Ch4 (arising in the nucleus basalis of Meynert).
(C) Pathways Ch5 and Ch6 (arising in the pedunculopontine and laterodorsal tegmental nuclei respectively).
(D) Ch7 arising in the medial habenula nucleus, and Ch8 arising in the parabigeminal nucleus.

#### 1.4. Anticholinesterases, organophosphorus compounds and nerve agents

Anticholinesterases (also sometimes referred to as cholinesterase inhibitors), inhibit the action of the cholinesterase family of enzymes either by a reversible or irreversible mechanism. There is in reality a continuum between these two extremes. What determines whether an anticholinesterase is a reversible or irreversible inhibitor is the rate at which it either disassociates or is broken down by the enzyme. The main action of the cholinesterases is to hydrolyse chemicals structurally related to esters of choline. The wider class of anticholinesterase compounds shows a wide diversity in chemical structure, some of which can be derived from biological sources (e.g. physostigmine) and others of

which are purely synthetic (e.g. parathion). This project will focus on carbamate and organophosphate (OP) anticholinesterase compounds.

The terms organophosphorus and organophosphate have been widely adopted for the sake of convenience to describe OP insecticides, but the compounds are typically phosphorothionates (central P atom is bonded to three oxygen atoms and a double bond to a sulphur atom e.g. chlorpyrifos), phosphorodithioates (structurally similar to phosphorothionates but with an additional sulphur atom in place of one of the oxygen atoms e.g. malathion), or phosphoroamidothiolates (the central P atom is bonded to nitrogen, oxygen (double bond) and sulphur atoms e.g. methamidophos) (Mileson *et al.*, 1998).

In their original states, many OPs are generally very weak inhibitors of AChE (Jokanovic, 2001). However, once within the body these compounds undergo biotransformation into oxygen analogs of the parent compound (oxons). This metabolic transformation occurs in both insects and mammals, but is more efficient in the former making them highly effective insecticides. Oxons such as paraoxon (parathion) and chlorpyrifos oxon (chlorpyrifos) exhibit a high affinity for AChE and as such are potent inhibitors of the enzyme causing the desired lethal effects of the insecticide (Kousba et al., 2004). The biotransformation from relatively innocuous parent compound to the bioactive oxon follows one of five known pathways; oxidative desulphuration of the thiophosphate group to form a phosphate compound, oxidation of a sulphide group to form sulphones or sulphoxides, oxidation of amide groups forming N-dealkylated or N-oxide compounds, hydroxylation of akyl groups forming cyclic phosphate esters or ketones, or by other non-oxidative mechanisms (Jokanovic, 2001). The various oxidative processes are mediated by cytochrome p450 and other enzymes such as the flavin-containing mono-oxygenases (Mileson et al., 1998; Jokanovic, 2001). The basic structures of two common OP pesticides and their respective oxons are shown in Figure 1.6.

OP compounds have found widespread use since first being developed the nineteenth century. Many OP compounds have found use as insecticides, as they exhibit high invertebrate toxicity with low vertebrate toxicity. However, poisoning with OP compounds in humans whether intentional (i.e. as an aid to suicide) or unintentional poses a very real challenge for clinicians worldwide. It is suggested that as many as 200 000 deaths in rural Asia could be attributed to intentional OP poisoning following ingestion of OP insecticides such as chlorpyrifos (Eddleston & Phillips, 2004).



#### Figure 1.6. Structures of the organophosphate insecticides.

These differ from the nerve agents by having a sulphur atom double bonded to the phosphorus atom instead of an oxygen atom. Also shown are the respective oxons. These are formed when the parent compound undergoes biotransformation within the body. Typically oxons are more potent inhibitors of AChE than the parent compound. This is due to the substitution of the sulphur atom with an oxygen atom (double bond).

Nerve agents are a family of highly toxic OP compounds which have found use as both military and terrorist weapons. There are two classes of nerve agents (identified by their NATO designation); G agents (G for German) which includes tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF); or V agents (Venomous) such as VX (Maynard, 1999). The basic structure of organophosphorus nerve agents is shown in Figure 1.7 and the structures of the individual compounds are shown in Figure 1.8. As with OP insecticides, the terms organophosphorus and organophosphate have been widely adopted, but the compounds are actually phosphoroamidates (tabun), phosphonofluoridates (sarin, soman, cyclosarin), and phosphorothiolates (VX).

The discovery of the nerve agents was born out of research into novel insecticides by Schrader and co-workers during the 1930s in Germany (Timperley, 2000;Schmaltz, 2006). A novel OP which had extremely high mammalian toxicity precluding it from safe use as a commercial insecticide was synthesised during this research. This was the compound which is now known as tabun. Further investigation of this series of compounds yielded the compounds sarin and soman. VX also shared a common ancestry with OP pesticides and was first synthesised in 1952. Whilst sharing similar chemical structures, the nerve agents have very diverse physical properties. The G agents tend to be volatile and therefore present mainly an inhalational hazard (although G agents can also be percutaneous hazards). This also makes them less persistent as the vapour is easily dispersed. In contrast VX is relatively involatile, making it a persistent agent and a percutaneous hazard. Whilst the early G agents (tabun, sarin and soman) were weaponised by Germany during the closing years of World War II, they were never deployed during this conflict. However, nerve agents have been used in other conflicts most notably the Iran-Iraq war (1980-1988) (Newmark, 2004), as well as internal conflicts in Iraq (Black *et al.*, 1994). Sarin has also been used as a terrorist weapon by the Aum Shinrikyo cult in attacks in Matsumoto and Tokyo in Japan (1994 and 1995 respectively). There is evidence that this group have also used VX in some of their activities (Centre for Nonproliferation, 2001).

The nerve agents and most organophosphorus compounds are potent inhibitors of cholinesterase enzymes (Maynard, 1999). Of prime importance is AChE which preferentially hydrolyses ACh into its constituent components choline and acetate, thereby limiting its availability for binding and therefore terminating the action of the neurotransmitter. A more detailed description of AChE is given in section 1.2.3. The reaction between AChE and a nerve agent is shown in Figure 1.9.



Compound	R1	R2
Tabun (GA)	CH <sub>3</sub> CH <sub>2</sub> O	(CH <sub>3</sub> ) <sub>2</sub> N
Ethyl <i>N</i> -		
dimethylphosphoramidocyanidate		
Sarin (GB)	CH <sub>3</sub> (CH <sub>3</sub> )CHO	CH <sub>3</sub>
Isopropyl		
methylphosphonofluoridate		
Soman (GD)	(CH <sub>3</sub> ) <sub>3</sub> C(CH <sub>3</sub> )CHO	CH <sub>3</sub>
Pinacolyl		
methylphosphonofluoridate		
Cyclosarin (GF)	(C₅H <sub>10</sub> )CHO	CH <sub>3</sub>
Cyclohexyl		
methylphosphonofluoridate		
VX	C <sub>2</sub> H <sub>5</sub> O	CH <sub>3</sub>
O-Ethyl-S-		
[2(diisopropylamino)ethyl]		
methylphosphonothioate		

#### Figure 1.7. Basic structure of organophosphorus nerve agents.

The table above shows the different groups present at positions  $R_1$  and  $R_2$  for each of the classical G agents. X can either be a CN group (tabun) or a fluorine atom, F (sarin, soman and cyclosarin), or SCH<sub>2</sub>CH<sub>2</sub>N(C<sub>3</sub>H<sub>7</sub>)<sub>2</sub> (VX).



#### Figure 1.8. Chemical structures of nerve agents.

These compounds are typically characterised by having a central phosphorus atom which is bound to a methyl group and an oxygen atom (double bond). The exception to this is tabun in which the methyl group is replaced by  $C_2H_6N$ .

Following nerve agent poisoning, various clinical signs become evident. Most if not all are due to overstimulation of the cholinergic system within both the peripheral and central nervous systems. This is also sometimes referred to as a cholinergic crisis. The order of the appearance of the signs and symptoms and time to onset is very much dependent on the route of exposure, time exposed and concentration of the agent compound. For example, following an inhalational challenge the signs and symptoms will present more quickly than those seen following a percutaneous challenge due to the slower penetration of the agent into the body. Overstimulation of muscarinic receptors within the sympathetic and parasympathetic systems results in some of the initial signs of the cholinergic crisis,
these being miosis, lachrymation, and an increase in secretions (rhinorrhea, sweating and bronchial secretions). Other signs of muscarinic overstimulation include gastrointestinal disturbances such as diarrhoea and abdominal cramps (which may develop into involuntary defecation and micturation) and bradycardia, may also present following poisoning. Overstimulation of peripheral nicotinic receptors results in signs and symptoms such as fasiculations, muscle weakness, flaccid paralysis of muscles (caused by neuromuscular blockade and muscle fatigue), tachycardia and attendant hypertension. CNS effects include restlessness, giddiness, confusion and



#### Figure 1.9. Reaction between AChE and a nerve agent

The initial reaction between the enzyme AChE and the nerve agent to form the enzyme-agent complex (1) is reversible. The next stage of the reaction is the phosphorylation of the enzyme (2) which releases the leaving group as a hydride (a fluorine atom in the case of sarin and soman, or a cyano group for tabun). At this point the enzyme is inactivated. The enzyme can be reactivated either spontaneously through the hydrolysis of the enzyme–agent complex or more likely by a nucleophilic attack by an oxime or related compound (3). Complete inactivation of the enzyme (called ageing) is achieved by the cleavage of an alkyl group from the R2 position (4). Once this reaction has occurred, the phosphorylated enzyme cannot be reactivated.

convulsions. The convulsions rapidly progress to a state which is similar to that seen in status epilepticus (Taylor, 1990;Somani *et al.*, 1992). Ultimately, If left untreated (and in severe intoxications) poisoning by organophosphates (including nerve agents) is lethal due to respiratory depression. This is caused by paralysis of the respiratory muscles and central inhibition of the respiratory control centres within the brainstem (Bird *et al.*, 2003).

In casualties who survive the initial cholinergic crisis the prognosis is generally good, although the seizures generated during the intoxication can cause extensive neuropathology within the brain (Shih *et al.*, 2003;Myhrer *et al.*, 2005). Therefore early termination of the seizure events is a high priority for the clinician. Disturbances in EEG have been reported in survivors who were exposed to sarin during the terrorist attacks in Tokyo (Yanagisawa *et al.*, 2006).

If the casualty can be supported through the initial cholinergic crisis, many may deteriorate into a state of muscular paralysis called the intermediate syndrome (Brown & Brix, 1998). This is especially associated with poisoning by organophosphorus insecticides. The intermediate syndrome is typified by rapid progression of paralysis of the muscles innervated by the cranial nerves, respiratory muscles and proximal limb musculature. Death by respiratory failure is not uncommon at this stage of the poisoning. The onset of the intermediate syndrome usually occurs between 1-4 days following exposure to high levels of organophosphates (Moretto, 1998). Whilst the underlying causes of this syndrome are not well understood, it has been shown that the magnitude of the exposure and muscle injury sustained during the initial cholinergic crisis is well correlated to the severity and incidence of the intermediate syndrome (John *et al.*, 2003). This condition is reversible but the casualty places a huge logistical burden on the secondary care providers until recovery is complete.

## 1.5. Effects of OP compounds in the central nervous system

The actions of organophosphorus compounds (including nerve agents) on the cholinesterase enzyme are well known. There are also reports that have highlighted other possible targets for interaction with organophosphates. Both the inhibition of the cholinesterase enzymes and the interaction of organophosphorus compounds with other non-cholinesterase targets will now be discussed.

## 1.5.1. Cholinesterases

As described in section 1.2.3, cholinesterase enzymes have two active sites (the esteratic and the anionic), both of which are involved in the binding and hydrolysis of ACh. The organophosphate compound is actively attracted to a serine hydroxyl residue within the esteratic site. When in close proximity to this serine residue, the electrophilic phosphorous atom undergoes nucleophilic attack and the halogen atom (or another group such as cyanide or thiocyanate at position X in Figure 1.7) is displaced in favour of the serine residue thus effectively phosphorylating or phosphonylating (depending on the OP)

the enzyme (Somani *et al.*, 1992). This is generally an irreversible reaction resulting in the loss of active enzyme, although the enzyme may be released by the actions of nucleophilic compounds such as oximes if they are administered soon after poisoning (Wong *et al.*, 2000). The loss of the catalytic activity of AChE leads to a build up of ACh within the synapse. This in turn causes a progressive overstimulation of the postsynaptic membrane leading to the overt signs and symptoms of the cholinergic crisis.

A further complication to the dissociation of the enzyme-agent complex is the cleavage of the O - C bond within the R<sub>2</sub> group resulting in a dealkylated complex (Figure 1.9) (Shafferman *et al.*, 1996). Dealkylation (also called ageing) can occur rapidly following poisoning with soman ( $t_{1/2}$  of between 1 and 8.5 minutes depending on species) (Talbot *et al.*, 1988), or more slowly in cases of intoxication with other nerve agents e.g.  $t_{1/2}$  sarin ~3 hours (Worek *et al.*, 2004). The resulting charge on the enzyme-agent complex resists the actions of oximes thereby inhibiting any regeneration of the enzyme. Oximes will be discussed in more detail in therapies section.

Recent advances in genetics have allowed the development of various animal models which have specific enzyme mutations. An AChE knockout mouse model (AChE -/-) has been used to study the role of BuChE following the loss of AChE. With this model it has been shown that survival without AChE is possible, although the AChE -/- mice were more sensitive to VX than the wild type mouse. Atropine failed to prevent lethality in the knockout mice, showing that either the mAChR of the AChE -/- mouse had become desensitised or that excess levels of ACh do not accumulate within their synapses (Duysen et al., 2001). As these mice are devoid of AChE the lethal effects of VX must be mediated by another target system such as BuChE. A study by Chatonnet et al., (2003) has shown that BuChE does indeed play a role (at least in the peripheral nervous system) in the survival of AChE -/- mice, however BuChE does not totally replace the activities of AChE within the CNS, especially at the sites of respiratory rhythm generation in the brainstem. Survival in this case is prolonged by a general down-regulation in the response of central respiratory neurones to cholinergic agonists (Chatonnet et al., 2003). Similarly, Minic et al., (2003) reported that BuChE does not replace AChE as the terminator of the cholinergic signal at the neuromuscular junction of AChE -/- mice, but instead acts to limit the release of ACh via a presynaptic mechanism (Minic et al., 2003).

A knockout mouse model which is devoid of plasma and tissue BuChE (BuChE -/-) has also been developed (Li *et al.*, 2006). In contrast to the AChE -/- mouse, the BuChE -/- appears to be relatively normal in its physiology, although changes in the function of mAChR have been reported (Li *et al.*, 2008). However, when exposed to the specific AChE inhibitors (-) - huperazine A and donezepil, the BuChE -/- mouse exhibited signs of

a pronounced cholinergic overstimulation (convulsions, fasiculations, and changes in core temperature) when compared to BuChE +/+ and AChE -/- mice. Whereas the BuChE +/+ and AChE -/- mice survived this challenge, all of the BuChE -/- mice were dead within 10 minutes of dosing with (-) – huperazine (1.5 mg/kg), and within 110 minutes of dosing with donezepil (10 mg/kg). Death usually occurred during a major tonic convulsion. The action of these compounds shows that BuChE is important in the AChE -/- mouse and has a role in controlling levels of ACh probably through hydrolysis of the agonist. Interestingly, exposure to the OP compounds chlorpyrifos oxon and ecothiopate affected all BuChE genotypes in equal measure (non-lethal) whereas it caused 100% lethality in the AChE -/- mice. It has been postulated that the reason for the apparent lack of effect of OP compounds in the BuChE -/- mouse is due to the high levels of circulating carboxylesterase which may act to scavenge the OP (Duysen *et al.*, 2007).

#### 1.5.2. Carboxylesterase

Carboxylesterase enzymes (EC 3.1.1.1) form a large and complex multigene family of enzymes (Satoh & Hosokawa, 1998). These enzymes have broad substrate specificity, and are responsible for the hydrolysis of various ester and amide containing compounds including many endogenous compounds such as acyl-glycerols, long chain acyl-CoA esters, and many drug compounds such as carbonates, pyrrolizidine alkaloids, cocaine and salicylates. The importance of carboxylesterases in the detoxification of organophosphorus insecticides and nerve agents has been well documented (Barata *et al.*, 2004;Jokanovic, 2001). The carboxylesterase enzymes are also responsible for converting many pro-drugs into their active forms. Carboxylesterase enzymes are present throughout the body with the highest levels being found in the liver (Satoh & Hosokawa, 1998), although lower concentrations can be found in the brain (Mori *et al.*, 1999), respiratory system (Barr *et al.*, 1998), gastrointestinal tract (Schwer *et al.*, 1997), reproductive system (Yan *et al.*, 1995), and the skin (Heymann *et al.*, 1993). Humans in contrast to rodents and guinea pigs, have no carboxylesterase in the plasma fraction of the blood (Li *et al.*, 2005).

Carboxylesterases are also inhibited by organophosphorus compounds. Whilst the reactions between the carboxylesterase enzyme and organophosphate compounds are largely non-specific, this class of enzyme exhibits higher inhibition rate constants for uncharged compounds such as paraoxon, sarin, and soman (Maxwell & Brecht, 2001). Various studies have concluded that the scavenging effect of carboxylesterase is an important factor in the toxicity of the organophosphate compounds. It is apparent that this protective element of carboxylesterase activity is more important in cases of poisoning with compounds that exhibit high AChE inhibition rate constants and therefore are highly

toxic (i.e. paraoxon, sarin, and soman), than those that have relatively lower inhibition kinetics and lower toxicities (i.e. DFP) (Dettbarn *et al.*, 1999;Maxwell, 1992).

In contrast to the cholinesterase enzymes, carboxylesterases do not readily undergo the ageing reaction and are able to spontaneously reactivate to an active state once bound to organophosphorus compounds (Maxwell & Brecht, 2001;Dettbarn *et al.*, 1999). There are six amino acid residues implicated in the ageing process (Masson *et al.*, 1997;Shafferman *et al.*, 1996;Maxwell & Brecht, 2001) which are highly conserved between AChE and BuChE, whereas carboxylesterase only contains three of the residues (Maxwell & Brecht, 2001). The reactivation of the enzyme is also dependent upon the physical properties of the organophosphate inhibitor. It has been reported that with VX, sarin and soman, the rate of reactivation decreased with an increase in the size of the inhibitor (Maxwell & Brecht, 2001).

## 1.5.3. Neuropathy Target Esterase

Neuropathy target esterase (NTE also known as NTE-lysoPLA) is a membrane protein found in all neuronal cell types as well as some non-neuronal cells, and has been shown to have a role in neural development (Glynn, 1999). It has been demonstrated that poisoning at sub-lethal doses with certain organophosphorus compounds (e.g. triorthocrysylphosphate (TOCP) and chlorpyrifos) can cause a delayed neurotoxic condition typified by axonopathy or degradation of distal regions of long axons within the peripheral and central nervous systems (Lotti, 2002). This results in ataxia and paralysis of the lower limbs which develops within 14 to 21 days of poisoning. This has been termed organophosphate induced delayed neuropathy (OPIDN) and has been demonstrated to be caused by dealkylation (ageing) of the organophosphate ester or amide which is covalently bound to the active site of NTE (Lotti, 2002;Glynn, 2003). Not all organophosphorus compounds can age NTE, with a notable exception being soman (Crowell et al., 1989). Other nerve agents such as sarin are also incapable of causing the degree of NTE inhibition required to initiate OPIDN (Crowell et al., 1989). It has been demonstrated that pre-treatment with a non-neuropathic inhibitor of NTE such as some carbamates (thiobencarb) will protect against the development of OPIDN following a subsequent exposure to a neuropathic inhibitor such as DFP. If given after exposure to a neuropathic inhibitor, however, the non-neuropathic inhibitor greatly increases the severity of the OPIDN produced and lowers the threshold of NTE inhibition required to produce the axonopathy (Moretto, 1998).

#### 1.5.4. Other serine hydrolase enzymes

Acylpeptide hydrolase is a serine hydrolase that has been reported to show a higher sensitivity for inhibition by DFP and dichlorvos than AChE (Richards *et al.*, 2000). Whilst the exact biological function of this enzyme is not known, it has been shown to catalyse the hydrolysis of *N*-acylated amino acids from short peptides and therefore may be involved in the latter stages of protein synthesis. This again maybe important in the cognitive deficits reported post OP exposure. However the inhibition of acylpeptide hydrolase is highly dependant on the OP inhibitor used. Whilst DFP is a potent inhibitor of the enzyme, sarin at a concentration that causes almost complete inhibition of AChE and BuChE in guineas pigs only partially inhibits the activity of acylpeptide hydrolase (Quistad *et al.*, 2005). This shows that it is likely that the inhibition of acylpeptide hydrolase is not of clinical importance in the nerve agent poisoned casualty. However, whilst some of the interactions may have a secondary importance to the primary inhibition of AChE, it is now being realised that certain serine hydrolase enzymes such as KIAA1363 (an acetyl monoalkylglycerol ether hydrolase found in the brain, heart, lungs and kidneys) have a role to play in the detoxification of OP compounds (Nomura *et al.*, 2008b).

Serine hydrolase enzymes are key to the normal functioning of many neurotransmitter systems. In addition to the cholinergic system, the cannabinoid system uses serine hydrolase enzymes to control the action of the endogenous agonists, anandamide and 2-arachidonyl glycerol (2-AG). These enzymes are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAG) respectively (Cravatt *et al.*, 1996;Karlsson *et al.*, 1997). Inhibition of FAAH and MAG by OP compounds leads to an accumulation of 2-AG and anandamide and a decrease in the levels of arachidonic acid. This in turns exhibits it self overtly as signs of CB<sub>1</sub> receptor activation such as deficits in cognitive ability (Nomura *et al.*, 2008a). This is secondary to the main AChE inhibition, however, as almost total inhibition of FAAH has been shown to be non lethal and without many overt signs in the mouse (Quistad *et al.*, 2002).

Other serine hydrolase enzymes including those that are involved in blood clotting (thrombin, plasmin and kallikrein) and digestion (trypsin,  $\alpha$ -chymotrypsin and elastin) have also been shown to be inhibited by OP compounds (Quistad & Casida, 2000). The IC<sub>50</sub> values for these reactions are in the order of several  $\mu$ M (50  $\mu$ M for trypsin inhibited by sarin) up to and in excess of 1mM for thrombin inhibited by sarin (Mounter *et al.*, 1963). These concentrations are several orders of magnitude greater than the IC<sub>50</sub> of erythrocyte AChE when exposed to sarin (0.015  $\mu$ M (Sivam *et al.*, 1984)). From these data it can be seen that the catalytic action of AChE is affected to a greater degree than other enzymes

of this type. It can therefore be concluded that the predominant effect of OPs especially the nerve agents, is to inhibit the cholinesterase enzymes leading to the cholinergic crisis.

#### 1.5.5. Neurotransmitter receptors

As well as inhibiting AChE, it has been demonstrated that some AChE inhibitors and nerve agents can also interact directly with neurotransmitter receptors including the muscarinic and nicotinic acetylcholine receptors, and cannabinoid receptors. In studies by Rocha et al., (1998) (Rocha et al., 1998), VX was demonstrated to increase the spontaneous release of GABA and glutamate as measured by an increase in the frequency and amplitude of miniature post-synaptic potentials in cultured hippocampal neurones. This was found to be TTX-insensitive i.e. action potential independent and dose dependent. The effect also persisted when AChE was inhibited by incubating the neurones with 2µM soman (this has been previously shown to inhibit all AChE). Therefore this effect must be due to a non-AChE mechanism although the authors have not elucidated further on any possible mechanisms such as receptor interactions. The same group have also reported that both VX (Rocha et al., 1999;Rocha et al., 1998) and sarin (Rocha et al., 1998) reduce the evoked release of glutamate and GABA from both cultured hippocampal neurones and hippocampal slices. This effect was abolished by the pre-incubation of the neurones with atropine, which suggests that the effect was mediated by a mAChR. Similar pre-incubation with various nAChR antagonists failed to block the effect. VX also reduced the rate of firing and amplitude of action potentials recorded from current-clamped hippocampal neurones in the presence of CNQX and picrotoxin (Rocha et al., 1999). Chebabo et al., (1999) reported in a similar study that sarin at low concentrations (0.3 –1nM) also affected the evoked release of GABA from neurones in the hippocampus and this was found to be mediated by pre-synaptic mAChR on GABAergic interneurons. However, sarin did not affect the TTX-insensitive release of GABA as had been observed with VX in previous studies (Chebabo et al., 1999).

