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Aminergic modulation of acetylcholine responses
recorded from an identified insect (*Periplaneta*
americana) motoneurone.

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(Ph.D.)

September 2000



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ACKNOWLEDGEMENTS

Hidden amongst the science presented here is four enjoyable years spent at the Gatty. To say I landed on my feet in St. Andrews would definitely be an understatement, and I have been extremely lucky in the people I have met and the friendships formed. Naturally, there are a few people that deserve a special mention and without whom this Ph.D. would never have happened or been so fulfilling.

Firstly, I would like to thank Bob Pitman for his wonderful support and encouragement over the last four years. He has provided thoughtful criticism and been a positive influence at every stage of this research, and always expressed interest in even my more bizarre notions. Without doubt he has greatly assisted my development into what I hope is a better scientist, and for that I am deeply indebted to him. He was also good enough to accept my rather graceless retirement from the squash court in the best manner. On the work front I would also like to thank Dr. Beulah Leitch and Sarah Judge for their generous input and assistance with the electron microscopy.

Secondly, I would like to thank my parents. Two incredible, individual people whom I love very much. They have always fully supported my journey into the murky world of scientific research, and it has been a source of much joy to me that they follow my progress with such keen interest.

Additionally I make no apologies for being distracted somewhat by love. Maybe the completion of this thesis would not have taken so long, but then it would not have been nearly so bearable as it was with the distraction of beautiful Hanna.

Then finally there are all the rest and please forgive the brief name checks: JB, Joe, Robbie, Dave, Irv, Ann, and Roland amongst a host of others for their friendships, fun and laughter. Finally I would like to thank the MRC (UK) for funding this research project, and also acknowledge the contributions made by my Grandma and Oddbins.

ABSTRACT

Neurones transmit and integrate information from sensory afferents to produce co-ordinated behavioural responses to environmental cues. Neurones transmit information in the form of rapid electrical signals. Communication between neurones and from neurones to muscles occurs at synapses, of which there are two types - electrical and chemical. In the former, there is direct electrical connection between the neurones. Whilst in the latter transmission of the signal from a presynaptic to postsynaptic neurone is reliant on the release, diffusion and binding of signal molecules, termed neurotransmitters. Alterations in the efficacy of transmission at chemical synapses is thought to impart flexibility to neuronal circuits and underlie the process of behavioural adaptation.

The aim of this research was to investigate whether neurotransmitters that act at G-protein-dependent metabotropic receptors can by the generation of intracellular second messenger molecules indirectly alter the efficacy of 'fast' synaptic transmission mediated by ionotropic receptors. The biogenic amines, dopamine, octopamine and serotonin are endogenous neurotransmitters that are known to act predominantly via metabotropic receptors to modulate neuronal signalling in a number of preparations. Bath application of all three amines suppressed nicotinic acetylcholine responses recorded from the fast coxal depressor (D_f) motoneurone of the cockroach *Periplaneta americana*. The presence of dopamine and serotonin immunoreactive neurones close to arborisations of D_f suggests that such modulation could occur *in vivo*. Electrophysiology combined with a pharmacological approach was used to determine the intracellular second messenger pathway by which the most potent amine, serotonin, suppresses nAChR responses. Modulation was G-protein-dependent and mimicked by an increase in intracellular cyclic nucleotide levels. Inhibition of protein kinases-dependent phosphorylation also prevented aminergic suppression of ACh responses. This suggests that monoamines, and in particular, serotonin can modulate the efficacy of nicotinic ACh transmission in the cockroach via an indirect G-protein dependent phosphorylation pathway, and that this represents a potential molecular mechanism for changes in the efficacy of cholinergic transmission.

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GENERAL INTRODUCTION

The ability of a finite number of neurones to integrate multiple, widely variable sensory inputs and produce defined behavioural patterns to suit the ongoing, ever changing environment is an incredible phenomenon evident in all but the most simple nervous systems. To produce a coherent behavioural response needs at the fundamental level a degree of temporal and spatial co-ordination of numerous neural circuits, that are at the same time sufficiently flexible to cope with novel situations. This requirement is reflected in the organisation of the neural networks – the developmental alignment of cells to form specific connections onto target neurones, and by the mode of communication between these individual neurones. This occurs predominantly at specialised structures termed either chemical (Sherrington, 1947) or electrical synapses. The former is characterised by a divide termed the synaptic cleft between the neurones which are characterised as presynaptic or postsynaptic depending on whether they respectively trigger or receive the signal. Such transfer of information is unidirectional unlike that usually mediated by electrical synapses. Electrical synapses are characterised by the close association of the neuronal membranes, and near continuous transfer of information from one cytoplasm to another via gap junctions, cell-to-cell pores that allow the free flow of ions and small molecules. Communication via such synapses is predominantly used to relay defined motor patterns such as escape responses, where the need for adaptability has been forsaken in favour of speed. A typical example of this is seen in the startle behaviour mediated by lateral giant interneurones in the crayfish (e.g. Kramer, Krasne and Wine, 1981).

The inter-neuronal communication at chemical synapses is reliant on the release of messenger molecules, termed neurotransmitters, from the presynaptic neurones in response to either an action potential (spiking synapses) or a graded response (non-spiking synapse), diffusion of the released transmitter across the synaptic cleft and its binding to transmembrane protein complexes, termed receptors, on the postsynaptic neurone, which, once activated, initiate a postsynaptic signal. Variability in the efficacy of synaptic transmission imparts a large degree of flexibility in neuronal circuits by conversion of the all-or-nothing action potential into chemical signalling, whose degree of operation is dependent on the extent to which the molecular mechanisms underlying chemical transmission function. Over recent years a great deal of interest has focused on the functional consequences of modulation of synaptic transmission, not only for its role in motor and behavioural plasticity but also as a means of flexibility in central nervous systems thought to underlie adaptive processes such as learning and memory formation.

This review is divided into three main sections, of which the initial section develops the broad concepts of neurotransmission at chemical synapses. The mode of communication is discussed, with particular reference to the sites of modulation. Towards the end of the first section the reasons for using a postsynaptic preparation are considered and the identified insect preparation introduced. The subsequent section concentrates on the neurotransmitter acetylcholine, and reviews the extensive literature from both vertebrate and invertebrate preparations relevant to studies on regulation of cholinergic transmission. The signalling systems proposed to regulate neurotransmission mediated by acetylcholine are reviewed in the final section, completing the mechanism underlying neuromodulation.

Section 1: NEUROTRANSMISSION AT CHEMICAL SYNAPSES.

1.1 Modulation of neuronal signalling at chemical synapses in central nervous systems.

Much of the pioneering work on transmission at chemical synapses was performed in the 1950s on frog neuromuscular synapses (for example see Fatt and Katz, 1951). This was due to the fact that it had the major advantage that the postsynaptic muscle fibres are large and accessible for electrophysiological recording, and further, that signal transmission was mediated by a single neurotransmitter. The morphology of vertebrate chemical synapses in the central nervous system was elucidated shortly afterwards (Palay, 1958) and the first electrophysiological studies conducted on chemical synapses in spinal cord preparations by Eccles and colleagues (see Eccles, 1964). Work on invertebrate synapses also developed in the 1950s and 1960s after the initial studies on squid giant fibre synapses by Young (1938)

Transmission at chemical synapses is reliant on the release and binding of neurotransmitters. A range of low molecular weight substances and neuroactive peptides have been classified as neurotransmitters according to whether they fulfil four criteria: (1) the neurotransmitter is synthesised in the neurone. (2) It is present in the presynaptic terminal and released in sufficient quantities to exert a defined effect on the postsynaptic target. (3) The neurotransmitter can be applied exogenously and mimic the actions of the endogenously released neurotransmitter and (4) there are specific mechanisms for the removal of the transmitter molecule from the synaptic cleft. Nine common, low molecular weight neurotransmitters satisfy most of these ‘classical’ criteria with a tenth grouping of the adenine compounds fulfilling a less defined neurotransmitter role (for example see White.

1988). With the exception of acetylcholine (ACh), the other 8 are all derivatives of amino acids. These include glutamate, glycine and γ -aminobutyric acid (GABA) as well as the group loosely associated as biogenic amines. This last group includes the catecholamines that are synthesised from tyrosine, the indolamines which are derived from tryptophan, and histamine a product of decarboxylation of histidine.

Neurotransmitters on release into the synaptic cleft, exert their effect via binding to specific glycoprotein macromolecules, termed receptors, which are expressed in the membranes of cells. The specificity of the neurotransmitter ligand-receptor interaction is ensured by the folding of the receptors protein structure to form binding sites complementary to the ligand structure. Receptors are divided into two categories according to how they control membrane ion channels: (1) 'direct' ionotropic receptors (ligand-gated ion channels) and (2) 'indirect' metabotropic (G-protein-linked) receptors.

Ionotropic receptors mediate fast synaptic transmission on a time scale of milliseconds and include the excitatory nicotinic acetylcholine receptor (nAChR) (Changeux, 1990), glutamate N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) / kainate receptors (Wisden and Seuburg, 1993; Ziff, 1999) and adenosine triphosphate- (ATP) gated cation channels (P2X receptors; Brake *et al.*, 1994; MacKenzie *et al.*, 1999) as well as the inhibitory GABA (Barnard, Darlison, and Seuburg, 1987) and glycine receptors (Rajendra, Lynch and Schofield, 1997). They are characterised by their pentameric structure, the individual subunits of the macromolecule arranged around the central ion-permeable channel, which opens on binding of the specific ligand to transduce the signal via flow of ions down their electrochemical gradient. The flow of ions across

the membrane generating either a positive (excitatory) or negative (inhibitory) shift in membrane potential, in the neurone expressing the receptor.

Metabotropic receptors consist of a single protein with seven α -helical transmembrane regions, which due to their reliance on activation of intracellular events rather than direct gating of an ion-permeable channel for the transduction of the ligand signal, operate over longer time courses than ionotropic receptors. Activation of metabotropic receptors stimulates or inhibits the production of intracellular second messengers via receptor-associated trimeric GTP-binding proteins (G-proteins). The term ‘second messenger’ includes a number of small diffusible metabolites such as adenosine 3’, 5’-monophosphate (cAMP), whose production leads to the activation of downstream enzymes and gating of receptors.

1.1.1 Presynaptic release of neurotransmitters.

Chemical synapses provide a plethora of processes with the potential to exhibit modulation corresponding to the three main stages of chemical signalling – transmitter release, diffusion and postsynaptic transduction. The initial process of transmitter release occurs in response to a presynaptic depolarisation of sufficient magnitude to cause opening of voltage gated calcium (Ca^{2+}) channels (Katz and Miledi, 1967; Miledi, 1973). If sufficient, the localised rise in intracellular Ca^{2+} stimulates vesicle release from the active zones of the presynaptic bouton – areas local to presynaptic dense bars where vesicle docking is facilitated, via molecular mechanisms that has been recently reviewed by Hilfiker, Greengard and Augustine (1999) (see also Bajjelieh, 1999). The extent to which vesicles containing neurotransmitter undergo exocytosis from presynaptic terminals can be regulated firstly by altering the functional properties of the membrane ion channels required

for the rapid and local increase in intracellular Ca^{2+} , secondly by modulating the machinery for synaptic vesicle fusion and release, and finally by altering the amount of transmitter primed for release via variability in the transport of neurotransmitter into vesicles and their subsequent trafficking to active release sites (for reviews see Starke, Göthert and Kilbinger, 1989; Miller, 1998; Liu, Krantz, Waites and Edwards, 1999).

Receptors play a fundamental role in regulating presynaptic processes, responding to chemical signals released from a variety sources including presynaptic afferents and retrograde transmission (see Starke, Göthert and Kilbinger, 1989 for review of the presynaptic terminal receptors or ‘autoreceptors’). The conductance of the presynaptic terminal to Ca^{2+} can be altered by activation of directly-gated ionotropic receptors (McGehee and Role, 1995). Inhibitory synaptic connections onto the presynaptic button in both invertebrate and vertebrate preparations have long been demonstrated to ‘shunt’ the afferent action potential by increasing chloride (Cl^-) ion conductance through ligand-gated ion channels (for reviews see Clarac and Cattaert, 1996; Rudomin and Schmidt, 1999). Ca^{2+} -permeable ionotropic receptors on presynaptic terminals, for example the vertebrate α_7 nicotinic acetylcholine receptor subtype, have also been demonstrated to facilitate vesicle release by increasing the intracellular Ca^{2+} concentration local to the site of vesicle release (for example see Gray *et al.*, 1996).

Presynaptic metabotropic receptors can also influence neurotransmission. A wide variety of G-protein-stimulated intracellular signalling cascades are known to affect vesicle exocytosis, amongst which are a number that are known to regulate the properties of ion channel proteins responsible for nerve terminal depolarisation (Reviewed by Miller, 1998). A well-characterised system involving presynaptic

metabotropic receptors is that of the *Aplysia californica* defensive gill-withdrawal reflex (see Kandel and Schwartz, 1982; Kandel *et al.*, 1983; Byrne and Kandel, 1996). The respiratory gill in *Aplysia* is protected by a mantle shelf. Withdrawal of the gill occurs in response to a tactile stimulus to the mantle shelf itself or the sensitive siphon process. The terminals of the mantle and siphon sensory neurones onto the interneurones and motoneurones responsible for gill retraction themselves receive presynaptic inputs from facilitating interneurones. The facilitating interneurones are stimulated by head and tail sensory interneurones (see Kandel and Schwartz, 1982 for review), and secrete the indolamine serotonin. Serotonin facilitates synaptic transmission between the mantle sensory neurones and gill motoneurones as proposed in the initial study by Brunelli *et al.* (1976) who also suggested that this action involved the second messenger cAMP. More recently a second intracellular messenger, diacylglycerol (DAG) has been implicated in serotonergic-dependent synaptic facilitation (Piomelli *et al.*, 1987; Braha *et al.*, 1990; Sossin and Schwartz, 1992; Sugita, Goldsmith, Baxter and Byrne, 1992). Both pathways exert their effects via enzymes termed protein kinases, which transfer a phosphate group from ATP to target proteins such as potassium (K^+) ion channels. Transfer of the phosphate group alters the protein conformation so producing a change in its activity. In the case of the *Aplysia* K^+ ion channel population, phosphorylation reduces gating of the ion pore and thus biases the membrane conductance towards increased Ca^{2+} conductance. The larger Ca^{2+} influx, triggered by the action potential in the presynaptic mantle and/or siphon sensory neurones increases the quanta of neurotransmitter vesicle exocytosis into the synaptic cleft (see Klein, Camardo and Kandel, 1982; Walsh and Byrne, 1989). The important points to take from this well studied preparation are that metabotropic receptors can

influence other membrane proteins via activation of intracellular second messenger systems and that phosphorylation plays a key role in the transduction pathways (Sweatt and Kandel, 1989).

1.1.2 Regulation of neurotransmitter concentrations in the synaptic cleft.

Levels of presynaptic neurotransmitter have been shown to be functionally regulated by neurotransmitter transporters and associated vesicle proteins, which are responsible for both transmitter concentrations in vesicles and the trafficking of the vesicles to sites of release (for reviews see Liu, Krantz, Waites and Edwards, 1999; Turner, Burgoyne and Morgan, 1999). Along with enzymes that degrade neurotransmitters, re-uptake transporters also play an important functional role in the maintenance of neurotransmitter concentrations in the synaptic cleft (see for reviews Amara and Kuhar, 1993; Beckman and Quick, 1998). Uptake transporters are membrane proteins that function to sequester the neurotransmitters into the neurones and glial cells bordering the synaptic site, where the ligands are then degraded by cytosolic enzymatic pathways. Interestingly, these neurotransmitter transporters are the site of action of a number of psychoactive drugs. In particular, a number of studies have elucidated the effect of the psychostimulant cocaine on the dopamine and serotonin transporters (Ritz, Cone and Kuhar, 1990; Rocha *et al.*, 1998). Cocaine inhibits monoamine uptake so causing a prolongation of the physiological effects of the amines both on the postsynaptic cell and via autoreceptors on the presynaptic terminus. Similarly the antidepressant fluoxetine, better known as ProzacTM, also forms an inhibitory interaction with serotonin transporters (see Miller and Hoffman, 1994). The importance of neurotransmitter transporters as a site for regulating synaptic transmission is increasingly apparent, particularly in the context

of developing new pharmacological agents for psychological disorders.

Furthermore, there is increasing evidence that transporters are subject to regulation by intracellular second messengers (Miller and Hoffman, 1994).

The second process for the removal of neurotransmitters from the synaptic cleft is by extracellular enzymatic degradation of transmitter (reviewed by Ladds, Hughes and Davey, 1998). ACh is the best understood ligand removed in this way. The enzyme acetylcholinesterase hydrolyses it to acetate and choline, the latter then been transported back across the presynaptic membrane for the resynthesis of ACh. The one other class of neurotransmitter known to be degraded extracellularly is the peptide neurotransmitter family which includes substance P. Degradation of these peptides is mediated by membrane bound, extracellularly orientated endopeptidases (for review see Konkoy and Davis, 1996).

1.1.3 Postsynaptic transduction of the neurotransmitter signal.

Binding of the neurotransmitter to specific postsynaptic receptors and transduction of the signal is the final stage of chemical transmission. Postsynaptic receptors, similar to their presynaptic counterparts, fall into two distinct classes: ionotropic receptors that directly gate a response by the opening of ion permeable channels, and metabotropic receptors that transduce their signal by indirect, intracellular pathways stimulated by activation of receptor-associated GTP-binding proteins.

Classically, chemical synaptic transmission alters the state of the postsynaptic cell via summation of the excitatory (EPSP) or inhibitory (IPSP) postsynaptic potentials transduced directly via the ionotropic receptors present on the postsynaptic surface. Activation of G-protein subunits by metabotropic ligands can also result in the opening of ion channels and thus produce summating EPSPs (see

Brown and Birnbaumer, 1990). G-protein subunits also interact with enzymes responsible for the generation of intracellular diffusible messenger molecules, termed second messengers. These include the cyclic nucleotides – cAMP, previously mentioned in the context of its presynaptic role in *Aplysia*, and guanosine 3', 5'-monophosphate (cGMP), as well as inositol triphosphate (IP_3). Such second messengers are known to activate protein kinases, which in turn control cellular events via phosphorylating specific protein substrates (Beavo, Bechtel and Kandel 1974; Krebs and Beavo, 1979). These target proteins include voltage dependent ion channels, apparently voltage and ligand-independent ion channels and ligand-gated ion channels (for reviews see Huganir and Greengard, 1990; Swope, Moss, Blackstone and Huganir, 1992; Raymond, Blackstone and Huganir, 1993; Smart, 1997). The regulation of the neuronal ion channels and receptors by a range of intracellular signalling pathways sensitive to the influence of metabotropic receptors is thought to contribute to the flexibility of synaptic transmission. Such plasticity in communication between neurones underlies the process of behavioural adaptation.

1.2 Preparations for the study of synaptic plasticity.

From the above discussion it is apparent that neurotransmission at chemical synapses is a highly dynamic process subjected to a number of diverse influences that can regulate each and every stage of signalling. It is also clear that receptors play a fundamental dual role in transmission, mediating both rapid changes at pre- and postsynaptic sites as well as slower time-course modulatory alterations.

Postsynaptic sites exhibit an abundant diversity of both metabotropic and ionotropic receptors and hence are ideal for the study of interactions between the two. Moreover due to the fact that postsynaptic sites, are more accessible for

electrophysiological study, the volume of literature on the topic greatly exceeds that devoted to presynaptic mechanisms.

The use of vertebrate *in situ* preparations to study behavioural adaptation at the molecular level has proved problematic due to the considerable problem of identifying particular cell populations. Instead, studies on modulation have relied on other approaches including dissociated cells (Chen, Stelzer, Kay and Wong, 1990; Cerne, Russin and Randic, 1993; Gillette and Dacheux, 1996), cell cultures (Blackstone *et al.*, 1994), or receptor systems expressed in *Xenopus laevis* oocytes (Huang, Morelli and Peralta, 1993; Hoffman Ravindran, and Huganir, 1994). Hence information from studies on vertebrate preparations has in the main consisted of hypotheses concerning molecular and cellular studies with little relation to the more global effects of modulation at the behavioural level. And it is only since the development of CNS slice technology and application of whole cell patch method of to vertebrate cells *in situ* that research has had the means to conduct such studies (for example see Mitoma and Konishi, 1996; Hsiao, Trueblood, Levine and Chandler, 1997; Mitoma and Konishi, 1999). This approach has allowed a degree of understanding of the plasticity exhibited by both pre- and postsynaptic neuronal processes, such as those long-term potentiation (LTP) in hippocampal neurones of vertebrates (see for review Bashir and Collingridge, 1992; Malenka, 1999). Indeed LTP and the related long-term depression (LTD) remain the only relatively understood phenomena underlying synaptic plasticity in a vertebrate neuronal circuit thought to underlie a definable behaviour process, that of learning and memory, and even this has not been shown conclusively when observed at the level of live animals performing learning tasks (see Holscher, 1999).

Recording from intact neural circuits capable of neuromodulation has not been such a problem with many invertebrate preparations, because of the accessibility, size and relatively small number of cells. Thus distinct neuronal circuits have been well characterised and individual, identifiable cells studied in some detail. Studies on neuromodulation at the level of the neuronal circuit have focused on well-defined central pattern generator systems such as the swimming pattern generator in the nudibranch mollusc *Tritonia diomedea*, and the decapod crustacean stomatogastric ganglion (reviewed in Bicker and Menzel, 1989; Katz, 1995; Katz and Frost, 1996). Not only are these systems ideal for the study of neuromodulation at the level of the neuronal circuit, where changes in behaviour are observable, but also due to the fact the constitutive cells are identifiable, they also provide a means for studying how changes in cellular and molecular properties contribute to adjustments to the entire circuit. Therefore, studies on invertebrate synaptic plasticity have an advantage over vertebrate research in providing an important means of relating the cellular and molecular aspects of neurotransmission to behavioural adaptation.

1.3 The fast coxal depressor (D_f) motoneurone of *Periplaneta americana*.

From what has been discussed so far it appears that an ideal system to investigate the hypothesis that metabotropic receptors can influence ionotropic receptors to modulate the efficacy of neurotransmission should exhibit a number of criteria. Firstly, it would be preferable to use an identifiable postsynaptic cell with pharmacologically defined populations of ionotropic receptors. Furthermore, the cell should be in an defined neuronal circuit responsible for a particular behavioural response that is known to be subject to modulation by endogenous ligands of metabotropic receptors.

A preparation that meets all the criteria listed above is the fast coxal depressor motoneurone (termed D_f by Pearson and Iles (1971) and corresponding to cell 28 as identified by Cohen and Jacklet (1967)) which is situated in the metathoracic ganglion of the cockroach *Periplaneta americana*. The soma of D_f is easily identifiable by its position and large size (approximately 80 – 90 µm in diameter). Furthermore, due to its peripheral location in the ganglion it is amenable to electrophysiological recording using two or more microelectrodes while pharmacological agents are applied locally to the cell body itself or perfused over the preparation. The accessibility of D_f for recording has allowed the biophysical and pharmacological properties of the neurone to be described in detail over recent years, as well as the presence of a number of neurotransmitter receptor populations (see Table 1).

The principle central neurotransmitters in insects are probably the excitatory neurotransmitter acetylcholine (ACh) and the inhibitory γ -aminobutyric acid (GABA) (see for reviews Pitman, 1971 and 1985; Gerschenfeld, 1973). Ionotropic receptors for both neurotransmitters on the soma membrane of D_f motoneurone have been pharmacologically identified and characterised (Pinnock, David and Sattelle, 1988; David and Sattelle, 1990). Brief application of ACh causes a transient depolarisation via a receptor sensitive to nicotine and to the antagonist alpha-bungarotoxin (α -bgt) similar to other nicotinic ionotropic AChRs identified in insect preparations (Eldefrawi and Eldefrawi, 1983; David and Sattelle, 1984). Furthermore, as for the well characterised nicotinic acetylcholine receptors (nAChRs) of the electric organ of *Torpedo californica*, the ACh current is carried predominantly by sodium and potassium ions but also to a lesser extent by calcium

Table 1. Receptor populations of the D_r motoneurone.

	Neuro-transmitter	Putative receptor	Postsynaptic response	Agonist profile [†]	Antagonist profile [†]
Soma of D _r motoneurone	α -bgt sensitive nAChR	Inward current via cation permeable ionotropic receptor. E _{ACh} : -35 mV	Nic>ACh>CCh>TMA	α -bgt> α -cotx> mecamylamine> dihydro- β -erythroidine	1,2,3
ACh	Mixed cholinergic AChR	Voltage-dependent (positive to -40 mV) inward current due in the main to reduction of IK _{C_A} .	Decamethonium *> oxotremorine> McN = (+)muscarine	Decamethonium *> PIR> QNB = atropine (α -bgt resistant)	4,5
	Muscarinic AChR	Slow onset, long duration depolarisation of membrane potential	Arecoline = oxotremorine	Scopolamine> atropine > PIR> 4-DAMP	6
GABA	GABAR	Hyperpolarisation via chloride permeable ionotropic receptor. E _{GABA} : -77 mV	GABA	Picrotoxin	7
	Glutamate GluR	Hyperpolarising response to L-glutamate mediated by chloride conductance.	L-glutamate > L-aspartate	Picrotoxin, γ -DGG	8
		Slow time course depolarising response	Kainate, quisqualate and L-Cysteate	-	8

Nic, nicotine; CCh, carbacholcholine; TMA, tetramethylammonium; α -cotx, α -cobratoxin; PIR, pirenzipine; QNB, quinuclidinyl benzilate; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine; γ -DGG, γ -D-glutamylglycine.

[†] Four most potent chemical agents quoted, see references for complete lists
* See David and Pitman (1993) for full explanation of the effects of decamethonium.

- | | | | | | |
|---|---------------------------|---|-------------------------|---|------------------------------------|
| 1 | David and Sattelle (1984) | 4 | David and Pitman (1995) | 7 | Pinnock, David and Sattelle (1988) |
| 2 | David and Sattelle (1990) | 5 | David and Pitman (1993) | 8 | Wafford and Sattelle (1989) |
| 3 | David and Pitman (1982) | 6 | Bai and Sattelle (1994) | | |

(David and Sattelle, 1990). The ACh response is abolished in low sodium (Na^+) saline and the outward current is sensitive to manipulation of external K^+ levels. Application of the amino acid GABA to the soma of D_f results in hyperpolarisation of the membrane potential (Pinnock, David and Sattelle, 1988). This response is mediated by an increase in Cl^- conductance and is blocked by picrotoxin. The D_f GABA receptor (GABAR) shows a pharmacological profile with similarities to both vertebrate GABA_A and GABA_C receptor agonists and antagonists (for reviews of vertebrate GABA receptors see Stephenson, 1988; MacDonald and Olsen, 1994; Bormann and Feigenspan, 1995). Similar to most insect GABARs the receptor is insensitive to the GABA_A antagonist bicuculline but is modulated by benzodiazepine flunitrazepam and the barbiturate sodium pentobarbital (for information of insect GABARs see Schnee *et al.*, 1997; Lees *et al.*, 1987).