It is not only the nerve agents that are capable of interacting with cholinergic receptors. Organophosphorus insecticides have also been shown to interact with the mAChR. Chlorpyrifos oxon, the activated oxygen analogue of chlorpyrifos, has been shown to interact directly with the M2 mAChR both in the striatum (Huff *et al.*, 1994) and in cardiac cell cultures (Bomser & Casida, 2001) at a site that does not prevent agonists from binding to the receptor. Chlorpyrifos oxon, malaoxon, and paraoxon have also been shown to interfere with the second messenger pathways associated with the M2 and M4 mAChRs. All three compounds affected the production of cAMP following stimulation with forskolin. This effect was blocked by atropine. It was deduced that the M2 and M4 receptor subtypes were involved in this effect as none of the oxons had any effect on

basal or agonist induced phosphoinostide hydrolysis which are both linked to the other subtypes of mAChR (Ward & Mundy, 1996).

The nAChR has also been shown to be directly affected by various AChE inhibiting compounds, both non-organophosphorus (Smulders et al., 2003; Di Angelantonio et al., 2004a; Albuquerque et al., 1984) and organophosphorus in nature (Albuquerque et al., 1985;Katz et al., 1997;Smulders et al., 2004a;Smulders et al., 2004b;Di Angelantonio et al., 2004b;Tattersall, 1990). It has been demonstrated that chlorpyrifos, parathion and their related oxons (chlorpyrifos oxon and paraoxon respectively) desensitise the nAChR of the electric organ of the Torpedo ray. This was shown by using a radiolabelled form of the non-competitive antagonist (<sup>3</sup>[H]-thienyl-cyclohexylpiperidine, <sup>3</sup>[H]TCP). Chlorpyrifos oxon significantly inhibited the agonist induced binding of  ${}^{3}$ [H]TCP (IC<sub>50</sub> 5  $\mu$ M). The remaining compounds also inhibited the binding of <sup>3</sup>[H]TCP but were far less potent than chlorpyrifos oxon. None of the compounds affected the binding of  $\alpha$ -bungarotoxin to the receptor, although pre-incubation with the compounds increased the inhibition of  $\alpha$ bungarotoxin binding by carbachol (Katz et al., 1997). This shows that the OP compounds do not bind to the main agonist binding site of the receptor and also that they increase the affinity of this site for carbachol. Such an increase in affinity is seen when the receptor is stabilised in a desensitised state and therefore it is postulated that the compounds act to desensitise the receptor (Tattersall, 1990).

Several organophosphorus and carbamate insecticides are capable of inhibiting the rat neuronal  $\alpha 4\beta 2$  nAChR (Smulders *et al.*, 2004a). Exposure to varying concentrations of the organophosphorus insecticide caused an inhibition of the current induced by the application of 1mM ACh, with the kinetics of the inhibition consistent with the actions of a non-competitive inhibitor. Interestingly it was shown that the oxon forms of malathion and parathion had no effect on this current, demonstrating that the thio- form of the compound is needed to affect the  $\alpha 4\beta 2$  nAChR (Smulders *et al.*, 2004a). Similar inhibition of the ACh-induced current was seen following exposure to carbamate insecticides which included fenoxycarb and carbaryl (Smulders et al., 2004b). Similar inhibition of other nAChR ( $\alpha$ 4 $\beta$ 4,  $\alpha$ 3 $\beta$ 2, and  $\alpha$ 3 $\beta$ 4) has also been reported with carbamate insecticides. The rank order of potency of the compounds differed between the different receptor subtypes showing that the molecular biology of the receptor is important in the inhibition caused by carbamate insecticides (Smulders et al., 2003). This may also be true for the organophosphate induced inhibition. It has been proposed that the interaction of the receptor with the inhibiting compound, be it an organophosphorus or carbamate, follows a sequential two step process. Firstly the inhibitor binds in a reversible non-competitive manner to the receptor. This is then followed by the receptor reverting to a more stable blocked or desensitised state (Smulders et al., 2004a; Smulders et al., 2004b). However it

is still not known if the organophosphates and carbamates are actually binding to the same site on the receptor or to separate sites which share similar functions. It should be noted however, that the modulation of nAChR function by AChE inhibitors is only reported at concentrations at which there would be total inhibition of AChE (Smulders *et al.*, 2003;Tattersall, 1990). Therefore this effect is likely to be of little clinical importance in the management of casualties.

Whilst the interaction of AChE inhibitors with both types of cholinergic receptor is well reported, there is also evidence that some inhibitors also act directly on non-cholinergic receptors. Soman (1mM, a concentration which completely inhibits AChE) has been reported to inhibit the NMDA-stimulated release of [<sup>3</sup>H]noradrenaline from cortical tissue via stimulation of the glutamate receptor and an increase in K<sup>+</sup> concentration. Similarly soman was also shown to inhibit release mediated by the activation of voltage gated sodium channels. Application of the NMDA receptor antagonists AP5, MK801, and ketamine, along with the NMDA receptor-ion channel blocker Mg<sup>2+</sup> was shown to modify the soman induced inhibition, identifying possible functional sites on the receptor which are modified by the application of soman. As neither atropine nor *D*-tubocurarine blocked this effect, it was concluded therefore that soman interacts directly with some of the modulatory sites on the NMDA receptor-ion channel complex. This study also suggested an interaction between soman and voltage gated sodium channels within the cell membrane (Tang & Cassel, 1998).

OP compounds are also reported to affect the cannabinoid  $CB_1$  receptor, which is prevalent throughout the CNS. Various analogues of methylfluorophosphonate have been shown to act as either partial or full agonists at this receptor (Martin *et al.*, 2000). This is in addition to their ability to inhibit the FAAH and MAG hydrolase enzymes as discussed in section 1.5.4.

#### 1.5.6. Gene expression

Several studies that have investigated changes in RNA (mRNA and total RNA) following exposure to organophosphorus nerve agents (Damodaran *et al.*, 2003;Williams *et al.*, 2003;Blanton *et al.*, 2004). Sarin has been shown to cause upregulation in the amount of AChE mRNA in the rat brain. This was a rapid increase (1-2 hours following exposure) which persisted in the cortex, cerebellum, and hippocampus for 24 hours before returning to pre-exposure levels. In the brainstem, AChE mRNA was found to be upregulated for more than seven days (Damodaran *et al.*, 2003). VX has also been shown to cause a rapid upregulation (1 – 2 hours post exposure) in expression of mRNA for many neuronal genes. This included genes coding for  $\beta 2$  and  $\alpha 6$  subunits of the nAChR, M4 subunit of

the mAChR, and subunits of the GABA<sub>A</sub>, 5-HT<sub>3A</sub>, 5-HT<sub>5B</sub> receptors, as well as the KA2 subunit of the ionotropic glutamate receptor (Blanton *et al.*, 2004). It should be noted however, that none of these studies carried out investigations into the levels of associated proteins. Therefore it is unknown whether the increase in RNA expression results in a functional change in protein expression.

Exposure to soman has been shown to cause an acute inflammatory response within the central nervous system (Williams et al., 2003;Zimmer et al., 1997b;Zimmer et al., 1997a). Following seizures, changes as early as one-hour post exposure can be seen within the brain, particularly in areas prone to seizure generation such as the piriform cortex. Zimmer et al. (Zimmer et al., 1997a) have reported that following a single exposure to soman (77  $\mu$ g/kg i.m, 0.9 x LD<sub>50</sub>) and the resultant seizures, an increase in the levels of the intermediate early gene protein product, c-Fos, could be seen as early as 30 - 45 minutes post exposure within the piriform cortex. By 24 hours post exposure, there were marked neuropathological changes within this area. This group has also shown that astrocytes and microglia within areas of the brain which express elevated levels of Fos post soman exposure such as the piriform cortex, undergo a rapid activation in response to the exposure (Zimmer et al., 1997b). Microglia are considered to be one of the primary immune response effector cells within the central nervous system, and as such a change of this cell type from a resting state to an active (and morphologically different) state can be interpreted as an inflammatory response to an insult. As well as the structural changes seen following a neuro-inflammatory response, changes in gene expression for factors known to be involved in an inflammatory response have also been recorded post exposure to soman. This included an increase in expression of the cytokines tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (Williams *et al.*, 2003). An increase in the expression of IL-1 $\beta$  protein has also been reported in the rat brain post soman exposure (Svensson et al., 2005). This is significant, as these cytokines are known to be involved in the activation of caspases involved in apoptotic cell death (Wang & Shuaib, 2002). Similar changes in the expression of three cell adhesion molecules (E-Selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule (VCAM)) have also reported (Williams et al., 2003).

#### 1.5.7. Other identified targets/effects

As well as the major targets of organophosphorus compounds identified above, other targets and effects caused by exposure to organophosphorus compounds have been identified. As shown in section 1.5.1 one of the main actions of OP compounds is to phosphorylate proteins. Whilst the phosphorylation of AChE and other cholinesterase

enzymes is relatively well understood, less is known about the actions of these compounds on other non serine hydrolase enzymes and proteins. An example of such a protein is the Ca<sup>2+</sup>/cAMP Response Element Binding protein (CREB). CREB is phosphorylated by cAMP-dependent kinases, which are in turn activated by an increase in synthesis of cAMP caused by activation of adenylyl cyclases. Once phosphorylated by these kinases, CREB is transported into the nucleus of the cell where it acts as a transcription factor for many genes. This translocation of CREB has been shown to be controlled (in part) by process mediated by nicotinic receptor activation (Hu et al., 2002). CREB has been implicated in many neuronal events such as synaptic plasticity and also in cell survival during development. Chlorpyrifos oxon has been shown to increase the phosphorylation of this protein (Schuh et al., 2002). It is unknown whether the increase in phosphorylation is deleterious to normal cell functioning, although by altering the ratio of CREB to phosphorylated CREB the transcription of genes necessary for synaptic function may be altered. This may contribute to the cognitive deficits seen following organophosphate poisoning. Alternatively, the increase in phosphorylation may be neuroprotective as an increase in levels of phosphorylated CREB has been seen in neurones post-ischemia (Riccio et al., 1999). Chlorpyrifos oxon may also alter the translocation process by interacting with the nicotinic receptors as previously described.

Exposure to soman (110  $\mu$ g/kg, ~1 x LD<sub>50</sub>) has been reported to cause various changes in both bone marrow and peripheral blood cells of mice that had also been treated with atropine methylnitrate (Collombet *et al.*, 2005). On the first day post poisoning, a significant decrease in the level of peripheral white blood cells and an increase in erythrocytes and platelets was reported. This was followed by a significant decrease on day four post poisoning in the levels of haematopoietic progenitor and precursor cells. This reduction was thought to be a result of a change in the replication rate of these cells. Soman intoxication also caused a later increase in the circulating levels of granulocytes and monocytes. This may correspond with the acute inflammatory response previously described post soman poisoning (Williams *et al.*, 2003).

In conclusion, it can be seen that organophosphorus compounds (and some of the carbamate compounds used for pre-treatment) may potentially have many other farreaching effects which cannot be simply attributed to inhibition of AChE and subsequent elevation of ACh levels within the synapse. Identification of these targets and their relevance to the clinical management of organophosphate poisoning remains a prime objective for research in this area.

### 1.6. Medical therapies

As discussed in the previous section, OP insecticides and nerve agents cause death primarily by inhibiting the AChE resulting in a cholinergic crisis. Many different approaches have been taken in the search for effective medical countermeasures against these compounds, although the vast majority have concentrated on the cholinergic aspect of the poisoning. There are two possible complementary avenues to the medical management of organophosphate poisoning. The first approach is to treat and ameliorate the cholinergic crisis following poisoning. The second uses pre-treatment with drugs that prevent the inhibition of AChE by the OP, which then complements the therapy approach. Both approaches will know be discussed in further detail.

## 1.6.1. Therapy

The primary role of this treatment regime is to counteract the cholinergic crisis and prevent deterioration of the condition of the casualty until more advanced secondary aid can be delivered. The build-up of unhydrolysed ACh following OP exposure results in a massive overstimulation of cholinergic receptors within the synapse causing the signs described previously. It is generally agreed that an anticholinergic drug is the most important part of the post poisoning therapy and atropine is the key drug of use in the clinical treatment of OP poisoned casualties (Eddleston et al., 2008). Atropine is a competitive non-subtype specific muscarinic antagonist that blocks the actions of the excess ACh within both the peripheral and central muscarinic synapses (Yamamura & Synder, 1974). By blocking the muscarinic receptors, atropine ameliorates the major signs of the cholinergic crisis (Douglas & Matthews, 1952), although the nicotinic symptoms such as fasiculations remain unaffected. In addition to this, atropine has been shown to be an effective terminator of OP-induced seizure activity both in vitro (Harrison et al., 2004) and in vivo (Shih & McDonough Jr, 1999;Capacio & Shih, 1991). However there is a time limit to the effectiveness of atropine as an anticonvulsant in organophosphate poisoning treatment. If atropine treatment is delayed beyond 40 minutes post poisoning, the seizures become refractory to treatment with the antimuscarinic drug (McDonough Jr et al., 2000).

The second component of effective post poisoning therapies is an oxime (such as pralidoxime or obidoxime) or oxime-like compound such as HI-6. Oximes are highly nucleophilic chemical compounds that are characterised by a –CH=NOH oxime moiety attached to either one or two bispyridinium rings. Oximes and allied compounds are able to varying degrees of efficiency to reactivate phosphorylated AChE (Smith *et al.*,

1981;Wong *et al.*, 2000). This is dependent upon the nucleophilic properties of the oxime group attached to the pyridinium ring. The oxime group conducts a nucleophilic attack on the phosphorous atom in the OP-enzyme complex, producing an oxime-phosphonate in the process. The formation of this conjugate reopens the active site of the enzyme to begin hydrolysis of ACh as usual. However this reactivation can only occur in OP-enzyme complexes which have not aged (dealkylated see section 1.4). Therefore oxime therapy must be initiated as soon as possible following intoxication. A further complication is that the efficacy of the oxime is dependent upon the OP causing the intoxication (Worek *et al.*, 2007).

Besides the enzyme reactivating effects attributed to these compounds, there is also increasing evidence for beneficial effects which are unrelated to enzyme reactivation (Smith et al., 1981). There are several reports of a direct action of the oxime on the ionchannel of the nAChR. This is characterised as a use-dependent open-channel block (Alkondon et al., 1988; Alkondon & Albuquerque, 1989; Tattersall, 1993). Tattersall (1993) reported that some oxime and non-oxime compounds such as TMB-4 and SAD-128 respectively, were able to exert a direct pharmacological action that was not related to the AChE reactivation normally associated with these compounds. The actions of these compounds were investigated using the phrenic nerve hemi-diaphragm preparation and single channel patch recordings of dissociated muscle cells. It was shown that there was a direct correlation between the neuromuscular recovery of the hemi-diaphragm (as measured by recovery of the tetanic contractions of soman-poisoned diaphragms) and the level of channel block caused by the compounds. In the single channel recordings these compounds were shown to cause a rapid flickering block of the channel openings. This would have the effect of decreasing the mean current flow through the ion channel without altering the burst opening length or channel activation. This then may relieve some of the effects caused by receptor overstimulation following the accumulation of ACh within the synapse post poisoning (Tattersall, 1993). A different mechanism has also recently been put forward. This study found that the noncompetitive block was responsible for restoring the decay of the endplate current allowing repolarisation of the endplate to occur. This is in turn relieved the depolarisation block (Turner, 2007).

The use of a benzodiazepine anticonvulsant (such as diazepam) is indicated for use post OP poisoning. Benzodiazepines act by increasing the mean open time of the chloride channel formed by the GABA<sub>A</sub> receptor (Twyman *et al.*, 1989). The increased influx of chloride ions into the cell increases the hyperpolarisation of the membrane, making the cell less likely to depolarise and fire an action potential. The electrographic seizures seen during the later stages of acute organophosphate intoxication are the result of excessive hypersynchronus discharges from populations of neurons within the brain. Diazepam and

other benzodiazepines have been shown to be effective in controlling the seizures caused by organophosphate intoxication even when administered up to 40 minutes post poisoning (McDonough Jr *et al.*, 2000). As already mentioned in the previous section, the early termination of these seizures is critical for the prevention of neuropathology and enhances the long term prognosis of the casualty (Shih *et al.*, 2003).

#### 1.6.2. Pretreatment

As previously stated, the main target of organophosphates is the AChE enzyme. Inhibition of this enzyme causes the majority of the symptoms of the acute cholinergic crisis seen following poisoning with OP insecticides or nerve agent. Protection of AChE by some means would allow neurotransmission to continue. One approach has been to use a reversible inhibitor of ACh to sequester a pool of the enzyme. The reversible binding of a carbamate to the active site of AChE will prevent the binding of an OP inhibitor to that site, thereby protecting the site from irreversible inhibition. Carbamates such as physostigmine and pyridostigmine bind to the esteratic site of AChE thereby prevent the binding of ACh and the subsequent hydrolysis of the transmitter molecule. In terms of pre-treatment it can then be seen that any circulating nerve agent will also be unable to bind to the active site of the enzyme molecule bound to a carbamate. Therefore a pool of AChE will be protected from the nerve agent challenge. The process of decarbamylation of the enzyme and subsequent hydrolysis of the carbamate inhibitor is relatively slow, and the entire process may take in excess of 4 hours to complete depending on which carbamate was being used. The newly decarbamoylated enzyme would then be free to hydrolyse ACh in the usual manner. It must be remembered that the decarbamolyation/hydrolysis process will be ongoing during the peak concentration of the OP and this helps to maintain a level of AChE, which may be able to hydrolyse the excess ACh present within the synapse. This approach was first demonstrated in 1946 by Koster, who showed that pretreating cats with the carbamate, physostigmine, prior to exposure to di-isopropylflurophosphate (DFP) protected cholinesterase activity post exposure to the OP (Koster, 1946).

Physostigmine has many unwanted side effects however, such as nausea (due in part to its CNS action), which preclude it from effective use as a pretreatment for OP poisoning. Therefore another candidate was required. Pyridostigmine, which has a long clinical history in the management of myasthenia gravis (Mestinon®), has also been found to provide protection against nerve agent-induced lethality when given as a pre-treatment to experimental animals prior to exposure. The current in service provision is that one 31.5mg tablet of pyridostigmine is taken at 8 hour intervals prior to a potential use of nerve agents, or during an attack (maximum of 94.5mg per 24 hour period). The dose is set so that approximately 30% of the AChE enzyme is inhibited (Lennox *et al.*, 1985), this is

much lower than that used to treat myasthenia gravis in which individual doses can range between 30 and 120 mg with a maximum daily dose of 0.3 to 1.2g (Joint Formulary Committee, 2008). Whilst it is highly effective in protecting peripheral enzyme from inhibition by organophosphates, pyridostigmine being a quaternary compound does not penetrate the blood-brain barrier under normal circumstances and therefore is better tolerated with fewer side effects than physostigmine. Pyridostigmine has however, been reported to cross the blood-brain barrier and cause regional brain AChE inhibition in animal models following exposure to stressors such as electrical shocks and forced swim tests (Beck *et al.*, 2003;Friedman *et al.*, 1996). This finding is however extremely controversial however, and there are many other reports showing that stress does not facilitate the penetration of pyridostigmine into the brain (Song *et al.*, 2002;Lallement *et al.*, 1998). Indeed, in some cases, stress may even cause changes within the blood-brain barrier which actually reduce the permeability of the barrier to pyridostigmine (Sinton *et al.*, 2000). It is apparent that the disputed penetration of pyridostigmine into the brain is very much dependent on the species and the nature of the stressor used.

Whilst pyridostigmine is effective at providing protection against the lethal effects of nerve agent exposure, it does little to counter the incapacitation seen post poisoning. Nerve agent induced incapacitation is believed to be caused in part by inhibition of AChE within the central nervous system. Therefore the current research effort is focussing on the use of a centrally active cholinesterase inhibitor such as physostigmine for use as a pre-treatment. Whilst physostigmine was one of the first carbamates to be shown to protect AChE against inhibition, it was found to have undesirable muscarinic side effects. Co-administration of physostigmine with the muscarinic antagonist scopolamine (hyoscine) has been shown to reduce the occurrence of the side effects. This pre-treatment combination has been shown to be highly effective in preventing lethality and minimising the severity of the incapacitation following nerve agent exposure (Miller *et al.*, 1993;Wetherell *et al.*, 2002).

Whilst pre-treatments such as pyridostigmine are highly effective in helping to protect AChE against the actions of nerve agents, they are only effective if given prior to poisoning and a sufficient period of time has elapsed to allow the drug to have an effect. If neither of these factors have occurred then the pre-treatment regime is of little or no use (Gordon *et al.*, 1978).

### 1.6.3. Current and future nerve agent therapies

Currently, the UK armed forces are equipped with pyridostigmine bromide (31.5mg) as a pre-treatment (Figure 1.10, Nerve Agent Pre-treatment Set (NAPS)), and a triple

component emergency therapy packaged within an auto-injector (Figure 1.11, Combopen®). The UK Combopen contains 2mg atropine, 500mg pralidoxime methanesulfonate (P2S), and 10mg avizafone. NAPS is taken prior to a potential nerve agent attack, with the Combopens used to counteract the effects of nerve agent poisoning and prolong life until the casualty can be removed to a clinical environment where secondary supportive care can be initiated. Avizafone is a water-soluble lysine conjugate pro-drug of diazepam which allows the three components of the therapy to be prepared in water, making the Combopen easier to store and use.

The development of effective medical countermeasures to nerve agent poisoning is an ever evolving process. The ultimate goal is a single shot therapy which has little or no reliance on pretreatment and which protects against both the lethal and incapacitating effects of nerve agents. The possible use of physostigmine as a component of the next generation of nerve agent therapies has once again been revisited. Whilst the current therapy is highly effective at preventing nerve agent induced lethality, there is still a level of incapacitation. Physostigmine when given in conjunction with scopolamine as a pretreatment is more effective at preventing incapacitation and lethality in guinea pigs challenged with a variety of nerve agents (Wetherell et al., 2002; Miller et al., 1993). The combination of scopolamine and physostigmine with the oxime HI-6 in a post poisoning therapy has also been evaluated. This combination also alleviated much of the incapacitation seen post subcutaneous poisoning (Wetherell et al., 2007;Wetherell et al., 2006). The addition of physostigmine to an already AChE compromised system would at first seem counterintuitive. It is hypothesised that the addition of scopolamine counters the central muscarinic effects of the cholinergic crisis until such point as physostigmine protected AChE is released. The oxime HI-6 is postulated to have two distinct actions. Firstly it regenerates inhibited AChE, and secondly it may have direct actions on the nAChR thus alleviating the neuromuscular block seen post nerve agent poisoning (Tattersall, 1993). The structures of existing and putative replacement compounds for treating nerve agent poisoning are shown in Figure 1.12.



#### Figure 1.10. Nerve agent pre-treatment set (NAPS).

This is currently in service with the United Kingdom armed forces (NAPS L1A1). This is a set of 21 tablets each containing 31.5 mg pyridostigmine bromide. The individual would commence taking the tablets when ordered to, based upon intelligence about the likelihood of contact with a nerve agent.



### Figure 1.11. The Combopen (L4A1).

This is the current in-service medical countermeasure for use post-exposure to nerve agents. It is an autojector device which contains 2mg atropine, 500mg P2S, and 10mg avizafone (lysine-conjugated diazepam) to be administered by intra-muscular injection. The top Combopen shows the device in its stored state. Before use, the grey safety cap must be removed (middle Combopen). The Combopen is now armed and is deployed by pushing the black end of the pen firmly against the thigh and holding it there until the injection is complete (5 seconds). The bottom Combopen shows the spring-loaded injection needle that is exposed during the injection.



Figure 1.12. Structures of current and proposed components of nerve agent pretreatment and therapy treatment sets.

## 1.7. In vitro models

The aim of this study is to develop an *in vitro* model which will be used to investigate the central actions of OP AChE inhibitors. Currently, there is a paucity of good models in which to study these actions. The selection of possible models for this study has been based on a number of criteria. These included ease of preparation of the model, the model having a good intrinsic cholinergic network, the model exhibiting a well-defined cholinergic response that can be modulated by anticholinesterases (preferably without the need to apply exogenous cholinergic agonists). The ability of anticholinesterase compounds to modulate the response recorded from the model is important as this more closely mimics the normal functioning of the brain, relying on existing levels of the endogenous cholinergic agonist rather than the effects of exogenous agonists artificially applied. The final requirement of a suitable model is the possible involvement of the area

in either incapacitation or lethal effects of organophosphate poisoning. This section will look at several candidate models in more detail.

## 1.7.1. Supraoptic Nucleus

The supraoptic nucleus (SON) is situated at the base of the forebrain and is responsible for controlling the release of vasopressin and oxytocin. Intracellular recordings from this area have shown that nAChRs mediate synaptic potentials elicited by paired pulse stimulation (Hatton & Yang, 2002). The synaptic potentials were blocked by methyllycaconititine (MLA) and  $\alpha$ -bungarotoxin ( $\alpha$ -BGT), both selective for neuronal  $\alpha$ 7 nAChR at the concentrations used, whereas dihydro-β-ethroidine (DHβE), an antagonist of  $\alpha 4\beta 2$  nAChR, had no effect. Hatton and Yang also reported dense  $\alpha$ -BGT binding in dendritic layer and glial lamina of the SON, suggesting a dense population of nAChRs (primarily  $\alpha$ 7). ACh when applied to neurones within this nucleus has also been shown to decrease the inhibition of the local network mediated by the release of GABA. ACh (100µM in the presence of TTX and CNQX) significantly decreased the frequency of miniature inhibitory postsynaptic currents (mIPSC) occurrence with no change in the amplitude of the events. The ACh induced decrease in mIPSCs was not affected by the addition of atropine and therefore was not mediated by mAChR, but it was blocked by the non-specific nAChR antagonist mecamylamine. Mecamylamine had no effect by itself on the appearance and kinetics of the mIPSCs (Li et al., 2001).

As a model, the SON allows a fast nicotinic response to be investigated in a slice that also retains the cholinergic input from the diagonal band of Brocca. Similar nicotinic responses may be present elsewhere in the brain in structures more involved in incapacitation, but are not as amenable to slice recording.

## 1.7.2. Frontal Cortex

The frontal neocortex has been exploited in much the same way as the work conducted in the SON as previously described. Using whole cell patch and intracellular recording techniques, a nAChR-mediated fast synaptic potential can be recorded from layer II/III pyramidal neurones. This was resistant to blockade by both excitatory amino acid (EAA) antagonists and the GABA<sub>A</sub> antagonist, bicuculline methyliodide (BMI). Currents evoked by electrical stimulation were reduced by around 50% in the presence of either 100 $\mu$ M DH $\beta$ E or 50 $\mu$ M MLA. The anticholinesterase neostigmine (10  $\mu$ M) prolonged the decay time constant of the EAA-resistant EPSC. The evidence presented all points to a nAChR controlling this current, although the exact identity of the nAChR cannot be deduced due

to the high concentration of DH $\beta$ E used. It has also been suggested that the potentials recorded may in fact originate from more than just one excitatory transmitter system and may not necessarily solely be mediated by the nicotinic acetylcholine receptor (Chu *et al.*, 2000).

#### 1.7.3. Nucleus Accumbens

The nucleus accumbens (NAcc) forms part of the ventral striatum and acts as an interface between many portions of the limbic system, including the hippocampus, prefrontal cortex and the amygdala. The NAcc has a primary output into the adjacent ventral pallidum, which is involved in the activation and regulation of voluntary movements. The amplitudes of glutamatergic EPSCs recorded from the NAcc were decreased when ACh or carbachol was bath applied in the presence of BMI. Conversely, in the presence of atropine, the application of ACh resulted in an increase in the amplitude of the glutamatergic EPSC. This was mimicked by the application of DMPP (a nicotinic agonist) and blocked by mecamylamine (Zhang & Warren, 2002). A combined slice incorporating the NAcc and the forebrain has also been used to investigate spontaneous epileptiform activity (caused by the application of BMI or picrotoxin) originated in the cortex (most likely from the adjoining piriform cortex) (Buckby & Lacey, 2001). Whilst this does not truly meet the requirements for a suitable slice model for the current project, it is interesting because it demonstrates a biphasic cholinergic effect on the main excitatory glutamatergic drive of this brain region. This may be important in the secondary phase of nerve agent induced seizures, in which it is known that other neurotransmitter systems (including glutamate) are recruited to maintain the epileptiform activity (McDonough Jr & Shih, 1997).

## 1.7.4. Substantia Nigra

The substantia nigra (SN) forms part of the basal ganglia within the mammalian brain, and is involved in motor control. Structurally it can be split into two adjoining areas: the substantia nigra pars reticulata (SNr) and the substantia nigra pars compacta (SNc). The SN receives cholinergic inputs from the pedunculopontine nucleus and has projections to the striatum. Neurones within the SN are lost during the progression of Parkinson's disease leading to the overt motor control problems that are symptomatic of the disease. A horizontal slice preparation which contained the SN and other surrounding structures has been used to investigate the effects of applied nicotine on the release of dopamine from neurones within the SN (Matsubayashi *et al.*, 2003;Matsubayashi *et al.*, 2004a;Matsubayashi *et al.*, 2004b;Yu *et al.*, 2000). Nicotine, when bath applied to dopaminergic neurones that are held in the whole cell patch current clamp configuration,

caused a depolarisation of the membrane potential. This resulted in an increase in spontaneous firing of the cell, which was independent of the external calcium ion concentration. The inward current seen during the application of nicotine could be blocked by the application of DH $\beta$ E showing that the current was mediated by the  $\alpha 4\beta 2$  nAChR (Matsubayashi *et al.*, 2003). Spontaneous miniature inward postsynaptic currents were also recorded from dopaminergic neurones within the SN. In the presence of atropine (mAChR antagonist), BMI (GABA<sub>A</sub> antagonist, I-glutamic acid diethyl ester (GDEE, a non-selective glutamate antagonist), and TTX it was shown that application of either MLA or DH $\beta$ E could reduce this activity, demonstrating that the activity was mediated by  $\alpha$ 7 and  $\alpha$ 4 $\beta$ 2 nAChRs, respectively (Matsubayashi *et al.*, 2004a). The same group have shown by single-cell polymerase chain reaction (PCR) that the mRNAs coding for  $\alpha$ 4 $\beta$ 2 and  $\alpha$ 7 nAChRs are present within the dopaminergic neurones of the SN (Matsubayashi *et al.*, 2004b).

#### 1.7.5. Hippocampus

The hippocampus is a part of the limbic system that lends itself well to in vitro studies as it has a well-defined structure with good delineation of cellular areas. It is thought to be involved in the encoding of memory (Teyler, 1987;Bliss & Collingridge, 1993), and is involved in some epileptiform seizures (Coulter, 1999). At present the hippocampal slice is the primary in vitro model used to study the effects of nerve agents (Wood & Tattersall, 2001;Chebabo et al., 1999;Harrison et al., 2000;Harrison et al., 2004) on the central nervous system. It has also been widely used as a neurotoxicity screen (Fountain et al., 1992), to study the cellular mechanisms of memory (Yamamoto & Chujo, 1978;Bliss & Collingridge, 1993) and epileptogenic seizure activity (Rutecki et al., 1985;Bradford, 1995). The hippocampus whilst not having an intrinsic cholinergic pathway, does receive cholinergic innervation from the septal nucleus and the diagonal band via the fimbria (Lewis et al., 1967;Liu et al., 1998), as well as via the entorhinal cortex (Colgin et al., 2003). During preparation of the slice however, the majority of this innervation is severed leaving only the distal terminals of the cholinergic afferents from the septal nucleus and the diagonal bandransection of this septo-hippocampal pathway in vivo, causes the almost complete loss of acetylcholinesterase and ChAT within the hippocampal formation(Lewis et al., 1967). Although the main cholinergic innervation is lost, there are still cholinoceptive cells that remain viable within the slice. The majority of these are GABAergic inhibitory interneurones that synapse onto either other interneurones, or onto the excitatory glutamatergic pyramidal cells. The interconnectivity of the inhibitory interneurones provides a complex modulation of the output of the local neuronal population (Buhler & Dunwiddie, 2001). It has been shown that nicotinic acetylcholine

receptors (especially  $\alpha$ 7) can be found in clusters on hippocampal interneurons (Kawai *et al.*, 2002). Studies have also shown that it is the  $\alpha$ 7 nAChR which is largely responsible for the cholinergic modulation of the inhibitory interneuron network, and has been shown to be involved in the inhibition and disinhibition of the pyramidal cells (Ji & Dani, 2000). The  $\alpha$ 4 $\beta$ 2 nAChR has also been reported to modify the level of inhibition of the GABAergic interneurones to a different degree than that caused by  $\alpha$ 7 nAChR activation (Alkondon & Albuquerque, 2001). It has also been shown that a small population of exclusively cholinergic interneurones exists within the rodent hippocampus, although the exact function of these neurones is still unknown (Frotscher *et al.*, 2000).

Organotypic slices of the hippocampus have been developed which allow longer-term studies to be conducted (Gähwiler, 1988); however, the organotypic hippocampal slice shares the same major shortcoming as the acute slice model, namely that the cholinergic (and GABAergic) afferent innervation is severed during preparation and undergoes subsequent axonopathy during culturing. The loss of the cholinergic afferents has been overcome by co-culturing an explant of the septal nucleus with the hippocampus. This has resulted in a viable cholinergic (Gähwiler & Brown, 1985) and GABAergic (Heimrich *et al.*, 1996) reinnervation of the hippocampus, mimicking the *in vivo* septo-hippocampal pathway.

Another major part of the hippocampal formation is the dentate gyrus. This area is innervated mostly by the medial and lateral perforant pathways arising in the adjoining entorhinal cortex, and also by afferents arising in the septal nucleus. The principal cell type of the dentate gyrus is the granule cell. The majority of the granule cell bodies are located in the granule cell layer of the dentate gyrus with their dendrites extending throughout the molecular layer, although ectopic granule cells have also been reported in the polymorphic layer of the dentate gyrus (Scharfman et al., 2003;Scharfman et al., 2007). Granule cells have been shown to release glutamate following their stimulation by the afferent fibres of the perforant path (Scharfman et al., 1990). There is also a body of evidence showing that these cells also express GAD (required for the synthesis of GABA) during early development stages, with the expression of the GABAergic phenotype decreasing rapidly during the maturation of the dentate gyrus. Also, the expression of the GABAergic phenotype can be upregulated by events such as seizures (Gutiérrez, 2003). The dentate gyrus has a long history of being used as a model to study complex activity within the brain. An example of this is long term potentiation (LTP) which is thought to underlie some of the processes involved in memory formation and was first demonstrated in the perforant pathway (Bliss & Lomo, 1973). It has been reported that the evoked response (glutamatergic) of cells within the molecular layer of the dentate gyrus can be modulated by application of the AChE inhibitor physostigmine (Colgin et al., 2003).

Application of  $5\mu$ M physostigmine caused a decrease in the magnitude of the fEPSP amplitude and slope that persisted after washing off the inhibitor. The effect of physostigmine was blocked by atropine showing that the effect was mediated by a muscarinic cholinergic receptor. Similarly, incubation with the cannabinoid CB<sub>1</sub> receptor antagonist AM251 prior to the application of physostigmine also prevented the decrease in response. Interestingly, the decrease in the fEPSP following the application of physostigmine was not observed in slices taken from animals that had received lesions of the septal-hippocampal pathway. This implies that the cholinergic input must arise from the septal nucleus/diagonal band complex (Colgin *et al.*, 2003). This is therefore an endogenous cholinergic response which can be modulated by anticholinesterases, and therefore fulfils the criteria as laid out in the introduction to this section.

## 1.8. Model selection

Historically the actions of organophosphorus compounds such as nerve agents have been mainly studied using *in vivo* models and *in vitro* models utilising peripheral nerve-muscle preparations. In contrast to this there are very few good *in vitro* models of central actions of nerve agents: *in vitro* studies of cholinergic systems have almost exclusively used application of an external cholinergic agonist such as carbachol to elicit the cholinergic response rather than rely on an innate source. In order to study the effects of cholinesterase inhibitors, a suitable model should have an intrinsic cholinergic response. Alternatively, a suitable model should exhibit an effect that is in direct response to the application of nerve agent. In this study, the hippocampal slice, specifically the dentate gyrus will be used.

#### 1.8.1. Approach and selection

Due to the nature of the funding of this PhD and the associated customer requirements (the work was conducted as part of a customer programme for the Defence Technology and Innovation Centre), a pragmatic approach to the selection of the model was taken to increase the likely success of this project. Factors for consideration include the recording techniques required, intricacy of the preparation of the slice, stability, and the number of slices obtained per animal (acute slices only). The quality and magnitude of the reported cholinergic effect was also considered. Ideally the selected model would be relatively easy to prepare, stable over many hours, with multiple slices being obtained from each animal allowing maximum benefit from its use. The models described in 1.7 were assessed against these criteria.

The supraoptic nucleus slice model was discounted as it required exogenous cholinergic agonists to be applied to show a full response. Also due to the size of the structure, only one slice can be reliably produced per animal which increases the technical risk. This would also increase the number of animals used during the study. In contrast, the size of the NAcc and SN structures allow several slices to be prepared from each animal. This has the obvious benefit of reducing the total number of animals used. Again, both of these models rely on the application of exogenous cholinergic compounds to elicit a response and so were discounted. The frontal cortex model has the benefit of allowing many preparations to be obtained from a single animal. Also the nicotinic responses as described can be elicited by electrical stimulation and not by applied exogenous agonists which is again one of the criteria for selection. As such this would make the frontal cortex a suitable model for this project. However the hippocampus was chosen in preference due to its well documented anatomical structure and pathways.

The dentate gyrus model has been shown to retain an intrinsic cholinergic response (Colgin *et al.*, 2003), which allows the classical effects of nerve agents i.e. inhibition of AChE, to be studied in a CNS preparation without the need to employ exogenous ACh. The hippocampal slice is also relatively easy to produce with many being obtained from a single animal thereby reducing the total number of animals used. Previous work has reported that the intrinsic response could be measured using extracellular recording techniques (Colgin *et al.*, 2003). This indicates that the dentate gyrus is well suited to the proposed investigation. For these reasons, the dentate gyrus slice model will be used during this study.

#### 1.9. Aim of the study

As stated in section 1.6.3, physostigmine is once again being investigated as a component of the next generation of nerve agent therapies. Physostigmine has shown promise as both a pretreatment and post poisoning therapy when used in conjunction with scopolamine. However, the mechanism by which physostigmine is exerting its effect is unclear.

This project will test two hypotheses. Firstly, it is hypothesised that physostigmine is an effective pretreatment for nerve agent poisoning and secondly, that physostigmine acts by sequestering a pool of AChE and protecting it from subsequent irreversible inactivation by nerve agent.

To allow both hypotheses to be tested, the dentate gyrus model has been developed and the cholinergic response characterised. Initially the project sought to confirm the findings reported previously in the rat dentate gyrus (Colgin *et al.*, 2003). After confirming the results of Colgin *et al.*, subsequent experiments were conducted in dentate gyrus slices from the guinea pig. The guinea pig has been identified previously as being more suitable than the rat for predicting the efficacy of nerve agent treatments in primate species (Dirnhuber *et al.*, 1979;Berry & Davies, 1970;Inns & Leadbeater, 1983;Gordon *et al.*, 1978). One reason for this could be due to species differences in the activity levels of the enzyme carboxylesterase. It has been reported that the activity of this enzyme in rat tissues is consistently higher than that measured in guinea pigs and marmosets (de Jong *et al.*, 1993). In this regard the guinea pig is closer to man (Li *et al.*, 2005), and is therefore considered to be a better model of nerve agent toxicity. This is important as carboxylesterase binds to OP compounds and nerve agents in a similar manner to AChE. Higher levels of activity of this enzyme will therefore mask the true extent of OP-AChE interactions.

Following successful development of the model in the guinea pig dentate gyrus slice, the interactions between physostigmine and the nerve agent sarin were investigated. The dentate slice model is better suited to investigate these interactions than a whole animal model, due to the level of control the experimenter has over the available concentrations of both compounds. The importance of the time of application of physostigmine relative to sarin and concentration of physostigmine was investigated and a correlation was established between the electrophysiological response and tissue ChE activity in slices treated with nerve agent.

2. Materials and Methods

# 2.1. Dentate gyrus slice model

## 2.1.1. Animal welfare

All procedures involving the use of animals were carried out under a relevant Home Office licence and in accordance with the Animals (Scientific Procedures) Act 1986.

# 2.1.2. Rat hippocampal slices

Male Porton-Wistar rats (130–260g, Animal Services, Dstl Porton Down) were anaesthetised with halothane and killed by decapitation. The brain was then cleared of overlying meninges and dissected free from the skull. Upon removal the brain was placed into an ice cold cutting solution artificial cerebrospinal fluid ( cutting ACSF) containing in mM, 189 Sucrose, 3 KCl, 5 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 0.1 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 10 D-glucose (gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub>). The brain was then hemisected into two blocks of tissue (one for each hemisphere). Horizontal slices (300 µm thickness) through the tissue block were then prepared using a Vibratome (Intracel, Royston, UK) whilst bathed in ice cold cutting ACSF. Slices were then stored in a holding chamber (BSC-PC, Medical Systems Corp) at room temperature in an ACSF solution, containing in mM, 120 NaCl, 3 KCl, 1.3 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 10 D-glucose, (gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub>). This solution was also used as the perfusate during recordings.

# 2.1.3. Guinea pig hippocampal slices

Male Dunkin Hartley guinea-pigs (300-400g, Harlan UK) were anaesthetised with halothane and killed by decapitation. The brain was the removed as described for the rat hippocampal slices. Both hippocampi were then dissected free from the surrounding cortical tissue. Each hippocampus was then trimmed at both the septal and temporal extremities leaving a central block of the hippocampus. This was then mounted onto a vibratome chuck and 300µm thick transverse slices were cut using a Vibratome. All other details for the preparation of the guinea-pig hippocampal slices were identical as those detailed above for preparation of rat hippocampal slices. The unused hippocampus was retained for subsequent cholinesterase activity assays.

### 2.1.4. Extracellular recording

Slices were left to recover for at least 1 hour before being transferred to a recording chamber (Zbisc chamber, Medical Systems Corp., New York, USA), where they were submerged and continually superfused (10 – 15 ml min<sup>-1</sup>) with gassed recording ACSF at 31℃ (±0.5℃, temperature controlled using an inline heat er (SH-27B Warner Instrument Corp., USA). During equilibration, baseline and drug applications the perfusing ACSF was recirculated through the bath. Following a further equilibration period of 30 minutes, a bipolar stimulating electrode (MCE-100, Harvard Apparatus, UK) was placed within the molecular layer of the dentate gyrus in a position approximate for the perforant pathway. A single glass extracellular recording pipette electrode (GC-150F (Harvard Apparatus, Edenbridge, UK) pulled using a Sutter P-97 Flaming/Brown micropipette puller (Sutter, Novato, Ca., USA) was also placed within the molecular layer of the dentate gyrus (approximate positions shown in Figure 2.1). The extracellular electrodes had tip resistances of between 2 and  $9M\Omega$  when filled with 2M NaCl. The slice was stimulated once every 30 seconds (pulse width 200 µsec, amplitude 180 - 300µA) and the amplitude of the evoked fEPSP was maximised by lowering the recording electrode into the slice until the response was maximal. Following optimisation of the response, the threshold and maximal stimulation currents were determined.

Using paired pulse stimulation (interpulse interval 50ms, stimulation current 90-150µA) the portion of the perforant pathway being stimulated was identified. It has been shown that paired-pulse stimulation of the medial portion of the perforant pathway results in an inhibition of the second evoked fEPSP. Conversely, if the lateral perforant pathway is being stimulated a facilitation of the second evoked fEPSP is seen (McNaughton & Barnes, 1977). The medial perforant pathway was used exclusively during these studies as it had been shown previously to be more sensitive to the actions of anticholinesterase compounds (Colgin *et al.*, 2003)

Following identification of the portion of the perforant path, the slice was stimulated every 30 seconds with stimulus amplitude that gave a response of approximately 70% of the maximal response previously determined. Stimulating the slice in this manner facilitated the production of stable fEPSPs. Baseline fEPSPs were recorded for at least 30 minutes before any compounds that were under investigation were applied to the slice. Slices which exhibited a  $\pm 10\%$  change in fEPSP amplitude during the baseline period were excluded from future analysis.



# Figure 2.1. Position of the stimulating ( $\bullet$ ) and recording electrode (\*) within the dentate gyrus.

This positioning allows stimulation of the medial perforant pathway and also recording of fEPSPs from the dendritic field of the molecular layer of the dentate gyrus.

# 2.1.5. Drug application

All compounds were added to the recording solution reservoir and allowed to perfuse for the required time. This solution was recirculated unless otherwise stated. Test compounds were removed by perfusing the slice with prewarmed and gassed drug-free ACSF. This wash solution was not recirculated and was allowed to drain into a collection vessel for disposal. Cumulative dosing was not used unless otherwise stated.

Time matched controls and vehicle controls were also carried out throughout the studies and were interleaved with appropriate treatment groups. For vehicle controls a concentration of the vehicle (dimethyl sulfoxide (DMSO) or ethanol), equivalent to that used to dissolve the maximum concentration of drug used was added to the reservoir. The maximum final bath concentration of the vehicles used was 0.06% DMSO and 0.10% ethanol.

# 2.1.6. Data capture and analysis

The programme LTP (v.2.30D, <u>www.ltp-program.com</u> (Anderson & Collingridge, 2001)) was used to control the stimulus, capture data and perform on- and off-line analysis. Data were captured at 10 kHz via an ADC-42 interface board (Pico Technologies Ltd, St. Neots, UK). Amplification of the signal was carried out using an AC-DC amplifier (Neurolog NL106, Digitimer, Welwyn Garden City, UK) and an Intra 767 electrometer (World Precision Instruments, Stevenage, UK) with a final gain of 500x DC. The amplitude of the stimulus was set using a NeuroLog NL510 system (Digitimer). Both the NL106 and NL510 were housed in a Neurolog NL900D (Digitimer).