The D_f motoneurone also exhibits a number of responses to glutamate agonists, suggesting that more than one glutamate receptors subtype could be expressed on the soma membrane (Wafford and Sattelle, 1989). One subtype of putative receptor exhibits a rapid hyperpolarising Cl^- conductance in response L-glutamate and could represent an ionotropic receptor (Raymond *et al.*, 2000). Application of kainate and quisqualate both elicit a slow depolarising response via an independent uncharacterised mechanism. The only other evidence for a glutamate receptor comes from a study by the same group (Sepulveda and Sattelle, 1989), who characterised a putative glutamate binding site using *Periplaneta americana* membrane extracts. No further evidence has been published as to the existence of glutamate afferents onto the D_f motoneurone, although more convincing evidence for glutamatergic transmission onto insect motoneurones, specifically motoneurones of the locust *Schistocerca gregaria*, has been published by Sombati and Hoyle (1984b)

and by Parker (1994). Until more research is conducted on glutamatergic transmission in the CNS of *P. americana*, the principal signalling role of glutamate should be regarded as an excitatory neurotransmitter at neuromuscular synapses (see Pitman, 1971).

As for metabotropic receptor populations, a putative receptor sensitive to ACh that initiates a voltage-dependent biphasic current at membrane potentials positive of -40 mV has been characterised by David and Pitman (1993). Like a number of other insect muscarinic G-protein-linked receptor controlled events, the resulting current is activated by muscarinic agonists and is resistant to α -bgt (for review see Trimmer, 1995). However, because it also exhibits an agonist response to nicotine, this ACh receptor been termed ‘mixed cholinergic’ or ‘ α -bgt-resistant’ by David and Pitman (1993). This metabotropic ACh receptor (mAChR) is linked to the inositol-1,4,5-tris phosphate (IP_3)/ Ca^{2+} pathway (David and Pitman, 1993; David and Pitman, 1994), and has been shown to modulate the activity of the ionotropic nicotinic AChRs as well as GABARs (David and Pitman, 1996b). A further population of muscarinic receptors has also been identified by Bai and Sattelle (1994), with a pharmacology different from that of the mAChR characterised by David and Pitman mentioned above. According to Bai and Sattelle, application of the muscarinic agonists arecoline and oxotremorine results in depolarisation at normal resting potentials, a response not observed by David and Pitman. However this muscarinic receptor is insensitive to the agonist McN-A-343 (McN) used to elicit the response observed by David and Pitman (1996).

A number of other putative metabotropic receptor ligands have been shown to be present in *P. americana* and more particularly in the metathoracic ganglion.

The biogenic monoamines dopamine, octopamine and serotonin are present throughout the central nervous tissue of *P. americana* (for review see Evans, 1980; also of interest Dymond and Evans, 1979; Fleming and Pitman, 1982; Bishop and O'Shea, 1983; Baker and Pitman, 1989). Furthermore, these amines are known to exert modulatory effects at multiple stages of the cockroach escape circuit of which the D_f motoneurone is a constituent part. All three monoamines modulate cholinergic excitatory postsynaptic potentials (EPSPs) recorded from identified interneurones in the metathoracic ganglion (Casagrand and Ritzmann, 1992b) as well as motor output recorded from nerve 5, which contains the D_f motoneurone axon (Goldstein and Camhi, 1991). These observations form the foundation on which the study to establish whether monoamines modulate D_f ionotropic receptor populations was based. If modulation occurs, the amenability of D_f to electrophysiology combined with a pharmacological approach should enable the processes underlying that modulation to be determined and should enable further understanding of how neurotransmitters interact to alter the responsiveness of neurones to particular inputs and so produce adaptability at the level of both neural circuits and behaviour.

Section 2: ACETYLCHOLINE.

2.1 Acetylcholine as a neurotransmitter

Acetylcholine is an important, ubiquitous neurotransmitter acting at central and peripheral synapses via both muscarinic metabotropic and nicotinic ionotropic receptor populations. Nicotinic acetylcholine receptors (nAChRs) represent a well-characterised family of ionotropic receptors due to the initial studies on the highly AChR-dense electric organs of *Torpedo californica* and *Electrophorus electricus*, and subsequent studies on receptor subtypes in a wide variety of both vertebrate and invertebrate tissues (for reviews concerning nAChRs see Sattelle, 1985; Role, 1992; McGehee and Role, 1995; Albuquerque *et al.*, 1997). Specifically, ACh has been shown to have a role as an important invertebrate signalling chemical (see for examples see Marder, 1976; Ascher, Marty and Neild, 1978; Marder and Paupardin-Tritsch, 1980; Witte, Speckmann and Walden, 1985), and in the context of this study has been demonstrated to be the major excitatory neurotransmitter in the central nervous system of insects (reviewed by Pitman, 1971).

A number of initial studies to determine a role for ACh as a neurotransmitter in insects were conducted on *Periplaneta americana* abdominal ganglia preparations by Roeder and colleagues in the 1940s and 50s. However they expressed considerable concern as to the high and apparently non-physiological concentrations required to elicit a discernible effect of ACh. Only when desheathed preparations incubated with the acetylcholinesterase (AChE) inhibitor eserine were used was the threshold for neurotransmission lowered to a relatively acceptable concentration of 10^{-4} M (Yamasaki and Narahashi, 1958). A subsequent investigation by Kerkut, Pitman and Walker (1969) using local ionophoretic application of ACh directly to

neuronal surfaces demonstrated that brief pulses of ACh exert excitatory effects on unidentified neurones near the dorsal midline of the cockroach ventral nerve cord, now termed 'DUM' neurones. They calculated from the threshold ionophoretic current required to elicit a depolarisation in response to ACh a probable threshold of 1.31×10^{-13} M, which is comparable to the sensitivity of other invertebrate preparations. Pitman and Kerkut (1970) further confirmed a role for ACh as an excitatory neurotransmitter in insects by comparing reversal potentials for EPSPs and ACh responses recorded from the cell bodies of DUM cells in the sixth abdominal ganglion of *P. americana*. The reversal potentials for EPSPs and ACh response were -45.3 ± 1.9 mV and -40.3 ± 1.6 mV respectively. These observations support the hypothesis that the observed EPSP were cholinergic in nature.

Cholinergic transmission is fundamental to all levels of integration in the cockroach escape circuit, a simplified circuit diagram of which is shown in figure 1 (see also the reviews by Ritzmann, 1984; Camhi and Levy, 1988). Briefly, sensory neurones associated with the fine hairs of the abdominal cerci respond to wind stimuli and synapse onto a set of ventral giant interneurones (vGIs) that run the length of the ventral nerve cord. In the thoracic ganglia the vGIs form direct synapses onto thoracic interneurones termed type A thoracic interneurones (TIA_s), which also receive other sensory inputs from a variety of loci (Ritzmann, Pollack, Hudson and Hyvonen, 1991). These, in turn, either directly stimulate motoneurones such as D_f or relay the signal to them via a further subset of local interneurones (Ritzmann and Pollack, 1990). Responses to acetylcholine have been elicited in the ventral giant interneurones (for example see Callec, David and Sattelle, 1982), whereas cholinergic transmission has been implicated as the means of chemical

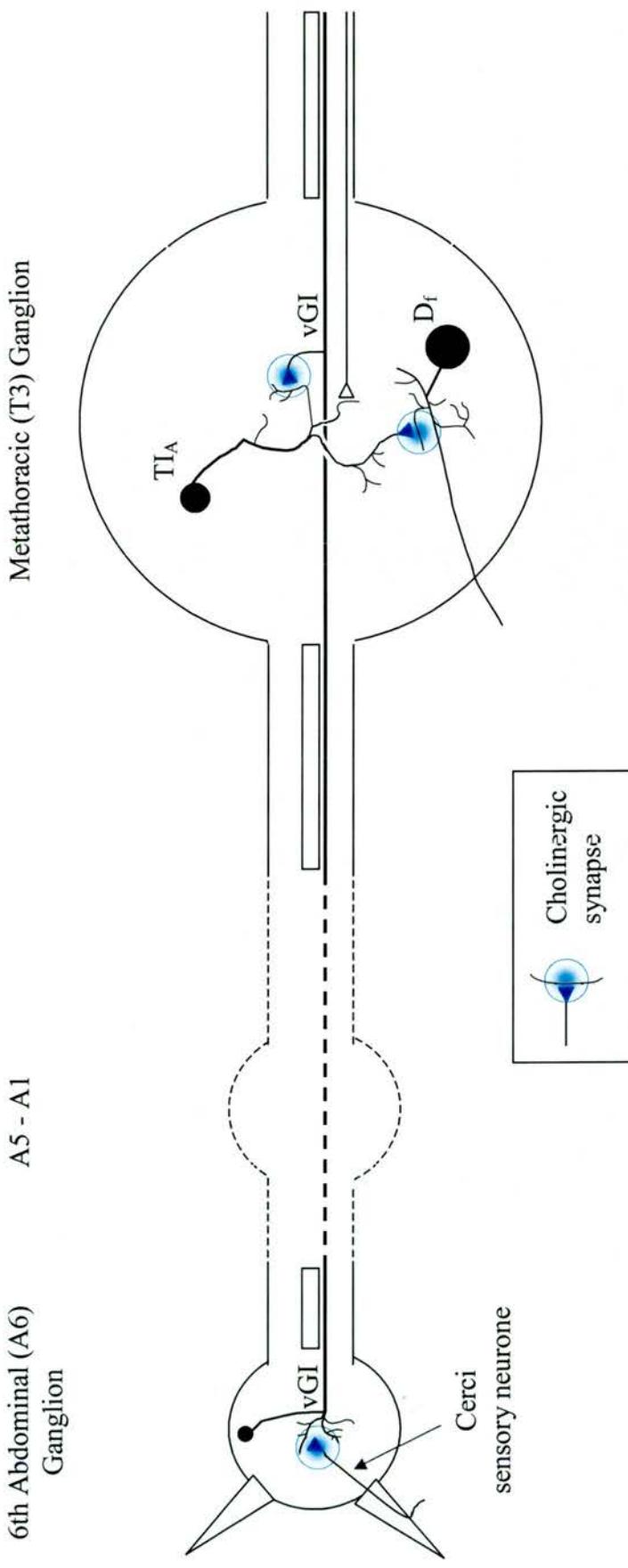


Figure 1 Simplified diagram showing the principle constituents of the cockroach (*Periplaneta americana*) escape circuit.

The escape circuit of the cockroach, *Periplaneta americana*, is triggered in response to wind or tactile stimuli activating sensory neurones of the antenna-like cerci. These form cholinergic synapses onto the giant interneurones, termed vGIs, that run the length of the ventral nerve cord (Callec, David and Sattelle, 1982; Daley and Camhi, 1988). The vGIs convey directional information to a variety of interneurone populations including in the metathoracic ganglia a population of 100 type A thoracic interneurones, TI_A s, which also receive inputs from other loci including auditory and light sensory information (Ritzmann and Pollack, 1990; Ritzmann, Pollack, Hudson and Hyvonen, 1991; Casagrand and Ritzmann, 1992a). The TI_A s form both direct and indirect synapses onto thoracic motoneurones to effect the correct, directed escape response.

transmission between the vGI and Tl_A interneurones (Casagrand and Ritzmann, 1992a) and motoneurones also exhibit nicotinic responses to ACh (David and Pitman, 1982; David and Sattelle, 1984; David and Pitman, 1996c).

Therefore it can be assumed that modulation of cholinergic transmission would have considerable behavioural consequences on the effectiveness of the cockroach escape circuit. A likely point for modulation to occur is the efficacy with which the ligand acetylcholine can activate nicotinic ionotropic acetylcholine receptors on the postsynaptic cells as previously discussed. In the next section the putative properties of nicotinic acetylcholine receptors (nAChRs) that could provide flexibility in the transduction of the ligand signal are discussed. However, as much of the information we have concerning the structure of nAChRs comes from vertebrate systems, the nature and importance of cholinergic transmission in vertebrates will be briefly discussed below (for an extensive review of vertebrate nAChR structure see Galzi, Revah, Bessis and Changeux, 1991).

In vertebrates, cholinergic transmission has been predominantly studied at neuromuscular synapses, in the main due to the high density of nicotinic acetylcholine on muscle fibres and electric organs (a modified skeletal muscle) of *Torpedo*. Molecular studies on the well-characterised nicotinic acetylcholine receptors of these systems have allowed subsequent identification of neuronal nAChRs and assisted the determination of a physiological role for nicotinic transmission at central synapses (reviewed by Luetje, Patrick and Seguela, 1990; Sargent, 1993; McGehee and Role, 1995; Albuquerque *et al.*, 1997). The nature of cholinergic transmission in the CNS was initially investigated using cultured hippocampal neurones (Aracava *et al.*, 1987) in which a number of pharmacologically distinct whole cell currents have been characterised (Alkondon

and Albuquerque, 1993). Taking this further, acetylcholine has been implicated in the hippocampal process of LTP predominantly acting at a high Ca^{2+} -conductance nicotinic AChR termed the presynaptic $\alpha 7$ nAChR subtype, and defined by the sensitivity of the receptors to α -bungarotoxin (Hunter *et al.*, 1994; Montgomery and Madison, 1999). Furthermore the effects of nicotinic ligands on the larger, live animal scale have been shown to be an important, contributing factor to memory formation in mammals (Bradley, Sigurami, Brucato and Levin, 1999; Mavil and Durkin, 1999).

2.2 Nicotinic acetylcholine receptors: structure.

Ionotropic nicotinic acetylcholine receptors belong to the ligand-gated ion channel superfamily of transmembrane proteins, specifically the group whose subunits exhibit four hydrophobic transmembrane domains (4TM). As previously mentioned the vast majority of data known about nAChRs receptors comes from vertebrate preparations and therefore reference to these is essential to any discussion of invertebrate nAChRs. Vertebrate nAChRs have been divided into two general categories on the basis of their pharmacology, subunit composition and location i.e. ‘neuronal’ nicotinic AChRs of central neuronal and ganglionic synapses (for example see Whiting *et al.*, 1991) and those from the electric organs and neuromuscular synapses termed ‘muscle’ nAChRs (see Table 2a).

2.2.1 Vertebrate nicotinic acetylcholine receptors.

Figure 2 shows a subunit model of the structure of a muscle nAChRs which, like the majority of ionotropic receptors characterised so far is a pentameric glycoprotein

Table 2a Vertebrate nicotinic acetylcholine receptor subtypes.

nAChR subtypes	ACh binding subunit	Structural subunits	Stoichiometry of functional receptors	α -bgt sensitive	+ Phos Subunit sites	+ Phos kinase		+ Phos refs.
						α	PKA	
Muscle	$\alpha 1$	β, γ, δ , and ε	$2\alpha : \beta : \delta : \varepsilon$ or γ	Yes	δ	β	PKA?, PTK, CaMK	1,2,4,6,9,16,17
Vertebrate					γ	α_3	PKA, PKC, PTK, CaMK	1,2,3,4,5,6,7,8,12,16,17
						α_4	PKA, PKC	2,5
Neuronal	$\alpha_2 - \alpha_6$	$\beta_2 - \beta_4$	$2\alpha : 3\beta$	No	β_3	PKC	PKA, PKC	6,10,13
					β_4	PKC	PKC	6,10,11
Neuronal	$\alpha_7 - \alpha_{10}$	-	α_5	Yes	α_7	PKA, PKC	PKA, PKC	6,10
							PKA, PKC	6,10
							PKA, PKC	14,15,18

+ Phos, Phosphorylation; PKA, Protein Kinase A; PKC, Protein Kinase C; PTK, Protein Tyrosine Kinase.

- 1 Huganir, Miles, and Greengard (1984)
- 2 Miles, Anthony, Rubin, Greengard, and Huganir (1987)
- 3 Ross, Green, Hartman, and Claudio (1991)
- 4 Safran, Sagi-Eisenberg, Neumann, Fuchs (1987)
- 5 Smith, Merlie and Lawrence (1989)
- 6 Swope, Moss, Blackstone and Huganir (1992)
- 7 Swope and Huganir (1994)
- 8 Hoffman, Ravindran and Huganir (1994)
- 9 Fuhrer and Hall (1996)
- 10 Huganir and Greengard (1990)
- 11 Visehakul, Figl, Lyte and Cohen (1998)
- 12 Huganir and Greengard (1983)
- 13 Vijayaraghavan *et al.* (1990)
- 14 J. Neurosci. (1999) 19(15): 6327-37
- 15 Moss, McDonald, Rudhard and Schoepfer (1996)
- 16 Smilowitz, Hadjian *et al.* (1981)
- 17 Nishizaki, Ikeuchi *et al.* (1997)
- 18 Boulter *et al.*(in press)

Table 2b Invertebrate nicotinic acetylcholine receptors.

nAChR Species	ACh binding subunits	Structural subunits	Stoichiometry of functional receptors	α -bungt Sensitive	kDa	+ Phos	Refs.
Insect	ALS, D α 2, D α 3	ARD, SBD D α 3/ARD	ALS/ D α 2 and D α 3/ARD	ALS : Yes [†] D α 2 : No D α 3 : Yes	R 250 – 300 kDa S 42*, 57, 65, 79 kDa S (D α 3) 85.4 kDa*	D α 3 : PKC, PTK but no PKA	2,8,9 10,11,12
<i>S. gregaria</i>	α L1 (ARL2)	ARL1	α L1 ₅ ARL1 ₅	Yes Yes	S (α L1) 60.6 kDa*		6,9
<i>L. migratoria</i>	loc α 1, loc α 2	loc β 1		loc α 2 : No	S 65 kDa*		3,5
	loc α 3		loc α 3 ₅ ?	Yes	S (loc α 1) 61.6 kDa S (loc α 2) 56.7 kDa S (loc α 3) 59.4 kDa S (loc β) 54.7 kDa	loc β 1 : PKA	
<i>P. americana</i>				Yes	R 200 – 300 kDa S 65 kDa*		1
<i>Manduca sexta</i>	MARA1			Yes		PKC, PTK?	4
Aphid	M α 1, M α 2			M α 1 : Yes			
<i>M. persicae</i>				M α 2 : ~ No			7

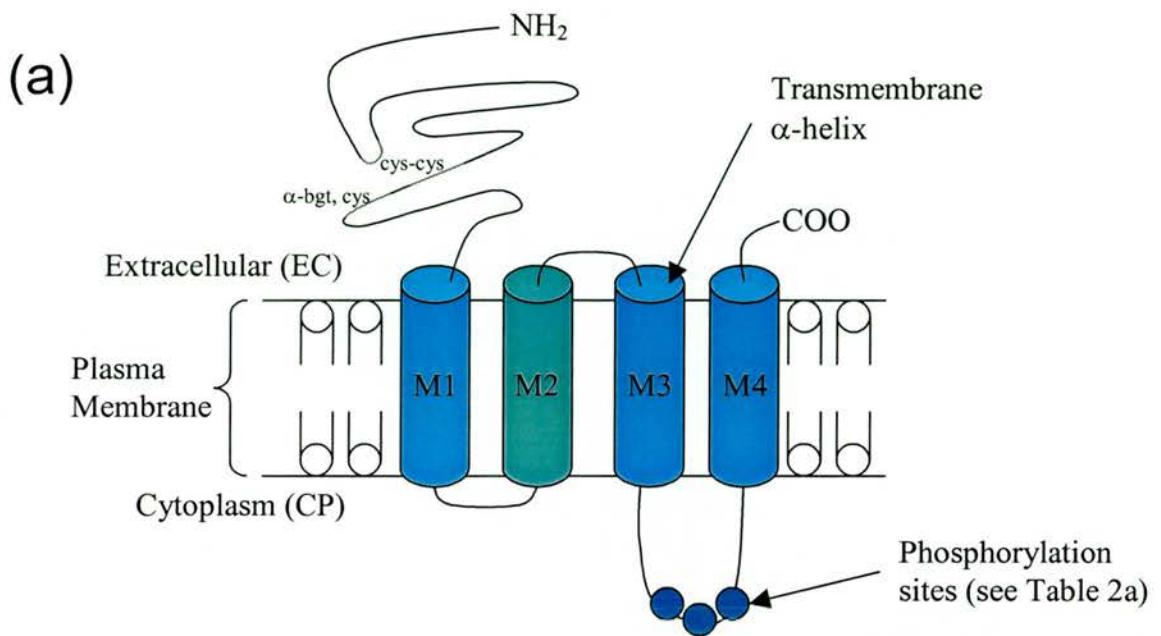
[†] see Marshall, Buckingham, Shingai *et al.* (1990) for slight contradiction in introduction.

* Identified α -bungarotoxin binding subunits of known molecular weight

- 1 Sattelle and Breer (1985)
- 2 Restifo and White (1990)
- 3 Breer *et al.* (1985)
- 4 Eastham, Lind, Eastlake *et al.* (1998)
- 5 Hernsen, Stetzer, Thees *et al.* (1998)
- 6 Amar, Thomas, Wonnacott and Lunt (1995)
- 7 Sgard, Fraser, Katkowska *et al.* (1998)
- 8 Gundelfinger and Hess (1992)
- 9 Gundelfinger (1992)
- 10 Marshall, Buckingham, Shingai *et al.* (1990)
- 11 Schulz, Sawruk, Mulhardt *et al.* (1998)
- 12 Sawruk, Hermans-Borgmeyer, Betz, and Gundelfinger (1988)

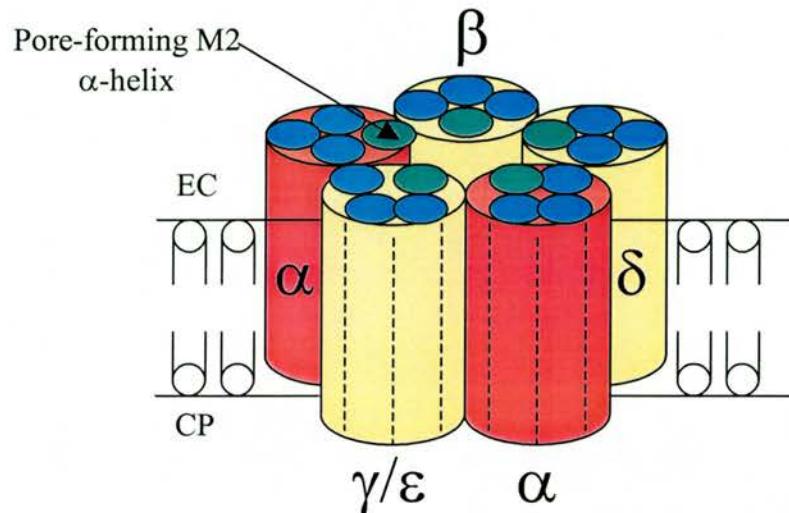
complex and more specifically is approximately 250 to 290-kDa in size with a five subunit stoichiometry of $2\alpha:\beta:X:\delta$ (Unwin, 1995). The stoichiometry represents subunits of varying molecular weight. ‘Muscle’ nAChRs are composed of four different kinds as opposed to neuronal receptors that are composed of only two (α and β). β , δ and X are structural subunits. X defines either the torpedo γ subunit or the developmentally expressed mammalian muscle adult ϵ or embryonic γ subunits (reviewed by Changeux, 1990; Karlin, 1993; for original texts see Reynolds and Karlin, 1978; Vandlen, Wu, Eisenach and Raftery, 1979; Huganir and Racker, 1982). The receptor oligomer also contains two α -bungarotoxin-sensitive acetylcholine binding sites one on each of the α subunits. Other subunits also contribute domains of the ligand binding sites and it is perhaps more correct to define the ACh binding sites as being at the α - γ/ϵ and α - δ interfaces (reviewed by Galzi, Revah, Bessis and Changeux, 1991). Theoretical studies utilising the complete cDNA coding sequences of *Torpedo californica* have revealed that all the subunits show a number of similar features in their transmembrane organisation (Figure 2a). Firstly, they all have four putative α -helical hydrophobic transmembrane domains (M1-M4). Secondly, they have a large amino terminal hydrophilic domain, and thirdly they possess a sizeable intracellular domain between the third and fourth transmembrane loops. It is this intracellular loop between M3 and M4 that has been determined as being functionally important for the regulation of nAChR signal transduction by intracellular factors. These predominantly exert their effect by phosphorylation of specific amino acid residues in the M3-M4 loop. The phosphorylation state of these residues determines the conformation and degree to which the nAChR functions (for reviews see amongst others: Levitan, 1988; Huganir and Greengard, 1990; Swope, Moss, Blackstone, and Huganir, 1992; Smart, 1997).

Figure 2. Model of the topology of an idealised nicotinic AChR.



(a) Predicted universal subunit structure. Cloned cDNA sequences of nAChR subunits all exhibit similar features. Firstly an amino terminal signal sequences for cell membrane localisation, that is cleaved in the mature protein. Secondly large hydrophilic domains near the amino terminal which contribute to ligand binding particularly that of the α -subunit. The proposed ACh binding domain of α -subunits is characterised by two highly conserved cysteine (cys192-cys193) residues near the start of the first α -helix amino acid sequence and a localised α -bungarotoxin (α -bgt) binding site (see Galzi, Revah, Bessis and Changeux, 1991; Gundelfinger, 1992). Two further amino terminal cysteine residues, cys128 and cys142, are proposed to form a disulphide bridge important for the correct tertiary structure of the ACh binding site exposed to the synaptic cleft. Hydropathy plots of the amino acid sequence of all AChR subunits have revealed four putative hydrophobic α -helical domains (M1 - M4) which are thought to form the transmembrane structure of the channel, see (b). In a number of cloned receptor sequences there is also the suggestion of a fifth amphipathic α -helix (see for examples Hermans-Borgmeyer *et al.*, 1986; Marshall *et al.*, 1990; and reviewed by Changeux, Devillers-Thiéry and Chemouilli, 1984). Finally all subunits have large hydrophilic regions between the M3 and M4 domains, which are highly divergent in their amino acid sequence and contain putative phosphorylation sites for a number of protein kinases (see text and table 2a, also the following individual references for examples of variability: Hermans-Borgmeyer *et al.*, 1986; Eastham *et al.*, 1998; Schulz *et al.*, 1998).

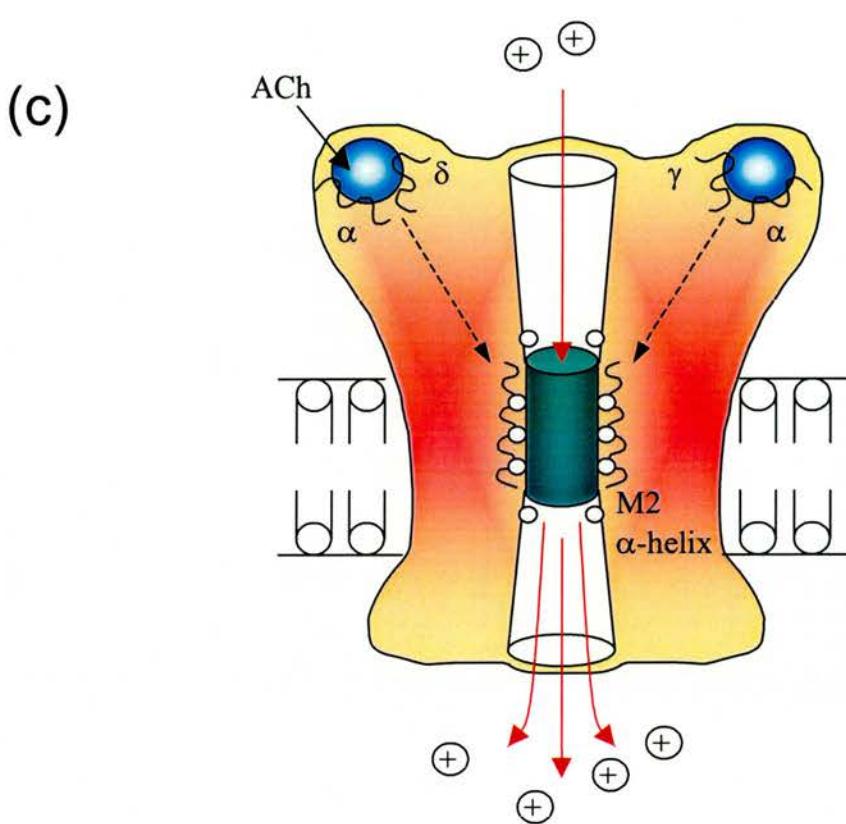
(b)



(b) Arrangement of individual subunits to form the cation permeable ligand-gated ion channel.

The diagram shows a hypothetical arrangement of *Torpedo*/muscle nAChR subunits to from the cation permeable pore. The receptor complex of *Torpedo californica* was investigated by Vandlen and colleagues (1979) and found to be composed of four subunits of different molecular weight. Subsequently these were termed α (40 kDa), β (48-50 kDa), γ (58-60 kDa) and δ (65 kDa) and the receptor formed of five subunits with the following stoichiometry $2\alpha:\beta:\gamma:\delta$. A further variant of the γ subunit has now been characterised and termed ϵ . The M2 α -helices (shaded blue-green) of all five subunits contribute to forming the central ion permeable pore and exhibit high homology between species (see below). The selectivity of the pore for cations is due to residue rings local to M2 and three within the α -helix (shown in bold below). The bold threonine 247 of the mouse γ -subunit is further explained in the text, section 2.4.

	238	M2	262
<i>Torpedo</i> α -subunit	DSG- E K	MTLSI S VLLSLTVFLLVIV	E ..
<i>Torpedo</i> β -subunit	DAG- E K	MSLSI S ALLAVTVFLLLAA	D ..
<i>Torpedo</i> γ -subunit	QAGG Q K	CTLSI S VLLAQTIIFLFLIA	Q ..
Mouse γ -subunit	E AGG Q K	CTVA T NVLLAQTVFLFVVA	K ..
<i>Drosophila</i> ARD	E AG- E K	VTLGI S ILLSLVVFLLVS	K ..



(c) Idealised map of the structure of the nicotinic AChR. The 3-D structure of the nAChR from *Torpedo marmorata* has been elucidated to a resolution of 25 Å using image analysis of crystalline arrays (Brisson and Unwin, 1985). The complex exhibits an approximate pentagonal symmetry of diameter 65 Å, protruding considerably (70 Å) into the synapse and only 30 Å on the cytoplasmic side.

Like muscle nAChRs, the neuronal subtype also mediates fast inward currents in response to binding of ACh and opening of a cation selective central pore (reviewed by Sargent, 1993). Structurally the neuronal nAChR is also a pentamer but is composed of only two broad categories of subunit type: ligand-binding α subunits of which there are at least 8 cDNA clones, and structural β subunits of which 5 variants have been identified (see Table 2a). The stoichiometry of most functional neuronal nAChRs is $\alpha_2\beta_3$ with both α and β subunits influencing functional properties of the receptor such as conductance, channel opening time and desensitisation (for reviews see Role, 1992; Sargent, 1993; McGehee and Role, 1995; Albuquerque *et al.*, 1997). Unlike muscle nAChRs these channels are insensitive to the snake toxin α -bgt, the only exception being homomeric neuronal receptors expressed in *Xenopus* oocytes which are composed of the $\alpha 7$, $\alpha 8$ or $\alpha 9$ subunits and a heteromer of $\alpha 7/\alpha 8$ subunits. These homo-oligomers which support high Ca^{2+} -conductance inward currents have only been characterised in transfection systems although there is some debate whether they exist as native receptors as α -bgt sensitive receptors in mammalian preparations (see McGehee and Role, 1995; Albuquerque *et al.*, 1997; Ospina *et al.*, 1998). These subtypes have further expanded the huge structural diversity of nAChR subtypes which was already daunting in its theoretical scope. However, the basic functions of these divergent receptor subtypes remains the same whether they are expressed at neuromuscular or central synapses, and whether or not they are homo- or heteromeric in structure. They are all pentameric protein complexes that gate cation-dependent currents in response to binding of their natural ligand ACh, and which can be subject to allosteric modulation via intra- and, or extracellular factors.

2.2.2 Insect nicotinic acetylcholine receptors.

ACh is present in relatively high amounts and is distributed throughout the CNS of insects (see Pitman, 1985), reflecting its functional importance as the major excitatory neurotransmitter in insect neuronal systems. A great deal of interest has focused on characterising insect ACh receptors principally for the development of pesticides. The first attempts to identify insect nicotinic acetylcholine receptors utilised the muscle nAChR-specific toxin, α -bungarotoxin, to identify putative ACh binding sites in the CNS. The affinity of this toxin for such sites allowed the subsequent isolation of the protein constituents of the putative nAChRs (Lummis and Sattelle, 1985; Sattelle and Breer, 1985; Gundelfinger and Hess, 1992). These initial studies purified large protein complexes of similar molecular weight to that of solubilized vertebrate nAChRs (typically in the range of 250-300 kDa), composed of single identifiable 65 kDa polypeptide subunits (see Sattelle and Breer, 1985; Gundelfinger 1992). This resulted in the hypothesis that insect nAChRs existed as homo-oligomers, and could represent ‘an ancestral ligand-gated ion channel’ (Gundelfinger, 1992).

More recently by using genetic probes developed from vertebrate nAChRs, a large diversity of both ligand-binding α -like subunits and structural β -like subunits have been cloned (see Table 2b). As a result of such studies, it is now apparent that insect nAChRs can functionally exist both as hetero-oligomers (Eastham, Lind, Eastlake *et al.*, 1998; HermSEN *et al.*, 1998; Schulz *et al.*, 1998) and homo-oligomers (Hermans-Borgmeyer *et al.*, 1986; Marshall *et al.*, 1990; Amar, Thomas, Wonnacott and Lunt, 1995). The insect nAChR, therefore, shows a range of different subunit compositions remarkably similar to that now known to exist with vertebrate neuronal nAChRs.

2.3 Nicotinic acetylcholine receptors: modulation.

Nicotinic acetylcholine receptors are subject to a wide range of modulatory influences that alter the ability of the receptor-channel complex to either bind ACh or gate the flow of ions across the plasma membrane. Figure 3 shows a diagrammatic representation of an nAChR showing the principle sites where the action of the receptor can be modulated. Changeux, Devillers-Thiéry and Chemouilli (1984) defined four general categories of modulation that appear to be common to the majority of both invertebrate and vertebrate ACh receptors characterised so far :

1- competitive interaction at the ACh binding site, 2- non-competitive allosteric modulation of ligand binding at low affinity sites, 3- non-competitive direct blocking of the ion pore and 4- allosteric change in structure via phosphorylation of intracellular sites. However, it is unlikely that the picture is as clear cut as that proposed by Changeux and colleagues, with an increasing number of neuro-active chemicals having been shown to interact with a wide diversity of sites on the receptor complex.

2.4 Modulation of nAChRs by allosteric and ACh-competitive agents.

The acetylcholine binding site of the α subunits of ACh ionotropic receptor have long been known to show high agonist selectivity for nicotine, differentiating them from the ACh binding site of the muscarine-sensitive G-protein-linked ACh receptors (Sattelle, 1985; Abood, Latham and Grassi, 1983). In muscle nAChRs the two binding sites at the $\alpha-\gamma/\epsilon$ and $\alpha-\delta$ interfaces exhibit slightly different pharmacological profiles; the latter have been shown to have a slightly higher affinity for agonists (Blount and Merlie, 1989). They can also be differentiated on the basis of their binding of antagonists, the $\alpha-\gamma/\epsilon$ site being shown to have a higher

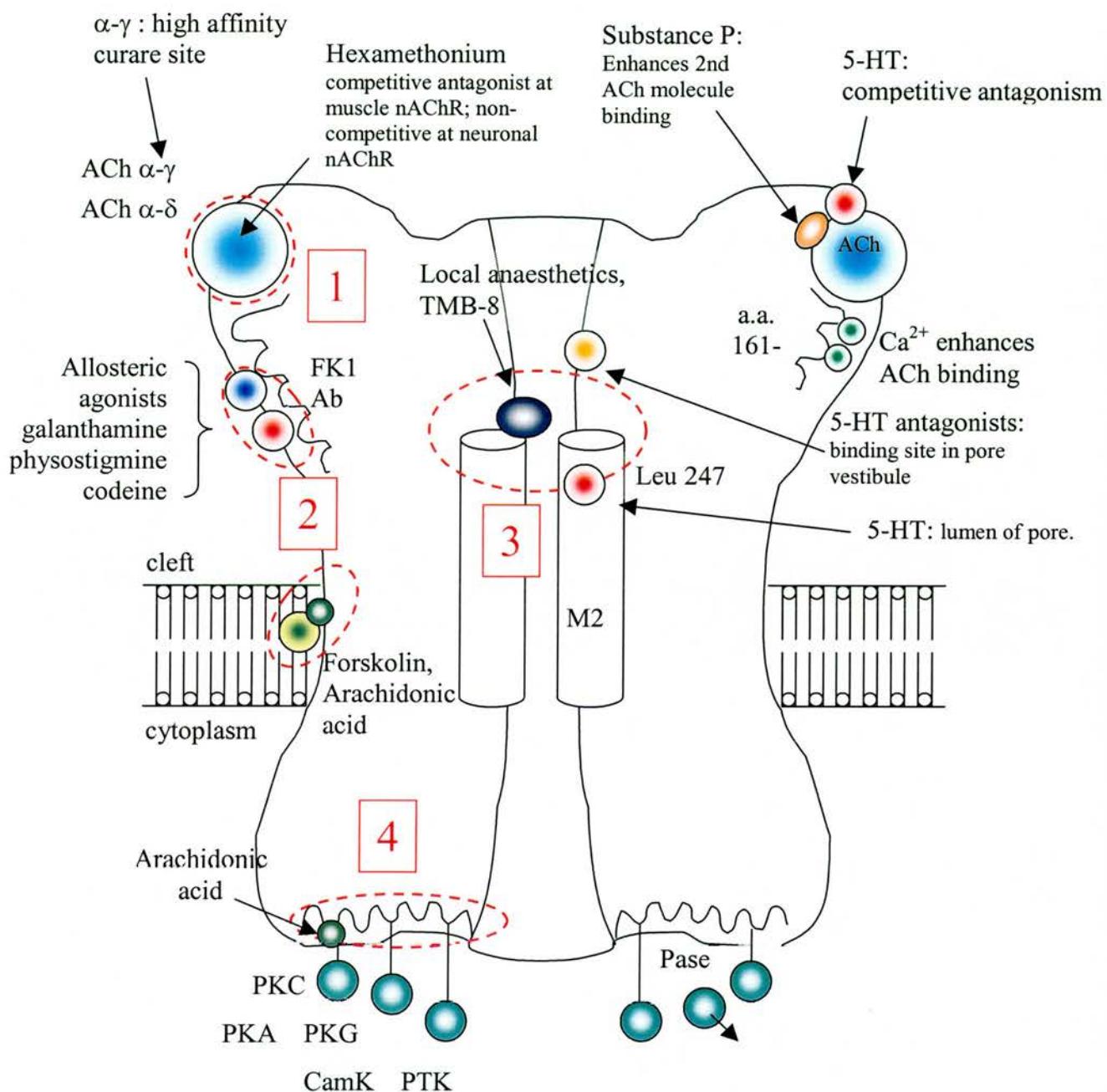


Figure 3. Principle sites of nAChR modulation.

Nicotinic acetylcholine receptors are subject to modulation by chemical agents acting at numerous extra- and intracellular sites (see text for full details). A number of ligands also act at the receptor membrane interface as with forskolin and arachidonic acid shown above. Both these agents have been shown to inhibit ACh currents, the former independently of its action on adenylyl cyclase (McHugh and McGee, 1986; Vijayaraghavan, Huang, Blumenthal and Berg, 1995; Ikeuchi, Nishizaki, Matsuoka and Sumikawa, 1996).

affinity for curare, a plant alkaloid that is a potent competitive antagonist of nicotine and ACh binding (O'Leary, Flitato and White, 1994; Bren and Sine, 1997). A number of agents exhibit specificity for either muscle or neuronal ACh binding sites including α -bungarotoxin (see tables 2a and 2b). Hexamethonium, on the other hand, acts as a competitive antagonist at muscle nAChRs but non-competitively at neuronal types (Garcia-Colunga and Miledi, 1999).

A wide variety of agents act allosterically, in the region of the ACh receptor site on the extracellular facet of the receptor macromolecule. These include Ca^{2+} , substance P and the reagents that bind to the FK1 antibody epitope which all enhance ACh ligand binding (see figure 3). The FK1 antibody targets a stretch of amino acids 118 to 137 of the α subunit of nAChRs, which is thought to exhibit a β -pleated configuration (Albuquerque *et al.*, 1997). The anti-acetylcholinesterases (anti-AChEs) galanthamine and physostigmine and the opioid codeine are thought to bind to the FK1 epitope and enhance the peak amplitude of non-saturated ACh currents, and prevent desensitisation of the ACh response in a manner similar to the action of benzodiazepines on GABA_A receptors. However the role of these agents as allosteric modulators is controversial and not supported by the recent findings that some anti-AChEs act to potentiate ACh currents in a competitive manner (Zwart *et al.*, 2000).

Calcium potentiates neuronal nAChR agonist binding by interacting with extracellular sequences expressed in the receptor amino-terminal (Amador and Dani, 1995; Galzi *et al.*, 1996). Further studies on neuronal nAChRs revealed that, although Ca^{2+} modulates the affinity for natural agonists, it does not affect antagonist binding. It does, however, increase the ability of agonists to cause desensitisation

indirectly by increasing the agonist-receptor interaction (Booker, Smith, Dodrill and Collins, 1998; Ospina *et al.*, 1998).

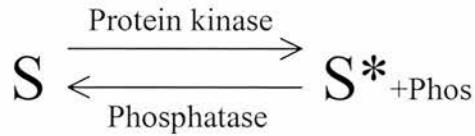
It is apparent from the literature that the degree of ACh interaction with the nAChR is modulated by a whole plethora of other potential endogenous or exogenous modulators. Serotonin (5-HT), apart from being the focus of this study, is a particularly interesting illustration of an endogenous neurotransmitter which interacts at a bewildering diversity of binding sites within a receptor for which it is not a ‘natural’ ligand. Apart from the acting at its own receptors, serotonin has been demonstrated to act as a competitive antagonist at the ACh binding site of bull-frog sympathetic ganglion cells (Akasu and Koketsu, 1986). Serotonin also exerts a non-competitive agonist effect at the FK1 antibody epitope of neuronal nAChRs (site 2 in figure 3) exerting an action similar to the anti-AChEs and codeine (reviewed in Albuquerque *et al.*, 1997). Furthermore serotonin and a number of serotonergic antagonists bind to specific sites lining the ion permeable pore of both muscle and neuronal nAChRs, behaving as high affinity non-competitive channel blockers (site 3 in figure 3)(Grassi *et al.*, 1993; García-Colunga and Miledi, 1999). The high affinity channel blocking site was initially regarded as a single locus characterised by Changeux *et al.* (1984) by its sensitivity to the frog toxin histrionicotoxin (HTX). However, it is now apparent that there exists more than one distinct high affinity locus and certainly it appears that serotonin and its antagonists act at separate discrete sites, the former acting at a site within the M2 transmembrane α -helix and the latter more towards the extracellular interface (García-Colunga and Miledi, 1999). This site has been further characterised in the $\alpha 7$ nAChR, with the threonine 247 residue of the M2 α -helix central to the actions of serotonin as a high affinity channel blocker. Interestingly if threonine 247 is mutated to leucine (L247T $\alpha 7$), it

results in the effect of serotonin being converted to that of a non-competitive agonist, albeit with different gating kinetics to receptors activation by the natural ligand ACh (Palma, Mileo, Eusebi and Miledi, 1996; Palma, Maggi, Eusebi and Miledi, 1997). Furthermore this property is unlikely to be confined to $\alpha 7$ nAChRs as the threonine 247 residue is conserved in a number of other nAChR M2 domains (see figure 2b, the bold red threonine of the mouse γ -subunit). Finally, whilst the direct effects of monoamines on AChRs are being discussed, dopaminergic antagonists and agonists have been shown by Nakazawa *et al.* (1994) to block the nAChR channels, although dopamine itself has no effect. The high affinity site in the pore is also proposed to be the target of a number of local anaesthetics and the ‘calcium antagonist’ TMB-8 (Bencherif *et al.*, 1995).

2.5 Modulation of nAChRs by phosphorylation of intracellular consensus sequences.

Apart from extracellular factors that act directly or allosterically to modulate nAChR function, the receptors are also subject to intracellular regulation. This principally involves phosphorylation and dephosphorylation of the receptor by specific protein kinases and phosphatases of threonine, serine or tyrosine residues of the cytoplasmic domains of the receptor subunits (site 4, figure 3)(for reviews see Levitan, 1988; Huganir and Greengard, 1990. Qu, Mortiz and Huganir, 1990, Swope, Moss, Blackstone, and Huganir, 1992. See also Smart, 1997). The extent of phosphorylation is a dynamic equilibrium dependent on the balance between kinase-dependent phosphorylation and the reverse process, cleavage of the phosphate group from the protein by phosphatases, which is best represented by the following

schematic: The altered state (S^*) represents the catalysed transfer of a phosphate group to the specific amino acids of the protein substrate.



Phosphorylation and dephosphorylation of nAChRs was initially reported on two separate electric organ preparations. Gordon *et al.* (1977a) demonstrated that membrane proteins of *Torpedo californica* were phosphorylated, whereas Teichberg and Changeux (1977) reported phosphorylation and dephosphorylation of proteins, proposed to be nAChR subunits, by endogenous kinases and phosphatases in *Electrophorus electricus*. The membrane phosphoprotein of *Torpedo* was subsequently identified as the nicotinic acetylcholine receptor (Gordon *et al.* 1977b). To complete the picture, a kinase capable of phosphorylating the receptor protein was identified by the same group in the early 1980s (Davis, Gordon and Diamond, 1982). Phosphorylation is a property not just confined to the electric organ nAChRs and has now been demonstrated in all forms of vertebrate nicotinic receptors (table 2a). It has also been tentatively proposed to regulate a number of invertebrate nAChRs (for insect receptors see table 2b). Successful transfer of a charged phosphate group from ATP to amino acids, catalysed by protein kinases, results in covalent modification of the protein structure and alterations of the receptor properties.

Phosphorylation of proteins is not indiscriminate, it is targeted to specific proteins by the presence of specific amino acid domains. Nicotinic AChRs from a variety of preparations express a range of putative domains in their M3-M4

intracellular loops that distinguishes the receptor as a target of phosphorylation. These motifs also determine what aspects of receptor-mediated transduction is modulated by phosphorylation. However the state of phosphorylation is regulated by the intracellular factors that regulate the kinases and phosphatases.

Kinases and phosphatases exert their action by regulating the state of the hydroxyl phospho-acceptor amino acid residues present in cellular proteins: serine (Ser, S), threonine (Thr, T) and tyrosine (Tyr, Y)(see table 3; Smart, 1997). Selectivity for the numerous phosphoenzymes is provided by the amino acids that form consensus sequences in the vicinity of the target amino acid. The selectivity provided by the additional amino acids is exemplified by the substrate sequences for the closely related cyclic nucleotide protein kinases, PKA and PKG. Superficially both sites are remarkably similar with the phospho-acceptor amino acids proceeded by an arginine (R) site, with the motif RRXS acting as the minimum recognition site required for both enzymes, where X is any amino acid (Smart, 1997). However, experimental evidence has revealed the often low activity of PKA on PKG substrates and vice versa (reviewed by Wang and Robinson, 1997). The primary determinant for such low activity is the local lysine (K) and phenylalanine (F) residues in the vicinity of PKG phosphorylation sites preventing catalytic PKA interaction (see Wang and Robinson, 1997). One important exception is the group of PKA and PKG substrate proteins that regulate the PP-1 family of phosphatases, for example the DARPP-32 protein (see section 2.5.3). DARPP-32 down-regulates PP-1 protein phosphatase activity but can be phosphorylated on the same threonine residue by either PKA or PKG, so causing the ability of the protein to inhibit phosphatase activity to be enhanced from a IC₅₀ of 1 μM to 2 nM (Herzig and Neumann, 2000).

Table 3. Putative phosphorylation sites of protein kinases.

Kinase type	Protein kinase	General consensus sequence	Example nAChR sites		References
			nAChR subunit	Identified and Putative + Phos sites	
Serine (S)/ Threonine(T) kinases	cAMP-dependent protein kinase (PKA)	RRXS/T RXS/T RXS/T	Muscle γ Muscle δ Neuronal $\alpha 7$ <i>Drosophila</i> ARD	³⁴⁶ KPQPRRRSSFGI ³⁵⁷ ³⁵⁵ LKLRSSSVGY ³⁶⁵ Ser 342 ⁴⁰⁶ RRESES ⁴¹¹	1,2,4,6,9
	cGMP-dependent protein kinase (PKG)	R/KR/KXST/T (RKXST) R/KXXS/T R/KXS/T	Drosophila ALS	³⁷⁵ KRFSG ³⁷⁹	2,3,4
	Protein kinase C (PKC)	R/KX ₁₋₃ S/TX ₁₋₃ R/K S/TX ₁₋₃ R/K R/KX ₁₋₃ S/T	Muscle α Muscle δ Neuronal $\beta 3$ Neuronal $\beta 4$ <i>Drosophila</i> ALS <i>Drosophila</i> D $\alpha 2$	³³⁰ KRASK ³³⁴ ³⁵⁵ LKLRSSSVGY ³⁶⁵ ³⁴⁷ KVSGKRR ³⁵² ⁴¹⁶ RLRSSGRFR ⁴²² ³⁷³ DSKRF ³⁷⁷ ⁴⁰¹ ATNRF ⁴⁰⁵	1,2,4,6
	Ca ²⁺ /CaM kinase II (CaMK II)	RXS/T			
Tyrosine Protein kinases (PTK) Src class kinases	Src	(E)DEEIYG/EEF)	Muscle γ	³⁵⁹ IKAAEEYILK ³⁶⁷	2,7
	Fyn		Neuronal $\alpha 2$	⁴²⁸ RALEGVQY ⁴³⁵	2,10,11
	Fyk		Neuronal $\alpha 4$	⁵⁶⁹ RAVEGVVOY ⁵⁷⁶	4,5,8
			<i>Drosophila</i> D $\alpha 3$	⁶⁸⁷ KAMDGVTY ⁶⁹⁴	
			<i>Drosophila</i> SBD	³⁷² KDSQDGAY ³⁷⁹	

1 Swope, Moss, Blackstone and Huganir (1992)

2 Smart (1997)

3 Wang and Robinson (1997)
4 Gundelfinger and Hess (1992)

5 Schulz *et al.*, (1998)

6 Safran, Sagi-Eisenberg, Neumann and Fuchs (1987)

7 Smilowitz, Hadjian, Dwyer and Feinstein (1981)
8 Viseshakul, Fig, Lyle and Cohen (1998)

9 Moss *et al.* (1996)

10 Huganir, Miles and Greengard (1984)

11 Mei and Huganir (1991)

Calmodulin kinase II is another kinase reported to modulate nAChR function in a number of preparations. However for this kinase there is a lack of evidence as to the presence of either identified or putative phosphorylation motifs expressed in nAChRs. The reviews by Hanson and Schulman (1992) and Smart (1997) identify RXXS/T as a CaMK II motif although this is identical to that of the other kinases (see table 3). Local glutamine (Q), glutamic acid (G) and aspartic acid (D) residues may provide increased specificity to CaMK. These are found in high concentrations in the region of CaMK phosphorylation sites (Hanson and Schulman, 1992).

As table 3 illustrates, a number of phosphorylation sites for the various kinases have been identified (red single letter amino acid code) or are proposed (blue) to be present on the M3-M4 intracellular loop of nAChRs and there is now increasing evidence that the phosphorylation state of these is important for receptor function.

As discussed, physiological agents acting at the extracellular facet of the nAChR can directly modulate receptor transduction by affecting ACh binding or can block or reduce the gating of the ion channel. These factors regulate the operation of the receptors expressed at the cell surface, whereas phosphorylation in addition to affecting channel properties has also been shown to control every stage of nAChR assembly and synaptic localisation. Phosphorylation thus provides an additional long-term means of adaptation in the efficacy of cholinergic transmission, specifically by controlling ACh receptor gene expression (Sapru, Zhou and Goldman, 1994; Walke *et al.*, 1994; De Koninck and Cooper, 1995; Carrasco-Serrano, Viniegra, Ballesta and Criado, 2000), receptor assembly (Ross, Green, Hartman and Claudio, 1991), synaptogenesis (Fuhrer and Hall, 1996; Dai and

Peng, 1998; Nimnuan *et al.*, 1998) and stability of nAChRs expressed at the cell surface (Caroni, Rotzler, Britt and Brenner, 1993).

There is experimental evidence to suggest that once protein complexes are expressed at the cell surface they are subject to considerable covalent modification resulting from phosphorylation. Phosphorylation can alter the conductance of membrane channel proteins, thus modulating the effectiveness of neuronal signalling. The membrane ion channels capable of phosphorylation include vertebrate and invertebrate potassium and calcium channels which are widely regulated by both serine-threonine and tyrosine kinases and phosphatases (Kostyuk, Lukyanetz and Doroshenko, 1992; Braha *et al.*, 1993; Huang, Morielli and Peralta, 1993; Wilson and Kaczmarek, 1993; Jonas and Kaczmarek, 1996; Aniksztejn, Catarsi and Drapeau, 1997), and vertebrate glutamate ligand-gated ion channels (Cerne, Rusin and Randic, 1993; Blackstone *et al.*, 1994; Wang and Salter, 1994). Similarly a number of studies have characterised in some detail phospho-regulation of GABA ionotropic receptor currents. Phosphorylation has been shown to be important for GABA gene expression (Thompson, Razzini, Pollard and Stephenson, 2000), maintenance of GABA currents (Chen, Stelzer, Kay and Wong, 1990), as well as current enhancement (Lin, Browning, Dudek and MacDonald, 1994; Kapur and MacDonald, 1996) and suppression (Heuschneider and Schwartz, 1989; Moss, Smart, Blackstone and Huganir, 1992; Gillette and Dacheux, 1996). Interestingly a recent study by McDonald and colleagues (1998) demonstrated that the differential regulation of GABA_A currents by cAMP-dependent kinase (PKA) is due to a small difference in number of phospho-acceptor serine sites between β 1 subunit. PKA suppressed currents carried by receptors containing β 1 subunits with a single serine amino acid (Ser409) within a conserved PKA phosphorylation consensus sequence,

*

Whereas PKA enhanced current carried by receptors expressing the $\beta 3$ subunit that exhibits two adjacent serines (Ser408 and Ser409) within the same motif. Similar double serine motifs are conserved within phosphorylation sites of muscle δ and neuronal $\beta 4$ nAChR subunits. In the former, PKA and protein kinase C (PKC) sites have been mapped to the same serine residue (ser361)(Table 3 and Safran, Sagi-Eisenberg, Neumann and Fuchs, 1987).