The LTP software program was used to measure two parameters of the fEPSP during data capture and off-line analysis. The first parameter measured was the amplitude of the fEPSP defined as the maximum deflection between the two time points approximate for the start and end of the fEPSP (typically measured between 1 and 20ms after the start of the stimulus). This is shown in Figure 2.2. The second parameter was the initial slope of the fEPSP. This was done by fitting a line to the computer generated representation of the captured fEPSP. An example is also shown in Figure 2.2. The slope of the fEPSP was measured as this can be interpreted as a good measure of synaptic strength. The initial slope is also less likely to be contaminated by other currents (such as those produced by feed forward GABAergic inhibitory interneurones) which may reduce the amplitude of the response. Data were then exported into Microsoft Excel 2003 (Microsoft Corporation, USA) and then into GraphPad Prism v.4.00 for Windows (GraphPad Software, San Diego, USA, www.graphpad.com) for further analysis.



Figure 2.2. Example of a fEPSP and the parameters measured using LTP.

(A) amplitude is measured between the maximum extent of the negative spike and a datum drawn across the top of the deflection. (B) the slope of the initial negative component of the fEPSP is measured as shown. Vertical scale bar 1.0mV, horizontal scale bar 25ms.

## 2.2. Cholinesterase assay

Guinea-pig hippocampi were used to determine the profile of inhibition of brain cholinesterases by the nerve agent sarin. Whole hippocampi were removed as described in section 2.1.3. The tissue was then chopped using a McIlwain tissue (Mickle Laboratory Engineering Co. Ltd, Gomshall, UK) chopper to a thickness of approximately 300µm. The chopped tissue was then held in a mesh bottomed chamber which was suspended in a known volume of recording ACSF. This solution was held at room temperature and was gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. Sarin was then added to the ACSF to give the required final concentration (either 0.01, 0.03, 0.10, 0.30 or 1.00µM). This was left in the chamber for 10 minutes, after which time the ACSF was exchanged for sarin-free ACSF. After another 2 minutes, the hippocampal tissue was frozen at -80°C until required for assay. Prior to assay samples were allowed to thaw out at room temperature for 30 minutes. Each tissue sample was homogenised in a volume of pH8 phosphate buffer to give a 10mg/ml tissue suspension, using a motorised pestle homogeniser (Heidolph, Wakefield, UK).

The assay used to determine the activity of cholinesterase is based on that described by Ellmann (Ellman *et al.*, 1961). Briefly acetylthiocholine is hydrolysed by active cholinesterase to yield thiocholine. This reacts with dithiobis(nitrobenzoate) (DTNB) to yield 5-thionitrobenzoate. The rate of production of this reaction product is then measured using a spectrophotometer at 412nm. For this assay 500µl of 3mM acetylthiocholine was added to 500µl of 250µM DTNB in a 2.5ml cuvette. 400µl of pH 8 phosphate buffer was then added before 100µl of the 10mg/ml tissue sample was added. The cuvette contents were then mixed thoroughly. The rate of reaction was measured at 412nm using a spectrophotometer (Libra S22, Biochrom Ltd, Cambridge, UK) set to record the absorbance at 10 second intervals over a 180 second run time. Each sample was run in triplicate and each concentration of sarin was repeated twice. Control slices which received no sarin were also carried out. The reaction rates obtained from this protocol were then corrected for the amount of protein present in the sample. The amount of protein was determined using the protocol in section 2.3. All assays were conducted at room temperature.

## 2.3. Protein determination

The amount of protein present within the 10mg ml<sup>-1</sup> tissue homogenate sample was determined using a modified Lowry assay (Lowry *et al.*, 1951). A detailed description of the method used is included in appendix A. The absorbance of the reaction product was measured at 700nM using a spectrophotometer (Libra S22). A standard curve using

known concentrations of protein (bovine serum albumin) was constructed to determine the amount of protein in the tissue homogenates.

# 2.4. Drug and reagent supply

The nerve agent sarin was synthesised by the Detection Department, Dstl Porton Down. This was supplied as a 5mg/ml solution diluted in isopropyl alcohol. The purity of this solution was ≥98.0%. A 1mM stock solution was made immediately prior to use and was diluted as required with a small volume of the recording ACSF.

All other drug compounds and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) with the exception of AM251 (Tocris Bioscience, Bristol, UK). Drug compounds were prepared using a suitable solvent (distilled water with the exception of picrotoxin (ethanol) and AM251 (DMSO) to form a stock solution. This was then frozen in aliquots at -20°C until required. Subsequent dilutions were made as required from this stock solution to obtain the desired final bath concentration of the compound.

# 2.5. Statistical analysis

Data are presented as the mean value  $\pm$  standard error of the mean (sem). Statistical analyses were performed using Graphpad Prism v4.00 for Windows. The tests conducted are stated in the results section of the following chapters. Statistical significance was determined when the value for the probability (*p*) was less than 0.05.

3. Characterisation of the dentate gyrus slice model

### 3.1. Introduction

This series of experiments planned to test the hypotheses that physostigmine is an effective pretreatment for nerve agent poisoning and secondly, that physostigmine acts by sequestering a pool of AChE and protecting it from subsequent irreversible inactivation by nerve agent. The study by Colgin *et al.*, (Colgin *et al.*, 2003) will be used as a basis for the set of experiments.

The dentate gyrus slice model was chosen for the reasons described in section 1.8.1, namely because it retains an innate cholinergic response after preparation and that this preparation is best suited to the planned study. The dentate gyrus is a part of the hippocampal formation, along with Ammon's horn areas 1-3 (cornu Ammonis (CA1-3)), entorhinal cortex, and the subiculum (see Figure 3.1). The dentate gyrus receives neuronal inputs from many different structures including other areas of the hippocampal formation, medial septal nucleus, thalamus, hypothalamus and the brainstem. There are also commissural projections to the contralateral dentate gyrus (for an in depth review see (Patton & McNaughton, 1995). The dentate gyrus can be divided into three areas. These are the molecular layer (further divided into the inner, medial, and outer thirds), granule cell layer and the hilus (also sometimes referred to as the polymorphic layer) and are arranged as shown in Figure 3.1.

One of the major inputs into the dentate gyrus is the perforant pathway from the entorhinal cortex. This originates in layers II and IV-VI of the entorhinal cortex. The perforant pathway has been shown to be mainly excitatory with glutamate as the main transmitter. The afferent fibres of this pathway terminate mainly in the outer two thirds of the molecular layer of the dentate gyrus, where they form synapses with the dendrites of the principle granule cells and also with the inhibitory basket cell interneurons. This pattern of innervation allows for fine control over the excitability of the granule cell population of the dentate gyrus. Whilst the majority of the main cholinergic projection from the medial septal nucleus enters the hippocampus via the fimbria, a small proportion of the afferent fibres directly innervate the dentate gyrus.

Electrical stimulation of the perforant pathway elicits fEPSPs which can be recorded by extracellular means in the molecular layer of the dentate gyrus. The magnitude of this response has been shown to be sensitive to the application of muscarinic antagonists and inhibitors of AChE (Melchers *et al.*, 1994;Colgin *et al.*, 2003). Loss of the septal cholinergic input to this area results in a decrease in the expression of AChE with in the dentate gyrus (Colgin *et al.*, 2003).

The experiments in this chapter have been devised to broadly characterise the response elicited in the dentate gyrus. For this reason, the majority of the experiments are conducted with only one concentration of agonist or antagonist. The concentrations used were chosen as they have been shown previously in the literature to be effective. They are also near maximal effective concentrations and in some cases may show less selectivity for certain receptor subtypes at the concentration used.



#### Figure 3.1. The hippocampal formation.

The dentate gyrus (DG) is comprised of three distinct layers, the molecular layer (m) in which the apical dendrites of the granule cells terminate, the granule cell layer (g) which contains the cell bodies of the granule cells, and the polymorphic layer or hilus (p) which contains the basal dendrites of the granule cells. The rest of the hippocampal formation is further divided into areas CA1, CA2 and CA3. These areas can be further subdivided into the stratum oriens (so), stratum pyramidal (sp) which contains the cell bodies of the pyramidal cells, stratum radiatum (sr) and the stratum lacunosum-moleculare (sl-m). Also shown is the fimbria (F) which contains the major afferent fibres into the hippocampus from structures such as the medial septal nucleus. The subiculum (Sub) which receives a major output from the hippocampus is also shown.
# 3.2. Methods

Rat and guinea pig hippocampal slices were prepared as previously described in section 2.1.2 and 2.1.3 respectively. All test compounds were prepared as described in section 2.4. Control slices were conducted at various points throughout the study and interspersed with treatment groups where appropriate.

Data recorded from each slice were manipulated as follows. The values recorded for each parameter during the 10 minute period immediately prior to the application of the first test compound were averaged (mean). This was used as a baseline value to which all data points were then normalised. Data are presented as percentage changes from this value. Data recorded during the application of test compounds and recovery were averaged into 1 minute bins. This smoothed the data removing any rapid fluctuations (sub 30 second) from the general trend. Recovery of the response was measured during two epochs following the wash out of the test compound(s). These were between 10 and 20 minutes and between 20 and 30 minutes post wash out. The level of recovery was determined as the maximum response during each epoch compared to the maximum decrease recorded during the application of the test compound.

#### 3.3. Effect of reversible inhibition of AChE by physostigmine

In the first series of experiments (Figure 3.2), eight rat dentate slices were exposed to a single 30 minute application of 5 $\mu$ M physostigmine followed by a period of washing with drug free ACSF. The application of physostigmine caused a rapid significant decrease in the amplitude of the evoked fEPSP to between 78.9 and 90.3 % of baseline (mean decrease to 85.7 ± 1.9 % of baseline, p<0.001 (unpaired t-test)). Similarly, application of 5 $\mu$ M physostigmine caused a significant decrease in the slope of the evoked fEPSP to between 78.1 and 88.8 % of baseline (mean decrease to 84.3 ± 1.8 % of baseline, p<0.01 (unpaired t-test)). The decrease was reversed by washing with fresh ACSF (mean amplitude recovery to 94.5 ± 3.2 % of baseline during 10-20 minute epoch (p<0.01), and to 96.2 ± 3.5 % of baseline during the 20-30 minute epoch (p<0.01). Recovery was also seen in the magnitude of the fEPSP slope at both epochs (94.6 ± 3.7 % of baseline (10-20 minute epoch, p<0.01) and 95.3 ± 3.9 % (20-30 minute epoch, p<0.01).





Figure 3.2. Effect of  $5\mu$ M physostigmine on perforant path responses recorded from the molecular layer of the dentate gyrus in the rat hippocampal slice.

(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  physostigmine treated slices n = 8,  $\blacksquare$  time matched controls n = 4, data presented as mean  $\pm$  S.E.M). Example traces are shown above. Traces i-iii are taken during baseline, exposure, and wash from control slices. Traces iv-vi are taken during baseline, exposure to 5µM physostigmine, and wash from exposed slices (vertical scale bar 1mV, horizontal scale bar 100ms).





# Figure 3.3. Effect of $5\mu$ M physostigmine on perforant path responses recorded from the molecular layer of the dentate gyrus in the guinea pig hippocampal slice.

Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  physostigmine treated slices n = 4,  $\blacksquare$  time matched controls n = 6, data presented as mean  $\pm$  S.E.M). Example traces are shown above. Traces i-iv are taken during baseline, simulated exposure, simulated second exposure, and wash from control slices. Traces v-viii are taken during baseline, exposure to 5µM physostigmine, addition of 10µM atropine and wash from exposed slices (vertical scale bars 1mV, horizontal scale bars 100ms) After confirming the effect in the rat dentate gyrus, the experiments were repeated in slices of the dentate gyrus taken from guinea-pigs. The guinea-pig hippocampus has been developed previously as a model to study the seizure-generating effects of OP anticholinesterase compounds (Harrison et al., 2000). The guinea-pig is the small laboratory animal of choice for nerve agent studies as it better models the enzyme behaviour of man. In the present study, the guinea-pig dentate gyrus was used to investigate other effects of acetylcholinesterase inhibitors on a response other than the generation of seizures as previously reported (Harrison et al., 2000). To compare the effects of the two acetylcholinesterase inhibitors between the two species, the work carried out previously in the rat dentate gyrus slice was repeated with guinea-pig slices (Figure 3.3). Physostigmine caused a significant decrease in both the amplitude (85.2  $\pm$ 2.9 % of baseline, p<0.05, unpaired t-test) and slope of the fEPSP ( $82.3 \pm 3.6$  % of baseline, p<0.05, unpaired t-test) recorded from the guinea-pig dentate gyrus (n=6). Example fEPSPs recorded during baseline, application of 10µM physostigmine and washout in guinea pig dentate gyrus slices are shown in Figure 3.4. There was no statistical difference in the magnitude of the effect of physostigmine between the species.





Trace A is baseline (average of the final 2.5 minutes of the baseline), trace B is the effect of a 10 minute application of  $10\mu$ M physostigmine (average of the final 2.5 minutes of the sarin application), trace C is the level of recovery achieved between 25 and 30 minutes post wash out of physostigmine (average of the final 2.5 minutes of this recovery epoch). Vertical scale bar 0.5mV, horizontal scale bar 25ms.

3.3.1. Effect of atropine on the physostigmine-induced decrease in the dentate gyrus

Previous experiments have shown that the decrease in the evoked EPSP can be prevented by application of a muscarinic antagonist (Colgin *et al.*, 2003). To confirm this, atropine (10µM) was applied 30 minutes after the application of physostigmine

(physostigmine was still present in the bath) in six rat dentate slices (mean physostigmineinduced decrease of the fEPSP amplitude to  $78.5 \pm 9.2$  % of baseline, mean physostigmine-induced decrease of the fEPSP slope to  $76.1 \pm 8.9$  % of baseline). Application of atropine resulted in a rapid but short-lived reversal of the physostigmineinduced decrease in the amplitude (10-20 minute epoch recovery to  $87.5 \pm 6.3$  % of baseline (p<0.05), 20-30 minute epoch recovery to  $83.8 \pm 9.9$  % of baseline (p<0.01). A similar pattern of recovery was seen in the slope of the fEPSP (10-20 minute epoch recovery to  $82.1 \pm 8.7$  % of baseline, 20-30 minute epoch recovery to  $79.3 \pm 9.6$  % of baseline), although this recovery was not statistically significant. Washing with fresh ACSF 30-minutes after the application of atropine, caused a further apparent increase in the level of recovery of both parameters. This also however did not reach statistical significance (Figure 3.5).

Atropine (10µM) was also applied 30 minutes after the application of physostigmine in guinea pig hippocampal slices. As was seen in the rat slices, atropine caused a rapid significant recovery in the evoked response (Figure 3.3). The amplitude of the fEPSP recovered to  $98.5 \pm 2.2$  % of baseline (p<0.01), and the slope recovered to  $98.2 \pm 2.0$  % of baseline (p<0.01) during the 10-20 minute epoch. In contrast to the rat, the level of recovery seen following application of atropine was sustained during the 20-30 minute epoch (amplitude 96.2 ± 1.7 % of baseline (p<0.01), slope 96.2 ± 2.7 % of baseline (p<0.001). Washing with drug-free ACSF did not cause any further significant recovery of either measures of the response compared to that seen following atropine.

Atropine (10 $\mu$ M) when applied for 30 minutes in the absence of physostigmine did not have any effect on either the slope or the amplitude of the fEPSP measured in the guinea pig dentate gyrus (Figure 3.6).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 6,  $\blacksquare$  time-matched controls n = 4, data presented as mean  $\pm$  S.E.M).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 6 data presented as mean  $\pm$  S.E.M).

3.3.2. Effect of irreversible inhibition of AChE by the nerve agent sarin

In order to investigate whether irreversible inhibitors of AChE have a different effect on the dentate gyrus, the nerve agent sarin (1 $\mu$ M) was applied to a series of guinea pig dentate gyrus slices for 30 minutes. Example fEPSPs recorded during baseline, application of

1 $\mu$ M sarin and washout in guinea pig dentate gyrus slices are shown in Figure 3.7. Sarin caused a decrease in both the amplitude (decrease to 83.7 ± 3.7 % of baseline (p<0.05 unpaired t-test)) and slope (reduction to 81.2 ± 3.8 % of baseline (p<0.01 unpaired t-test)) of the fEPSP (Figure 3.8), the magnitude of which was broadly similar to that seen previously with physostigmine.





Trace A is baseline (average of the final 2.5 minutes of the baseline), trace B is the effect of a 10 minute application of  $1\mu$ M sarin (average of the final 2.5 minutes of the sarin application), trace C is the level of recovery achieved between 25 and 30 minutes post wash out of the nerve agent (average of the final 2.5 minutes of this recovery epoch). Vertical scale bar 0.5mV, horizontal scale bar 25ms.

Atropine (10µM) was applied 30 minutes after the application of sarin and caused a recovery in both parameters of the fEPSP (10-20 minute epoch; amplitude recovery to  $97.6 \pm 4.8$  % of baseline (p<0.001), slope recovery to  $96.4 \pm 5.2$  % of baseline (p<0.001). The recovery caused by atropine was sustained during the 20-30 minute recovery epoch (both parameters p<0.01). Washing with drug free ACSF did not further increase the level of recovery seen compared to that caused by atropine (Figure 3.8). Figure 3.9 illustrates the effect of sarin when applied by itself. The effect of sarin is covered in more detail in Chapter 4.





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. The decrease is modified by the application of  $10\mu$ M atropine (a drug treated slices n = 4, a time-matched controls n = 6 data presented as mean ± S.E.M).



Figure 3.9. Effect of 1 $\mu$ M sarin on the perforant path responses recorded from the molecular layer of the dentate gyrus in the guinea pig hippocampal slice. This is an example trace showing the effect of 1 $\mu$ M sarin on the magnitude of the amplitude (A) and slope (B) of the fEPSP in one dentate gyrus slice. Slices exposed to sarin do not recover as well as those exposed to physostigmine (note shorter sarin duration compared to Figure 3.8).

#### 3.4. Cholinergic characterisation of the response

As can be seen from the results presented so far, the depression of the amplitude and slope of the fEPSP by inhibitors of acetylcholinesterase can be reversed by the non-specific muscarinic antagonist atropine. Further studies were conducted to characterise the cholinergic component of this effect.

#### 3.4.1. Effect of cholinergic agonists

To test the hypothesis that the inhibition of AChE by physostigmine or the organophosphates was causing a build up of ACh, which in turn was stimulating post-synaptic cholinergic receptors thereby in turn reducing the output of the perforant path, the cholinergic agonist carbachol was applied to guinea pig dentate slices. Carbachol (10µM) caused a rapid decrease in the amplitude and slope of the fEPSP (amplitude 73.8  $\pm$  0.8 % of baseline (p<0.001 unpaired t-test), slope 69.5  $\pm$  1.0 % of baseline (p<0.001, unpaired t-test)). This was a much larger effect than that seen following application of the AChE inhibitors (Figure 3.10). The carbachol induced decrease was reversed by application of 10µM atropine (10-20 minute epoch recovery of fEPSP amplitude to 93.8  $\pm$  1.4 % of baseline (p<0.01), recovery of slope to 92.6  $\pm$  1.9 % of baseline (p<0.01). Although the level of recovery dropped with time (20-30 minute epoch recovery of amplitude to 89.7  $\pm$  1.4 % of baseline, recovery of slope to 89.0  $\pm$  1.9 % of baseline) both measures remained significantly different from those recorded during carbachol application (both measures p<0.01).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. The decrease is modified by the application of  $10\mu$ M atropine (**a** drug treated slices n = 4, **a** time-matched controls n = 6 data presented as mean ± S.E.M).

3.4.2. Effect of subtype selective muscarinic receptor antagonists

The effect of atropine would suggest that the depressive effect of AChE inhibition is mediated by muscarinic acetylcholine receptors (mAChR). The subtype of these receptors can not be resolved using the broad acting antagonist atropine. Therefore, a

series of experiments utilising receptor subtype selective antagonists of mAChRs were conducted using guinea pig dentate gyrus slices. The concentrations used were chosen to include the approximate dissociation constant (K<sub>i</sub>) of the two compounds (telenzepine 0.94nM, methoctramine ~40nM). Following application of 1 $\mu$ M sarin for 30 minutes, either the M1 selective antagonist telenzepine or the M2 selective antagonist methoctramine was added to the recording ACSF. Both compounds were added at a starting concentration of 0.03 $\mu$ M for 15 minutes. The concentration was then increased to 0.30 $\mu$ M by cumulative dosing. After a further 15 minutes the concentration was increased to 3.00 $\mu$ M. Following 15 minutes of application of the antagonist at the highest concentration, all compounds were washed off the slice.

Sarin caused a decrease in the amplitude and slope of the fEPSP compared to control slices (reduction to  $83.1 \pm 1.0$  % of baseline amplitude, reduction to  $79.8 \pm 1.2$  % of baseline slope). Telenzepine at the lowest concentration ( $0.03\mu$ M) caused a small yet significant recovery in the amplitude of the fEPSP (recovery to  $85.2 \pm 1.1$  % of baseline (p<0.05) although the slope was unaffected. When the concentration was increased to 0.30µM of telenzepine, there was a small recovery in both the amplitude (recovery to 89.6  $\pm 1.8$  % of baseline, p<0.05) and slope (recovery to  $85.7 \pm 1.6$  % of baseline, p<0.05) of the fEPSP. Finally, increasing the concentration to  $3.00\mu$ M caused a further increase in the recovery of both the amplitude (recovery to  $91.7 \pm 1.3$  % of baseline, p<0.01) and slope of the fEPSP (recovery to  $87.6 \pm 2.0$  % of baseline, p<0.01). The level of recovery reached a plateau during the time of antagonist application at the highest concentration. The level of recovery was sustained following washing of the slice (Figure 3.11).

Application of the M2 selective antagonist methoctramine (0.03µM) following 1µM sarin (causing a reduction to  $84.3 \pm 1.1$  % of baseline amplitude and  $79.8 \pm 1.0$  % of baseline slope) caused a small recovery in both the amplitude (recovery to  $86.2 \pm 0.5$  % of baseline (p<0.05) and slope (recovery to  $82.5 \pm 0.7$  % of baseline (p<0.05) of the fEPSP (Figure 3.12). However, this recovery was absent at the next concentration tested (0.30 µM). A small recovery was recorded following application of  $3.00\mu$ M of methoctramine in both the amplitude and slope of the fEPSP (recovery to  $84.4 \pm 1.2$  % of baseline amplitude and recovery to  $81.4 \pm 0.9$  % of baseline slope). This however was not statistically significant.





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 6 data presented as mean  $\pm$  S.E.M).



Figure 3.12. Effect of the M2 mAChR subtype selective antagonist methoctramine on the sarin induced decrease of the fEPSP recorded from the molecular layer of the dentate gyrus in the guinea pig hippocampal slice. (A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 6 data presented as mean  $\pm$  S.E.M).

# 3.4.3. Involvement of nicotinic receptors

The dentate gyrus also has a large number of nicotinic receptors, which are mainly located on inhibitory interneurons within the molecular layer (Frazier et al., 2003) such as basket cells (Jones & Yakel, 1997;Frazier *et al.*, 2003). To investigate whether there was a nicotinic component to the effect of physostigmine, the non-specific nicotinic antagonist

mecamylamine was used instead of atropine following application of 5µM physostigmine in four slices from both species. In the rat dentate gyrus slice, mecamylamine ( $30\mu$ M) had a small yet statistically significant effect on the amplitude of the fEPSP (physostigmine induced reduction of amplitude to 88.8 ± 2.0 % of baseline (p<0.05 unpaired t-test), recovery of amplitude to 91.2 ± 2.2 % of baseline during 10-20 minute epoch of mecamylamine administration (p<0.05)). This effect was transient as the level of recovery of the amplitude recorded during the 20-30 minute epoch had reduced. A similar pattern of recovery was recorded in the slope of the fEPSP (Figure 3.13). Washing with drug-free ACSF did not cause any further recovery of either parameter.

In the guinea pig dentate gyrus, following application of 5µM physostigmine (physostigmine induced decrease in amplitude to  $82.5 \pm 2.6$  % of baseline (p<0.01 unpaired t-test), physostigmine induced decrease in slope to  $80.2 \pm 3.9$  % of baseline (p<0.01 unpaired t-test), 30µM mecamylamine caused a small yet significant recovery in the amplitude and slope of the fEPSP during the 10-20 minute epoch (recovery of amplitude to  $88.5 \pm 2.6$  % of baseline (p<0.05), recovery of slope to  $87.4 \pm 3.6$  % of baseline (p<0.05)). In contrast to the rat slices, the level of recovery was sustained during the 20-30 minute epoch at a significant (p<0.05 paired t-test) level. Washing with drugfree ACSF caused a further increase in recovery of 95.8 ± 4.6 % of baseline (p<0.05)). A smaller increase in the recovery of the slope parameter was also recorded but this was not statistically significant compared to that achieved with mecamylamine alone (Figure 3.14).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 4, data presented as mean  $\pm$  S.E.M).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 6, data presented as mean  $\pm$  S.E.M).

## 3.5. Involvement of cannabinoid systems

There are two subtypes of cannabinoid receptor which can be found in mammalian tissues. These are classified as  $CB_1$  and  $CB_2$ .  $CB_1$  receptors are found throughout the CNS, whereas the  $CB_2$  receptor is mainly confined to the immune system (for review see (Howlett *et al.*, 2004)). Expression of the  $CB_1$  receptor is high in the hippocampus (Moldrich & Wenger, 2000) with dense immunoreactivity for the receptor in the molecular layer of the dentate gyrus and pyramidal cell layer of CA1 and CA3 (Tsou *et al.*, 1998). Further studies have shown that the  $CB_1$  receptor is located presynaptically on the terminals of GABAergic interneurons within the molecular layer of the dentate gyrus (Katona *et al.*, 2000). The  $CB_1$  specific antagonist AM251 (8µM) has been previously reported to block the depression of the fEPSP in the rat dentate gyrus caused by the administration of 5µM physostigmine (Colgin *et al.*, 2003).

#### 3.5.1. Effect of AM251 on the physostigmine-induced decrease

Initially, the study by Colgin et al., was repeated in the guinea pig dentate gyrus (Colgin et al., 2003). Slices were pretreated with 8µM AM251 for 30 minutes prior to the application of 5µM physostigmine. Application of 8µM AM251 did not cause any significant changes in the amplitude or slope of the fEPSP when compared to control slices. Application of 5µM physostigmine 30 minutes after the start of the AM251 perfusion resulted in a decrease in both the amplitude and slope comparable with that seen in earlier studies (reduction in amplitude to  $83.8 \pm 1.3$  % of baseline (p<0.05 paired t-test), reduction in slope to 79.3 ± 1.2 % of baseline (p<0.05 paired t-test)). Washing with drug-free ACSF did not result in any significant changes in either parameter (Figure 3.15). In a second series of slices, the concentration of AM251 was raised to 30µM (Figure 3.16). Again, AM251 did not cause any significant changes in either parameter measured. Physostigmine (5µM) applied 30 minutes after AM251 again resulted in a significant decrease in the amplitude of the fEPSP (reduction of amplitude to  $83.4 \pm 0.9$  % of baseline (p < 0.05). The slope of the fEPSP was not significantly affected by the application of physostigmine. Washing with drug-free ACSF resulted in a significant recovery of the amplitude (recovery to  $93.1 \pm 1.2$  % of baseline (p<0.01) and slope (recovery to  $90.5 \pm 2.5$  % of baseline (p<0.05) of the fEPSP.





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 6, data presented as mean  $\pm$  S.E.M).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 6, data presented as mean  $\pm$  S.E.M).

## 3.5.2. Effect of AM251 on the sarin-induced decrease

These studies were then repeated using the irreversible AChE inhibitor sarin. As reported before,  $8\mu$ M AM251 did not have any significant effects on either the amplitude or slope of the fEPSP (Figure 3.17). Application of  $1\mu$ M sarin caused a significant reduction in the

amplitude of the fEPSP (reduced to  $86.3 \pm 2.0$  % of baseline (p<0.05)). Whilst there was also a reduction of a similar magnitude in the slope (reduced to  $83.4 \pm 1.7$  % of baseline) this was not statistically significant. Washing with drug-free ACSF caused a recovery in both parameters (amplitude recovered to  $98.8 \pm 3.5$  % of baseline (p<0.05), slope recovered to  $95.2 \pm 4.0$  % of baseline (p<0.05)). Increasing the concentration of AM251 to  $30\mu$ M did not affect the amplitude or slope (Figure 3.18). The increased concentration of AM251 also failed to modify the reduction in both the amplitude (reduced to  $84.1 \pm 1.1$  % of baseline (p<0.05)) and slope (reduced to  $84.3 \pm 0.1$  % of baseline (p<0.05)) caused by the application of  $1\mu$ M sarin. Washing with fresh ACSF did not significantly increase the level of recovery seen in both parameters.





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 3,  $\blacksquare$  time-matched controls n = 6,  $\blacksquare$  sarin treated slices n = 4 (data adjusted to align sarin application between groups), data presented as mean  $\pm$  S.E.M).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 3,  $\blacksquare$  time-matched controls n = 6,  $\blacksquare$  sarin treated slices n = 4 (data adjusted to align sarin application between groups), data presented as mean  $\pm$  S.E.M).

3.5.3. Investigation of species specific differences in the involvement of cannabinoid receptors

To ensure that the apparent lack of effect of AM251 in this study was not species related (rat dentate gyrus slices were used in the study by Colgin *et al.,*); this experiment was

repeated in rat dentate gyrus slices. As observed in the guinea pig dentate gyrus slices,  $8\mu$ M AM251 did not have any effect on the amplitude or slope of the fEPSP (Figure 3.19). Application of 5 $\mu$ M physostigmine 30 minutes after AM251 caused significant decreases in both the amplitude and slope (amplitude reduced to  $81.9 \pm 2.0$  % of baseline (p<0.01), slope reduced to  $81.0 \pm 3.4$  % of baseline (p<0.01)). Washing with drug-free ACSF caused a small recovery in the both parameters, however only the recovery of the amplitude was significant (p<0.05 paired t-test). Increasing the concentration of AM251 to 30 $\mu$ M again did not have any effect on either the amplitude or slope of the fEPSP (Figure 3.20). Application of 5 $\mu$ M physostigmine 30 minutes later caused a significant decrease in both the amplitude and slope of the fEPSP (amplitude reduced to 79.1  $\pm$  1.4 % of baseline (p<0.05), slope reduced to 77.5  $\pm$  1.3 % of baseline (p<0.05)). Washing with drug-free ACSF caused an apparent recovery in both parameters, however only the recovery recovery recorded in the slope was statistically significant (p<0.05).

The solvent DMSO was used to produce solutions of AM251. When applied for 30 minutes at a concentration equivalent to that used for the highest concentration of AM251 (0.06% v/v), DMSO did not effect the amplitude or slope of the fEPSP when compared to normal control slices.





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 4, data presented as mean  $\pm$  S.E.M).



Figure 3.20. Effect of the CB<sub>1</sub> antagonist AM251 ( $30\mu$ M) on the physostigmine induced depression of perforant path responses recorded from the molecular layer of the dentate gyrus in the rat hippocampal slice.

(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  time-matched controls n = 4, data presented as mean  $\pm$  S.E.M).

#### 3.6. Involvement of GABAergic pathways

There is anatomical evidence for mAChR being located on GABAergic neurons within the hippocampus and the dentate gyrus (van der Zee & Luiten, 1993). These neurons are inhibitory interneurons which regulate the output of the granule cells of the dentate gyrus.

It has been shown that cholinergic agonists are able to exert excitatory effects directly on these neurons (Reece & Schwartzkroin, 1991). To investigate whether the depression of the fEPSP following application of sarin was mediated by GABAergic systems, the non-competitive GABA antagonist picrotoxin ( $20\mu$ M) was added 10 minutes prior to the application of  $1\mu$ M sarin. As can be seen in Figure 3.21, picrotoxin did not significantly affect either amplitude of slope of the fEPSP (the apparent increase in the amplitude of the fEPSP was not statistically significant compared to vehicle control). Application of  $1\mu$ M sarin 10 minutes after the picrotoxin did not have any effect on either parameter measured. When both compounds were washed off, there was a gradual decline in both the amplitude and slope of the fEPSP. This however was not significant across both the 10-20 and 20-30 minute wash epochs (with exception of the slope during the 20-30 minute epoch, p<0.05). Vehicle controls were also carried out using ethanol (0.1% v/v). This did not have any significant effect on the amplitude of the fEPSP, however there was a significant difference in the slopes of slices sham exposed and those that were exposed to ethanol (p<0.01 unpaired t-test).





(A) Effect on the amplitude of the evoked fEPSP. (B) Effect on the magnitude of the slope of the fEPSP. ( $\blacksquare$  drug treated slices n = 4,  $\blacksquare$  vehicle 0.1% ethanol controls n = 4, data presented as mean  $\pm$  S.E.M). Note that the application time of sarin has been reduced to 10 minutes. This is due to the experimental protocol used and the results discussed in Chapter 4.

# 3.7. Discussion

The aim of the present study was to determine the effects of acetylcholinesterase inhibition on the evoked response recorded from the molecular layer of the dentate gyrus. This study has confirmed and extended the findings of Colgin *et al.* (Colgin *et al.*, 2003), and demonstrates that the inhibition of acetylcholinesterase by physostigmine can modify the output of the perforant path in the dentate gyrus, recorded as a decrease in the magnitude of the evoked fEPSP. In addition, the OP anticholinesterase compound, sarin, was also shown to be capable of causing a depression in the magnitude of the fEPSP.

The depression of the fEPSP recorded from the molecular layer of the dentate gyrus following administration of physostigmine or soman, was almost fully reversed by the muscarinic antagonist atropine. The exact mechanism by which this effect is produced is as yet unclear although the involvement of cholinergic mechanisms has been clearly demonstrated. The muscarinic antagonist atropine has been previously reported to reverse the decrease in the fEPSP caused by physostigmine and other cholinesterase inhibitors (Melchers et al., 1994;Colgin et al., 2003). Application of the cholinergic agonist carbachol (10µM) to the dentate gyrus mimicked the decrease in the amplitude of the fEPSP recorded following application of physostigmine. As with the decrease caused by physostigmine, the carbachol-induced decrease was reversed upon application of atropine. This has been previously reported as only affecting fEPSPs caused by activation of the medial and not the lateral component of the perforant pathway (Kahle & Cotman, 1989). The actions of atropine and carbachol in this model show that the anticholinesterase compounds are causing an increase in the concentration of acetylcholine within the synapse by blocking its hydrolysis, rather than having a direct agonist-like action on the perforant path or granule cells of the dentate gyrus.

Inhibition of acetylcholinesterase by both OP and non-OP compounds has been shown to produce many different effects in the hippocampus (Williamson & Sarvey, 1985;Wood & Tattersall, 2001). Previous work from this laboratory by Harrison et al has confirmed that soman (100nM) is capable of causing seizure-like activity that can be recorded from the pyramidal cell layer in the CA1 region of the hippocampus (Harrison *et al.*, 2000;Harrison *et al.*, 2003;Harrison *et al.*, 2004). This activity could be could be blocked to a degree by the application of muscarinic antagonists such as atropine. Interestingly the rank order of effectiveness of muscarinic antagonists selective for the M1 and M2 receptors was similar to that reported here for the dentate gyrus, namely that antagonists of the M1 receptor (i.e. telenzepine) were more effective at resolving soman-induced seizures than those selective for the M2 receptor such as AF-DX 116 (Harrison *et al.*, 2004). The effectiveness of M1 (and M3) receptor antagonists in blocking seizure activity has also been reported in the neocortex (Potier & Psarropoulou, 2004).

From a review of similar studies, the mechanism by which this action is mediated appears to involve the M1 muscarinic receptor. The depression seen following application of a

cholinesterase inhibitor was blocked by application of pirenzipine (Melchers *et al.*, 1994) or reduced in a concentration dependent manner (Burgard *et al.*, 1993). A similar level of antagonism was seen following application of the M3/M1 antagonist 4-diphenylacetoxy-*N*-methyl-piperidine methiodide (4-DAMP) (Burgard *et al.*, 1993). However, the M2 receptor antagonists methoctramine and gallamine showed no apparent antagonism of the cholinesterase inhibitor-induced depression (Colgin *et al.*, 2003;Melchers *et al.*, 1994). This is in agreement with what is reported from the current study. A caveat must be applied to these results, however, as the concentrations of antagonists used may also have non-selective effects on other mAChR subtypes.

Further evidence to support this conclusion comes from immunohistochemical and lesion studies which have shown that M1 receptor can be found throughout the molecular and granule cell layers of the dentate gyrus (Levey et al., 1995). The levels of immunoreactivity for the M1 receptor are not affected by lesions of the entorhinal cortex affecting the perforant path projections. However following lesions of the granule cell layer, M1 immunoreactivity is completely abolished (Rouse et al., 1998). This shows that the M1 receptor is located postsynaptically on the axons and dendrites of the granule cells. A similar pattern of expression was also seen for the M3 receptor. The M2 and M4 receptors were shown to be presynaptically located on the projection fibres of the medial perforant path and also presynaptically on other afferent sources within the outer and inner thirds of the dentate gyrus (Rouse et al., 1998). The relative lack of effective of the M2 antagonist methoctramine in this study is surprising as it all but rules out a role for presynaptic M2 mAChR heteroreceptors on the afferent fibres of the perforant pathway. The presence of a presynaptic muscarinic receptor on the afferents of the medial perforant pathway within the molecular layer of the dentate gyrus has been reported previously (Kahle & Cotman, 1989). A novel M2-like mAChR which controls the release of glutamate in hippocampal synaptosomes has also been reported (Marchi & Raiteri, 1989). Muscarinic receptors (mainly M2) have also been shown to modulate the release of glutamate from other afferent fibres such as those in the spinal cord (Zhang et al., 2007). The M4 mAChR is related to the M2 mAChR and acts to inhibit neuronal activity by hyperpolarising the cell (Hasuo et al., 1995). The M4 mAChR has also been identified as being located presynaptically on the fibres of the perforant pathway (Rouse et al., 1998).

As stated above, functionally the results from the current study show that the cholinergic effect of AChE inhibitors is likely to mediated by an M1-like mAChR, although this is somewhat complicated by studies which have shown M2 or M4 mAChR localised on the afferent terminals of the perforant pathway fibres. One explanation may lie with the antagonists and concentrations used. Methoctramine which is described as being more selective for the M2 subtype also shows affinity for other mAChR subtypes with very little

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difference between subtypes ( $K_i$  13nM M2, 31nM M4 and 50nM M1 (Dorje *et al.*, 1991)). Similarly , telenzepine has been shown to be more selective for the M1 subtype ( $K_i$  0.9nM M1 *vs* 17.8nM at the M2 mAChR (Galvan *et al.*, 1989)). In the current study, the apparent effect of telenzepine at 0.3 and 3µM (Figure 3.11) may in fact be due to a non-specific effect on the M2 or putative M4 heteroreceptors on the afferent terminals of the perforant pathway thereby modulating the release of glutamate. The interaction of telenzepine with M4 receptors is possible, as although it is classed as being more selective for the M1 mAChR, it has a very similar profile at the M4 mAChR. Lazareno *et al.*, (1990) have reported that telenzepine has a pK<sub>i</sub> value of 8.89 ± 0.05 at the M1 mAChR compared to 8.61 ± 0.05 at a putative M4 receptor in the chicken heart (Lazareno *et al.*, 1990). The similarity in these values show that the selectivity of this compound for the M1 mAChR over the M4 mAChR is marginal and that at the concentrations used in this study it may be acting at presynaptic M4 receptors on the perforant pathway afferent fibres. The likely identity of this receptor could be uncovered by repeating the current experiment with a more selective M4 antagonist such as tropicamide.

This putative role for the M4 (or M2) receptor acting as a presynaptic modulator of glutamate release should be accepted with caution. The endogenous source of ACh in the dentate gyrus has been accepted to be the cholinergic septohippocampal afferent fibres. These fibres form synapses with all cell types within the hippocampus, with the main termination of the fibres occurring in the supragranular layer (inner third of the molecular layer directly beneath the granule cell layer) and also some fibres terminating in the hilus (polymorphic layer) (Storm-Mathisen, 1977) There is little evidence for large numbers of axo-axonic synapses on these fibres in the molecular layer close to the perforant pathway fibres (Clarke, 1985) and the distance between these two locations is such that ACh released synaptically by the septohippocampal fibres is unlikely to act via volume transmission on the terminals of the perforant pathway (Brunner & Misgeld, 1994).

The apparent lack of effect of the nicotinic antagonist mecamylamine is a surprising, in this study. Nicotinic acetylcholine receptors (nAChRs) are expressed in great numbers throughout the CNS and the hippocampal formation is no exception to this. Within the hippocampus and dentate gyrus, nAChR are expressed on the interneurons rather than the principal cells (McQuiston & Madison, 1999;Jones & Yakel, 1997). There is evidence that cholinergic activation of nAChR expressing interneurons is responsible for the regulation of the output of the principal cells of CA1 (pyramidal cells) and the dentate gyrus granule cells (Rózsa *et al.*, 2008;Frazier *et al.*, 1998;Frazier *et al.*, 2003). It has been shown that activation of  $\alpha$ 7 subunit containing nAChR within the subgranular interneurons of the hilus region of the dentate gyrus results in GABAergic inhibitory post synaptic potentials in granule cells (Frazier *et al.*, 2003). The relative lack of effect of

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mecamylamine may be due to limitations of the experimental protocol and also the method by which the anticholinesterases are acting to reduce the output of the granule cells. Whilst ACh is an agonist of the nAChR, the product of its hydrolysis, choline, is a far more potent agonist of nAChR especially those containing the  $\alpha$ 7 or  $\alpha$ 9 subunits (Pereira *et al.*, 2002).

The physiological properties of postsynaptic mAChRs in the dentate gyrus (both M1 and M3 (Rouse *et al.*, 1998;Rouse & Levey, 1997)) have yet to be fully elucidated. Direct activation of M1 mAChRs present on the dendrites and axons of the granule cells would in theory cause excitation of the granule cells. This theoretical effect is hard to reconcile with the results presented in this chapter and those reported in the literature. However, a role for the M1/M3 mAChR present on the granule cells may lie in the control of the release of endocannabinoids from the granule cells (Ohno-Shosaku *et al.*, 2003).

Endocannabinoids include anandamide and 2-arachidonoyl-glycerol (2-AG) and are synthesised in, and released by neuronal cells in response to depolarisation (Di Marzo et al., 1994), especially that caused by cholinergic mechanisms (Bisogno et al., 2005). The synthetic enzyme required for 2-AG formation, diacylgylcerol lipase  $\alpha$  (DGL-  $\alpha$ ), has been shown to be expressed in the principal cells of the hippocampus including the granule cells of the dentate gyrus. In contrast this enzyme appears to be absent from the interneurons (such as the basket cells) of the dentate gyrus (Katona et al., 2006). Postsynaptic M1 and M3 on hippocampal neurons have been reported to be responsible for modulating a retrograde signal which is facilitated by endocannabinoids (Ohno-Shosaku et al., 2003). Endocannabinoids released by stimulation of the granule cells are able to interact with presynaptic CB<sub>1</sub> receptors on GABAergic interneurons resulting in a decreased release of GABA. This process is called depolarisation induced suppression of inhibition (DSI) and has been recorded in the GABAergic basket cell interneuron of the dentate gyrus (Isokawa & Alger, 2005). The presence of DGL- α and M1/M3 mAChR on the granule cells would suggest further evidence for postsynaptic mAChR playing a role in modulating the release of endocannabinoids.

The hypothesis that endogenous cannabinoids act as retrograde messengers to inhibit the release of glutamate from the perforant path was investigated. This has been previously suggested in earlier reports (Colgin *et al.*, 2003). It contrast to the study by Colgin, the depressive effect of physostigmine (and also sarin) was not blocked by the CB<sub>1</sub> antagonist AM251 in the current study. However, it appeared that the fEPSP recorded from slices pretreated with AM251 prior to exposure to 1 $\mu$ M sarin recovered quicker than those that did not receive AM251 (Figures 3.17 and 3.18). This was only statistically significant (p<0.01 unpaired t-test) in the amplitude and slope of the fEPSP recorded during the 10-

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20 minute recovery epoch in slices pretreated with 8  $\mu$ M AM251. The lack of statistical significance in other measures may be an artefact of the low sample number (n=3). The effect on the recovery of physostigmine exposed slices could not be measured due to the lack of suitable slices exposed to 5  $\mu$ M physostigmine alone. Species differences in the efficacy of AM251 between the rat and guinea pig can be ruled out as there was no effect of this antagonist in either species in the current study. Other studies have also reported the presence of presynaptic CB<sub>1</sub> receptors in the guinea pig hippocampus (Schlicker *et al.*, 1997). One possible reason for the conflicting results is purely practical; i.e. a problem with the formulation of the AM251 used in the current study. This could be ruled out by repeating the experiments with a fresh batch of the antagonist or using a different CB<sub>1</sub> antagonist compound.

Despite these conflicting results, there is also some doubt about the validity of Colgin's hypothesis. There is a paucity of evidence indicating that endocannabinoids can directly modulate the release of glutamate from the perforant pathway afferent fibres. In fact, endocannabinoid modulation of glutamate release has recently been shown to occur only at the mossy cell - granule cell synapse and not at synapses involving the medial or lateral perforant pathways (Chiu & Castillo, 2008). CB<sub>1</sub> receptors have been shown to be expressed in large densities in the molecular layer of the dentate gyrus (Herkenham et al., 1991;Dove Petit et al., 1998), especially on presynaptic terminals of GABAergic interneurons within the molecular layer of the dentate gyrus (Hajos et al., 2000). The vast majority of CB<sub>1</sub> receptors are expressed by cholecystokinin (CCK) containing basket cells in the dentate gyrus and their activation inhibits the release of GABA (Katona et al., 1999). Localisation of the receptor on GABAergic neurons allows cannabinoid agonists to decrease the release of GABA through DSI thereby fine tuning neuronal output (Hofmann et al., 2006). A small number of nonGABAergic neurons in the mouse dentate gyrus (described as possibly being afferent neurons although not identified conclusively) have also been reported (Marsicano & Lutz, 1999).



# Figure 3.22. A simplified schematic of the connections between the neuronal cells of the dentate gyrus.

The cell shown are the granule cells, the CA3 pyramidal cells, excitatory mossy cells, and the various inhibitory interneurons (simplified). This diagram shows the relative topography across the layers of the dentate gyrus (M-molecular layer, G-granule cell layer, H-hilus (polymorphic layer). Also shown are the two main neurotransmitters present in the dentate gyrus. GABA is the main inhibitory transmitter (-) and glutamate (Glu) is the main excitatory transmitter (+). For clarity the hilar interneurons represented here include the hilar commissural-association pathway-related (HICAP), molecular layer perforant path-associated (MOPP) and hilar perforant path-associated (HIPP) subtypes. Another class of interneuron, the chandelier cell, has been omitted for clarity. These cells form inhibitory synapses with other GABAergic interneurons within the inner portions of the molecular layer. This complicated pattern of innervation allows for fine control of the output of the granule cells which is important for the generation and maintenance of hippocampal rhythms (Houser, 2007).