Nicotinic AChRs have long been known to be phosphoproteins and therefore it is perhaps no surprise that the channel kinetics and receptor properties of nAChRs are modulated by phosphorylation, in a manner similar to that reported for GABA receptors. Like GABARs, signal transduction by nAChRs can be either up- or down-regulated depending on the site of phosphorylation. Phosphorylation potentiates nicotinic currents by increasing the proportion of functional receptors in a neuronal preparation, switching them from an inactive non-ligand binding state to an ACh-sensitive state, (Margiotta, Berg and Dionne, 1987a). Certainly it is likely that this is not a property exclusive to this system as other studies on both muscle-type and neuronal nAChR preparations have highlighted kinase pathways capable of potentiating nicotinic transmission (Briggs, 1992; Nishizaki, Ikeuchi, Matsuoka and Sumikawa, 1997). Phosphorylation has also been proposed to enhance agonist binding (Mileo *et al.*, 1995), and even in *Torpedo* to increase spontaneous channel opening in the absence of an agonist (Ferrer-Montiel, Montal, Díaz-Munoz, and Montal, 1991).

Phosphorylation can also reduce acetylcholine currents (Eusebi, Molinaro and Zani, 1985; Papp and Hoyer, 1996). Functionally this underlies the phenomenon of desensitisation exhibited by AChRs at neuromuscular synapses. Prolonged exposure of the muscle nAChR to nicotinic agents results in a decrease of the

receptor conductance (for example see Feltz and Trautmann, 1982), the nAChR undergoing a conformational change from active, conducting form to a non-conducting, desensitised conformation. One hypothesis is that the cation conductance of the receptors (particularly via Ca^{2+} influx) triggers intracellular phosphorylation pathways that feedback onto the nAChRs as a means of auto-regulation, preventing the potentially damaging effects of excessive depolarisation in the continual presence of agonists (Miles, Audiger, Greengard and Huganir, 1994; Nishizaki and Sumikawa, 1998). Certainly the hypothesis is supported by a number of studies which have demonstrated phosphorylation can promote desensitisation (Albuquerque, Deshpande, Aracava, Alkondon and Daly, 1986; Downing and Role, 1987; Hoffman, Ravindran and Huganir, 1994), but the simplified picture is complicated by additional evidence that phosphorylation pathways are also important for the recovery from desensitisation to the active state of the nAChR (Khiroug, Sokolova, Giniatullin, Afzalov and Nistri, 1998; Paradiso and Brehm, 1998; Fenster *et al.*, 1999).

The multiple modulatory effects of phosphorylation are not attributable to one single kinase-phosphatase pathway with a number of differentially regulated signalling processes all targeting nAChRs. These interact with different sites to exert distinct modulatory actions.

2.5.1 Phosphorylation of nAChRs by serine-threonine kinases.

The diversity of effects of phosphorylation are reflected in the number of kinases known to interact with nAChRs. Table 3 shows the further categorisation of serine, threonine and tyrosine kinases into subsets based on the intracellular second messengers responsible for their activation (reviewed by Edelman, Blumenthal and

Krebs, 1987; Scott and Soderling, 1992; Swope, Qu, and Huganir, 1995). Serine-Threonine kinases have differing structures, reflecting whether they are stimulated by either elevations in cyclic nucleotides (cyclic AMP and cyclic GMP dependent kinases (PKA and PKG)), or by intracellular Ca^{2+} pathways dependent on either diacylglycerol (PKC) or calmodulin (CaMK). The triggering, dynamics and regulation of these second messenger pathways will be dealt with in the next section, as will whether they and the downstream kinases have specific functions in modulating nAChRs.

Cyclic AMP-dependent protein kinases are activated by the binding of four cAMP molecules to the enzyme complex. The kinase consists of two regulatory subunits of which four isoforms have been identified (RI α and RI β (primarily cytoplasmic), RII α and RII β) and two of the three known catalytic regions (C α , C β , and C γ) to give a holoenzyme complex of stoichiometry R₂C₂ (Edelman, Blumenthal, and Krebs, 1987; Scott and Soderling, 1992). There is strong evidence that neuronal forms of cAMP-dependent protein kinase phosphorylate vertebrate nAChRs (see table 2a; see also Miles *et al.*, 1987; Huganir and Greengard, 1983). However the effects of PKA-dependent phosphorylation on nAChR function can be mixed, reflecting the diversity of regulatory functions that have already been discussed in the introduction to this section. PKA has been implicated in desensitisation of muscle nAChRs, mediated by phosphorylation of serine residues on the γ and δ subunits (Albuquerque, Deshpande, Aracava, Alkondon and Daly, 1986; Hoffman, Ravindran and Huganir, 1994). A number of studies have revealed the requirement for a high concentration of extracellular calcium for PKA regulation of desensitisation (e.g. see Nojima, Kimura and Kimura, 1994) and therefore this effect could be a result of cross-talk between cAMP-dependent pathways and those

regulated by intracellular concentration of Ca^{2+} (for example Sugita, Baxter, and Byrne, 1997).

There is more evidence for PKA catalysis of phosphorylation as a positive influence on transmission at ACh receptors. It has been shown to increase the number of functional receptors independent of a change in gene expression and the number of receptors expressed at the cell surface (Margiotta, Berg and Dionne, 1987b). The potentiation of neuronal nicotinic currents is achieved by phosphorylation of the ligand binding α subunit (Margiotta, Berg and Dionne, 1987; Vijayaraghavan, Schmid, Halvorsen, and Berg, 1990). PKA also accelerates embryonic nAChRs recovery from desensitisation (Paradiso and Brehm, 1998). Furthermore, in direct contrast to the desensitisation results, PKA phosphorylation of *Torpedo* γ and δ subunits has been shown by Ferrer-Montiel and colleagues (1991) to increase spontaneous nAChR gating. Additionally forskolin, the activator of the cAMP generating enzyme adenylyl cyclase, increases nAChR subunit assembly and enhances receptor expression at the cell surface (Ross, Green, Hartman and Claudio, 1991), a process likely to enhance the sensitivity of neurones to ACh.

Although two types of kinase sensitive to the guanosine cyclic nucleotide, cGMP, have been identified in mammalian tissue (PKG-I α/β and PKG-II) - predominantly expressed in the cerebellum (Wang and Robinson, 1997), there is little direct evidence of cGMP-dependent kinase (PKG) interaction with nAChRs. Gordon, Davis and Diamond reported that phosphorylation was independent of PKG in the *Torpedo* nAChR, while Moss *et al.* (1996) reported a similar situation for neuronal $\alpha 7$ subunits. In contrast, Huganir and Greengard (1983) did report PKG-dependent phosphorylation of *Torpedo* γ and δ subunits, although the effect was only observed at significantly higher concentrations of cGMP than that required for

cAMP-PKA phosphorylation. This could therefore, represent non-specific interaction of the cGMP-dependent enzyme at the PKA site. The literature on the physiological effects ofPKG activity consists of one tentative publication by Briggs (1992) who reported potentiation of nicotinic transmission in rat superior cervical ganglia after application of cGMP analogues, and an abstract published by Drozdova and Pivovarov (1995) reporting nitric oxide-cGMP induced desensitisation of ACh responses in the snail *Helix lucorum*.

There is considerably more evidence for a role for protein kinase C (PKC) - dependent phosphorylation, as opposed to that of PKG, in modulating nAChR function (Safran, Sagi-Eisenberg, Neumann and Fuchs, 1987; Nishizaki and Sumikawa, 1998). Activation of PKC is dependent on the phospholipid second messenger diacylglycerol (DAG), PKC actually representing a family of nine enzymes (α , β I, β II, δ , ε , γ , η , ξ and L) of which only four isoforms (α , β I, β II, and γ) also require Ca^{2+} to function. The role of PKC-mediated phosphorylation of nAChRs seems to be primarily one of accelerating desensitisation (Eusebi, Molinaro, and Zani, 1985; Downing and Role, 1987; Papp and Hoyer, 1996). There is also increasing evidence that the physiological trigger for such an effect could be activation of nAChRs. The resultant cation conductance, predominantly that of Ca^{2+} , activating PKC and thus providing a means of autoregulation (Miles, Audigier, Greengard and Huganir, 1994; Nojima, Tsuneki, Kimura and Kimura, 1995; Nishizaki and Sumikawa, 1998). It does appear that agonist activation of cation conductance through nAChRs does directly stimulate phosphorylation pathways responsible for the activation of gene expression (Walke *et al.*, 1994) and receptor stabilisation at synaptic sites(Caroni, Rotzler, Britt and Brenner, 1993). Additional factors apart from nicotinic receptor cation conductance promote PKC activity

including the lipid, DAG, and phorbol esters. Pharmacological stimulation of PKC phosphorylation by the latter has been proposed to (a) regulate neuronal nAChR gene expression (Carrasco-Serrano, Viniegra, Ballesta and Criado, 2000), (b) to aid recovery of nAChRs desensitisation (Khiroug, Sokolova, Ginitatullin, Afzalov and Nistri, 1998; Fenster *et al.*, 1999), (c) to increase agonist binding affinity at nAChRs (Mileo *et al.*, 1995) and (d) to affect receptor clustering at synapses (Nimnuual *et al.*, 1998). There are also a number of reports implicating PKC activation but not PKC-dependent phosphorylation in the potentiation of nicotinic transmission (Limatola, Palma, Mileo and Eusebi, 1996; Nishizaki, Matsuoka, Nomura and Sumikawa, 1997).

The fourth main neuronal serine-threonine kinase type is the Ca^{2+} -CaM dependent protein kinase II (CaMK II) (for review see Hanson and Schulman, 1992). Initial activation is dependent on Ca^{2+} and CaM but, once activated, the CaMK II can auto-phosphorylate and adopt a Ca^{2+} -independent conformation (See Hanson and Schulman, 1992; Scott and Soderling, 1992). Like the three other kinase types, CaMK II has been demonstrated to have important neuronal effects (see Llinas, McGuinness, Leonard, Sugimori and Greengard, 1985; Kano, Kano, Fukunaga and Konnerth, 1996; Nakanishi, Zhang, Baxter, Eskin and Byrne, 1997). For example CaMK II function is important for initiation and maintenance of long term potentiation (Malenka *et al.*, 1989; Silva, Stevens, Tonegawa and Wang, 1992; Lisman, 1994). CaM-dependent phosphorylation of *Torpedo* nAChRs was initially demonstrated by Smilowitz, Hadjian, Dwyer and Feinstein (1981) and confirmed in a later study by Huganir and Greengard (1983). The physiological data on the effects of CaMK II however comes from studies on neuronal nAChRs in line with its proposed role in more central neuronal functions. CaMK-catalysed phosphorylation

enhances ACh currents (Nishizaki, Ikeuchi, Matsuoka and Sumikawa, 1997), prevents desensitisation (Liu and Berg, 1999) and also up-regulates $\alpha 7$ subunit gene expression (De Koninck and Cooper, 1995) but as yet has not been shown to induce desensitisation of ACh responses.

What is apparent from the literature at present is that the various neuronal serine-threonine kinases can exert multiple effect on nAChRs. It is likely that different kinases can interact at the same phosphorylation motif, such as Ser361 of muscle δ subunits, to exert similar functions. Additionally, there is extensive interaction and crosstalk between the kinases and between their activation pathways. This added level of complexity allows integration of divergent signals to produce the desired effect on the activity of the receptors and ion channels at the cell surface.

2.5.2 Phosphorylation of nAChRs by tyrosine kinases.

Protein tyrosine kinases (PTKs) are commonly associated with the transduction of hormone signals, particularly those of growth factors, where they operate as integral parts of the hormone receptors. However, there are also numerous PTKs that are not associated with specific activating hormone receptors. These like the serine-threonine kinases discussed above, are thought to play important functional roles in regulating neuronal transmission (for recent reviews see Boxall and Lancaster 1998; Luttrell, Daaka and Lefkowitz, 1999). Furthermore, it is this group of tyrosine kinases that have been demonstrated to interact strongly with nAChRs in particular members of the *Src* cytoplasmic class of PTK (Fuhrer and Hall, 1996). *Src* protein tyrosine kinases are identified according to the presence of a myristylation site, SH2 (Src homology 2) and SH3 domains and a catalytic region. PTK phosphorylation was initially demonstrated on *Torpedo* nAChRs by Huganir *et al.* (1984) and

subsequently two members of the Src kinase family, termed *Fyn* and *Fyk*, were shown to closely associate with the receptor (Swope and Huganir, 1994; Swope, Qu and Huganir, 1995). The action of PTKs has only been demonstrated in muscle type nAChRs although *Fyn* and *Fyk* are expressed in the brain of *Torpedo* as well as in the electric organ and skeletal muscle (Swope and Huganir, 1993). The principal role of PTKs appears to be to regulate aggregation of nAChRs at synapses (Qu, Moritz and Huganir, 1990; Mei and Si, 1995), specifically by strengthening the association of the receptor with the cytoskeleton in an agrin controlled process (Meier, Perez, and Wallace, 1995; Wallace, 1995). One report has also implicated PTK phosphorylation in regulation of nAChR channel properties, in particular desensitisation (Hopfield *et al.* 1988), though there is no additional evidence for such a PTK role. There is however evidence of a protein tyrosine phosphatase (Mei and Huganir, 1991), one of the group of enzymes which opposes the actions of kinases by removing phosphate groups.

2.5.3 Dephosphorylation, the role of neuronal phosphatases in cholinergic synaptic plasticity.

As discussed above, phosphorylation of receptor proteins can modulate their function, similarly, the reversal process is also an important factor in synaptic plasticity (for reviews see Nairn and Shenolikar, 1992; Herzig and Neumann, 2000). However, little is known about phosphatase regulation of cholinergic transmission. Protein tyrosine phosphatases (PTPases) represent the best characterised group of phosphatases known to modulate nAChRs, but even so, remarkably little is known about their function. As mentioned in the previous section, a novel PTPase has been shown to interact with the *Torpedo* nAChR (Mei and Huganir, 1991; Mei, Liao and

Huganir, 1994). Functionally PTPase has been implicated in muscle ϵ subunit gene expression (Sapru, Zhou and Goldman, 1994) and to oppose the action of PTK in nAChR clustering (Dai and Peng, 1998) but that is the extent of the literature at the moment. PTPase does, however, regulate a variety of other membrane proteins involved in neurone excitability. It has been shown to affect gating of an *Aplysia* voltage-gated cation channel and its responsiveness to PKA (Wilson and Kaczmarek, 1993), illustrating firstly that phosphatases can actively regulate conductance, and secondly modulate the regulatory influence of kinases.

The story is even briefer for serine-threonine phosphatases (PPase) with only one study identifying a PPase interaction with nAChRs. The study by Hardwick and Parsons (1996) pharmacologically defined a protein phosphatase 2B (PP2B), calcineurin, that prolongs the recovery of carbachol currents from desensitisation. Calcineurin is a Ca^{2+} -CaM dependent phosphatase important in neuronal modulation (for review see Yakel, 1997), and one of 9 identified subtypes of mammalian phosphatase (Herzig and Neumann, 2000). Along with calcineurin, PP1, PP2A, and PP2C are the best understood phosphatases, whose neuronal functions have been defined using a number of specific protein and non-protein inhibitors (Nairn and Shenolikar, 1992; Price and Mumby, 1999; Herzig and Neumann, 2000). Table 4 shows the principal features of these enzymes and a number of characteristics that distinguish them from one another. Of particular interest, the PP-1 family of phosphatases have been shown to be regulated by PKA- and PKG-substrate proteins including the inhibitor-1 protein (I-1) which was originally characterised in a muscle preparation (Huang and Glinsmann, 1976) and the similar DARPP-32 which is mainly expressed in mammalian brains (Hemmings and Greengard, 1986). Phosphorylation of these proteins enhances their interaction with regulatory domains

Table 4. Characteristics of the principle neuronal phosphatases.

	PP-1	PP-2A	PP-2B (Calcineurin)	PP-2C	References
Gene family	PPP	PPP	PPP	PPP	1
Catalytic subunits	1 α ,1 δ ,1 γ_1 ,1 γ_2	C α ,C β	CNA α_1 , CNA α_2 , CNA β_1 , CNA β_2 , CNA β_3 , CNA γ	C α_1 , C α_2 , C α_3 , C β_1 , C β_2	1
No. of identified regulatory subunits	16	16	6	-	1
Requirement for divalent cations	No	No	Yes (Ca ²⁺ /Ca ²⁺ -CaM)	Yes (Mg ²⁺)	2,4,5
Inhibition by PKA/PKG (I-1, DARPP-32)	Yes	No	No	No	1,2,4
Inhibition by Okadaic acid	Yes (IC ₅₀ 272 nM)	Yes (IC ₅₀ 1.6 nM)	Yes (IC ₅₀ 3.6 μM)	No	1,3,4, *
Inhibition by Cantharidin	Yes (IC ₅₀ 473 nM)	Yes (IC ₅₀ 40 nM)	Weak (>30 μM)	No	1, **

- 1 Herzig and Neumann (2000) 4 Cohen (1989)
 2 Nairn and Shenolikar (1992) 5 Yakel (1997)
 3 Hardie (1993)

Other sources of information: * RBI® information sheet for Okadaic acid; ** ALEXIS™ catalogue and information sheet.

of the PP-1 phosphatases, attenuating their activity. The inhibitory effect of I-1 protein on PP-1 is attenuated by activation of calcineurin, which dephosphorylates the I-1 PKA motif, suggesting that these proteins allow interaction between cyclic nucleotide and Ca^{2+} -dependent pathways (Cohen, 1989). Undoubtedly there remain numerous phosphatases, yet to be characterised, that down-regulate the effect of the various kinases on nAChR function. Certainly more is known about phosphatases that regulate voltage-dependent ion channels (which will not be covered here, see Herzig and Neumann, 2000). Application of the knowledge derived from these studies should enable further understanding of the effects of phosphorylation on cholinergic systems.

Certainly the balance of kinase and phosphatase activity is fundamental to the regulation of neuronal signalling. The dynamic flux of the phosphorylation state provides a molecular mechanism for flexibility. Importantly, it allows intracellular events to determine the responsiveness of neurones to extracellular influences. In the next section the dynamics of these intracellular events, particularly those known to be regulated by metabotropic receptors will be discussed.

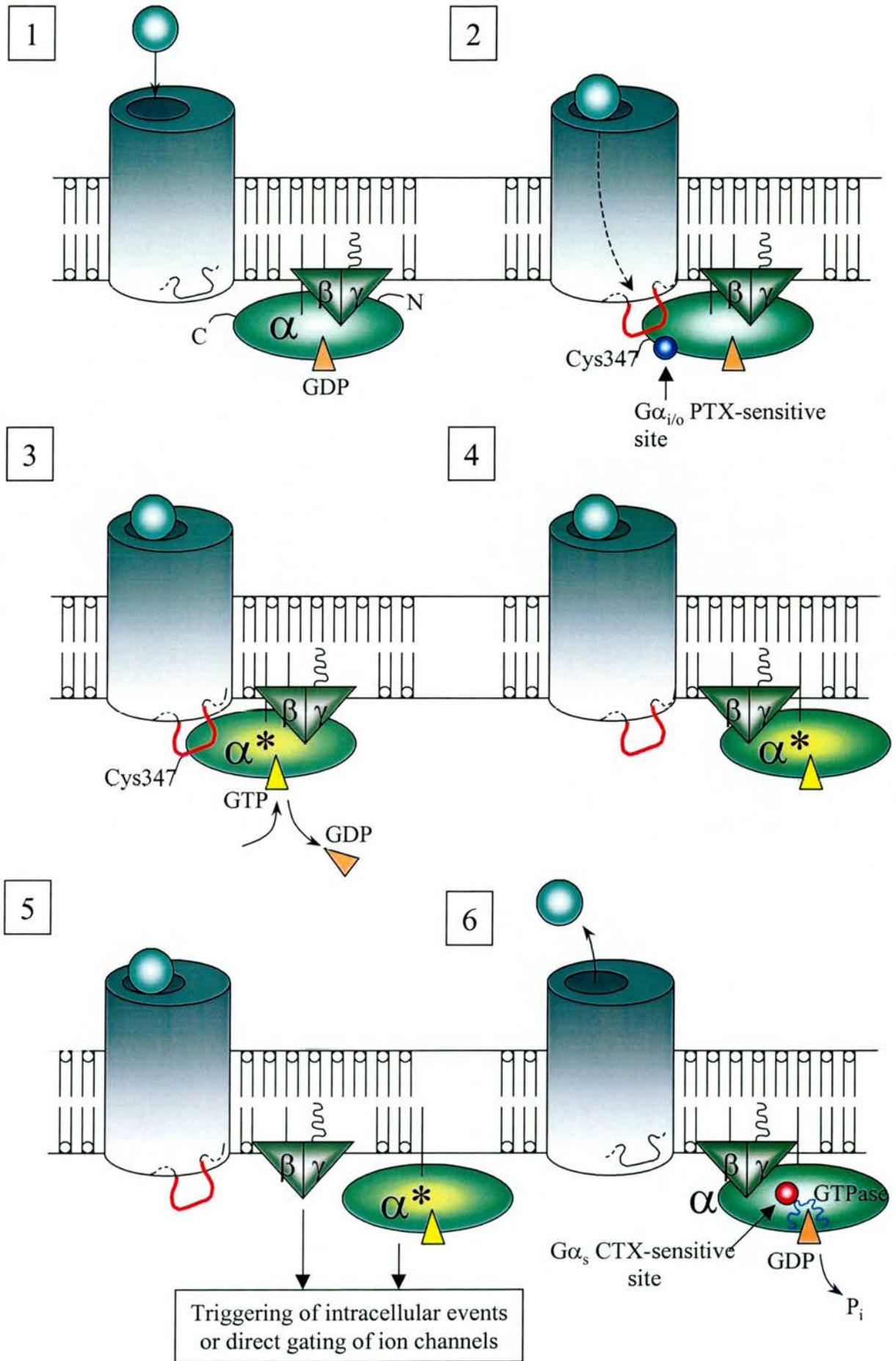
Section 3: MONOAMINE RECEPTOR-ACTIVATED SECOND MESSENGER SYSTEMS.

The broad family of nicotinic acetylcholine receptors are all highly modulated proteins subject to the diverse array of influences, a number of which have been discussed in the previous section. The focus of this review is the mechanism by which endogenous metabotropic receptor ligands modulate postsynaptic nicotinic receptors. Metabotropic ligands exert their effects on cellular events by binding to G-protein-linked receptors (Birnbaumer, 1990). An incredible range of ligands act through such signalling systems, the activation of G-proteins by ligand-receptor complexes being ubiquitous to living organisms (see Gudermann, Kalkbrenner and Schultz, 1996). The mechanics of G-protein signalling are shown in figure 4: the receptor-activated heterotrimeric G-protein subunits dissociate to interact with protein localised to the plasma membrane either to exert a direct activating action on channel proteins (reviewed by Brown and Birnbaumer, 1990; Schneider, Igelmund and Hescheler, 1997) or stimulate the enzymatic turn-over of a plethora of intracellular signalling molecules (Gudermann, Kalkbrenner and Schultz, 1996). As discussed already, the latter act via phosphorylation-dependent pathways to control cellular events including the functional properties of voltage and ligand-gated ion channels involved in neuronal transmission. The direct effect of G-proteins on ion channels is termed either ‘cytoplasmically independent’ or ‘membrane-delimited’. G-proteins form such interactions with a wide diversity of K^+ (e.g. Scornik, Codina, Birnbaumer and Toro, 1993; Lee *et al.*, 1997) and Ca^{2+} (Ikeda, 1996; Yan, Song, and Surmeier, 1997) ion channels.

Figure 4. Receptor coupling to G-protein signal transduction.

Binding of guanosine nucleotides and GTPase activity are inherent to G-proteins. Heterotrimeric G-proteins are, distinguishable from other members of the GTPase family firstly by their organisation into an α subunit incorporating GTPase activity and guanosine nucleotide binding, and a $\beta\gamma$ dimer that increases the affinity of the α subunit for receptors, and can itself regulate cellular enzymes. Secondly, they are distinguished by their association with seven transmembrane helical metabotropic receptors.

1. The G-protein heterotrimers are classified according to the type of α subunit, of which at present a number of families have been identified based on primary sequence homology: $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12}$ (Gudermann, Kalkbrenner and Schulz, 1996). The inactive GDP-bound form of the α subunit associates with the $\beta\gamma$ dimer principally via its amino terminus, while the carboxyl terminus is important for interaction with receptors in the surface membrane. The $\beta\gamma$ dimer is tightly complexed, the phenylated γ subunit is functionally important for localisation of the dimer at the plasma membrane interface and interaction with α subunits (Casey, 1995).
2. Binding of a specific agonist to the metabotropic receptor results in a conformational change in its structure and exposure of a high affinity site for the G-protein α subunit. In the $G\alpha_{i/o}$ family the site on the α subunit that interacts with the high affinity receptor can be disrupted by pertussis (PTX) toxin ADP-ribosylation of the Cys347 amino acid residue (Rens-Domiano and Hamm, 1995).
- 3,4. Interaction of the quiescent G-protein α,β,γ timer with the exposed high affinity receptor site results in release of GDP and its exchange for GTP. The α subunit then undergoes a conformational change to the active form which dissociates from the $\beta\gamma$ dimer.
5. The dissociated, activated $G\alpha$ subunits interact with a variety of effectors. They can act ‘indirectly’ by activating enzymes including adenylyl cyclase ($G\alpha_s$) cGMP phosphodiesterase ($G\alpha_i$) and phospholipase C ($G\alpha_{q/11}$) to exert an effect, or alternatively via G-proteins ‘directly’ gating ion channels ($G\alpha_{k/i}$) (e.g. Scornik, Codina, Brinbaumer and Toro, 1993). The $\beta\gamma$ dimer also regulates cellular functions including type I, II and IV adenylyl cyclases and PLC α isoforms and K^+ and Ca^{2+} channels (Brown and Birnbaumer, 1990; Pieroni, Jacobowitz, Chen and Iyengar, 1993; Ikeda, 1996; Schneider, Igelmund and Hescheler, 1997).
6. G-protein activation of the effectors is terminated by the inherent GTPase activity of α subunit. This is often activated by interaction with the effector enzymes, for example PLC- $\beta 1$ functions as a GTPase-activating protein (GAP; Berridge, 1993). The inactive α form re-associates with the $\beta\gamma$ dimer, until further binding of the ligand to the metabotropic receptor initiates the whole cycle again. The bacterial cholera toxin (CTX) ADP-ribosylates $G\alpha_s$ to reduce the GTPase action of the GDP-GTP site so potentiating the action of the G-protein.