From the results presented in this series of experiments it can be seen that endogenous acetylcholine is responsible for the decrease in the amplitude of the fEPSP recorded from the molecular layer of the dentate gyrus. However, as shown in Figure 3.21, this is facilitated by GABA<sub>A</sub> receptors. The non-competitive GABA<sub>A</sub> receptor antagonist picrotoxin blocked the decrease in the fEPSP usually associated with exposure to  $1\mu$ M sarin. GABA<sub>A</sub> receptors are expressed by dentate granule cells and are thought to be responsible for providing both tonic and phasic inhibition of the cell (Nusser & Mody,

2002;Mtchedlishvili & Kapur, 2006). GABA<sub>A</sub> receptors have also been reported on to be present on the hilar mossy cells and could play a role in maintenance of the excitability of these cells (Nahir *et al.*, 2007). GABA<sub>B</sub> receptors have further been shown to be important for maintaining tonic inhibition of the granule cell output *in vivo* (Canning & Leung, 2000). Excitation of the interneuron and the granule cell together has been shown to cause a shunt in the charge from the excitatory postsynaptic current which abolishes the excitatory NMDA excitatory post synaptic current. This is modulated by post synaptic GABA<sub>A</sub> receptors (Staley & Mody, 1992). Such a situation as this can be brought about by stimulation of the medial perforant pathway afferent fibres, which form excitatory synapses with both the granule cells and the inhibitory interneurons (Zipp *et al.*, 1989).



#### Figure 3.23. Cholinergic innervation of the dentate gyrus.

Cholinergic septohippocampal projection fibres (yellow arrows) terminate mainly in the hilar and supragranular layer regions of the dentate gyrus as well as CA3. Released ACh excites the mossy cells and CA3 pyramidal cells causing an increase in the activity of the hilar interneurons. This in turn causes an inhibition of the granule cells. ACh also causes a smaller depolarisation of the granule cells which also leads to an increase in the level of recurrent inhibition of the granule cells (key as in Figure 3.21).

It is proposed that the following scheme of events occurs during the experiments reported here. Electrical stimulation of the perforant pathway afferent fibres causes a release of glutamate. This excites both the granule cells and also the numerous inhibitory interneurons. Depolarisation of the granule cells then facilitates the release of glutamate at the mossy fibre-mossy cell and mossy fibre-CA3 pyramidal cell synapses. The
feedback loops provided by the dendritic fibres of the mossy cells and CA3 pyramidal cells cause an increase in the activity of the hilar interneurons (Figure 3.22). The interneurons release GABA which binds to post synaptic GABA<sub>A</sub> and GABA<sub>B</sub> receptors on the granule cells causing hyperpolarisation of the cell (Piguet, 1993). There is also evidence for recurrent inhibition between interneurons (Freund & Buzsáki, 1996). Following release of endogenous ACh (Figure 3.23), the overall level of inhibitory drive is greatly increased. This is due to the increase in activity of the inhibitory interneurons driven by direct activation of mAChR on the interneurons themselves (Dougherty & Milner, 1999), and also on the excitatory CA3 pyramidal cells and mossy cells (Deller *et al.*, 1999). Application of cholinergic agonists causes an increase in the level of bursting of inhibitory interneurons (EPSPs) and IPSP formation in the granule cells themselves (Brunner & Misgeld, 1994;Harney & Jones, 2002) and this activity is modulated by both mAChR (van der Zee & Luiten, 1993) and nAChR (Jones & Yakel, 1997). This produces a profound inhibition of the granule cell output witnessed as a decrease in the magnitude of the fEPSP.

Although not shown by the results presented here, it is postulated based on the available literature, that endocannabionids may be released by the depolarisation of the granule cell. The released endocannabinoid compound then acts at presynaptic CB<sub>1</sub> receptors on the GABAergic interneurons to depress the further release of GABA by the process of DSI thereby modulating the level of granule cell inhibition. Endocannabinoids may also play a role in decreasing the release of glutamate by a process called depolarisation-induced suppression of excitation (DSE). This is similar to DSI with the exception that it is the release of glutamate which is inhibited rather than GABA (Straiker & Mackie, 2005). This process has been reported at the mossy cell-granule cell synapse and is likely to be enhanced by the cholinergic septohippocampal fibres (Chiu & Castillo, 2008). Further evidence for this comes from the identification of CB<sub>1</sub> receptors in locations within the inner third of the molecular layer of the dentate gyrus corresponding with the location of mossy cell-granule cell synapses (Katona *et al.*, 2006).

A novel action of AChE inhibitors on the GABAergic septohippocampal afferent fibres in a slice model of the medial septal diagonal band has also been reported. Physostigmine and other AChE inhibitors have been shown to increase the rate at which these afferent fibres fire. Conversely, impulse flow in the cholinergic septohippocampal afferents is greatly decreased (Wu *et al.*, 2003). As the GABAergic fibres preferentially innervate interneurons within the hippocampus it would provide a strong signal to disinhibit the granule cells. This increase in the output of the granule cell would be seen as a maintenance or increase in the amplitude of the fEPSP and not the decrease seen in the present study. The differing results may be due in part to the greater density of

cholinergic septohippocampal fibres in the dentate gyrus slice compared to their GABAergic counterparts thus tipping the balance overall towards inhibition of the granule cells.

In conclusion, the results from this study have confirmed that the output from the perforant path in the dentate gyrus can be modified by the acetylcholinesterase inhibitor physostigmine. This was seen as a decrease in the amplitude of the evoked field potential recorded from the molecular layer of the dentate gyrus in the rat and guinea pig, with no significant differences between the two species. This finding was extended by using the nerve agent sarin that resulted in a decrease similar to that observed following application of physostigmine in the guinea pig. The physiological pathways involved in this response are complex and probably represent a series of inter-related processes which are occurring at the same time. This study has confirmed that the dentate slice model retains an intrinsic cholinergic response which is most likely to be mediated by afferent terminals of cholinergic septohippocampal projection fibres. This means that this model is therefore well-suited for investigating the effects of nerve agents and other compounds on a model of a CNS cholinergic response.

4. Investigation of the interactions between physostigmine and the nerve agent sarin

#### 4.1. Introduction

This study has been designed to begin to investigate the mechanisms of the interaction between physostigmine and the nerve agent sarin in the dentate gyrus slice model. This builds upon the characterisation of the model and the effects of the individual compounds.

Physostigmine has been proposed as a component of the next generation nerve agent therapy and pretreatment sets. This is not a new concept of use for physostigmine as it was first shown in 1946 to offer protection against the organophosphate DFP in cats (Koster, 1946). Physostigmine is a tertiary amine and is able to cross the blood brain barrier and inhibit AChE within the CNS. It is thought that this inhibition of central AChE is responsible for the protection against the lethal actions of organophosphates such as the nerve agents. However, the inhibition of central AChE also causes many unwanted side effects such as vomiting, disruption of balance and motor control (Proudfoot, 2006). These are the result of overstimulation of the cholinergic systems within the CNS. These complications led to the better tolerated pyridostigmine being introduced as a pretreatment to counter nerve agent poisoning as it is unable to cross into the CNS in normal situations and is largely devoid of the side effects reported with physostigmine use.

Due to ongoing research into replacement therapies that are less reliant on pretreatment, the use of physostigmine as a therapy for nerve agent poisoning is being investigated once more. To counter the undesirable cholinergic side effects associated with the use of physostigmine, the addition of the non-specific muscarinic antagonist scopolamine has been proposed. The use of physostigmine in this way appears at first to be counterintuitive. The addition of another inhibitor of AChE into an already compromised system would at first glance be expected to compound the effects of the nerve agent. However, in recent animal studies this combination has been proven to be more efficacious than current treatments at preventing lethality and improving the level of incapacitation seen post poisoning with a range of nerve agents (Wetherell *et al.*, 2006).

#### 4.2. Methods

Guinea pig hippocampal slices were prepared as previously described in section 2.1.3. Physostigmine and sarin were prepared as described in section 2.4. The optimal concentration of physostigmine or sarin was determined by applying a single of concentration of the compound following the timeline shown in Figure 4.1. This was repeated with several different concentrations of each compound (0.03 – 30.00µM

physostigmine, and  $0.003 - 1.00\mu$ M sarin). Cumulative dosing was not used, and each data point was recorded from a single slice. The time line in Figure 4.2 was used to investigate any potential interactions between the two compounds.



Figure 4.1. Timeline used for experiments determining the concentration-effect relationships of physostigmine or sarin.



Figure 4.2. Timeline used for experiments investigating the interactions between sarin and physostigmine.

Data recorded from each slice were manipulated as follows. The values recorded for each parameter during the 10 minute period immediately prior to the application of the first test compound were averaged (mean). This was used as a baseline value to which all data points were then normalised. Data are presented as percentage changes from this value. Data recorded during the application of test compounds and recovery were averaged into 1 minute bins. This smoothed the data removing any rapid fluctuations (sub 30 second) from the general trend. Recovery of the response was measured during two epochs following the wash out of the test compound(s). These were between 10 and 20 minutes and between 20 and 30 minutes post wash out. The level of recovery was determined as the maximum response during each epoch compared to the maximum decrease recorded during the application of the test compound.

Sigmoidal curves were fitted to the concentration-effect data using Graphpad Prism V.4.00.using the sigmoidal dose-response (variable slope) function. The equation for this fit was  $Y = Bottom + (Top-Bottom)/(1/10^{((LogEC50-X)*Hill slope))}$ , where X is the

logarithm of concentration and Y is the response.  $IC_{50}$  values for both compounds were then derived from this curve.

#### 4.3. Characterisation of the effect of physostigmine in the dentate gyrus

To allow determination of a suitable concentration of physostigmine for use in subsequent experiments, a concentration-effect relationship for physostigmine in the dentate gyrus slice was derived. A generic timeline for the experiments is shown in Figure 4.1. Briefly, a baseline period lasting at least 30 minutes was recorded before a single concentration of physostigmine (0.03-30µM) was applied to the slice for 10 minutes. Cumulative dosing was not used. The average amplitude of the fEPSP recorded during the baseline was 2.5  $\pm$  0.1 mV and the average slope was 2.6  $\pm$  0.2 mV msec<sup>-1</sup>. After 10 minutes the recording solution was changed for a pre-warmed drug-free recording solution. The slice was then washed for at least 60 minutes. These data are shown in Table 4.1 and 4.2. The maximum depression of both the amplitude and slope of the evoked fEPSP during the period whilst physostigmine was present was determined and used to construct a concentration-effect relationship for physostigmine (Figure 4.3). The maximum concentration of physostigmine tested (30µM) caused the amplitude of the fEPSP to decrease to  $85.9 \pm 0.5\%$  of baseline levels (p<0.01 compared to control slices n=4). A similar decrease was also observed in the slope of the fEPSP ( $82.8 \pm 1.3\%$  of baseline, p<0.01 compared to control slices n=4). The concentration of physostigmine which caused a 50% depression relative to the maximal inhibition of the fEPSP (IC<sub>50</sub>) was derived as 1.86µM for the effect on the amplitude and 2.22µM for the effect on the slope. The recovery of each slice parameter during the washout of the test compounds was measured at two adjacent epochs. These were between 10 and 20 minutes post start of washing, and between 20 and 30 minutes post start of washing. The maximum recovery seen during each epoch was then compared with the maximum depression seen during the period where the drug was present. The slice response (both amplitude and slope) fully recovered to baseline levels during the wash period at all concentrations of physostigmine used (Tables 4.1 and 4.2).



Figure 4.3. Concentration-effect relationship for physostigmine in the guinea pig dentate gyrus.

Physostigmine when applied for 10 minutes caused a concentration dependent decrease in the amplitude (A) and slope (B) of the evoked fEPSP (mean  $\pm$  SEM, n=4).

Concentration	Maximum effect	Maximum recovery	Maximum recovery
(µM)	(% of baseline	achieved at 10-20 min	achieved at 20-30 min
	compared to	post washing (% of	post washing (% of
	control slices)	baseline)	baseline)
0.030	95.6 ± 1.4**	99.6 ± 2.1*	100.3 ± 3.5
0.100	91.6 ± 0.7**	100.8 ± 1.3**	102.3 ± 5.5
0.300	95.1 ± 1.2**	105.1 ± 3.7*	102.3 ± 4.6
1.000	92.5 ± 1.8**	107.3 ± 3.0*	107.5 ± 4.0
3.000	87.0 ± 0.7	105.9 ± 3.4**	103.9 ± 4.0*
10.000	83.7 ± 1.0 <sup>™</sup>	103.9 ± 2.8**	101.4 ± 1.5**
30.000	85.9 ± 0.5	99.2 ± 1.9**	100.3 ± 2.1**

## Table 4.1. The effect of physostigmine at a range of concentrations (0.03- $30.00\mu$ M) on the amplitude of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs* control slices), paired t-test (recovery *vs* maximum effect)).

Concentration	Maximum effect	Maximum recovery	Maximum recovery
(µM)	(% of baseline	achieved at 10-20 min	achieved at 20-30 min
	compared to	post washing (% of	post washing (% of
	control slices)	baseline)	baseline)
0.030	94.77 ± 2.07**	99.96 ± 2.46*	100.90 ± 3.64
0.100	88.17 ± 1.98**	98.32 ± 0.89*	101.50 ± 5.88*
0.300	92.30 ± 2.55**	$104.60 \pm 4.00^{**}$	101.10 ± 4.77*
1.000	91.06 ± 1.99**	106.90 ± 2.98*	106.70 ± 4.14
3.000	83.66 ± 1.54	104.00 ± 3.23**	102.20 ± 3.28**
10.000	79.07 ± 3.32"	103.20 ± 2.42*	100.80 ± 1.85*
30.000	82.76 ± 1.26**	97.72 ± 1.52**	96.87 ± 1.54*

## Table 4.2. The effect of physostigmine at a range of concentrations (0.03-30.00µM) on the slope of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs* control slices), paired t-test (recovery *vs* maximum effect)).

#### 4.3.1. Characterisation of the effect of sarin in the dentate gyrus

A concentration-effect relationship for sarin was also constructed. The average amplitude of the fEPSP recorded during the baseline was  $2.3 \pm 0.1$  mV and the average slope was  $2.2 \pm 0.1$  mV·msec<sup>-1</sup>. Initially a single concentration of sarin (0.003–1µM) was applied for 10 minutes. When applied for 10 minutes, sarin also caused a concentration dependent decrease in the amplitude (p<0.01 at all sarin concentrations compared to equivalent data from time matched control slices) and slope of the fEPSP. These data are shown in Table

4.3 and 4.4. From these results a concentration-effect relationship was derived and is shown in Figure 4.4. From this data  $IC_{50}$  values of  $0.09\mu$ M (amplitude) and  $0.02\mu$ M (slope) were derived. In contrast to slices exposed to physostigmine, both the amplitude and slope of the fEPSP recorded from sarin exposed slices did not recover to pre-exposure levels following washing of the slice. This lack of recovery was most pronounced in the slope of slices exposed to sarin.

However, it appeared that at lower concentrations the circulating sarin may not have reached full equilibrium within the slice. This was seen as a maximal depression of both measures of the fEPSP which did not plateau during the application of the nerve agent. Therefore a second concentration-effect curve was constructed from slices which were exposed to sarin for 30 minutes (Tables 4.5 and 4.6, and Figure 4.5). The average amplitude of the fEPSP recorded during the baseline was  $2.2 \pm 0.1$  mV and the average slope was  $2.1 \pm 0.1$  mV·msec<sup>-1</sup>. Application of 1µM sarin caused the amplitude of the fEPSP to decrease to  $84.2 \pm 2.0\%$  of baseline. Again the slope showed a decrease of a similar magnitude ( $81.9 \pm 2.3\%$  of baseline). The only IC<sub>50</sub> value for this longer application that could be derived from the data was that of  $0.05\mu$ M for the effect on the fEPSP amplitude. No value for the IC<sub>50</sub> for the effect on the slope could be mathematically derived as the line did not converge. As seen in the shorter 10 minute application, the fEPSP recorded from slices exposed to sarin showed no recovery in either the amplitude or slope.

The data recorded from slices exposed to sarin for 30 minutes was not statistically different from that recorded in slices exposed to sarin for 10 minutes. Therefore, to maximise the experimental time available, it was decided that slices would be exposed to sarin for 10 minutes in subsequent experiments.





Sarin when applied for 10 minutes caused a concentration dependent decrease in the amplitude (A) and slope (B) of the evoked fEPSP (mean  $\pm$  sem, n=4).

Concentration	Maximum effect (% of	Maximum recovery	Maximum recovery
(µM)	baseline compared to	achieved at 10-20	achieved at 20-30
	control slices)	min post washing (%	min post washing (%
		of baseline)	of baseline)
0.003	97. 1 ± 0.8**	100.3 ± 1.4	100.0 ± 1.7
0.010	96.6 ± 0.6**	98.8 ± 1.2	97.6 ± 1.5
0.030	96.6 ± 0.4**	99.0 ± 1.8	95.6 ± 1.9
0.100	89.8 ± 1.2	90.4 ± 1.3	89.7 ± 1.8
0.300	86.2 ± 0.8	91.9 ± 0.8*	91.7 ± 1.4*
1.000	84.1 ± 1.8	90.0 ± 2.6*	89.3 ± 3.4

# Table 4.3. The effect of a 10 minute application of sarin at a range of concentrations ( $0.003-1.000\mu$ M) on the amplitude of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs* control slices), paired t-test (recovery *vs* maximum effect)).

Concentration	Maximum effect (% of	Maximum recovery	Maximum recovery
(µM)	baseline compared to	achieved at 10-20 min	achieved at 20-30
	control slices)	post washing (% of	min post washing (%
		baseline)	of baseline)
0.003	95.9 ± 0.9	99.6 ± 2.0	99.3 ± 2.3
0.010	96.0 ± 1.2*	96.4 ± 1.9	94.5 ± 1.4
0.030	97.4 ± 2.7	96.5 ± 2.3	93.4 ± 2.4
0.100	87.6 ± 2.4	89.3 ± 0.6	89.4 ± 1.4
0.300	84.4 ± 1.8**	88.1 ± 1.4	87.1 ± 1.1
1.000	77.9 ± 2.7**	85.9 ± 3.9*	85.2 ± 4.8

# Table 4.4. The effect of a 10 minute application of sarin at a range of concentrations ( $0.003-1.000\mu$ M) on the slope of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs* control slices), paired t-test (recovery *vs* maximum effect)).



# Figure 4.5. Concentration-effect relationship for sarin in the guinea pig dentate gyrus.

Sarin when applied for 30 minutes caused a concentration dependent decrease in the amplitude (A) and slope (B) of the evoked fEPSP (mean  $\pm$  sem, n=4).

Concentration	Maximum effect	Maximum recovery	Maximum recovery
(µM)	(% of baseline	achieved at 10-20 min	achieved at 20-30 min
	compared to	post washing (% of	post washing (% of
	control slices)	baseline)	baseline)
0.003	92.5 ± 1.5**	97.4 ± 2.7*	96.0 ± 3.0
0.010	91.8 ± 1.5**	100.6 ± 5.2	98.8 ± 5.7
0.030	89.5 ± 1.3**	96.2 ± 1.6*	94.3 ± 1.2*
0.100	83.0 ± 0.6**	89.7 ± 1.3*	89.8 ± 1.6*
0.300	81.2 ± 2.3**	86.9 ± 3.4*	86.3 ± 3.5*
1.000	80.8 ± 1.0**	89.9 ± 2.1*	89.6 ± 2.1*
3.000	84.2 ± 2.0**	92.1 ± 3.7	90.2 ± 4.4

## Table 4.5. The effect of a 30 minute application of sarin at a range of concentrations ( $0.003-1.000\mu$ M) on the amplitude of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs* control slices), paired t-test (recovery *vs* maximum effect)).

Concentration	Maximum effect	Maximum recovery	Maximum recovery
(µM)	(% of baseline	achieved at 10-20 min	achieved at 20-30 min
	compared to	post washing (% of	post washing (% of
	control slices)	baseline)	baseline)
0.003	90.5 ± 2.7**	97.0 ± 4.0	94.9 ± 4.3
0.010	88.2 ± 1.6**	99.3 ± 7.2	97.4 ± 7.6
0.030	88.5 ± 2.0**	93.5 ± 3.4*	91.7 ± 3.1
0.100	80.4 ± 1.6**	87.5 ± 2.0**	87.4 ± 1.4**
0.300	74.2 ± 5.3**	82.9 ± 5.4*	80.4 ± 5.4
1.000	76.6 ± 1.2**	86.8 ± 3.1	85.5 ± 3.7
3.000	81.8 ± 2.3**	89.1 ± 3.5	86.2 ± 5.1

Table 4.6. The effect of a 30 minute application of sarin at a range of concentrations ( $0.003-1.000\mu$ M) on the slope of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect vs control slices), paired t-test (recovery vs maximum effect)).

## 4.4. Interactions between physostigmine and sarin

To investigate any potential time-dependent interactions between the two anticholinesterase compounds, physostigmine was applied at various time points relative to the start of the sarin exposure. A timeline for this part of the study is shown in Figure 4. 2. A low concentration of physostigmine (0.100µM) was chosen as this had little effect on

the amplitude of the fEPSP by itself as shown in Figure 4.3. This concentration also approximates a concentration achievable in the hippocampus following administration of 0.2mg/kg physostigmine in the guinea pig. This is the highest dose of physostigmine given in current *in vivo* assessments of the proposed next generation therapy (Wetherell *et al.*, 2006). This value is also approximate for a concentration of physostigmine that can be recorded in the rat hippocampus (Somani & Dube, 1989;Hallak & Giacobini, 1986). The average amplitude of the fEPSP recorded during the baseline was  $2.2 \pm 0.1$  mV and the average slope was  $2.4 \pm 0.2$  mV·msec<sup>-1</sup>.

## 4.4.1. Effect of a concurrent application of physostigmine on the sarin-induced depression of the fEPSP

Physostigmine when applied concurrently with sarin had no additional effect on the magnitude of the sarin-induced depression of the fEPSP amplitude. This can be seen in concentration-effect relationship shown in Figure 4.6 and also in the IC<sub>50</sub> of the concentration-effect relationship derived from these data (0.02µM for both the amplitude and slope of the fEPSP). This supports the hypothesis that both compounds are acting in the same way in the dentate gyrus and also that the maximum effects reported in Chapter 3 are in fact the total effect on the cholinergic system present within the slice model. When both compounds where washed off the slice (Figure 4.7), there was an initial small yet statistically significant recovery in the amplitude of the fEPSP during the 10-20 minute recovery epoch recorded from slices exposed to sarin concentrations of 0.03 (p<0.05), 0.30 (p<0.01) and  $1.00\mu M (p<0.05)$ . The significance of this recovery was only maintained at the two highest concentrations during the next recovery epoch. The slope of the fEPSP only showed a significant recovery at  $1.00\mu$ M sarin (p<0.05) during the first epoch. This was not maintained through into the second recovery epoch. Slices exposed to 0.30µM sarin showed a significant recovery in the slope of the fEPSP when measured during the second epoch (p<0.05). However the recovery observed in slices exposed to sarin at concentrations greater than 0.03µM did not reach baseline amplitudes.





Sarin concentration (µM)	Maximum effect (% of baseline compared to control slices)	Maximum recovery achieved at 10-20 min post washing (% of baseline)	Maximum recovery achieved at 20-30 min post washing (% of baseline)
0.010	98.8 ± 0.7	103.8 ± 1.2**	102.8 ± 1.3*
0.030	97.7 ± 0.4	104.3 ± 2.0	103.4 ± 1.8
0.100	93.9 ± 1.6	96.6 ± 1.5	96.2 ± 1.7
0.300	86.0 ± 1.1**	93.9 ± 2.3*	95.1 ± 1.9**
1.000	83.3 ± 2.2**	90.0 ± 1.5**	89.8 ± 1.9**

## Table 4.7. The effect of a concurrent application of 0.100µM physostigmine on the sarin induced depression of the amplitude of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs* control slices), paired t-test (recovery *vs* maximum effect)).

Sarin concentration (µM)	Maximum effect (% of baseline compared to control slices)	Maximum recovery achieved at 10-20 min post washing (% of baseline)	Maximum recovery achieved at 20-30 min post washing (% of baseline)
0.010	97.5± 0.6	102.1 ± 1.4*	101.8 ± 1.3
0.030	97.0 ± 0.3	102.4 ± 2.2	101.8 ± 2.6
0.100	93.9 ± 0.9	94.6 ± 2.3	94.4 ± 2.4
0.300	86.6 ± 0.6**	93.3 ± 1.8*	94.6 ± 2.1*
1.000	80.6 ± 3.2**	88.2 ± 2.5**	87.6 ± 2.6**

## Table 4.8. The effect of a concurrent application of 0.100µM physostigmine on the sarin induced depression of the slope of the evoked fEPSP.

Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect vs control slices), paired t-test (recovery vs maximum effect)).



**Figure 4.7.** The effect of a concurrent application of sarin and physostigmine. 0.100µM physostigmine was added at the same time as sarin. Amplitude (A) and slope (B) of the evoked fEPSP (sarin concentrations shown in graph legend; data presented as a mean of 4 slices with error bars omitted for clarity).

4.4.2. Effect of applying physostigmine before or after exposure to sarin

To mimic the use of physostigmine as a pretreatment,  $0.100\mu$ M physostigmine was applied 10 minutes before the sarin challenge. The average amplitude of the fEPSP recorded during the baseline was  $2.0 \pm 0.1$  mV and the average slope was  $2.0 \pm 0.1$ mV·msec<sup>-1</sup>. Again, the addition of physostigmine at this concentration had no additional effect on the magnitude of the sarin-induced depression of the fEPSP (85.1 ± 1.4% amplitude and 78.4 ± 2.2% slope). This observation was seen across the range of sarin concentrations tested previously and is confirmed in the concentration-effect relationship shown in Figure 4.8. However in contrast to slices exposed to sarin or sarin concurrently with physostigmine, slices pretreated with 0.100 $\mu$ M physostigmine 10 minutes prior to sarin exposure showed a large recovery in both the amplitude and slope of the fEPSP at all concentrations of sarin tested (Figure 4.9). The levels of fEPSP recovery observed are compared in Figures 4.10, 4.11, 4.12, and 4.13. With the exception of slices exposed to 0.10 $\mu$ M sarin, all slices showed a robust recovery which started almost immediately after both compounds were washed off the slice. The recovery seen in slices exposed to 0.30 $\mu$ M sarin (amplitude 101.9 ± 2.9% of baseline, slope 100.3 ± 1.4% of baseline) and 1.00 $\mu$ M sarin (amplitude 104.7 ± 43.1% of baseline, slope 109.4 ± 3.4% of baseline) sarin was highly significant (0.30 $\mu$ M both parameters p<0.01; 1.00 $\mu$ M both parameters p<0.001). This recovery was sustained across both recovery epochs and also until the end of the experiment.

To further test the time-dependency of this finding, a small number of slices (n=4) were pretreated with 0.100µM physostigmine either 1 minute or 30 seconds prior to exposure to 1µM sarin. Four slices were also treated with 0.10µM physostigmine 1 minute post sarin exposure. This is shown in Figure 4.14. Altering the time of physostigmine application relative to the sarin challenge did not modify the magnitude of the sarin induced depression of the fEPSP (with the exception of the slope of slices pretreated with physostigmine 1 minute before sarin challenge p<0.05). Whilst the level of recovery seen in slices pretreated with no physostigmine, this was not statistically significant during either epoch (with the exception of the slope of slices pretreated 1 minute before sarin challenge of slices pretreated 1 minute section 9.005).





The data for slices exposed to sarin alone are shown for comparison (•). As before sarin caused a concentration dependent decrease in the amplitude (A) and slope (B) of the evoked fEPSP (mean  $\pm$  sem, n=4).





Amplitude (A) and slope (B) of the evoked fEPSP (sarin concentrations shown in graph legend; data presented as a mean of 4 slices with error bars omitted for clarity).

Sarin concentration (µM)	Maximum effect (% of baseline compared to control slices)	Maximum recovery achieved at 10-20 min post washing (%	Maximum recovery achieved at 20-30 min post washing (%
		of baseline)	of baseline)
0.010	94.7 ± 1.1**	103.9 ± 3.6*	106.1 ± 4.4*
0.030	94.9 ± 0.6**	101.5 ± 0.7***	101.2 ± 3.2
0.100	88.6 ± 1.4**	98.6 ± 4.9	99.2 ± 4.9
0.300	87.1 ± 1.7**	101.9 ± 2.9*	100.3 ± 1.4**
1.000	85.1 ± 1.4**	104.7 ± 4.1**	109.4 ± 3.4**

Table 4.9. The effect of a single application of  $0.100\mu$ M physostigmine 10 minutes prior to the application of sarin on the amplitude of the evoked fEPSP. Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs* control slices), paired t-test (recovery *vs* maximum effect)).

Sarin	Maximum effect	Maximum recovery	Maximum recovery
concentration	(% of baseline	achieved at 10-20 min	achieved at 20-30 min
(µM)	compared to	post washing (% of	post washing (% of
	control slices)	baseline)	baseline)
0.010	94.3 ± 1.0**	106.1 ± 4.4*	104.0 ± 5.6
0.030	94.5 ± 3.0**	101.2 ± 3.2**	100.4 ± 3.9*
0.100	88.1 ± 1.5**	99.2 ± 4.9	96.0 ± 5.6
0.300	84.9 ± 1.5**	100.3 ± 1.4***	100.2 ± 2.2**
1.000	78.4 ± 2.2**	109.4 ± 3.4**	110.3 ± 3.8**

Table 4.10. The effect of a single application of 0.100 $\mu$ M physostigmine 10 minutes prior to the application of sarin on the slope of the evoked fEPSP. Also shown is the maximum recovery achieved during washing with drug-free ACSF (mean  $\pm$  sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 unpaired t-test (maximum effect *vs*)

control slices), paired t-test (recovery vs maximum effect)).





Data recorded during the 10-20 minute epoch post start of washing in slices that have received either no physostigmine (white bar),  $0.10\mu$ M physostigmine 10 minutes before sarin (hatched bar), or a concurrent application of  $0.10\mu$ M physostigmine (solid black bar). Results are shown for the amplitude (A) and slope (B) of the fEPSP compared to the maximum effect of 1µM sarin on each parameter (mean ± sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 paired t-test).





Data recorded in slices that have received either no physostigmine (white bar), 0.10 $\mu$ M physostigmine 10 minutes before sarin (hatched bar), or a concurrent application of 0.10 $\mu$ M physostigmine (solid black bar). Results are shown for the amplitude (A) and slope (B) of the fEPSP compared to the maximum effect of 1 $\mu$ M sarin on each parameter (mean ± sem, n=4, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 paired t-test).



## Figure 4.12. Comparison of the level of recovery achieved during the 10-20 minute epoch post start of washout.

(A) amplitude and (B) slope of the fEPSP. Slices were exposed to either sarin ( $\bullet$ ), 0.10µM physostigmine and sarin ( $\bullet$ ), or 0.10µM physostigmine 10 minutes before sarin ( $\bullet$ ). Data expressed as mean ± sem, n=4.





(A) amplitude and (B) slope of the fEPSP. Slices were exposed to either sarin ( $\bullet$ ), 0.10µM physostigmine and sarin ( $\bullet$ ), or 0.10µM physostigmine 10 minutes before sarin ( $\bullet$ ). Data expressed as mean ± sem, n=4.





Amplitude (A) and slope (B) of the fEPSP after a single challenge with  $1.00\mu$ M sarin. Physostigmine ( $0.10\mu$ M) was added 10 minutes before ( $\blacksquare$ ), 1 minute before ( $\blacksquare$ ), 30 seconds before ( $\blacksquare$ ) and 1 minute after the sarin challenge ( $\blacksquare$ ). Slices exposed to  $1.00\mu$ M sarin with no physostigmine are also shown for comparison ( $\blacksquare$ ). Data presented as a mean of 4 slices with error bars omitted for clarity. Data have been adjusted so that the sarin application acts as a datum across conditions.

4.4.3. Effect of increasing the concentration of physostigmine

A small number of studies were carried out using 3.00µM physostigmine to investigate if the concentration of physostigmine as well as time of application was important in the recovery of the amplitude of the fEPSP in slices exposed to sarin. Four slices were pretreated with 3.00µM physostigmine 30 seconds prior to challenge with 1.00µM sarin

(Figure 4.15). Increasing the concentration of physostigmine to  $3.00\mu$ M significantly reduced the magnitude of the sarin-induced depression of the amplitude of the fEPSP compared to that recorded from slices that had received no physostigmine (89.8 ± 0.6% of baseline amplitude compared to 84.1 ± 1.8% of baseline amplitude in slices which received no physostigmine, p<0.05). The slices showed significant recovery in both parameters measured during the 10-20 minute recovery epoch (p<0.01) compared to slices which received no physostigmine. This recovery was sustained during the next epoch (amplitude p<0.05 and slope p<0.01).

In a separate study, four slices were concurrently exposed to  $1.00\mu$ M sarin and  $3.00\mu$ M physostigmine (Figure 4.16). The increase in physostigmine concentration had no effect on the magnitude of the sarin-induced depression of the fEPSP when measured in either parameter. Despite an apparent recovery during both the first recovery epoch (10-20 minutes), there was no statistical difference between both physostigmine concentration used and that recorded from slices exposed to sarin alone. However the recovery observed in slices which received  $3\mu$ M physostigmine concurrently with the sarin challenge reached a statistically significant level during the 20-30 minute recovery epoch.





Amplitude (A) and slope (B) of the fEPSP. Physostigmine at  $3.00\mu$ M was added 30 seconds before challenge with  $1.00\mu$ M sarin. Data from slices pretreated with  $0.10\mu$ M physostigmine 30 seconds before sarin challenge and slices which received no physostigmine are also shown for comparison. Data presented as mean  $\pm$  sem n=4.





Amplitude (A) and slope (B) of the fEPSP. Physostigmine at  $3.00\mu$ M (-) was added concurrently with  $1.00\mu$ M sarin. Data from slices which  $0.10\mu$ M physostigmine concurrent with  $1.00\mu$ M sarin challenge (-), and slices which received no physostigmine (-) are also shown for comparison. Data presented as mean  $\pm$  sem n=4.

#### 4.5. Discussion

The aim of this study was to investigate the actions of the carbamate physostigmine and the organophosphorus nerve agent sarin in the dentate gyrus slice model. The previous chapter has shown that this model retains an innate cholinergic response. The use of the dentate gyrus slice model builds upon previous work which has utilised slices of the hippocampus (of which the dentate gyrus is part) to study the effects of nerve agents and other cholinesterase inhibitors on the production of seizures (Harrison *et al.*, 2000;Harrison *et al.*, 2004).

To allow a full understanding of the effects of both compounds in the dentate gyrus model, the concentration dependent effects of these compounds have been characterised. The production of a concentration-effect relationship for sarin in this model has not been reported before and is a novel piece of information furthering our understanding of the CNS effects of this compound. The range of concentrations tested includes those at which previous *in vitro* work has been conducted in the hippocampal slice (Harrison *et al.*, 2004). Both compounds caused concentration dependent decreases in the output of the medial perforant path measured as a fEPSP in the molecular layer of the dentate gyrus. The effect of physostigmine 10µM on the amplitude of the fEPSP (reduction to 83.7  $\pm$  1.0% of baseline) is of a similar magnitude to that reported previously in the rat dentate gyrus slice (Colgin *et al.*, 2003). The effect reported in the present study has been shown to be consistent and robust, and therefore reinforces the suitability of the guinea pig for use in OP and nerve agent studies.

A similar study which used the anticholinesterase compound S27 has been reported. This compound is based on the nerve agent soman, and has been structurally altered so that it resembles an aged version of the parent agent compound. This makes it a potent inhibitor of AChE which is resistant to intervention by existing therapies such as oximes. The results obtained for sarin in the present study compare favourably with those reported for S27 (reduction to 84.1  $\pm$  1.8% of baseline compared to 81.0  $\pm$  4.0% reported for S27) (Melchers *et al.*, 1994). These results taken with those presented in chapter 3 show that the depression of the fEPSP in the dentate gyrus is mediated by the inhibition of AChE. The resultant excess of ACh then acts to stimulate inhibitory GABAergic transmission within the dentate gyrus. This results in a decrease in the excitability of the principal granule cells, which is recorded *in vitro* as a decrease in the amplitude of the fEPSP (Canning & Leung, 2000).

The concentration-effect relationships shown in Figures 4.2, 4.3 and 4.4 show that increasing the concentration of the AChE inhibitor to  $30.00\mu$ M physostigmine and  $3.00\mu$ M sarin, does little to cause further reductions in the magnitude of the fEPSP. Comparing the findings of the present study and those reported by others in the literature (Melchers *et al.*, 1994) (Colgin *et al.*, 2003), it would appear that a reduction of the amplitude of the fEPSP by approximately 20% can be considered the maximum depression achievable by AChE inhibition. However, another study using slices taken from guinea pigs reported a much larger depression (~50%) when the cholinergic agonist carbachol (20µM) was applied (Kahle & Cotman, 1989).

The protective actions of physostigmine in this model are believed to be due to its ability to reversibly inhibit the actions of AChE. Physostigmine acts as a reversible pseudoinhibitor of AChE (the term pseudo-inhibitor is used as the inhibition of AChE by physostigmine is not truly reversible as the carbamate is metabolised via a process called decarbamolyation and is therefore altered during the interaction). Physostigmine (and other carbamates) sequesters a pool of AChE which is effectively inactive during the challenge with the nerve agent. This sequestered enzyme is therefore protected from being phosphorylated by circulating nerve agent which would result in the enzyme being irreversibly inhibited. Once the metabolism of the reversible inhibitor has been completed, the AChE molecule is free to interact with either ACh or another inhibitor molecule. This process is ongoing during the nerve agent challenge and effectively produces a supply of active AChE to allow normal termination of the cholinergic signal. The organophosphorus compound whilst quickly reaching a high plasma concentration is rapidly metabolised to the phosphonic acid metabolite and excreted. It is desirable therefore to select a reversible inhibitor of AChE which has a half life longer than the period when the nerve agent is at its peak concentration. The half-life  $(t_{1/2})$  for the decarbamolyation of physostigmine in the guinea-pig is 32 minutes (erythrocyte). It is possible that approximately 5% of physostigmine-inhibited AChE will be made available within 1-2 minutes following application (Wetherell & French, 1991). The protective inhibition of a pool of AChE by physostigmine has been shown theoretically by Green using a dynamic kinetic model (Green, 1983). This has recently been confirmed in vitro by (Eckert et al., 2006).

The results from the present study have shown that the effectiveness of physostigmine treatment in reversing a nerve agent induced change in the slice response increases with the amount of time it is present before the nerve agent challenge. The protective action of physostigmine has been described as prophylactic and has been shown theoretically (Green, 1983) and is in agreement with the findings of the present study. This has been shown to also be the case *in vivo*. Koster showed that physostigmine given before DFP

protected cats against the lethal effects of the organophosphate (Koster, 1946). More recent studies by Berry and Davies (Berry & Davies, 1970) and Gordon *et al.*, (Gordon *et al.*, 1978) have confirmed this and reported that the optimum time between application of carbamate (physostigmine, pyridostigmine, mobam, or decarbofuran) and organophosphate was between 10 and 60 minutes depending on the carbamate used (physostigmine most effective following 10 minutes of pretreatment (Berry & Davies, 1970;Gordon *et al.*, 1978)). This correlates well with the results of pharmacokinetic studies in rats which showed that following an intramuscular injection of 650µg/kg [<sup>3</sup>H]physostigmine, the plasma ratio of physostigmine in the brain peaked after 15-22 minutes before decreasing (Somani & Khalique, 1986).

This time course also approximates to the time required for a steady state of inhibition (where inhibition of AChE is balanced by the reactivation of previously inhibited AChE) of blood AChE to be reached in guinea pigs (Dawson 1981). The deacylation step in the decarbamolyation process is the rate limiting step in this reaction (Triggle et al., 1998). The application interval of 10 minutes used in the present study would therefore allow the AChE present within the dentate gyrus to approach this steady state of inhibition. In contrast, organophosphates being more potent inhibitors of AChE are likely to reach a steady state of inhibition much quicker following application. Decreasing the time between physostigmine application and nerve agent challenge as in the present study, therefore changes the dynamic relationship between the two inhibitors allowing a smaller pool of AChE to be protected by physostigmine. This appears to be independent of the concentration of physostigmine used. Increasing the concentration of physostigmine to 3.00µM from 0.10µM in the present study did not affect the recovery seen in slices exposed to 1.00µM sarin and the higher concentration of physostigmine. This would suggest that the relationship between the two inhibitors when applied together is not competitive. Again this finding supports the hypothesis that proposed by Green (Green, 1983).

Residual AChE activity can be measured in samples which have been treated with supramaximal concentrations of anticholinesterase compounds. Following inhibition with an irreversible inhibitor of AChE, this activity is depressed but will undergo limited recovery following removal of the inhibitor. If the same assay is pretreated with physostigmine, the level of residual activity is increased and the AChE activity undergoes a rapid recovery following removal of both inhibitors (Eckert *et al.*, 2006). The time course of this effect is similar to that reported for fEPSP recovery in the present study. It is possible that the proportion of AChE not affected by the inhibitor may maintain enough control over the level of ACh, to allow transmission from the perforant path into the molecular layer of the dentate gyrus. Delaying the application of physostigmine would

therefore reduce the amount of AChE available to undergo spontaneous reactivation. This would therefore explain the depressed recovery seen in slices which received physostigmine either concurrent to, or after the sarin challenge.

As well as an effective pretreatment against nerve agent poisoning, physostigmine has also been shown to have beneficial effects when applied post poisoning. Berry and Davies (Berry & Davies, 1970) reported that a single application of physostigmine (28-54 $\mu$ g/kg) 1 minute post challenge with soman effectively increased the LD<sub>50</sub> of the nerve agent two-fold. Physostigmine has been used successfully as part of a novel immediate therapy combination that prevents lethality and lessens the severity of incapacitation observed in guinea pigs challenged with a range of nerve agents (Wetherell *et al.*, 2006;Wetherell *et al.*, 2007). As reported above, the relative inability of physostigmine to reverse the sarin induced depression of the fEPSP when given concurrently or 1 minute after sarin challenge in the current study is intriguing. In the current study, two concentrations of physostigmine (0.10 and 3.00 $\mu$ M) were administered concurrently with the sarin challenge. A significant level of recovery was only seen in slices that received the higher 3.00 $\mu$ M application of physostigmine. No significant recovery was seen in slices treated post sarin challenge.

The differences between these findings and those of the *in vivo* studies may be due to the inclusion of other components of the immediate therapy. In the *in vivo* studies physostigmine was combined with the cholinergic antagonist scopolamine, and the oxime HI-6. The effect of scopolamine in this therapy is two-fold. Primarily, it counters the cholinergic effects of physostigmine and organophosphates (excess ACh) and secondly it has limited anticonvulsant properties if given immediately post poisoning. Oximes have the ability to dephosphorylate AChE, thereby removing the nerve agent complex and reactivating the enzyme. As well as the action on AChE, several studies have suggested that various oximes have other beneficial effects not related to the reactivation of AChE. These include direct interaction with the nicotinic ion channel (Tattersall, 1993;Tattersall, 1990), and interactions between HI-6 and physostigmine decreasing the rate at which AChE is carbamoylated (Dawson, 1994). The actions of both of these compounds may be acting on processes that are not visible using the dentate gyrus slice model and are relevant to the whole animal model. Oximes were not included in the current slice study due to a lack of time available to conduct the necessary experiments.

In the military and law enforcement context, pretreatment is a feasible option available to the commanding officer in most situations where there is a possible or probable exposure of personnel to nerve agents or similar compounds, and the timings used in this project reflect this to a large degree. In contrast whilst the use of OP pesticides is widespread around the world, the use of any form of pretreatment or prophylaxis is unlikely to occur. This may be due to several factors including logistical burden, cost, accountability, and the ethical consideration of taking a drug with known side effects on the off chance of exposure. For these reasons, agricultural exposures of humans to OP compounds is more likely to continue to be treated as a hospital case with antimuscarinic, anticonvulsant and oxime compounds. Similarly in the event of a deliberate release of nerve agent or other OP (i.e. terrorist attack), there would be no opportunity for pretreatment and the treatment given post-exposure is also likely to follow the antimuscarinic, anticonvulsant, and oxime regime.

In conclusion this study has shown that physostigmine effectively reverses the effects of the nerve agent sarin in a model of a central cholinergic response. This effect was dependent on the time between application of physostigmine and the challenge with sarin. Whilst the relative lack of effect of a concurrent or delayed application of physostigmine agrees with the kinetic model proposed by Green (Green, 1983), the differences between this slice model and the results reported *in vivo* require further investigation. The addition of other components of the proposed next generation therapy (HI-6 and scopolamine) would also further extend these findings and allow a better mechanistic understanding of the actions and interactions of the therapy compounds in a model of the CNS.

# 5. Effect of sarin on the activity of AChE in the hippocampus

### 5.1. Introduction

The experimental findings discussed in the previous chapters have demonstrated a nerve agent induced change in an excitatory response recorded from the dentate gyrus. This effect is mediated by inhibition of AChE, thus prolonging the effect of ACh within the dentate gyrus slice. To date there is a paucity of published data which quantitatively links AChE inhibition to a change in an electrophysiological response recorded *in vitro*. The following work was conducted to investigate the level of inhibition of AChE within slices exposed to sarin at the concentrations used in Chapter 4. This was then compared with the level of decrease seen in the fEPSP recorded from the molecular layer of the dentate gyrus. This will be a novel interpretation of the data and will allow the level of AChE inhibition needed to have a functional effect in this model to be determined.

The main mechanism of action of OPs and nerve agents is to inhibit AChE. This in turn leads to the physical signs of poisoning and detriment to normal functioning. The rate at which tissue cholinesterases (ChE) present with the dentate slice model are able to metabolise suitable substrates was measured using a modified Ellman assay (Ellman *et al.*, 1961). This is a two step assay and the principle reactions of this assay method are shown in Figure 5.1. Briefly, a thiol-bearing substrate (acetylthiocholine or butyrylthiocholine) is hydrolysed by ChE to yield thiocholine. This then reduces the chromagen 5,5'- dithiobis-2-benzoate (DTNB) to yield the coloured (yellow) dianion, 5-thio-2-nitrobenzoic acid (TNB). The production of TNB is then measured spectrophotometrically (change in absorbance) at 412nM. The rate at which the intensity of the yellow product increases is a direct measure of the rate at which ChE is breaking down acetylthiocholine or other substrate. In this assay no inhibitors of other cholinesterases such as BuChE were added, so the results presented here are a combination of both AChE and BuChE activity.



Figure 5.1. Principle of the Ellman method for the spectrophotometric determination of cholinesterase activity.
## 5.2. Methods

Whole hippocampi were sliced as described in Chapter 2. The slices were then exposed for ten minutes to sarin across the same concentration range as used in Chapter 4 (0.003µM-1.000µM), followed by a two minute wash in drug-free recording ACSF. At least two hippocampi from separate animals were exposed separately to each concentration of nerve agent. The tissue was not pooled. Three replicates were carried out on each hippocampal homogenate. Control slices were treated in the same manner as nerve agent treated slices but were not exposed to sarin. The amount of protein present in each homogenate was determined using the method detailed in chapter 2. All procedures were carried out at ambient room temperature (approximately 20°C).