Compared to ionotropic receptor-mediated transmission, transduction via G-proteins is on a slow time course but has the inherent advantage of signal amplification and the ability to regulate diverse cellular properties over a long time course. One of the best systems for the study of G-protein dependent pathways is serotonergic presynaptic facilitation exhibited by sensory neurones of the marine mollusc *Aplysia* (see section 1.1.1). The actions of the monoamine serotonin on the presynaptic nerve terminal are exerted by two G-protein-dependent intracellular pathways, one involving activation of PKC by DAG and Ca^{2+} , the second mediated by a rise in cAMP concentration (see figure 5). Both pathways converge on K^+ channels populations to attenuate their gating. They also modulate spike-duration independent (SDI) processes thought to involve vesicle mobilisation and exocytosis. The end effect is to increase the Ca^{2+} conductance and amount of transmitter primed for release (reviewed by Byrne and Kandel, 1996). This model system demonstrates how a single initial ligand signal recruits two pathways to modulate several properties of neuronal signalling. Amplification of the ligand signal is provided by G-protein mediated activation of adenylyl cyclase (AC) and phospholipase C (PLC) cascades. These enzymes, once activated, catalyse production of second messenger molecules, resulting in their rapid accumulation in the cytoplasm. These second messengers do not, however, operate via straightforward linear pathways like those shown in figure 5, since both systems interact with the constitutive stages of each others signalling mechanism. For example protein kinase C is known to inhibit AC in *Aplysia* (see figure 5). This inhibition of AC by Ca^{2+} has also been observed in mammalian preparations and appears to be a conserved mechanism by which Ca^{2+} regulates cAMP accumulation. In contrast to these reports the activity of a number

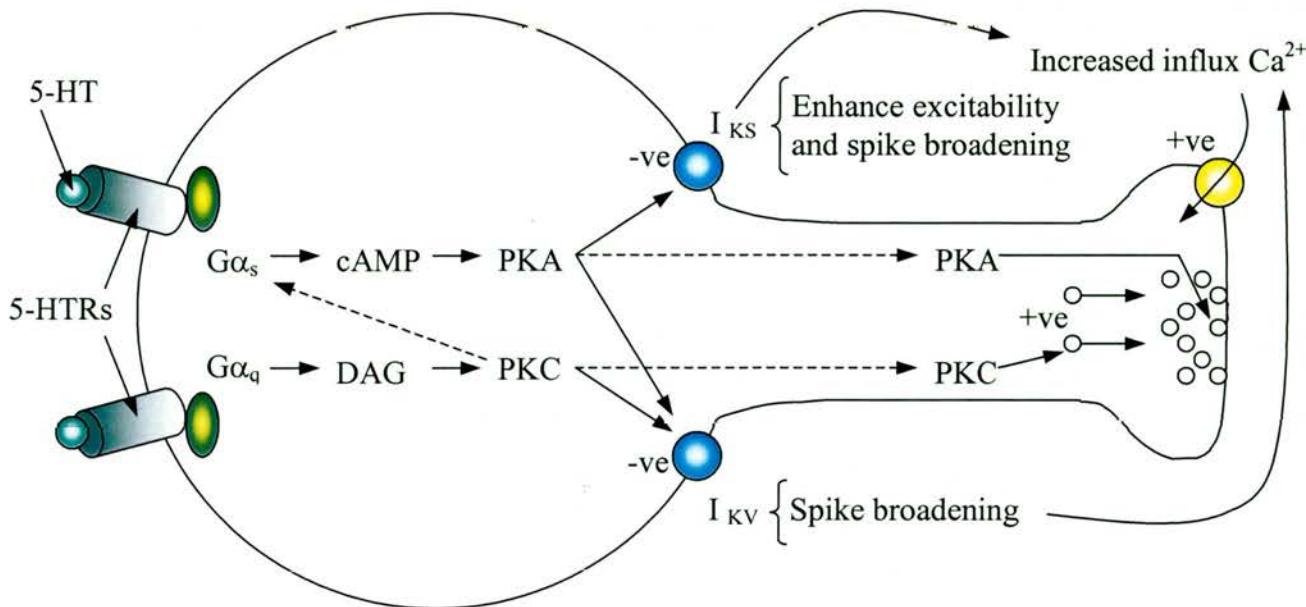


Figure 5. Multiple G-protein dependent processes mediate serotonergic presynaptic facilitation of *Aplysia* sensory neurones (adapted from Byrne and Kandel, 1996).

Aplysia is a model system for the study of metabotropic receptor activated modulation of synaptic transmission, which provides insights into the complex interactions that exist between second messenger systems. The monoamine serotonin (5-HT) is released from facilitatory interneurones and binds to two independent populations of serotonin receptor (5-HTR) expressed on the surface of sensory neurone presynaptic terminals. The first population of 5HTRs interact with a G_s -like protein to stimulate an increase in intracellular cAMP levels (Walsh and Byrne, 1989). The second type of serotonin receptor increases phosphoinositol turnover by activating phospholipase C (PLC) producing the PKC activator diacylglycerol (DAG)(Sacktor and Schwartz, 1990). The cAMP-dependent protein kinase (PKA) and PKC both reduce voltage-dependent K^+ conductance (I_{KV}) to prolong the duration of the action potential (Braha, Dale, Klein, Abrams and Kandel, 1990; Sugita, Goldsmith, Baxter and Byrne, 1992), increase Ca^{2+} influx at the nerve terminal and enhance vesicle release. PKA additionally phosphorylates a population of K^+ channel that mediate a second rectifying serotonin-sensitive K^+ conductance (I_{KS})(Klein, Camardo and Kandel, 1982), to bias the membrane potential towards a more depolarised state. Both kinases additionally directly increase the quantity of neurotransmitters released via ‘spike-duration independent’ processes. PKC increasing the number of vesicles primed for release and PKA facilitating the processes of Ca^{2+} -dependent exocytosis (Byrne and Kandel, 1996).

It is proposed that the two intracellular pathways provide different state and temporal means of synaptic modulation (see Byrne and Kandel, 1996). PKA is predominantly active in non-depressed synapses and after brief exposure to serotonin, whilst PKC is important for the reverse situations.

of mammalian AC isoforms is enhanced by rises in intracellular Ca^{2+} (for review see Cooper, Mons and Karpen, 1995).

The finding that the monoamines act by mobilisation of cAMP and DAG-dependent processes is an often reoccurring theme throughout the literature on neuromodulation. Monoaminergic systems of vertebrates are extensive and regulate a wide variety of neuronal functions. They have been extensively investigated, therefore, as potential sites of clinical intervention for psychological disorders, drug dependency and a number of neuropathologies (for example see Rocha *et al.*, 1998; Piccini *et al.*, 1999). Consequently aminergic transmission is well characterised at the molecular level in vertebrate preparations, with particular receptor subtypes characterised by cloning, pharmacology and their putative modes of signal transduction. There is, however, little physiological evidence as to the processes underlying modulation at the behavioural level, with only a few relatively simple vertebrate preparations such as the *Xenopus laevis* tadpole (for example McDearmid, Scrymgeour-Weddeburn and Sillar, 1997) and adult lamprey *Petromyzon marinus* (Kemnitz, Strauss, Hosford and Buchanan, 1995) providing experimental evidence as to the mechanisms of aminergic modulation at a level of detail available for some invertebrate systems like that of *Aplysia*. Aminergic neuronal circuits form highly complex modulatory systems key to orchestrated regulation of behaviour. A complete understanding of the mode of action of these substances from the molecular level up to the level of the whole organism is essential if we are to understand neuronal processing.

In the remainder of this section the nature of monoaminergic transmission will be discussed with reference to the similarities in the mode of signal transduction between vertebrate and invertebrate monoamine receptors. In the final section the similarities in function will be discussed with particular attention to the possibility

that monoamines, specifically dopamine, serotonin and octopamine, modulate cholinergic transmission.

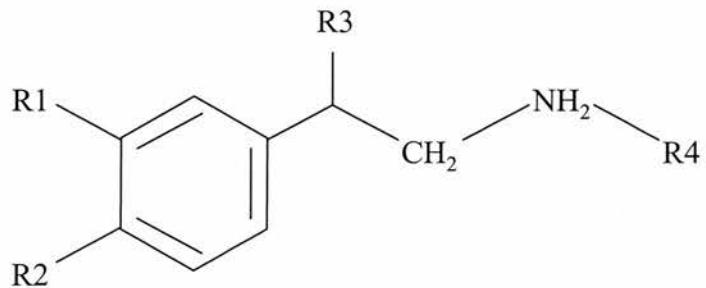
3.1 Monoamines.

The term monoamine encompasses a range of functionally important transmitters all synthesised from the common amino acids. A number of them are also characterised by a similar structure of a single aromatic ring and variable side chains (figure 6), with the exceptions of serotonin and histamine. The scope of this investigation is limited to the catecholamine dopamine, the indolalkylamine serotonin, and the principally invertebrate transmitter octopamine, since these appear to be far the most important monoamines in invertebrates as judged by their high concentrations and proposed modulatory roles (for reviews see Pitman, 1971 and 1985; Evans, 1980; Bicker and Menzel, 1989; Roeder, 1994). In the specific context of this thesis these amines have been chemically identified in the CNS of *Periplaneta americana* (Dymond and Evans, 1979; Evans, 1980), localised in neurones throughout the ventral nerve cord (Fleming and Pitman, 1982; Bishop and O'Shea, 1983; Baker and Pitman, 1989; Milton, Verhaert and Downer, 1991) and been implicated in modulation at the neuronal and organism level (Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992b; Washio and Tanaka, 1992; Weisel-Eichler and Liberstat, 1996).

3.1.1 Dopamine receptors

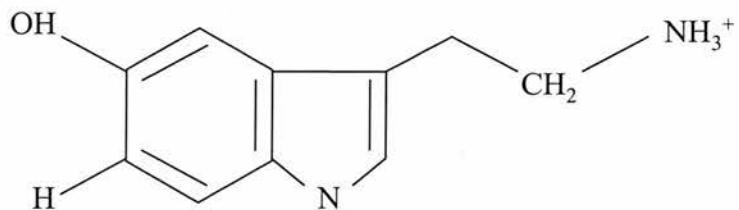
Evidence for a role for dopamine as a neurotransmitter has been provided in nervous tissue from a wide variety of animals by the detection of the amine itself as well as enzymes responsible for its synthesis, and because it has definable specific effects on cells expressing receptor sites with high affinity for dopamine.

Figure 6. Monoamine neurotransmitters

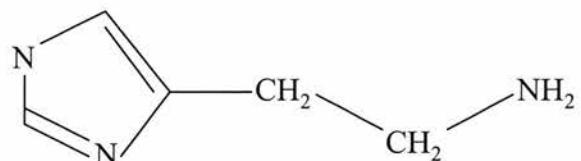


Monoamine	R1	R2	R3	R4
Tyramine	H	OH	H	H
Octopamine	H	OH	OH	H
Dopamine	OH	OH	H	H
Noradrenaline	OH	OH	OH	H
Adrenaline	OH	OH	OH	CH ₃

* precursor amino acid = tyrosine



Serotonin, precursor amino acid = tryptophan



Histamine, precursor amino acid = histidine

Two types of vertebrate dopamine receptor (DAR) were originally identified according to their pharmacological profile and linkage to the cAMP second messenger pathway (see review by Kebabian and Calne, 1979). D1 receptors were considered to be positively coupled to adenylyl cyclase (AC) activity via G_s proteins and sensitive to dopamine in the μM range. D2 receptors, on the other hand, were characterised as being sensitive to dopamine in the nM range and causing inhibition of AC. Additionally they promote phospholipase C activity by coupling to G_{i/o} proteins. More recently synthetic pharmacological agents have been developed that further support the receptor classification: D1-type SCH23390-sensitive receptors and D2-type spiperone-sensitive sites (for example see Kokay and Mercer, 1996). Gene cloning has further isolated subtypes of these two broad families (table 5a), such that five distinct receptor types are now recognised: D₁ and D₅ (alternatively known as D_{1b}) of the D1 family, and D₂, D₃ and D₄ receptors belonging to the D2 family. All five receptor genes are widely expressed in a variety of neuronal tissues.

A number of insect dopamine receptors (DARs) have been identified (table 5b). Of note is the fact that the insect DARs often show poor selectivity for specific vertebrate D1- and D2-type pharmacological agents. For example the dDA1 and DmDop1 receptors show poor affinity for D1-selective benzazepine agonists like SKF38393 (Sugamori, Demchyshyn, McConkey, Forte, Niznik, 1995; Gotzes and Baumann, 1996). The difference in agonist and antagonist properties is proposed to be attributed to variability between insect and vertebrate receptors in the ligand binding N-terminus (Gotzes and Baumann, 1996). The cloned insect receptors do, however, typically exhibit sequence homology to vertebrate receptors in a range of 40 - 60 % in the hydrophobic putative transmembrane sequences. The mode of

Table 5a. G-protein linked mammalian dopamine receptors.

Receptor	G-protein	Effect	Agonist profile	Antagonist profile	Localisation
D ₁	G _s	↑AC	(-) Apomorphine > β-Fenoldopam = Pergolide > SCH38393	SCH23390 >> Haloperidol = Chlorpromazine	CNS: CP, NA, FC PNS: LIM, CV
D _{5/1b}		↑AC	SCH38393 > β-Fenoldopam	SCH23390 >> Haloperidol > Chlorpromazine	CNS: HIP, HYP PNS: LIM
D _{2S}	G _{i/o} *	↓AC, ↑PLC	(+/-) Apomorphine > Pergolide > Naxagolide > Quinpirole	Spiperone > Haloperidol > Raclopride	CNS: CP, NA, C (low) PNS: LIM, CV, PIT
D _{2L}		↓AC, ↑PLC	7-OH-DPAT > Pergolide > Bromocryptine	Nemonapride > Spiperone > Chlorpromazine	CNS: NA, C (low) PNS: LIM
D ₃	G?	autoreceptor	(+/-) Apomorphine > Naxagolide > Quinpirole	Spiperone > Nemonapride > Haloperidol	CNS: FC, AMY, MED PNS: LIM, CV
D ₄	G?	-			

* The short form D_{2S} is more effective at coupling to G_i and inhibits AC to a greater degree than D_{2L} (Dal Toso, Sommer *et al.*, 1989).

AC, adenylyl cyclase; PLC, phospholipase C.

CNS localisation abbreviations: AMY, amygdala; C: cortex; CP, caudate putamen; FC, frontal cortex; HIP, hippocampus; HYP, hypothalamus; MED, medulla; NA, nucleus accumbens
PNS localisation: CV, cardiovascular; LIM, limbic; PIT, pituitary.

References:

- Seeman and Van Tol (1994)
- Sokoloff, Giros *et al.* (1990)
- Sibley and Monsma, Jr. (1992)
- Dal Toso, Sommer *et al.* (1989)
- Grandy and Civelli (1992)
- Kebabian and Calne (1979)

Table 5b. Pharmacology of insect dopamine receptors.

Class of Receptor	Preparation	Receptor subtype	Agonist pharmacology	Antagonist pharmacology	Effector	References
D1 family	<i>Drosophila</i>	DmDop1	DA > SKF38393	BTC = cis-FPX. not SCH23390	↑cAMP	1
	DopR99B		*DA > NE > EP > TYR no effect: 5-HT or OA		↑[Ca ²⁺] _i ; ↑cAMP	2,3
dDA1			DA > NE >> 5-HT † no effect: OA, or TYR	BTC > SCH23390 > cis-FPX	↑cAMP (EC ₅₀ 300nM)	4
<i>Apis mellifera</i>	D1 site			SCH23390 > cis-FPX > BTC > SPIP		5
	D1-like			CPZ > cis-FPX > BTC > SCH23390 > HAL	↑cAMP	6
D2 family	<i>Apis mellifera</i>	D2 site		SPIP > FPZ > BTC > domperidone		5
Antennal lobes	<i>P. americana</i> D ₃ MN	D2-like	DA, but not TYR, OA, 5-HT	SPIP=FPZ > SCH23390		7
Unclassified		D1/D2	DA = ADTN > Ergometrine > APO	FPX = HAL > BTC > FPZ	Volt-depend inward current, does not involve cAMP	8

* Agonist profile of elevated Ca²⁺ response. † also very weak D1-agonist effects of benzazepines e.g. SKF-38393 and SKF-82526.

APO, apomorphine (D2) BTC, butaclamol (D1/D2); cis-FPX, cis-flupentixol (D1/D2); EP, epinephrine; FPZ, fluphenazine (D1/D2); HAL, haloperidol (D2); NE, norepinephrine; SCH23390 (D1); SKF38393 (D1); SPIP, spiperone (D2); TYR, tyramine.

1 Gotzes and Baumann (1996)

2 Reale, Hannan, Hall and Evans (1997)

3 Feng *et al.* (1996)

4 Sugamori *et al.* (1995)

5 Kokay and Mercer (1996)

6 Blenau, Erber and Baumann, (1998)

7 Kirchhlof and Mercer (1997)

8 Davis and Pitman (1991)

transduction of insect DARs is similar to that of D1 vertebrate receptors, the majority of insect DARs exerting their action via G-proteins-dependent elevations in cAMP concentration. There is an apparent lack of reported D2-type receptors in insect systems although a number of spiperone-sensitive binding sites have been labelled in *Apis mellifera* (Kokay and Mercer, 1996).

A few insect DARs do exert their effect via pathways independent of AC stimulation. For example the mixed D1/D2 pharmacology DAR of the D3 inhibitory motoneurone of *Periplaneta americana* which exerts its effect independently of cAMP (Pitman and Baker, 1989; Davis and Pitman, 1991). Neither the cAMP-analogue Db-cAMP nor the AC-stimulant forskolin mimic the depolarising action of dopamine (Pitman and Baker, 1989). Another receptor, the cloned DopR99B D1-like receptor, when expressed in *Xenopus* oocytes couples to multiple G-protein pathways and generates a rapid, transient rise in intracellular Ca^{2+} concentration as well as a more sustained elevation in cAMP (Reale, Hannan, Hall and Evans, 1997). Similar multiple G-protein coupling has also been reported in vertebrate systems (for example see Kimura, White and Sidhu, 1995; Sidhu, Kimura, Uh, White, and Patel, 1998). Taken with the fact that the different subunits of the trimeric G-protein can interact with a diverse array of effectors (figure 4) it is plausible that the activation of divergent intracellular pathways is a property of most DARs.

3.1.2 Serotonin receptors.

Initial studies on serotonin identified both excitatory and inhibitory effects of applying the amine locally to preparations such as cat cerebral cortex neurones (Roberts and Straughan, 1967). The diverse effects of serotonin in vertebrate systems (Anwyl, 1990) were identified pharmacologically as acting at multiple

receptors, of which seven families are now recognised (for review see Julius, 1991; Hoyer *et al.*, 1994). Serotonin receptors (5-HTRs) couple to a diverse range of G-proteins (table 6a) with the exception of the 5-HT₃ family which are cation-permeable ionotropic receptors. Vertebrate DARs are principally defined on the basis of whether they stimulate or suppress AC activity. Serotonin receptors can be similarly classified according to their mode of signal transduction via an array of intracellular pathways. Receptors of the 5-HT₂ family activate phospholipase C (PLC) intracellular pathways via G_q proteins, whereas further subtypes couple either positively (5-HT₄, 5-HT₆, and 5-HT₇), or negatively (5-HT₁ family) to adenylyl cyclase.

In contrast to dopaminergic systems, this diversity of vertebrate receptors is also maintained in insect preparations with 5-HTRs coupling to PLC as well as AC (for example Berridge and Heslop, 1981), and putative 5-HT_{2/3} receptors being reported by Bermudez *et al.* (1992) in *Locusta migratoria* (see table 6b; also Hen, 1992; Saudou and Hen, 1994). However, the pharmacology of the insect receptors is not as clear-cut as that of the corresponding vertebrate subtypes; specific antagonists are often ineffective, unlike classical serotonergic broad spectrum agents such as lysergic acid diethylamide (LSD) and dihydroergotamine (DHE)(e.g. Wedemeyer, Roeder and Gewecke, 1992).

3.1.3 Octopamine receptors

Octopamine is present at low levels in some vertebrate systems, predominantly occurring in the hypothalamus and brain stem. However a physiological role for this amine in vertebrates has proved elusive, due in part to its co-localisation with

Table 6a. G-protein-linked mammalian serotonin receptors

Receptor	G-protein	Effect	Specific agonists antagonists	Localisation (Human/rat/mouse)
5-HT _{1A}		↓AC, hyperpolarisation	8-OH-DPAT, buspirone, 5-CT	CNS: HIP, SEP, AMY, RN
5-HT _{1B}		↓AC, inhibit nt release	Sumatriptan, CP93129, 5-CT	CNS: BG, STR, FC, SN; PNS
5-HT _{1D}		↓AC, inhibit nt release	Sumatriptan, L694247, 5-CT	CNS: CP, HIP, C, SN
5-HT _{1E}		↓AC	-	Only CNS: CP
5-HT _{1F}		↓AC	LY334370, 5-CT	CNS, PNS
5-HT ₁ -like		↓AC, smooth muscle contract.	Sumatriptan, 5-CT	PNS
5-HT _{2A}	G _{q/11}	↑PLC,	Ketanserin	CNS: C, BG; PNS
5-HT _{2B}		↑PLC	α-methyl 5-HT	CNS: AMY, SEP, HYP; PNS
5-HT _{2C}	G _{q/ii}	↑PLC	α-methyl 5-HT, BW723C86	SB200646, SB204741
5-HT ₄	G _s	↑AC, potentiate nt release	α-methyl 5-HT	Mesulergine, SB200646
5-HT _{5A}	G _s	↓AC	BIMU8, RS67506	CNS: CP
5-HT _{5B}	?	?	-	RS23597-190, GR113808
5-HT ₆	G _s	↑AC (HEK 292 cells)	Methiothepin	CNS: STR, HIP, FC; PNS
5-HT ₇		-	Methiothepin	CNS: C, HIP, CER
		-	Methiothepin	CNS: STR, AMY, HIP, C
		-	Methiothepin	CNS, PNS

AC, adenylyl cyclase; nt, neurotransmitter; PLC, phospholipase C.

CNS localisation abbreviations: AMY, amygdala; BG, basal ganglia; C, cortex; CER, cerebellum; CP, caudate putamen; FC, frontal cortex; HIP, hippocampus; HYP, hypothalamus; RN, raphe nuclei; SEP, septum; SN, substantia nigra; STR, striatum.

References:

- Markstein *et al.* (1999) Alexander and Peters (1997) Hoyer *et al.* (1994) Eglen *et al.* (1995)
- Saudou and Hen (1994) Humphrey, Hartig and Hoyer (1993) Hen (1992) Julius (1991)
- Anwyl (1990)

Table 6b. Pharmacology of insect serotonin receptors.

Vertebrate class of receptor	Preparation	Receptor subtype	Agonist pharmacology	Antagonist profile	Effector	References
5-HT ₁	<i>Drosophila</i>	5-HT _{dro2A}	5-HT*, no effect: DA, EP, NE, OA, TYR, HIST	DHE > <i>d</i> -BTC >> PRA > MSD > <i>l</i> -BTC >> 8-OH-DPAT	↑PLC, ↓AC. Pertussis toxin sensitive	1,2,8
	<i>Locusta migratoria</i>	5-HT ₁ site		LSD = BOL not KET or MIA	G-protein	3
5-HT ₂	<i>Drosophila</i>	5-HT _{dro2B}	5-HT*, no effect: DA, EP, NE, OA, TYR, HIST	DHE > <i>d</i> -BTC >> PRA > MSD > <i>l</i> -BTC >> 8-OH-DPAT	† G _q ↑PLC Pertussis toxin sensitive	1,2,4,8
5-HT _{2,3}	<i>Locusta migratoria</i>	5-HT ₃ like: I(5-HT) _K I(5-HT) _{Na} I(5-HT) _X	5-HT, 2-Me5-HT 5-HT, 2-Me5-HT, ICS 5-HT	KET = RIT >> MDL MDL	↓I _K , Inward current ↑I _{Na} , Inward current 4-AP sensitive current	5
5-HT _{4,6,7}	<i>Drosophila</i>	5-HT _{dro1}	5-HT*, no effect: DA, EP, NE, OA, TYR, HIST	DHE > <i>d</i> -BTC >> MSD > PRA > <i>l</i> -BTC >> 8-OH-DPAT	G _s , AC, ↑cAMP	1,2,4,8
	<i>Schistocerca gregaria</i>		Low affinity for 5-HT (K _i = 0.209 μM)	DHE = BTC >> 8-OH-DPAT = KET	Homology to dro1, G-protein coupled	6
	<i>Periplaneta americana</i>	5-HT sensitive AC	5-HT > BOL > LSD**	BOL > LSD > CYPRO‡	Stimulation of AC	7

* Affinity for 5-HT: dro1 (K_a = 1.6 μM) > dro2b (2.1 μM) > dro2a (16 μM). † Saudou *et al.* (1992) dro2B coupled to inhibition of AC.

** Agonist affinity: LSD > 10⁻⁷ M (K_a = 4 μM), BOL > 5.10⁻⁸ M (1.5 μM). ‡ Cyproheptadine (CYPRO) not so specific for 5-HT-sensitive AC over those of DA, OA or NE

2 Me5-HT, 2 methyl 5-HT (5-HT₃); 8-OH-DPAT (5-HT_{1A}); DHE, dihydroergotamine (5-HT); ICS, ICS205930 (5-HT₃); KET, ketanserin (5-HT₂); MDL72222 (5-HT₃); MIA, miaserin; MSD, methysergide; PRA, prazosin (α1 adrenergic antagonist); RIT, ritanserin (5-HT₂).

1 Saudou, Boschert *et al.* (1992) 3 Hiripi and Downer (1993) 5 Bermudez, Beadle and Benson (1992)

2 Hen (1992) 4 Obosi, Schuette *et al.* (1996) 6 Wedemeyer *et al.* (1992) 8 Saudou and Hen (1994)

5 Nathanson and Greengard (1974)
8 Saudou and Greengard (1974)

noradrenaline and apparent action at adrenoceptors (for review see David and Coulon, 1985). The current hypothesis is that the octopaminergic system of invertebrates is homologous to the adrenergic systems of vertebrates and certainly there are considerable similarities in their behavioural roles and receptor pharmacology (reviewed by Roeder, 1999).

Insect octopamine receptors (OARs) almost exclusively stimulate adenylyl cyclase, the only exception being OARs isolated from the moths *Heliothis virescens* and *Bombyx mori* which inhibit AC activity (von Nickisch-Rosenegk *et al.*, 1996). In this sense they are similar to vertebrate β -adrenoceptors, but their pharmacology is more akin to that of α -adrenergic receptors (see table 7a and 7b).

Octopamine is associated with midline neurones in insect ventral nerve cords (Roeder, 1999), and is present in the dorsal unpaired median (DUM) neurones of *Periplaneta americana* (Dymond and Evans, 1979). The neurotransmitter has been shown to selectively stimulate an adenylyl cyclase in *P. americana* (Nathanson and Greengard, 1973) as well as regulate motor output (Goldstein and Camhi, 1991; Weisel-Eichler and Libersat, 1996). It is also present in higher concentrations than dopamine in the metathoracic ganglion of this species (Dymond and Evans, 1979), where they both exhibit similar potentiating effects on EPSPs recorded from interneurones (Casagrand and Ritzmann, 1992b). In the same preparation serotonin exhibits an opposing, suppressive effect.

3.1.4 Commonalities in transmission mediated by different monoamines in insects.
Before discussing the cAMP- and Ca^{2+} -dependent intracellular signalling pathways stimulated by aminergic metabotropic receptors it is perhaps worth tying up a

Table 7a. G-protein linked adrenoceptors

Receptor	Potency order.	G _i -protein	Effect	Selective agonists	Antagonists	Ref.
α_{1A}	NA ≥ A		↑[Ca ²⁺] _i via PLC, also interact with PLA ₂ , PLD and cAMP.	A61603	PRA	1, 3, 4
α_{1B}	A = NA	G _{q/11}		-	PRA, chloroethylclonidine	1, 3, 4
α_{1D}	A = NA			-	PRA	1, 3, 4
α_{2A}	A > NA		↓AC. Stimulate Ca ²⁺ influx, activate	CLO, Oxymetazoline	ATI > IDA > YOH	2, 3, 4
α_{2B}	A > NA	G _{i/o}	K ⁺ channels, PLA ₂ .	CLO	ATI > YOH > CHL	2, 3, 4
α_{2C}	A > NA			CLO	YOH > ATI > IDA	2, 3, 4
β_1	NA > A			Denopamine	BET > METO > ATE, PRO	3, 4
β_2	A > NA			Procaterol, zinterol	ICI > BET > ATE > METO, PRO	3, 4
β_3	NA = A	G _s *	↑AC	BRL37344, SR58611A	BRU, PRO	4
β_4	NA ≥ A			-	BRU, PRO	4

* β_3 receptors also couple to G_{i/o} protein.

ATE, atenolol; ATI, atipamezole; BET, betaxolol; BRU, brupranolol; CLO, clonidine; CHL, chlorpromazine; ICI, ICI118551; IDA, idazoxan; METO, metoprolol; PRA, prazosin; PRO, propranolol; YOH, yohimbine.

References:

- 1 Hieble *et al.* (1995)
- 2 Nicholas, Hokfelt, and Pieribone (1996)
- 3 MacDonald, Kobilka and Scheinin (1997)
- 4 Trends in Pharmacology (1999)

Table 7b. Pharmacology of insect octopamine receptors.

Receptor subtype	Preparation	Agonist profile	Antagonist profile	Effect	References
OAR ₁	<i>Schistocerca am. gregaria</i>	CLO >> NAP	PHE > CHL > YOH > MIA	Modulates myogenic rhythm	1
OAR _{2A}	<i>Schistocerca am. gregaria</i> (presynaptic) DUMETi autoreceptor	NAP >> TOL ~ CLO TOL > CLO	MET > MIA > PHE > CHL	Modulates amp of SETi twitch tension	1,2
	<i>Locusta migratoria</i>	NC7 > NCS5 > NAP > TOL > CLO	MIA > PHE > PRO		3
OAR _{2B}	<i>Schistocerca am. gregaria</i> (postsynaptic)	NAP ~TOL >> CLO	PHE > MET > MIA > CHL	Rate of relax of SETi & FETi twitch tension	1
OAR ₃	Locust brain		MIA > PHE > CHL > MET > YOH		4,5
	Locust air sac	AC-6 > CLO > Compl	MIA > PHE > MET > CHL*. But not YOH	AC, ↑cAMP	6
Unclassified	<i>Periplaneta americana</i>	OA > XAMI	MIA > PHE. Not PRO.	AC.	7,8
	<i>Drosophila</i> Mushroom bodies	OA >> TYR = NE. Not DA, 5-HT, HIST.		AC, ↑cAMP. ↑[Ca ²⁺] _i	9
	<i>Drosophila</i> Neuromuscular synapse	OA	YOH	Decreased excitatory currents: pre & postsynaptic	10

* CHL, Chlorpromazine, enhances agonist binding at low concentrations

CLO, clonidine; Compl, Compound 1; MET, metoclopramide; NAP, naphazoline; NC5/7, phenyliminoimidazolines; PHE, phentolamine; PRO, propranolol (β -adrenergic) XAMI, imidazoline XAMI; YOH, yohimbine.

1 Evans (1981)

2 Howell and Evans (1998)

4 Roeder (1992, 1995)

5 Roeder and Nathanson (1993)

7 Nathanson and Greengard (1973)

8 Orr *et al.* (1991)

6 Zeng, Jennings and Loughton (1996)

9 Han, Millar and Davis (1998)

10 Nishikawa and Kidokoro (1999)

number of key points about the initial transduction mediated by insect aminergic receptors that are common to invertebrate systems. A number of these observations have lead some researchers to label invertebrate receptors as ‘primordial’ (e.g. Sugamori, Demchyshyn, McConkey, Forte, Niznik, 1995) but such categorisation can be misleading. The actions of monoamines in insect systems are transduced predominantly via G_s-like proteins that couple positively to adenylyl cyclase. Adenylyl cyclases responsive to amines have been identified in a diverse range of invertebrate preparations (for examples see Uzzan and Dudai, 1982; Evans, 1984; Deterre, Paupardin-Tritsch and Bockaert, 1986; Cappaso *et al.*, 1991) including the metathoracic ganglion of *P. americana* (Nathanson and Greengard, 1973, 1974). These represent potentially important sites of aminergic transduction and targets for pharmacological intervention. Additional studies on invertebrate systems will, no doubt, elucidate further modes of transduction possibly in line with the intriguing study by Reale *et al.* (1997). Certainly the apparent simplicity of insect aminergic receptors almost exclusively coupling to adenylyl cyclase is likely to be unfounded. With the increasing number and importance of aminergic receptors linked to ACs in vertebrates (e.g. Eglen, Wong, Dumuis and Bockaert, 1995; Baker *et al.*, 1998; Shimizu, Nishida, Zensho, Miyata and Yamawaki, 1998; Markstein *et al.*, 1999), invertebrate preparations provide ideal model systems for prising the mechanics of the cAMP transduction system apart, principally because it will be easier to identify and record from the individual neurones involved.

One stumbling block is that invertebrate aminergic receptors do not conform to the pharmacology of classical vertebrate subtypes. Non-specific antagonists represent the best tools for identifying receptors. For example LSD, methysergide and dihydroergocryptine are potent antagonists at a number of invertebrate serotonin

sites (for example Nathanson and Greengard, 1974; Saudou *et al.*, 1992; Mar and Drapeau, 1996) whereas subtype specific antagonists such as ketanserin often exhibit only partial effects (e.g. Mar and Drapeau, 1996) or non-specific actions (e.g. Deterre, Paupardin-Tritsch and Bockaert, 1986). Invertebrate receptors do show stronger selectivity for endogenous agonists, for instance *Drosophila* 5-HT_{dro1}, 5-HT_{dro2A}, and 5-HT_{dro2B} receptors do not respond to dopamine, octopamine, tyramine and histamine (Saudou *et al.*, 1992), whereas the dopamine receptors DopR99B and dDA1 show marked specificity for dopamine over serotonin (Reale, Hannan, Hall and Evans, 1997; Sugamori, Demchyshyn, McConkey, Forte, Niznik, 1995).

3.2 G-protein activated second messenger pathways.

The aim of the following sections is to review the present literature on the intracellular messenger pathways that link the G-protein aminergic receptors to the putative protein kinases that modulate nicotinic acetylcholine function, thus completing the link from metabotropic to ionotropic receptor and laying the hypothetical basis for this investigation.

In brief, this review focuses in more detail on the two pathways that involve the membrane localised enzymes - phospholipase C (PLC) and adenylyl cyclase (AC), but not the membrane-delimited direct coupling of G-proteins to the activation of ion channels (e.g. Yan, Song and Surmeier, 1997; reviews by Levitan and Irwin, 1988; Brown and Birnbaumer, 1990). There is also recent evidence of a direct dopamine receptor interaction with GABA_A ligand-gated ion channels (Liu *et al.*, 2000) but there is little additional evidence of a role for such direct interactions in signal transduction. Of the enzyme pathways, the AC enzymes are regulated by both G_{i/o} and G_s G-proteins whilst PLC is stimulated by G_{q/11} proteins. G-protein βγ

dimers of a wide range of G-proteins also exert effects on these two enzymes.

Aminergic G-protein-linked receptors also influence other intracellular enzymes indirectly via these two pathways including the Ca^{2+} -dependent activation of guanylyl cyclases (for example Tohda and Nomura, 1990).

3.2.1 Phospholipase C-dependent second messengers.

The membrane-localised phosphoinositidase phospholipase C, specifically PLC- β 1, is stimulated by pertussis toxin-insensitive G_α subunits of the $G_{q/11}$ G-protein subtype. The PLC- β 2 enzyme is stimulated by G-protein $\beta\gamma$ dimers whereas two further members of the PLC family, PLC- δ and PLC- γ , are stimulated by Ca^{2+} and receptor-associated tyrosine kinases respectively (Berridge, 1993).

PLC is an important enzyme whose hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) to produce two intracellular second messengers, the cytosolic inositol 1,4,5-triphosphate (IP_3) and membrane lipid diacylglycerol (DAG), links metabotropic receptors with both Ca^{2+} - and DAG-dependent intracellular events. Both Ca^{2+} and DAG converge on and activate protein kinase C (PKC), whilst a rise in intracellular Ca^{2+} alone binds to CaM and can stimulate calmodulin kinase (reviewed in Edelman and Blumenthal, 1987; Hanson and Schulman, 1992; Scott and Soderling, 1992; Chakravarthy, Morley, and Whitfield, 1999).

Phosphorylation catalysed by PKC and CaMK regulates a range of widely differing cellular events (Chakravarthy, Morley and Whitfield, 1999), and, as already discussed, modulates the function of nicotinic AChRs.

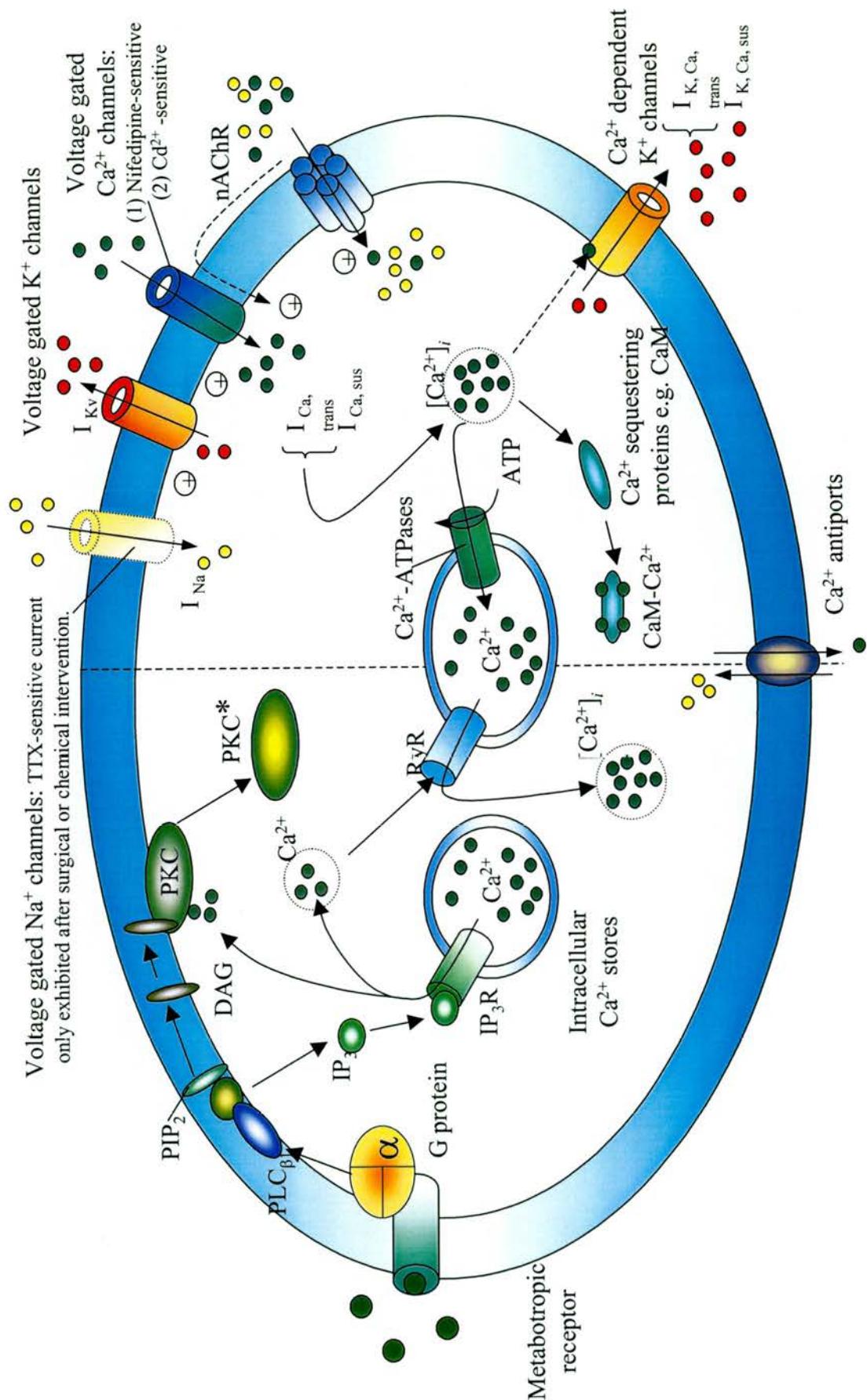
The divalent cation calcium is fundamental to neuronal function (for reviews see Augustine and Neher, 1992; Ghosh and Greenberg, 1995; Simpson, Challiss and Nahorski, 1995; Dolphin, 1996) and is implicated in both presynaptic and postsynaptic plasticity and signalling (reviewed by Racay and Lehotsky, 1996; Zucker, 1999), its intracellular concentration is controlled by a variety of pathways (figure 7). Metabotropic receptors manipulate intracellular calcium levels through the PLC enzyme and more specifically by generation of the cytosolic second messenger IP₃ (figure 7, left-hand side). IP₃ triggers a rise in intracellular Ca²⁺ by binding to receptors on the membranes of intracellular stores. The resultant upsurge in calcium can further initiate Ca²⁺-release (CDCR) via Ca²⁺-sensitive ryanodine receptors also present on the membranes of intracellular Ca²⁺ stores (Berridge, 1993; Simpson, Challiss and Nahorski, 1995; Mikoshiba, 1997).

Inositol triphosphate (IP₃) acts as a second messenger in the insect CNS (for example David and Pitman, 1993, Wegener, Hanke and Breer, 1997), and has been shown to be stimulated by muscarinic ligands (David and Pitman, 1994; Timmer and Qazi, 1996). One such muscarinic-IP₃-Ca²⁺ pathway has been demonstrated to modulate the function of D_f motoneurone ionotropic receptors and ion channels, including the nicotinic AChR (David and Pitman, 1995, 1996b, and 1996c). This importantly showed that the nicotinic receptor population of D_f is subject to intracellular modulation initiated by putative G-protein-linked receptors.

Apart from release from intracellular stores, calcium levels of neurones are also dynamically regulated by a variety of calcium channels and transporters (see Ghosh and Greenberg, 1995). A simplified diagrammatic representation of the D_f motoneurone Ca²⁺ fluxes is shown in figure 7.

Figure 7. Calcium pathways in the D_f motoneurone.

The left-hand side of the diagram represents the intracellular pathways activated by metabotropic receptors that are responsible for an increase in intracellular Ca^{2+} concentrations, the asterisks denoting the activated form of PKC. The right-hand side illustrated ionotropic and voltage-dependent events. Calcium concentrations additionally controlled by sequestering proteins such as CaM and antiport ion-exchange proteins.



3.2.2 Adenylyl cyclase and cAMP second messenger pathways.

Sutherland's studies in the late 1950s on the breakdown of glycogen by the liver in response to adrenaline and glucagon first demonstrated the involvement of cyclic adenosine 3',5'-monophosphate (cAMP) and the signal transduction pathway through which it operates, so establishing the concept of second messengers. Cyclic AMP concentrations are up-regulated by the enzyme adenylyl cyclase, that catalyses formation of the cyclic nucleotide from ATP. This messenger, once formed can be degraded by phosphodiesterase which hydrolyses the cAMP phosphodiester bond. Adenylyl cyclases are predominantly integral membrane glycoproteins which are stimulated by the α -subunits of G_s proteins and by forskolin, but inhibited by G_{ai} protein (see reviews by Iyengar, 1993; Pieroni, Jacobowitz, Chen, and Iyengar, 1993). More recently soluble adenylyl cyclases have also been described that are insensitive to G-protein regulation (Kawabe *et al.*, 1996; Buck, Sinclair, Cann and Levin, 1999) and which could have similar roles in signal transduction to the well-characterised soluble guanylyl cyclases (Hardman and Sutherland, 1969; Hurley, 1998).

Of the membrane-localised adenylyl cyclases, nine mammalian isoforms have been characterised (see table 8), which are variably regulated by G-protein α and $\beta\gamma$ subunits, Ca²⁺, CaM and by the CaMK and PKC kinases (reviewed by Iyengar, 1993; Pieroni, Jacobowitz, Chen, and Iyengar, 1993; Cooper, Mons and Karpen, 1995). A number of adenylyl cyclases have been described in invertebrates (see section 3.1.4), including the dopamine-sensitive enzyme in the corpora allata of the larval tobacco hornworm (Granger *et al.*, 1995) and the similar serotonin-responsive enzyme of *Aplysia* (see Kandel, Abrams, Bernier *et al.*, 1983; also

Table 8. Mammalian adenylyl cyclase and their regulation by G-proteins and Ca^{2+} .

Adenylyl Cyclase	Activation by forskolin	Intracellular signals effecting AC function		
		$G_{\alpha s}$ protein	G-protein By dimer	Ca^{2+}
AC1	Yes	Mild activation	Inhibits	Stimulates**
AC3	Yes	Activates	?	Stimulates**
AC8	Yes	Mild activation*	?	Stimulates**
AC2	Yes	Activates	Stimulates	No effect
AC4	Yes	Activates	Stimulates	No effect
AC7	Yes	Activates	?	No effect
AC5	Yes	Activates	No effect	Inhibits
AC6	Yes	Activates	No effect	Inhibits
AC9	No	?		
sAC	No	Insensitive	Insensitive	?

SAC, soluble/cytosolic adenylyl cyclase as opposed to AC1-9 which are membrane localised isoforms. Exogenous Ca^{2+} and $G_{\alpha s}$ act synergistically to activate AC8.

** Stimulation is mediated by CaM, in the cases of AC1 and AC8 Ca^{2+} -CaM activation is 4 times more effective than that by $G_{\alpha s}$. † AC5 is potently activated *in vivo* by phorbol-insensitive, Ca^{2+} -independent PKC ζ .

The majority of the data for this table is taken from the review by Cooper, Mons, and Karpen (1995). See also reviews by Iyengar (1993); Pieroni, Jacobowitz, Chen and Iyengar (1993); Hurley (1998). Additional sources of information: Buck, Sinclair, Schapal, Cann and Levin (1999)

reviewed by Bodnaryk, 1982). Although a number of these enzymes have been shown to be regulated by Ca^{2+} and CaM, their regulation has not been investigated in any depth. An important system for study of invertebrate aminergic adenylyl cyclase and cAMP systems has proved to be *Drosophila*, since it enables genetic approaches to be brought to bear (for review see Dubnau and Tully, 1998). Expression of a number of key genes for olfactory learning and memory in the fruit fly has revealed that they are enzymes of the cAMP cascade. These include an isoform of PDE (*dunce* mutation) and adenylyl cyclase (*rutabaga*), suggesting that such proteins are important for synaptic plasticity in the mushroom body of this insect. A recent study utilising the *dunce* PDE mutation has further demonstrated an involvement of cAMP in the regulation of presynaptic processes of cholinergic transmission in *Drosophila* (Lee and O'Dowd 2000). The *dunce* mutant exhibited increased frequency of quantal release and hence cholinergic EPSPs, that was mimicked in the wild type by application of cyclic nucleotide analogues.

3.2.2 Guanylyl cyclase – a role in aminergic transmission?

Catalysis of the conversion of GTP to guanosine 3', 5'-monophosphate (cGMP) by guanylyl cyclase (GC) was initially reported by Hardman and Sutherland in 1969. However it was apparent that the guanylyl cyclase system was not regulated in the same direct way as that proposed for adenylyl cyclase (Hardman and Sutherland, 1969; Goldberg and Haddox, 1977). Indeed, unlike the predominantly membrane-associated adenylyl cyclases, different guanylyl cyclase enzymes are localised to other cellular compartments including the cytoskeleton and cytoplasm (see review by Schulz *et al.*, 1989). The membrane bound forms of the enzyme appear to be monomeric, peptide-activated cell-surface receptors (Singh *et al.*, 1988; Schulz *et al.*, 1989) of which a number of isoforms have been characterised in rat atrial

myocardial extracts (Waldman *et al.*, 1984) and spermatozoan membranes (Singh *et al.*, 1988). Soluble cytoplasmic GCs, on the other hand, are heterodimers activated by free radicals and nitrovasodilators.

Cyclic GMP, like cAMP, stimulates the activity of a wide range of downstream signalling proteins. The diversity of the transduction machinery for cGMP signals is elegantly demonstrated in cGMP-dependent visual transduction systems which involve cGMP-dependent protein kinase (PKG), cGMP-gated ion channels, and cGMP-stimulated phosphodiesterases (for reviews see Stryer, 1986; Maelick, 1990; Yarfitz and Hurley, 1994).

The cyclic GMP pathway is involved in synaptic plasticity and important for the mechanisms underlying long term depression (LTD) (Boulton *et al.*, 1994; Reyes-Harde *et al.*, 1999; Santschi *et al.*, 1999). One of the key roles for cGMP in nervous systems, apart from visual transduction, appears to be to mediate retrograde alterations in neurotransmitter release produced by nitric oxide (NO). NO-dependent elevations in presynaptic cGMP enhance transmitter release between cultured hippocampal neurones (Arancio *et al.*, 1995) and cholinergic transmission at excitatory synapses in *Aplysia* (Mothet *et al.*, 1996a and 1996b). More recently Lewin and Walters (1999) have shown that a NO-cGMP-PKG pathway is required for long term hyperexcitability in *Aplysia* nociceptive sensory neurones. The enzymes for cGMP signalling are present in insect sensory and motor systems (Bicker and Schmachtenberg, 1997) and have been shown to modulate synaptic transmission. For example Wildemann and Bicker (1999) have demonstrated that vesicle release at *Drosophila* larval neuromuscular junctions is sensitive to NO and cGMP.

There is limited evidence that monoamines also interact with cGMP-dependent signalling pathways, as well as activating adenylyl cyclase or

phospholipase C pathways. Serotonin has been demonstrated to stimulate cGMP synthesis in a number of systems. Tohda *et al.* (1990, 1991) have extensively characterised serotonin stimulated cGMP production in cell cultures. Two serotonin - cGMP pathways have been demonstrated to co-exist in these cells. Firstly, a fast, Ca^{2+} -dependent 5-HT₃ receptor pathway and secondly a slower Ca^{2+} -independent pathway. The latter 'slow' pathway is transduced via a membrane associated GC (Tohda and Nomura, 1990). Both pathways involve the production of NO and activation of NO-sensitive GCs (Arima *et al.*, 1996). A putative Ca^{2+} -dependent pathway involved in the up-regulation of cGMP levels by serotonin has also been described in molluscan neurones by Paupardin-Tritsch *et al.* (1986). Similarly, in the dissected pig choroid plexus, activation of serotonin 5-HT_{2C} receptors results in cGMP formation via a Ca^{2+} and phospholipase A₂-dependent pathway (Kaufman *et al.*, 1995; Kaufman and Hirata, 1996). Conversely serotonin 5-HT_{1A} receptors have been implicated in inhibition of cGMP production in rat cerebellum via inhibition of a rise in the postsynaptic intracellular Ca^{2+} concentration mediated by NMDA/AMPA receptors.(Maura and Raiteri, 1996). It must be noted that, with the exception of slow Ca^{2+} -independent pathway described by Tohda and Nomura (1990), the majority of serotonin-cGMP pathways are dependent on intracellular Ca^{2+} .

3.2 Do monoamines modulate cholinergic transmission via intracellular phosphorylation-dependent pathways?

Cholinergic transmission via nicotinic acetylcholine receptors is important in the central nervous systems of insects, for neuromuscular transmission in vertebrates and emerging as being of increasing importance in central processes in higher organisms. Therefore any alteration in the efficacy of signalling at cholinergic synapses is likely

to have fundamental behavioural consequences. The monoamine neurotransmitters are ubiquitous in neuronal systems and have been demonstrated to regulate a diverse array of cell functions through G-protein-dependent intracellular pathways. It is evident from the literature that amines stimulate the production of second messengers, and furthermore that these signals trigger the activity of kinases known to modulate nAChR function via phosphorylation-dependent processes. However, aminergic modulation of cholinergic transmission via activation of an identified intracellular phosphorylation pathway has not been established. The purpose of this study is to determine whether monoamines acting via metabotropic receptors influence transmission at postsynaptic ionotropic receptors, specifically those of the nicotinic acetylcholine receptor family. Furthermore it is important to establish whether modulation of nAChRs by amines is mediated by the same or a different pathway to that produced by activation of muscarinic ACh receptors.

EXPERIMENTAL AIMS.

The aim of this study was to investigate aminergic modulation of nicotinic acetylcholine responses recorded from the fast coxal depressor (D_f) motoneurone of the cockroach *Periplaneta americana*. Cholinergic and aminergic neurotransmitter systems are ubiquitous to nervous systems, and an understanding of their interactions is of primary importance for a full understanding of neuronal function both at the cellular and organism level. The research implicates a signal transduction pathway by which metabotropic amine receptors can influence the activity of nicotinic acetylcholine receptors, distinct from the previously reported direct blocking actions of amines on nicotinic receptors expressed in a variety of systems.

A combined electrophysiological and pharmacological approach was used to elucidate the modulatory mechanism. Having established an initial role for aminergic modulation, light and electron microscopy immunohistochemistry was used to probe for potential aminergic afferents onto the D_f motoneurone, to provide a physiological basis for the electrophysiology data, prior to commencing further electrophysiology. The following bullet points represent the principle elements of the research programme:

- Single electrode recording and two electrode voltage-clamp techniques were used to establish the modulatory actions of the monoamines serotonin, octopamine and dopamine on nAChR-mediated effects of the D_f motoneurone of *Periplaneta americana*.

- Electron microscopy in conjunction with immunocytochemistry was used to localise aminergic terminals in contact with, or in the proximity of the motoneurone under investigation.
- Pharmacological agents which act on intracellular signalling pathways were applied externally to the preparation or injected intracellularly in order to determine the mechanism by which the amines modulate nAChR function. The experiments conducted focused on Ca^{2+} -dependent and cyclic nucleotide-dependent pathways that have proved to underlie modulatory processes in other preparations, most notably in regulating the presynaptic release of neurotransmitter in *Aplysia* sensory neurones.

MATERIAL AND METHODS.

1.1 Preparation.

Adult male cockroaches (*Periplaneta americana*) were used for all experiments to eliminate any variation in responsiveness to the monoamines between sexes. The cockroaches were kept in mixed sex colonies maintained at a temperature of 25 °C and fed regularly on an artificial diet of moist dog biscuits, with water *ad libitam*.

Cockroaches were dissected and prepared for electrophysiological recording as detailed below, at all times the preparation was kept moist with saline of the following composition (mmol/l): 214.0 NaCl; 3.1 KCl; 9.0 CaCl₂; and 10.0 TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) (pH 7.2, adjusted with 1.0 mol/l NaOH). On capture the animal was decapitated and all its legs removed. It was then pinned ventral side up onto a wax block and placed under a binocular dissecting microscope (magnification ×10 to ×30). The exoskeleton was removed from the ventral surface of the thoracic and abdominal segments to expose the section of ventral nerve cord from the mesothoracic (T2) to third abdominal ganglion (A3). Peripheral nerves were cut away to allow easy removal of the required section of nerve cord, which, once isolated, was attached ventral side up to a small plastic slide. The nerve sheath of the ventral surface of the metathoracic (T3) ganglion was stained using 5% methylene blue and removed using fine forceps. Excess methylene blue was washed off with saline and the preparation was immediately transferred to a Perspex bath of circulating saline bubbled with 100% oxygen. The time taken from initial capture of the cockroach to placement of the isolated nerve cord in the saline bath typically took no longer than 10 minutes. The preparation was then left for a minimum of half an hour prior to neurone impalement.