## 5.3. Effect of sarin on hippocampal cholinesterase activity

Homogenates of control slices had a mean ChE activity of  $0.004 \pm 0.0005 \ \mu mol mg^{-1} min^{-1}$  (minimum  $0.003 \ \mu mol mg^{-1} min^{-1}$ , maximum  $0.005 \ \mu mol mg^{-1} min^{-1}$ , corrected for protein, n=4). Exposure to sarin at the three lowest concentrations tested ( $0.003 \ \mu$ M,  $0.010 \ \mu$ M and  $0.030 \ \mu$ M) caused little change in the level of ChE activity, with an apparent increase in activity seen in slices exposed to  $0.010 \ \mu$ M and  $0.030 \ \mu$ M sarin. This however was not statistically significant. In contrast, ChE activity was significantly reduced in the homogenates from slices exposed to the three highest concentrations of sarin (p<0.05 unpaired t-test). The activity data are shown in Figure 5.2.



## Figure 5.2. The effect of increasing sarin concentration on the activity of cholinesterase within hippocampal slice homogenates.

Sarin at concentrations above  $0.100\mu$ M significantly decreased the level of ChE activity recorded (\*p<0.05, \*\*p<0.01, unpaired t-test compared to control activity). Activity values have been corrected for the amount of protein present in the homogenate. Data presented are the mean of three replicates from two separate hippocampi  $\pm$  sem (n=4 control (0 $\mu$ M), n=2 for sarin exposed slices).

# 5.4. Correlation of ChE inhibition and functional effect in the dentate gyrus slice

The relationship between hippocampal ChE inhibition and changes in the evoked fEPSP was investigated. The level of ChE inhibition (as a percentage of control slices) was plotted against the changes recorded in the size of the fEPSP (presented as percentage of maximum change) following exposure to sarin (Figure 5.3).

Whilst the effect of sarin on the amplitude of the fEPSP (-2.9 to -15.9 % difference from control (Table 4.3)) was significantly different from control slices at all concentrations tested, this covered a range of ChE inhibition from +22.8% (increase in activity compared

to control samples) to -82.2%. As seen in Figure 5.2 the inhibition of ChE in this model was only statistically different from control when it reached a level of between -71.7% to –



Figure 5.3. The relationship between ChE inhibition and functional response. Effect of ChE inhibition on the amplitude (A) and slope (B) of the fEPSP recorded from the molecular layer of the guinea pig dentate gyrus (mean  $\pm$  sem, n=4).

82.2% of control. This was achieved with sarin concentrations of between  $0.100 - 1.00\mu$ M. The greatest effect on the amplitude of the evoked fEPSP was also recorded following exposure to these concentrations of sarin. A similar result was seen in the magnitude of the slope of the fEPSP.

#### 5.5. Discussion

This study has shown that the nerve agent sarin inhibits the activity of ChE within the hippocampus in a concentration dependent manner. The inhibition of ChE can then be related to the changes in the fEPSP recorded in the dentate gyrus and presented in the

preceding chapters. Interestingly the level of ChE inhibition does not appear to be directly correlated with the magnitude of the depression seen in the fEPSP recorded post sarin exposure. As shown in Chapter 4, sarin exposure caused a significant decrease in the amplitude of the fEPSP at all concentrations tested (Figure 4.4 and Table 4.3). However in comparable hippocampal slices exposed to sarin, the level of ChE inhibition was not significantly different from controls until the concentration of sarin exceeded 0.10µM and the relative activity of ChE was inhibited by approximately 71%.

One interpretation of the data shown in Figure 5.3 is that the perforant pathway is relatively tolerant of low level ChE inhibition. However at higher levels of ChE inhibition, the system becomes less tolerant and functional deficits become more obvious. This raises the possibility that there is reserve capacity of ChE enzymes (including AChE), in the hippocampus. In making any interpretation of this data, however, one must remember that this is drawn from a small number of slices (n=2). To fully understand the relationship between the level of ChE inhibition and effect on the fEPSP, more experiments are required. As well as increasing the overall number of replicates, further concentrations of sarin which caused inhibition of ChE activity by between 25 and 50% of control would need to be tested. This would allow for the true slope of the relationship shown in Figure 5.3 to be determined.

If, as postulated here, there is reserve capacity in the functional level of ChE activity in this system, it would be a very useful characteristic. Reserve capacity implies that a proportion of available enzyme could effectively be removed from use without any significant detriment to function. In the rat, the absolute minimum level of AChE activity at the neuromuscular junction following excess OP inhibitor has been defined as 3% of normal (Eckert *et al.*, 2006). In the guinea pig it has been reported that sarin ( $42\mu g/kg$  (~1 x LD<sub>50</sub>)) caused a rapid inhibition of blood (both red blood cell and white blood cell) AChE to between 5 and 10% of control values after 10 minutes. However in the hippocampus AChE activity was only reduced to a minimum of approximately 35% of control activity after 15 minutes (Shih *et al.*, 2005). This level of inhibition agrees well with that reported in the current study, although it must be remembered that the concentration of agent may have been different once metabolic processes *in vivo* have been taken into consideration. Reserve capacity in the level of ChE enzymes has been reported elsewhere within the mammalian nervous system including the NMJ (Wood & Slater, 2001).

AChE and the related enzyme BuChE can be found throughout the central and peripheral nervous systems (PNS). There is also evidence that as well as hydrolysing choline esters, these enzymes are also involved in many non-cholinergic processes (Balasubramanian & Bhanumathy, 1993;Whittaker, 1993). In the CNS, AChE can be

found both within areas rich in markers of cholinergic transmission and other areas such as the substantia nigra which have little or no cholinergic activity (Holmes *et al.*, 1997;Llinás & Greenfield, 1987). BuChE is also found in relatively high concentrations in both the CNS and PNS and has been shown to be located in neurons, glia, and endothelial cells (for review see (Darvesh *et al.*, 2003). In the hippocampus, neurons staining for BuChE and AChE can be visualised in the stratum pyramidale and stratum oriens as well as the polymorphic layer of the dentate gyrus (Darvesh *et al.*, 1998). In studies using AChE knockout mice, BuChE has been shown to be important for survival by modulating the action of ACh within the synapses of central respiratory centres (Chatonnet *et al.*, 2003;Duysen *et al.*, 2001). However it is reported that BuChE is not simply replacing AChE and hydrolysing ACh as it appears to act by reducing the release of ACh via a presynaptic modulatory process (Chatonnet *et al.*, 2003). This has also been reported at the neuromuscular junction of this mouse model (Minic *et al.*, 2003).

The level of ChE inhibition caused by physostigmine in the study reported here was not investigated. Physostigmine acts to reversibly inhibit AChE. The bound enzyme then becomes available as the complex undergoes a decarbamolyation reaction. As this process will be continuing as the samples are prepared, it is impossible to have high levels of confidence in the levels of inhibition recorded from physostigmine-treated hippocampi; the variability between technical and biological replicates will be substantial dependent upon the bound/unbound state of the physostigmine.

Comparing the data presented in Chapters 4 and 5, there is an interesting anomaly between the two sets of experiments. The data presented in Chapter 4 (Table 4.3) clearly shows that sarin at 0.003, 0.01 and 0.03µM significantly reduces the amplitude of the fEPSP. However the data presented in this chapter shows that there are no significant changes in the activity of ChE in slices exposed to these same concentrations of sarin. There are two possible explanations for this. Firstly, ChE inhibition may not be the only process involved in reducing the amplitude of the fEPSP. One explanation for the data obtained from slices exposed to the lower concentrations of sarin, is that the sarin may be acting directly on the granule cells possibly via muscarinic receptors. The direct interaction between anticholinesterase compounds and muscarinic receptors has been reported previously (Huff et al., 1994; Bomser & Casida, 2001). It is conceivable that such a process may be taking place in tandem with that mediated by ChE inhibition. Further experiments in which slice ChE was totally inhibited before the application of sarin, would be required to test this theory. The second explanation for this discrepancy is due to the limitations of the Ellman method and also the small number or replicates used in the current study.

The relative activity of ChE within the samples used is lower than most of those reported in the related literature. This may be largely due to the fact that the data presented in this study comes from tissue sample which included the total protein fraction and not that collected after centrifugation. Centrifugation and analysis of the resultant pellet would result in a lower volume of protein per unit activity thereby raising the level of activity per unit volume. The use of a detergent such as Triton-X 100 may also increase the level of activity as a result of solubilising the cell. The results presented in the current study should be taken with the caveat that the number of samples is low and there is evidence of variability between samples that received the same treatment. Further work is required to confirm these preliminary results.

The current study is novel in that it has addressed the relationship between ChE inhibition in the dentate gyrus and changes in an electrophysiological response in CNS tissue. Previous studies have shown that ChE inhibitors such physostigmine as can modify the output of principal cells of the hippocampus such as the pyramidal cells of CA1 and the granule cells of the dentate gyrus (Misgeld *et al.*, 1989;Colgin *et al.*, 2003;Harrison *et al.*, 2004). However, ChE activity was not recorded in these slices. Further work is required to fully understand the relationship between ChE inhibition and decrease in functional response (as measured in the fEPSP).

## 6. Discussion

## 6.1. Introduction

This study was designed with two primary objectives. Firstly, to investigate the mechanisms of action of anticholinesterase compounds in an *in vitro* model of a cholinergic response in the CNS, and secondly to investigate interactions between two different anticholinesterase compounds.

To achieve these aims a model of a cholinergic response in the CNS needed to be identified that fulfilled a set of criteria including the ease of preparation, the presence of a good intrinsic cholinergic network, and the retention of cholinergic activity which can be modulated by anticholinesterase compounds *in vitro*. Based on the findings of a literature review, a brain slice model of the hippocampus, specifically the dentate gyrus, was chosen for use in this study. Dentate gyrus slices exhibit an excitatory (glutamatergic) response following electrical stimulation of the perforant path which can be modified by application of compounds which inhibit AChE (Colgin *et al.*, 2003;Melchers *et al.*, 1994;Colgin *et al.*, 2003) and cholinergic agonists (Kahle & Cotman, 1989). The effect of carbamate AChE inhibitor physostigmine was first confirmed in slices prepared from the rat, before being transferred to the guinea pig. This work was then extended by using the nerve agent sarin. The guinea pig was the preferred species to use as it has been shown previously to better predict the efficacy of nerve agent treatments in primate species than other small laboratory animals such as the rat (Gordon *et al.*, 1978;Berry & Davies, 1970;Dirnhuber *et al.*, 1979).

## 6.2. Discussion of model characterisation

Several models were highlighted during the initial literature survey, which were of potential use for this project. These are described in chapter 1. Whilst all of the models fulfilled the main criteria required of the final model, a slice model of the hippocampus and dentate gyrus was chosen. The dentate gyrus is a well established model which is relatively easy to produce and maintain during experimentation.

A large part of this project was devoted to the characterisation of the fEPSP response recorded from the molecular layer of the dentate gyrus following stimulation of the medial perforant path. Application of cholinergic agonists or AChE inhibitors caused an apparent decrease in the amplitude of the glutamatergic fEPSP recorded from the molecular layer of the dentate gyrus. From the studies undertaken, several possible separate or interrelated processes were identified. Firstly, glutamate release from the perforant

pathway afferents could have been reduced by activation of a presynaptic mAChR. A decrease in the amount of glutamate released would cause a decrease in the amplitude of the recorded fEPSP. Secondly, a GABA<sub>A</sub> mediated component responsible for the decrease in amplitude of the fEPSP was identified.

#### 6.3. Discussion of physostigmine-sarin interactions

This project was designed to test two related hypotheses. These were that physostigmine is an effective pretreatment, and the second hypothesis was that physostigmine acts by sequestering a pool of AChE by reversibly binding to it, therefore preventing binding and inhibition by any circulating nerve agent. The results presented in Chapter 4 strongly support both hypotheses. The results reported in this chapter clearly show a statistically significant effect of time between application of physostigmine and subsequent application of the nerve agent sarin on the recovery of the amplitude of the fEPSP. This was most significant when physostigmine was applied 10 minutes prior to the sarin. This is similar to that reported in vivo (Berry & Davies, 1970;Gordon et al., 1978). In theory, the period of time between physostigmine and sarin applications could be extended and the beneficial effect of physostigmine should remain. The data presented in the current study lends support to this hypothesis. The extended pretreatment period would only be beneficial if it did not extend past the half-life for the decarbamoylation of physostigmine inhibited AChE. In the guinea pig the half life for red blood cell AChE inhibited by physostigmine is 32 minutes and that of plasma ChE is 52 minutes (Wetherell & French, 1991). Further experiments would be needed to fully confirm that an increased interval between application of physostigmine and sarin furthers this beneficial effect.

Another factor which has an important role in the pretreatment effect of physostigmine is bioavailability. Using the octanol:water partition coefficient method it can be shown that sarin is more lipophillic than physostigmine (log *P* sarin 0.3, physostigmine 1.6) and therefore is quicker to enter the tissue of the slice (Czerwinski *et al.*, 2006;Dahlin & Björk, 2008). For this reason physostigmine, especially at the relatively low concentration used in this study (0.100µM) needs to be in circulation prior to the sarin to allow it to reach (or approach) equilibrium within the tissue before the rapid influx of sarin into the slice begins. To overcome the disparity of the lipophilicity of the two compounds, physostigmine if added in excess will have a beneficial effect on the recovery post sarin challenge by outcompeting the sarin molecules for available AChE; despite the advantage that sarin has regarding increased lipophilicity. This is in effect what has happened in the experiment in which the concentration of physostigmine was increased to 3.00µM. A recent *in vivo* study has also shown that far greater concentrations of physostigmine are needed for it to

be effective against nerve agent poisoning when given post exposure (Wetherell *et al.*, 2007).

## 6.4. Recommendations for future work

This project has demonstrated the interactions between a reversible and irreversible inhibitor of AChE (physostigmine and sarin respectively) in a model of a CNS cholinergic response. However, as shown in previous chapters there are several other pathways/systems which can be affected by OPs and nerve agents. Long term effects are also reported in animal studies and human exposure case studies. Understanding the mechanisms behind these effects may aid the medical management of casualties and identify novel targets for therapeutic intervention.

Firstly, the current study reported in this document could be extended as follows. To properly identify the mAChR present on the presynaptic membrane of the perforant path, a more considered concentration-effect experiment would need to be conducted. In this a wider range of compounds which are more selective for the different mAChR subtypes would be used across a wider range of concentrations. This would address the limitations of the current study. Secondly, an *in vivo* study in which the effect of depressing the output of the perforant pathway in the guinea pig should be considered. This would involve *in vivo* recording of the fEPSP recorded from the intact dentate gyrus and its response to differing concentrations of OP or physostigmine given to the animal. Once this dose has been reliably calculated, it would then be given to freely moving guinea pigs and their behaviour analysed. A further refinement of this proposed study would require slices of the dentate gyrus to be prepared from these animals and examined as was done previously in Chapter 3. This would allow the physiological effect of depressing the perforant pathway fEPSP to be determined with greater confidence.

Using a slice model such as the dentate gyrus used in this study is ideal for use in short term studies (lasting several hours). After this the viability of the slice declines rapidly. Longer term studies which were designed to study the long term effects on neuronal function after a single high exposure or repeated low-level exposures to a nerve agent would be beneficial. The terrorist releases of sarin during 1994 and 1995 in Japan are amongst the best documented accounts of the effects of sarin in humans. An acute exposure to sarin resulted in changes to the structure of the brains of exposed survivors (decrease in volume of the insular cortex and the hippocampus (Yamasue *et al.*, 2007)) and also prolonged changes in the EEG (Yanagisawa *et al.*, 2006). It would be beneficial to understand the mechanisms behind changes in the brain following such exposures.

Such knowledge may aid the treatment of poisoned casualties leading to a better prognosis. Whilst whole animal studies have the benefit of allowing the researcher to investigate the whole system, it is sometimes difficult to focus on specific areas or mechanisms.

One approach to overcome this problem is to use organotypic cultures of tissues of interest. To understand the effects of either chronic low level or single exposure of sarin on an area of the brain identified as susceptible could be carried out using an organotypic culture of the hippocampus. Typically, thin slices of the brain structure of interest are taken from neonatal animals and allowed to grow in a cell culture environment. A viable slice culture retaining the structure of the original brain area is then able to be maintained over several weeks rather than the few hours usually associated with acute brain slices. Organotypic slice cultures (OTSC) have been routinely used to study damage caused by ischemia (McManus et al., 2004), seizures (Bausch et al., 2006), and neurogenesis (Sadgrove et al., 2006; Raineteau et al., 2004). Another benefit of the OTSC is that cocultures of related or adjoining structures can be grown together. This allows neuronal connections normally lost during the production of slices to be maintained. An example of this is the septo-hippocampal culture in which an explant of the septal nucleus is cultured alongside a hippocampal slice. This allows a cholinergic/GABAergic pathway to be reestablished in the culture which is usually lost due to the spatial separation of the two structures in vivo (Fischer et al., 1999;Gähwiler, 1988;Gähwiler & Brown, 1985;Gähwiler et al., 1991). It is proposed that the current physostigmine-sarin interaction study reported in Chapter 4 is repeated in an OTSC system containing a co-culture of the septal nucleus and hippocampus. This would allow the study of the long term (weeks) effect of physostigmine alone (as a pretreatment) or in combination with a nerve agent on the neuronal infrastructure and level of AChE in the hippocampus.

The current study has shown that at least in the dentate gyrus model, the effects of nerve agents are predominately mediated by mAChR (Chapter 3). However, it has also been reported that anticholinesterases can affect neuronal nAChRs (Nagata *et al.*, 1997;Smulders *et al.*, 2003;Smulders *et al.*, 2004a;Smulders *et al.*, 2004b). The relative lack of effective of the nicotinic antagonist mecamylamine was surprising as the dentate gyrus has a population of nAChR (Frazier *et al.*, 2003). Whilst the various subtypes of nAChR are found throughout the CNS, very little is known on how OPs interact with this class of receptor. This is a huge contrast to that which is known about the interactions of peripheral nAChR at the neuromuscular junction and OPs. A study which mirrors that reported in Chapter 3 should be carried out using a model (preferably a slice model or OTSC) which exhibits a robust nicotinic response. During the literature review for the current study, several models came to light which exhibit a response mediated by nAChR.

These include the medial habenula nucleus (Girod *et al.*, 2000), hippocampus (Frazier *et al.*, 1998;Alkondon & Albuquerque, 2001;Dani *et al.*, 2000;Jones & Yakel, 1997;McQuiston & Madison, 1999;Radcliffe *et al.*, 1999;Vogt & Regehr, 2001), nucleus accumbens (Fu *et al.*, 2000), and cortex (Chu *et al.*, 2000). The majority of these models were disregarded due to technical challenges in setting up the model or recording from the system. However, this decision should be revaluated in light of the results presented in the current study. If the involvement of neuronal nAChR is similar to that of the muscle-type nAChR in the periphery, the targeting of therapies against neuronal nicotinic targets may be of future interest especially in counteracting the respiratory depression and incapacitation seen post nerve agent poisoning.

Finally, as the current study and review has shown, nerve agents and OPs have other effects besides causing seizures. A further in vitro study which would enhance our knowledge of one of these effects is proposed. This is the role of inflammation in brain injury and recovery following nerve agent poisoning. Currently, the role of the inflammatory process in nerve agent poisoning is poorly understood and may have a bearing on the treatment and prognosis of casualties. The control of inflammation plays an important part of the homeostasis of the CNS (Wang & Shuaib, 2002;Stollg & Jander, 1999) and can be activated by a variety of stimuli such as infection (Koontz et al., 2008), trauma (Toft-Hansen et al., 2007), seizures (Wang & Shuaib, 2002; Vezzani & Granata, 2005), and chemical insults (Monnet-Tschudi et al., 2007). Recent reports have shown that the nerve agents soman and sarin cause an acute inflammatory response within the CNS which results in the activation of astrocytes and microglia within the brain (Williams et al., 2003; Chapman et al., 2006). These immune cell types once activated release many different chemical mediators including cytokines and eicosanoids. Following nerve agent exposure large increases in the levels of mRNA for pro-inflammatory cytokine markers such as IL-1β have been reported (Svensson et al., 2005). Certain cytokines have been shown to affect neuronal cell signalling and play an important role in the activation of pathways which lead to cell death. Prolonged inflammation therefore may be deleterious to cell survival. If the loss of cells is sufficiently severe, this may result in loss of certain functions and other pathophysiological changes in the casualty. A co-culture model of the hippocampus and macrophages has been described (Brana et al., 1999) which will allow the interactions between nerve agents and immune cells of the CNS to be studied. Again, better understanding of these underlying processes may result in novel treatments to manage the OP poisoned casualty.

## 6.5. Conclusion

This study has shown that anticholinesterase compounds including physostigmine and sarin are capable of modulating the output of the glutamatergic granule cells of the dentate gyrus. The results reported in Chapters 3 and 4 show that ACh and GABA are responsible for the modulation of the output of these cells. Three possible locations for this interaction have been identified (Figure 6.1).



**Figure 6.1. Proposed sites of action of anticholinesterases in the dentate gyrus.** This diagram shows the sites likely to be involved in the modulatory effects of anticholinesterases in the dentate gyrus. Sources of endogenous ACh are represented by the yellow arrows.

Released ACh causes excitation of the CA3 pyramidal cells (1) and also hilar interneurons (2) resulting in an increase in inhibition of the granule cells (note excitation of CA3 pyramidal cells will also lead to direct excitation of hilar interneurons). Inhibition of AChE in these locations will lead to a continued cholinergic excitation of the two cell types. A third possible location for the action of the anticholinesterases is at the post synaptic terminals of the perforant pathway afferent fibres (3). It is postulated that they may

prolong the action of ACh on pre-synaptic autoreceptors (possibly M4-like) resulting in a decreased release of glutamate. Any one of these three actions (or a combination of them) will decrease the excitability of the granule cells. This is recorded as a decrease in the amplitude and slope of the fEPSP.

This study has begun to address the knowledge gap that exists between what is known of nerve agent effects in the peripheral nervous system and those in the CNS. The approach taken has been novel in as much as most studies looking at the problem of nerve agent and OP poisoning tend to focus on the seizure activity typical of this type of poisoning. Identification of a non-seizure effect allows for a greater understanding of the underlying processes that occur following intoxication in the CNS. The data presented in this study is the first direct evidence of the mechanism of action of physostigmine protection against nerve agent in the CNS. This is an important step towards the identification and introduction of more centrally effective treatments which will build upon the existing therapies to the benefit of the OP poisoned casualty.

# 7. Appendix A

## Reagents

- 1. 0.3M NaOH
- 2. 0.6M NaOH
- 3. 2% NaCO<sub>3</sub>
- 4. 1% Na-K tartrate in 0.5% CuSO<sub>4</sub> (pH adjusted to 7.4 with NaOH if precipitate forms)
- 5. 2mg/ml BSA in water
- 6. 1N Folin and Ciocalteaus phenol reagent (dilute stock 1:1 with water)

## Method

1. Make up standards and unknowns as follows: Dilute BSA 1:1 with 0.6M NaOH to give 1mg/ml in 0.3M NaOH

Dilute unknowns 1:1 with 0.6M NaOH

2. To duplicate test tubes add the following (all volumes  $\mu$ I)

0.3M NaOH	1mg/ml BSA	Unknown	Protein (µg)
	in 0.3M NaOH	diluted 1:1 in	
		0.6M NaOH	
200	0		0
195	5		5
190	10		10
175	25		25
150	50		50
100	100		100
50	150		150
0	200		200
180	-	20	?
140	_	60	2

140	-	60	?
100	-	100	?

- 3. Mix 49 ml of reagent 3 with 1 ml reagent 4
- 4. Add 1 ml of the above to all tubes and incubate at RT for 15 mins
- 5. Add 0.1 ml of reagent 6 to all tubes
- 6. Incubate for 30 min at RT
- 7. Read A<sub>700</sub> using a spectrophotometer (zeroed on blank)
- Plot standard curve and determine unknowns (results will be μg protein in original sample (10, 30, 50 μl)

N.B. used 200µl tissue

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