1.2 Bath set-up and visualisation of the D_f motoneurone.

Figure 8a shows a three dimensional diagrammatic representation of the experimental chamber used in all experiments. It consisted of a Perspex block from which was milled a central inclined bath (1) into which the preparation slide is placed. Oxygen bubbled into the side chamber (2; blue arrow) circulating the saline in the direction of the black arrows as shown in figure 8b. Pharmacological agents were injected into this side compartment (red arrow) to mix with the oxygenated saline before entering the chamber containing the preparation. Alternatively fresh saline could be perfused through the chamber to wash out drugs applied to the preparation. Under such conditions saline feeds under gravity from a reservoir into a second side chamber on the left-hand side (3), and the level in the bath is maintained by aspiration of saline through a side tube into a wash bottle. At times the rate of saline flow through the bath was such that the oxygenation of the preparation saline was disrupted, and a hypoxic response was observed. To prevent such an undesirable effect the rate at which the oxygen was bubbled into the bath was increased, counteracting the drag of saline against the flow of oxygenated saline (saline flow shown in figure 8c).

The left-hand side chamber was additionally connected via an agar bridge to a well at the rear of the Perspex block (5). This was filled with normal saline and into which the earth wire was connected. The physical but not electrical isolation of the earth wire probe prevented the recording of artefacts due to fluctuations in saline level or changes in the ionic composition of the preparation bath.

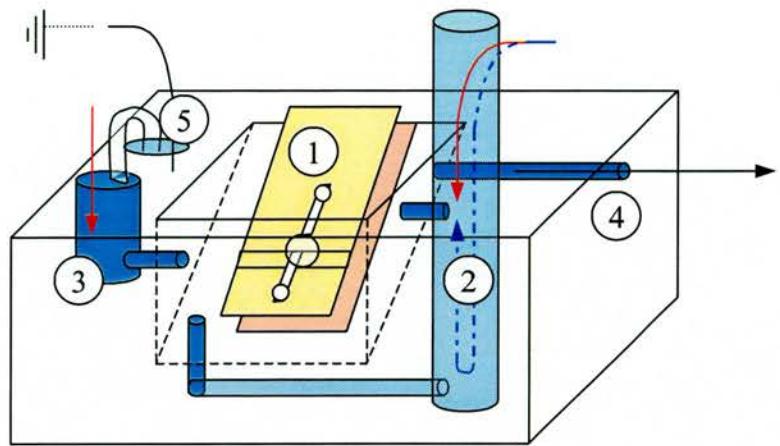
The D_f motoneurone was visualised using a binocular microscope (magnification $\times 18$ to $\times 80$) mounted in front of the bath. It was readily identified by

Figure 8. Bath set-up and protocols for saline flow.

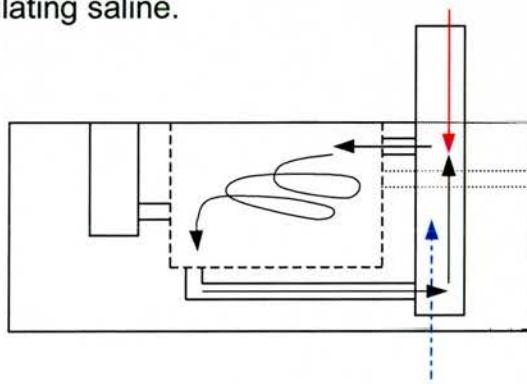
(a) Shows a diagrammatic representation of the Perspex bath in a 3-dimensional view. The isolated section of *Periplaneta americana* ventral nerve cord was placed on the slide in a central inclined bath (indicated by the number 1), which along with the connecting pipes had previously been filled with normal saline. The saline was circulated in an anti-clockwise direction through the central bath and the tubes by bubbling oxygen into an adjoining chamber (dashed blue arrow, 2). This set-up and flow of saline through the bath was referred to as the ‘circulating saline’ protocol. The flow of saline through the chambers is more clearly demonstrated in (b) (black arrows). Pharmacological agents were pipetted into the adjoining chamber (2) as indicated by the red arrows. There, they mixed with the oxygenated saline and were rapidly circulated throughout the bath set-up. The ‘circulating saline’ protocol allowed accurate analysis of the effects of varying concentrations of chemical agents, however in order to remove the drug from the preparation the bath had to be perfused with fresh saline. This sudden change in flow often created artefacts in the traces recorded.

Under continual perfusion (c), fresh saline could be introduced from a reservoir that fed into a third, connected chamber (3) on the left hand side of the Perspex block. The level of saline in the bath was maintained by aspiration of the saline through a duct on the right-hand side of the main chamber (4). The locality of the saline outlet (4) to the pipe (2) whereby oxygenated saline, containing the drugs entered the main chamber was not ideal as often, the suction was sufficient to remove the saline before it had time to come into contact with the preparation. To counteract such an eventuality, the flow of oxygen into the adjoining chamber was increased as was the concentration of drug applied.

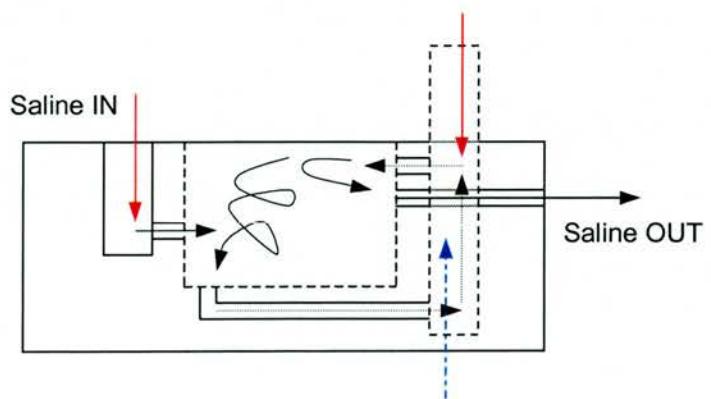
(a) Bath set-up.



(b) Circulating saline.



(c) Perfusion with fresh saline.



its size (80 – 90 µm in diameter) and position on the ventral surface of the metathoracic ganglion relative to tracheal landmarks.

2.1 General protocol for electrophysiology.

The cell body of the D_f motoneurone was impaled with borosilicate glass thin-walled, fibre-filled microelectrodes (Cat. no. GC150TF-10, Clark Electromedical, Pangbourne) filled with 2 M potassium acetate. Voltage electrodes had a resistance of 15 – 20 MΩ and current electrodes 10 – 15 MΩ. Transmembrane current was measured using a virtual earth current monitoring device, the earth wire of which was placed in the isolated well as mentioned. The data were displayed on a Tektronix 5000 oscilloscope and recorded on VHS video tape using a JVC recorder and Sony digital audio processor. An instant hard copy was provided by a Gould 220 pen recorder.

To elicit acetylcholine responses from the D_f motoneurone, a Picospritzer II (General Valve Corporation) was used to deliver pressure pulses of acetylcholine chloride (10^{-1} M; dissolved in saline) every one or two minutes from a microelectrode positioned locally to the motoneurone soma. The duration of the pressure pulses (1 Bar, ranging between 20 and 500 ms in different experiments) was adjusted to evoke a membrane depolarisation of approximately 10 – 15 mV under current clamp or an inward current of 2 – 4 nA under voltage clamp. Only cells whose resting potentials were in the range of –70 and –90 mV were used. For measurements under voltage clamp the holding potential was –80 mV.

In a number of experiments the cell body of the D_f was surgically isolated from its axon and synaptic inputs. A sharpened pair of fine iridectomy scissors were used to cut around and under the soma, although it was not physically removed from

the ganglion. Previously this 'undercutting' method has been shown to be effective in isolating the soma of D_f (Hancox and Pitman, 1992).

Pharmacological agents dissolved in saline were applied directly to the side chamber as described, in 20 µl aliquots. In a number of preparations, control application of 200 µl aliquots of saline were found to disrupt ACh responses - indiscriminately increasing or reducing the control ACh responses. This effect was apparently only due to the quantity of drug applied to the preparation and therefore, where possible, application of agents was conducted in 20 µl aliquots. Chemicals dissolved in dimethyl sulfoxide (DMSO) were diluted in saline prior to application such that the final bath concentration of DMSO was less than or equal to 0.05%. Concentrations greater than 0.05% were found to alter the control ACh responses. At none of the DMSO concentrations tested did the agent reduce the responsiveness of the neurone to the modulatory action of the amines. Chemicals dissolved in 95% ethanol were diluted such that the final bath concentration of the ethanol was less than 0.01%. Figure 9 shows traces from two control experiments in which ethanol diluted in normal Ringer solution was applied to the D_f motoneurone. High concentrations of ethanol depolarised the membrane potential and increased spontaneous synaptic activity, as well as attenuated the control acetylcholine response. All the effects of ethanol were reversed after 5 –10 minutes washout with normal saline. All solutions added to the bath were adjusted to pH 7.2 using 10 mmol/l NaOH or HCl.

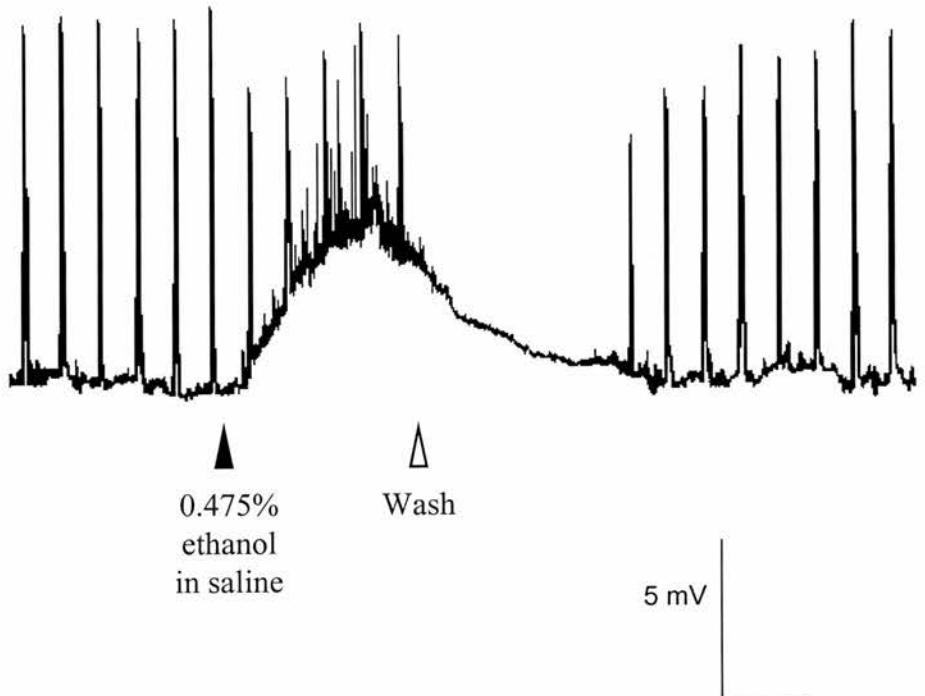
Pharmacological agents were applied after a minimum of 6 constant control acetylcholine responses and half a minute prior to the subsequent ACh application. The effects of the amines were tested under the two protocols illustrated in figure 8. In the first, aminergic modulation was investigated during continual perfusion of the

Figure 9. Effect of ethanol on the ACh response of the D_f motoneurone.

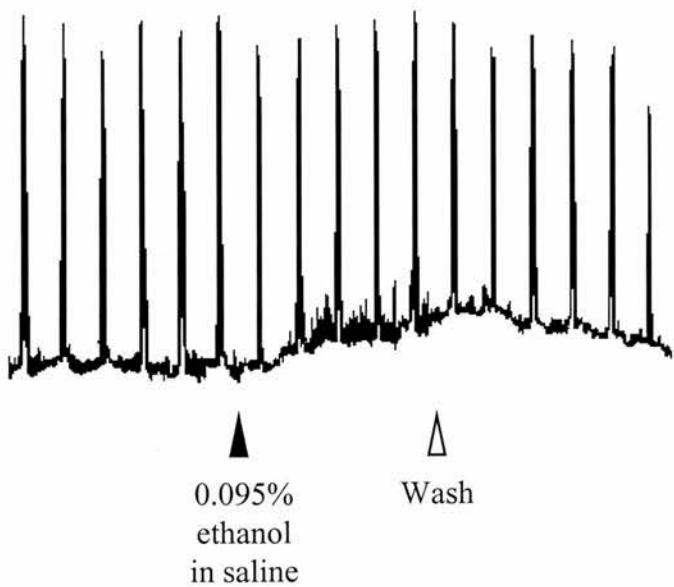
(a) Demonstrates the effect of adding a 20 µl aliquot of 47.5% ethanol to the preparation (final bath concentration 0.475% ethanol), recorded using single electrode current clamp. The amplitude of the ACh responses (seen as the large vertical deflections from the baseline) declined after addition of the ethanol (resting potential of the cell was approximately -73 mV). Also evident was a prolonged depolarisation of the membrane potential and an increase in the spontaneous synaptic activity – the deflections observed between the ACh responses.

The action of ethanol was dose-dependent. Addition of 0.095% ethanol to the bath resulted in a reduced effect of the agent on the motoneurone, an example of which is shown in (b). Although there was a depolarisation of the membrane potential on addition of ethanol, it was not as pronounced as that observed at the higher concentrations. Both the reduction in the amplitude of the ACh responses and increase in synaptic activity were similarly less marked. When pharmacological agents were dissolved in ethanol they were diluted with normal saline such that the final bath concentration of ethanol was less than 0.01%.

(a)



(b)



saline through the bath at a rate of 5 to 9 ml min⁻¹ (figure 8c). However under this protocol the responses exhibit by the neurone were often extremely variable, and only elicited by concentrations of the drug towards the upper limit of physiological concentrations. This was due to the process of perfusion of saline resulting in inconsistencies in the amount of drug reaching the cell. The design of the bath such that the drug entered the main chamber adjacent to the point where the saline was removed, meant that some of the agent did not even reach the preparation. The rate of washout of drugs applied under continual perfusion was estimated for each preparation by application of aliquots containing fast green dye. The time taken for the complete turnover of fast green was taken as an approximate time during which the preparation was exposed to the amine. The benefits of this protocol were that the cell was only briefly exposed to the amine. The continual flow of saline and drugs through the bath also provided information as to the time-course of modulation. This allowed the processes underlying reversal of aminergic modulation to be investigated.

Under the second protocol, the effect of the amines was measured under conditions in which the experimental chamber was not continuously perfused with fresh saline (figure 8b). Instead, the pharmacological agents were carried to the preparation from a side-well of the experimental chamber by the flow of circulating saline. Typically the amines were washed from the preparation after 10 minutes exposure time. Reversibility of the actions of pharmacological agents varied considerably; if the effect of the drug persisted after half an hour of washing with fresh Ringer solution it was deemed irreversible. The second method had the advantage that the concentration of drug reaching the preparation could be controlled precisely. However, the disadvantage with this protocol is that the flow of fresh

saline that occurs during drug wash-out, can itself alter the amplitude of ACh responses.

A number of cell membrane-impermeable drugs were injected into the soma of the D_f motoneurone. The GDP analogue GDP-β-S was injected intracellularly using 3 – 5 pressure pulses (0.2 – 1.0 bar, 500 ms). The micro-pipette (resistance of 8 – 12 MΩ) contained 10⁻² M GDP-β-S dissolved in 10⁻¹ M KCl. The Ca²⁺-chelator BAPTA was injected by ionophoresis using 200 ms pulses sufficient to hyperpolarise the neuronal membrane potential by 40 mV (2-10 nA) delivered at 0.1 Hz for 20 minutes. Microelectrodes were filled with solution containing 10⁻¹ M BAPTA dissolved in 10⁻¹ M KCl.

2.2 Protocol for testing of pharmacological agents.

In order to elucidate the signal transduction mechanism underlying aminergic modulation of nicotinic acetylcholine responses, a range of pharmacological agents were utilised, the majority of which have previously been shown to interfere with specific aspects of intracellular signalling pathways. However, it was essential that the observed effects of the drugs could be attributed to direct effects on the pathway under investigation, and not to non-specific phenomena or rundown of the cells normal physiology. Therefore great care was taken to ensure that only results that met a set of criteria were used to compile the data upon which the final conclusions were based: results were discarded if the resting potential of the cell did not remain within a range of –70 to –90 mV, and if the cell did not exhibit robust ACh responses throughout the course of the entire experiment. The protocol for the testing of pharmacological agents is discussed in more detail below, but, if preparations were not stable for the entire course of the protocol then they were also

discarded, since accurate comparison between control and test results was not possible. Figure 10 diagrammatically represents a hypothetical experiment to test the effects of a drug of aminergic modulation. The ACh responses are represented as vertical deflections from a stable resting potential. Prior to incubation with the test agents, control applications of the amine were conducted to establish that the D_f motoneurone exhibited a ‘typical’ response in the absence of the drug (figure 10a). Furthermore to ensure that the effect of incubation with the test drug (figure 10b) could not be attributed to a non-specific decline in aminergic modulation, post-washout amine controls were also conducted (figure 10c). The significance of the drug’s action could then be directly compared with responses recorded before application of the test agent and after it has been removed to provide conclusive evidence as to the role of the agent in modulation, and whether or not the observed effect was reversible.

2.3 Sources of chemicals.

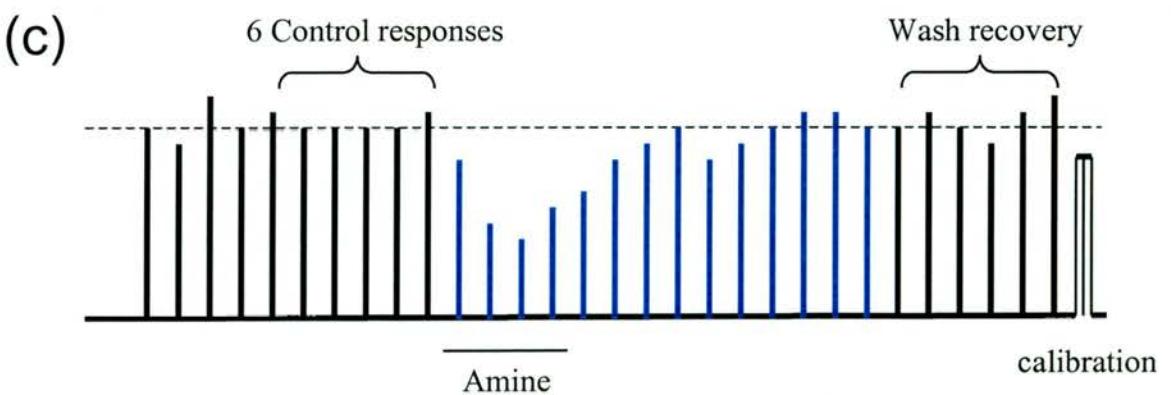
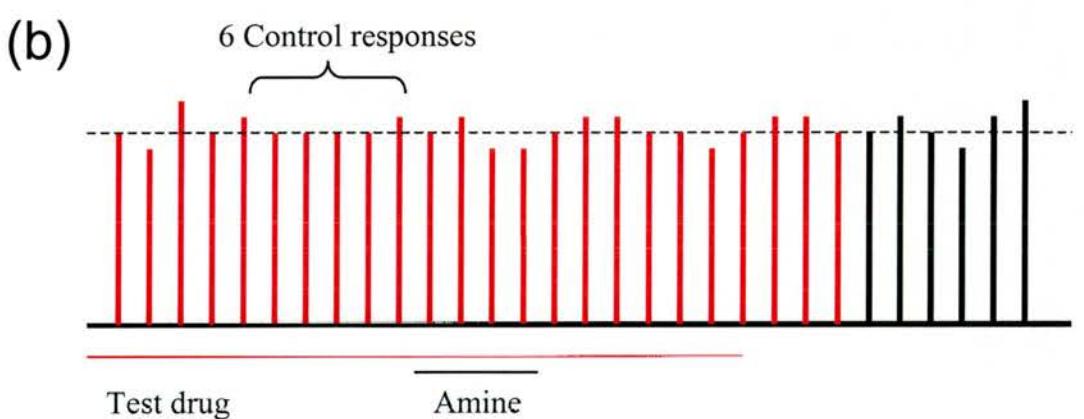
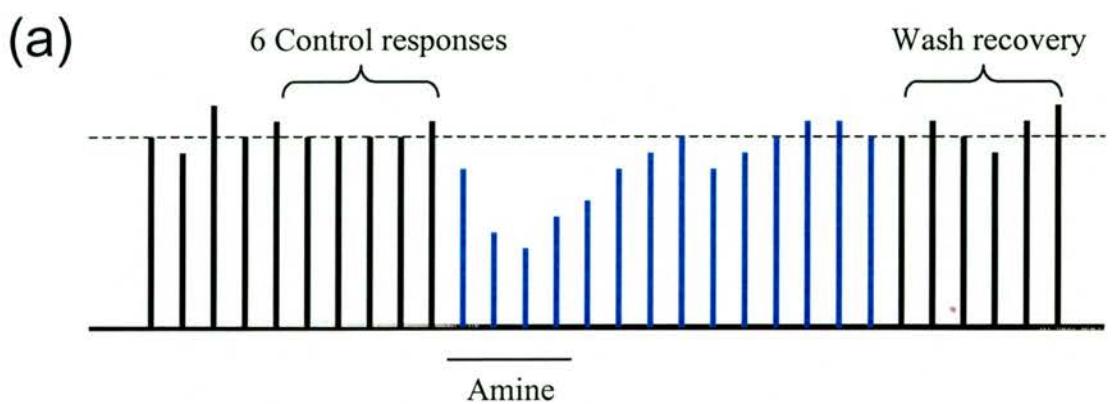
Chemical agents for electrophysiology were obtained from the following sources: RS23597/190 HCl - Tocris (UK). Staurosporine – Calbiochem-Novabiochem (USA). Rp-8-Br-cAMPS and Rp-8-Br-cGMPS – Biolog Life Science Institute (Germany). Cantharidin – Alexis corporation (USA). SNAP was kindly donated by Professor Keith Sillar (University of St. Andrews). All other reagents were obtained from Sigma Chemical Co. Ltd (UK).

2.4 Analysis of electrophysiology results.

Acetylcholine responses were played back using the JVC video and measured on a Gould Digital Oscilloscope (DSO)160A. The amplitudes were calibrated using

Figure 10. Experimental protocol for a hypothetical electrophysiology experiment. In order to test the effectiveness of pharmacological agents to regulate aminergic suppression of the ACh response, the experiment was divided into three stages (a, b and c). The ACh responses are represented as the vertical deflections in all three panels. Applications of the amine in the absence of the pharmacological agent being tested are indicated in blue. These ‘control’ runs were conducted prior to (a), and after washing out (c) of the test agent. All chemical agents, including the amines, were added to the bath after a minimum of 6 constant ACh responses were recorded.

Preparations were incubated with the test drug (red deflections) for a minimum of 10 minutes prior to test application of the amine (b). The preparation was then washed for a prolonged period prior to the final ‘control’ application of the amine (c). Once the experiment had been successfully completed the trace was calibrated using a depolarisation of either 10 mV, duration 5s for voltage traces or 2 nA, duration 5s for current traces.



depolarisations of either amplitude 10 mV, duration 5 s under current clamp or amplitude 2 nA, duration 5 s under voltage clamp performed at the end of each experiment. A minimum of 6 control ACh responses were averaged and the standard deviation determined. Preparations in which the standard deviation of the control ACh response was greater than an arbitrary value of 10% of the mean were discarded prior to commencing any experiments. All subsequent responses were expressed as percentages of the averaged control response. These percentage data were arc-sine transformed before calculation of the standard error of the mean. The arc-sine transformation was not applied to values over 100%; in instances where values exceeded 100% non-transformed mean and standard errors were quoted. Significance was assessed by application of a two-tailed t-test (Minitab, Version 11).

3 Immunocytochemistry and electronmicroscopy.

The general protocol for fixing and embedding the nervous tissue for electronmicroscopy and immunocytochemistry was kindly provided by Dr Beulah Leitch (University of Durham) who also generously donated the antibodies used in the study. The method of tissue fixation for use with the serotonin paraformaldehyde-conjugated antibody was adapted from the paper by Sun, Tolbert and Hildebrand (1993). The protocols are detailed in the flow charts shown on pages 98 - 101 (figure 11), and are described below. The end aim of the study was to identify aminergic afferents onto the D_f motoneurone at the ultrastructural level. Motoneurones were labelled by intracellular injection of horseradish peroxidase, the resultant product of the diaminobenzidine (DAB)-peroxidase reaction creating identifiable electron dense profiles. Aminergic terminals were labelled with specific amine primary antibodies and gold secondary antibodies.

3.1 Horseradish peroxidase (HRP) labelling of the D_f motoneurone.

Cockroaches were dissected as described in the general protocol (Material and Methods section 1.1) and impaled with a single microelectrode whose tip was filled with 4 – 8 % HRP (HRP Sigma VI) in 0.2 M Tris buffer (pH 7.4) and the shank filled with normal electrode solution. HRP was intracellularly injected by ionophoresis using 500 ms pulses of 0.5 - 2 nA depolarising current applied at 1 Hz for 30 – 40 minutes. After injection, the HRP was allowed to diffuse throughout the cell for a further half hour prior to transfer to the initial fixative.

After initial fixation in either glutaraldehyde or paraformaldehyde primary fixative (see below) the sections were repeatedly washed in Sorenson's phosphate and then Tris buffer before being immersed in 0.5% cobalt chloride in Tris buffer for

Figure 11. Flow diagrams of immunohistochemistry protocols.

Figure 11a: Protocol for immunofluorescent staining and light microscope analysis.

Figure 11b: Horseradish peroxidase labelling of the D_f motoneurone.

Figure 11c: Post-embedding staining with antibodies.

Figure 11d: Pre-embedding technique for applying antibodies.

Abbreviations:

1°, 2°...etc.: Primary, secondary

Para: Paraformaldehyde

BSA: Bovine serum albumen

RT: Room temperature

Glut: Glutaraldehyde

SPB: Sorenson's phosphate buffer

HRP: Horseradish peroxidase

TEM: Transmission electron microscope

NGS: Normal goat serum

Details of buffers and solutions:

- *Sorenson's phosphate buffer:*

0.1M = 36ml 0.2M Na₂HPO₄ + 14ml NaH₂PO₄ + 50ml deionised distilled water (DDW), pH 7.2 (+sucrose = 13.6g/100ml SPB).

- *Tris Buffer:*

0.2M = 2.42g Tris (mwt 121.1g) per 100 ml DDW; adjusted to pH 7.2 with HCl.

- *Incubation medium for DAB reaction:*

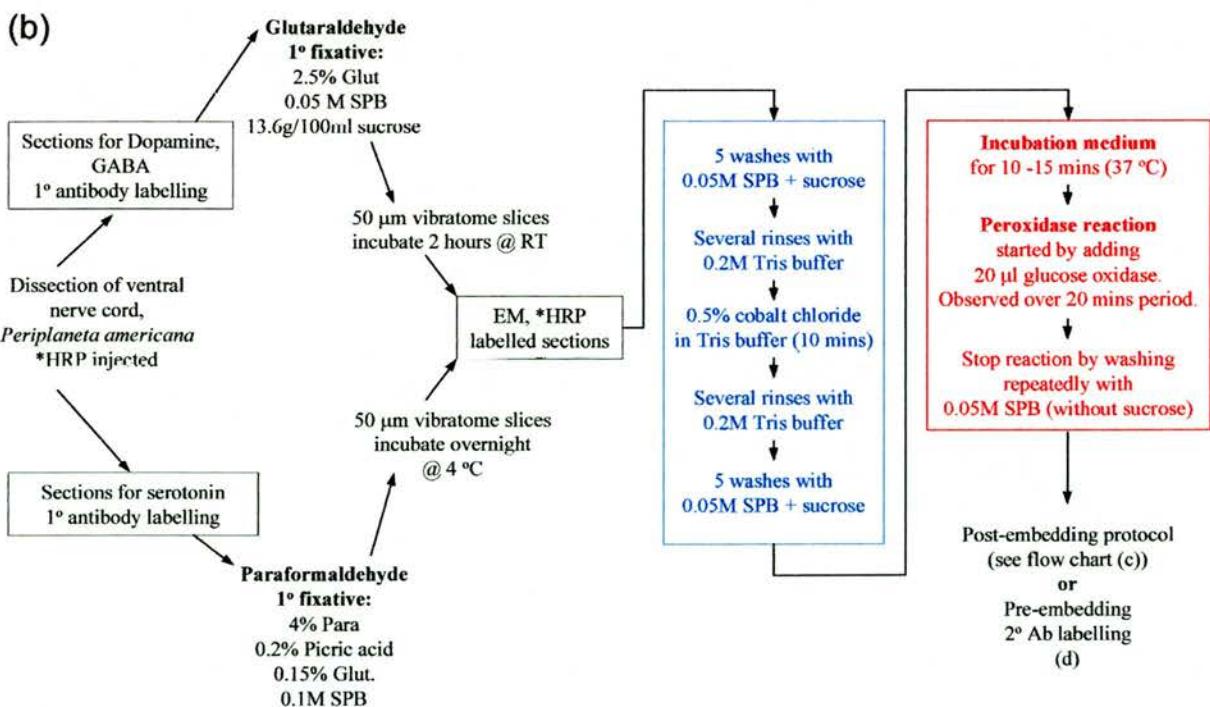
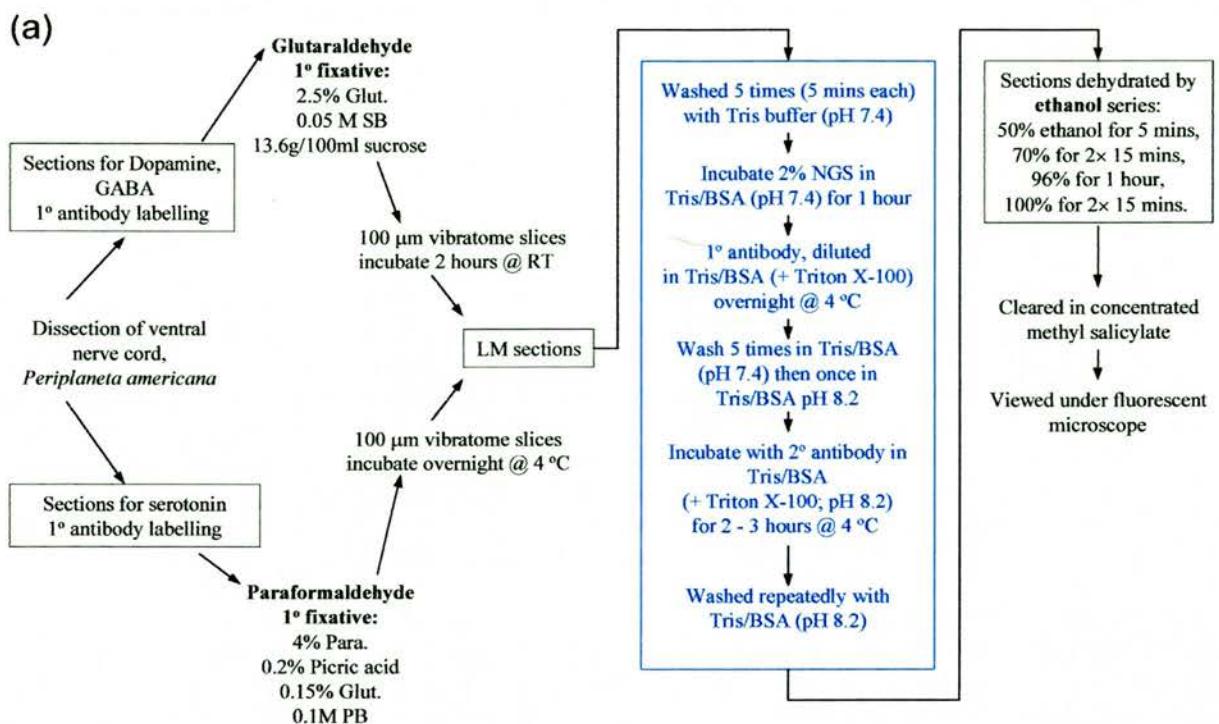
Add 20mg ammonium chloride and 100mg β-D glucose to 50ml 0.05M SPB without sucrose. Add 9ml of this solution to 1ml DAB.

- *Normal goat serum:*

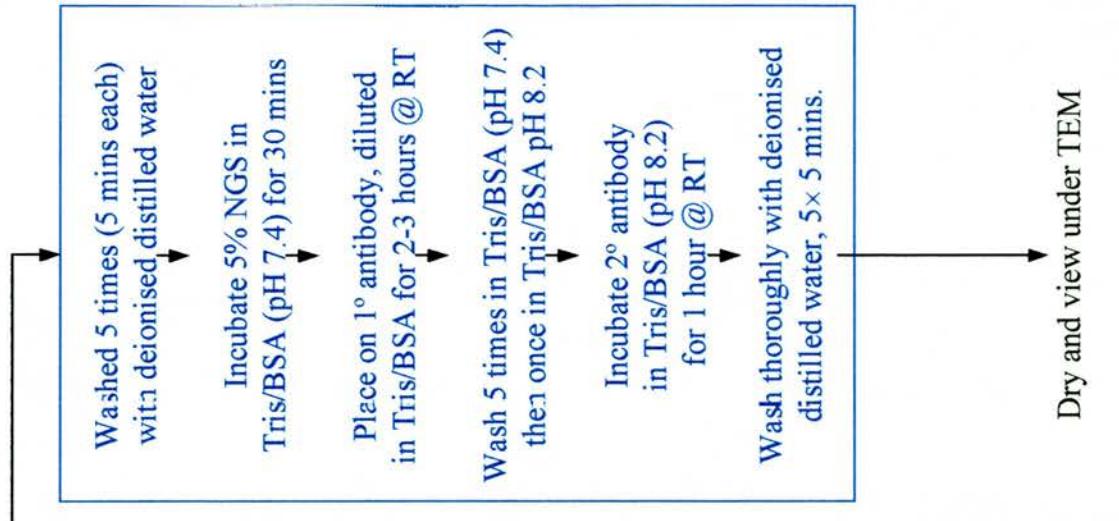
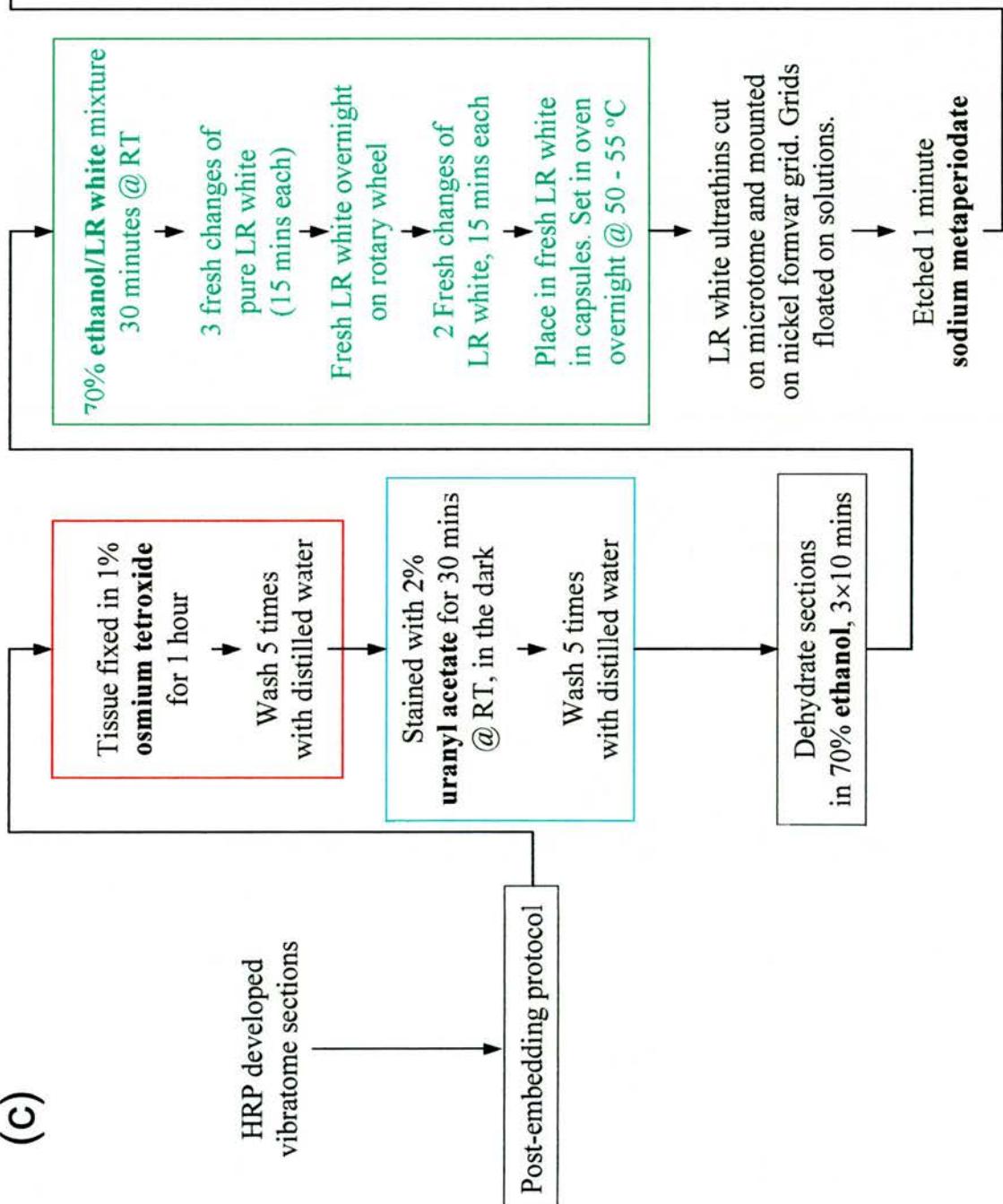
Heat inactivated at 56 °C for 30 mins in order to destroy complement. 0.5 ml serum added to 4.5 ml Tris buffer.

- *Tris/BSA buffer:*

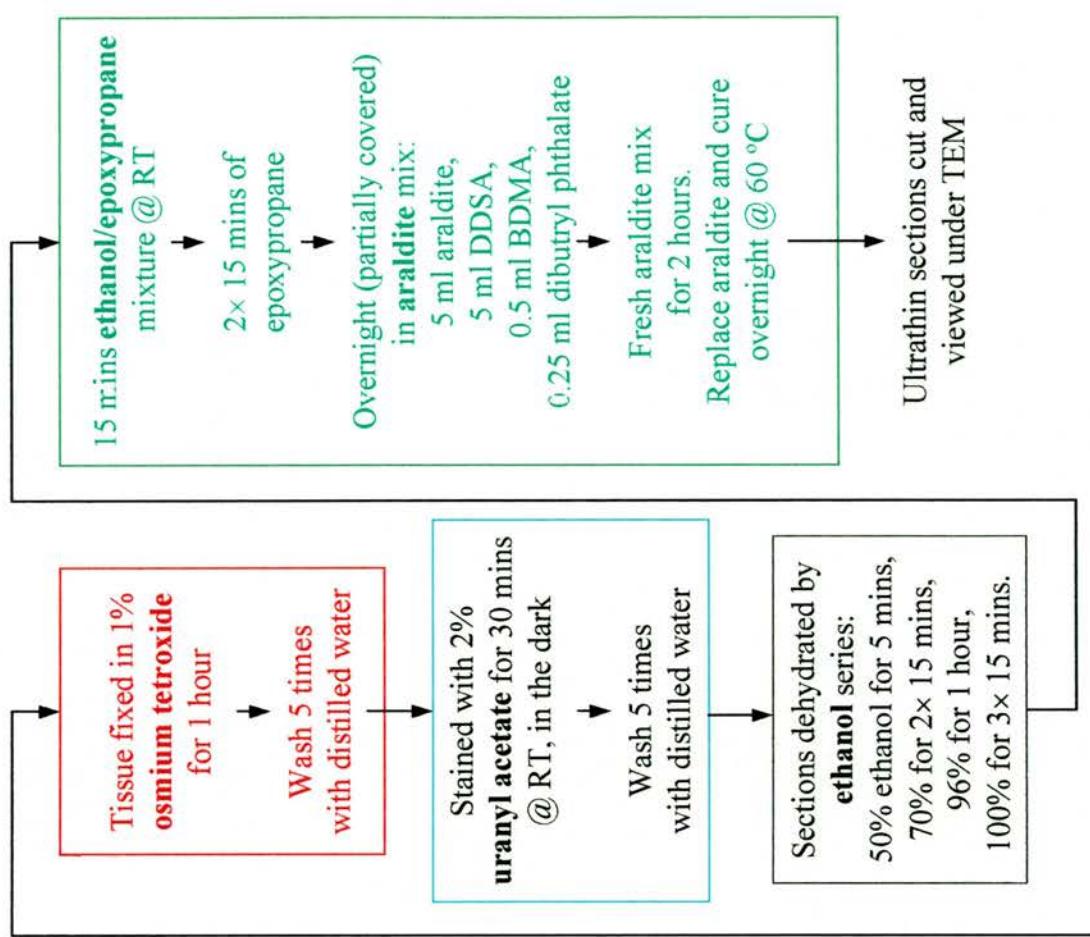
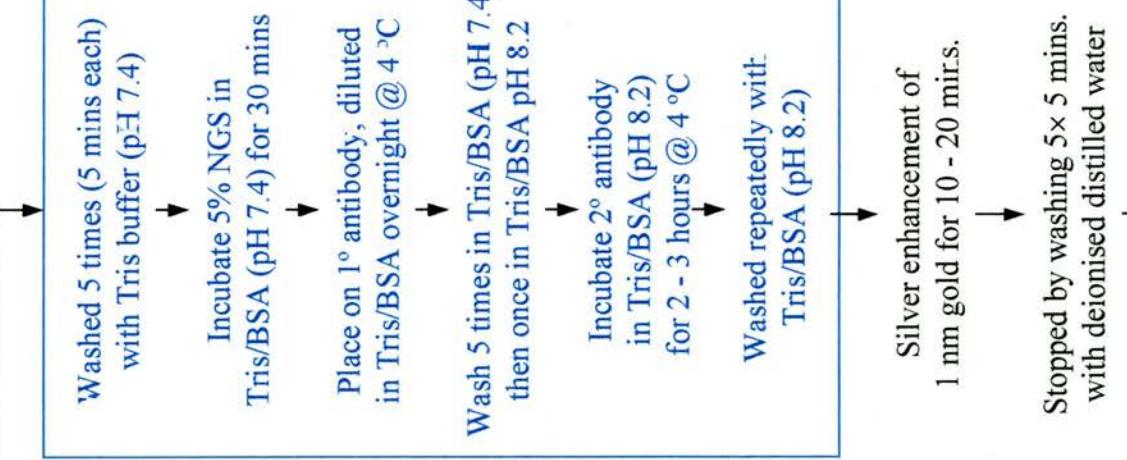
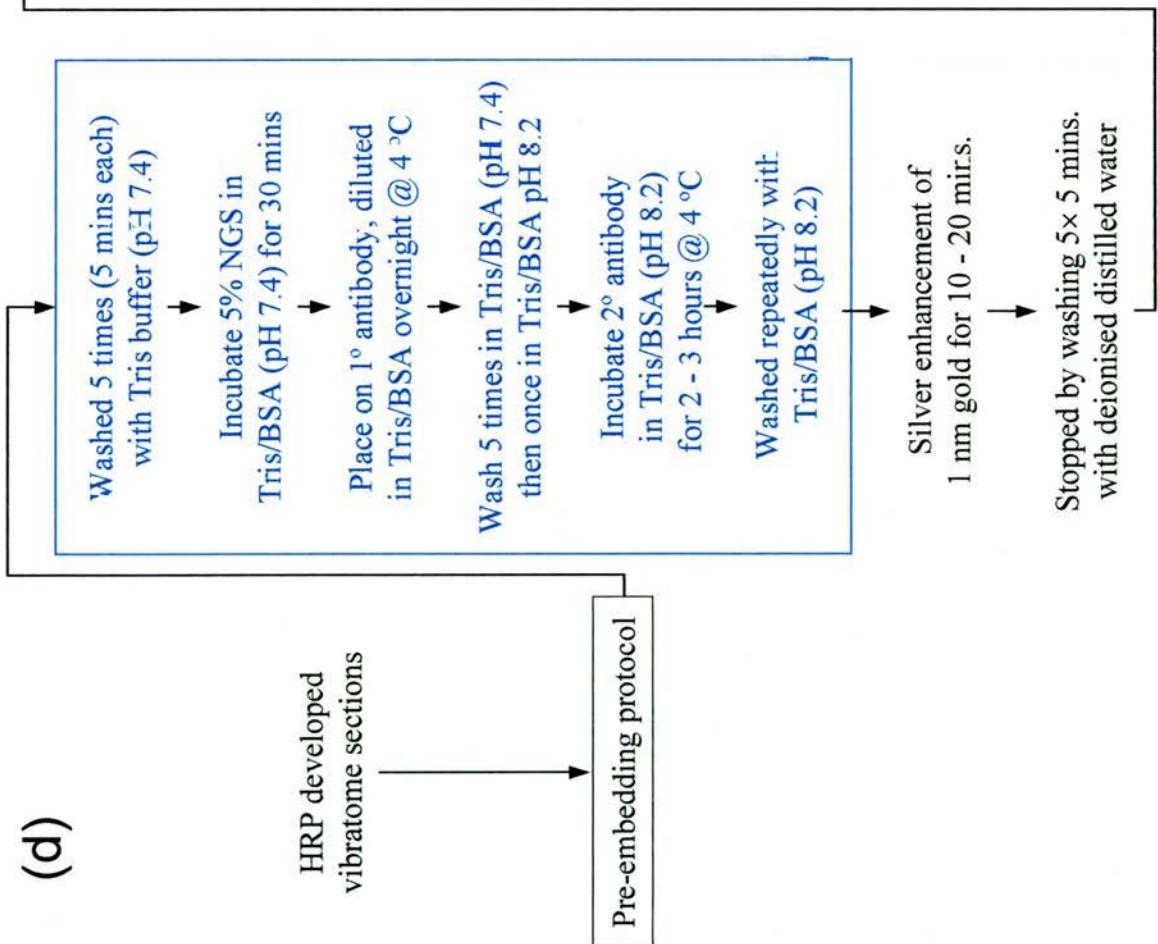
0.242g Tris, 0.9g NaCl and 0.1g bovine serum albumen in 100ml DDW. 50ml adjusted to pH 8.2, 50 ml adjusted to pH 7.4 with HCl.



(C)



(d)



10 minutes. The sections were then washed with Tris buffer followed by phosphate buffer prior to incubation (10 – 15 minutes, 37 °C) in a medium of the following composition: 9 ml of a 50 ml Sorenson's buffer without sucrose, containing 20 mg ammonium chloride, 100 mg D-β glucose added to 1 ml DAB. The peroxidase reaction was initiated using 20 µl glucose oxidase (Sigma V), and checked regularly for development of the black reaction product. Typically the process was terminated after 20 – 30 minutes with several washes of phosphate buffer without sucrose.

Tris buffer was composed of (g / 100 ml deionised distilled water): 0.242 g Tris, 0.9 NaCl, and 0.2 bovine serum albumen; the pH was adjusted to either 8.2 or 7.4 using HCl. 100 ml Sorenson's buffer contained 36 ml 0.2 Na₂HPO₄, 14 ml NaH₂PO₄ and 50 ml deionised distilled water.

3.2 Fixation of tissue for immunocytochemistry and electronmicroscopy.

Fixation of the tissue was varied according to the conjugation of the primary amine antibody. The polyclonal amine antibodies were obtained from Diasorin (MN 55082, USA) whereas the GABA antibody obtained from Sigma. All antibodies were generated in rabbits, the neurotransmitters coupled to bovine serum albumin (BSA). The serotonin antibody is conjugated to BSA with paraformaldehyde whereas the GABA and dopamine antibodies conjugated to BSA with glutaraldehyde.

The initial fixative for the glutaraldehyde-conjugated antibodies was 2.5 % glutaraldehyde in 0.05 M Sorenson's phosphate buffer with sucrose (13.6 g sucrose per 100 ml buffer). Whereas the sections for labelling with the serotonin antibody were fixed in a paraformaldehyde fixative of the following composition: 4 % paraformaldehyde, 0.2 % saturated picric acid, 0.15 % glutaraldehyde, 0.1 M

phosphate buffer. To ensure rapid fixation of the tissue the ganglia were sectioned after HRP injection using an Oxford G502 vibratome, following brief incubation in the initial fixative. 100 µm slices were cut for the fluorescent staining and viewing under the confocal microscope, whereas for electronmicroscopy 50 µm slices were cut to increase the potential antigen surface. After sectioning the tissue was returned to the fixative in the case of the paraformaldehyde-fixed sections overnight at 4 °C, and for the glutaraldehyde ones for a further two hours at room temperature.

3.3 Preparation of the tissue for immunocytochemistry.

Glutaraldehyde and paraformaldehyde rapidly fix the protein constituent of the tissue. Tissue was then labelled with antibodies using three different protocols: (1) Immunofluorescent staining for confocal microscopy, (2) post-embedding antibody staining for electron microscopy and (3) pre-embedding immuno-staining for EM. In all protocols the GABA antibody was used as a control for successful antibody labelling since the same antibody has previously been shown to specifically label GABAergic processes in insect tissue using the protocol as described here (Leitch and Laurent, 1993 and 1996; Watson, Burrows and Leitch, 1993).

3.3.1 Immunofluorescent staining:

In the initial studies the D_f motoneurones were not HRP labelled. After vibratome sectioning the 100 µm slices were immediately stained with antibodies. The sections were not resin embedded, but incubated with primary antibodies and secondary goat anti-rabbit rhodamine and fluorescein conjugated antibodies (Chemicon International Inc., CA 92590, USA) and viewed under a Biorad scanning confocal laser microscope. Prior to labelling, the sections were incubated with 2 % normal goat

serum (NGS) in Tris buffer (pH 7.4). The NGS was obtained from Chemicon and heat inactivated to destroy complement (56 °C for at least 30 minutes). The slices were incubated with the primary antibody (1:1,000) overnight in Tris buffer (pH 7.4) containing Triton X-100, then thoroughly rinsed prior to secondary incubation with the fluorescent antibodies (1:50 in Tris/Triton X-100 buffer; pH 8.2) for a couple of hours. The non-bound antibody was then washed off with repeated applications of Tris buffer (pH 8.2) then the labelled sections dehydrated with a graded ethanol series, placed in cavity slides and cleared with methyl salicylate. Initial viewing to determine the extent of the fluorescence was under a fluorescent microscope, the slides were then viewed under the confocal microscope.

3.3.2 Secondary gold labelling and electronmicroscopy.

For ultrastructural study of aminergic afferents onto the D_f motoneurone the sections were embedded in either LR White (Agar code R1281) or Araldite resin (Agar Scientific, UK) according to the antibody labelling protocol used. Ultrathin sections were cut using a glass knife (prepared using LKB knifemaker type 7801A) on a Reichart Om U2 ultramicrotome. The sections were mounted on formvar-coated nickel grids and viewed under a Philips 301 transmission electron microscope. Toluidine blue thin sections were prepared after the ultrathins had been cut and viewed under a Leitz Laborlux 11 light microscope.

Initial studies were performed using LR White and post-embedding antigen labelling. Having initially fixed the sections using either glutaraldehyde or paraformaldehyde primary fixation, secondary fixation was achieved by an hour long incubation in 1% osmium tetroxide. The tissue was then stained with 2 % aqueous uranyl acetate. After incubation with 2% uranyl acetate the sections were rinsed

with distilled water and partially dehydrated using an ethanol series. After 70% ethanol the sections were placed in a 70% ethanol - LR white mixture for 30 minutes. After this the mixture was replaced with 100% pure LR white three times, at which point it was left in fresh LR white overnight on a rotary wheel. A further two changes of LR white (15 minutes each) and the sections were set in airtight capsules, incubated overnight in an oven set at 50 – 55 °C.

The advantage of LR white is that the resin does not require complete dehydration prior to embedding and accepts sections from 70% ethanol. Furthermore in order to set it is cured at 50 - 55 °C, both factors contributing to preserve the antigenicity of the tissue. LR white ultrathin sections are also suitable for sodium metaperiodate etching and subsequent immuno-labelling. Ultrathin sections cut on the microtome were mounted on formvar-coated nickel grids and then etched for 1 minute with saturated sodium metaperiodate. Etching was stopped by repeatedly washing the grids with distilled water, after which point they were floated on 5% normal goat serum (NGS) in Tris buffer (pH 7.4) for 30 minutes. Primary labelling was achieved by floating the grids on primary antibody diluted in Tris buffer (pH 7.4) for 2 – 3 hours. They were then washed with Tris buffer and incubated with 10 nm gold-conjugated goat anti-rabbit secondary antibodies (British Biocell International, UK) diluted 1:100 in pH 8.2 Tris buffer. Once labelled, the grids were washed thoroughly with deionised distilled water and viewed under the electron microscope.

Immuno-labelling for amines was also conducted using pre-embedding antibody incubation. After the peroxidase reaction the sections were washed with Tris buffer (pH 7.4) and then incubated with the primary antibody overnight. The next day the excess primary antibody was rinsed off using further Tris buffer and

then the sections incubated with the secondary gold antibody. 1 nm gold-conjugated goat anti-rabbit antibodies (British Biocell International, UK) were used to improve penetration of the secondary antibody into the tissue section. To improve the visibility of the small gold particles under the electronmicroscope the sections were incubated with silver enhancing solution (Cat. no. SEKL15; British Biocell International, UK) for 10 – 20 minutes prior to fixation with osmium tetroxide. The sections were embedded in araldite resin. Great care was taken to dehydrate the sections gradually until several changes of 100% ethanol have been achieved at which point they were placed for 15 minutes in an epoxypropane - 100% ethanol mix prior to transfer to epoxypropane only. The tissue was then transferred to partially covered vials of araldite resin overnight (araldite mix consisted of 5ml araldite : 5 ml DDSA : 0.5 ml BDMA hardener : 0.25 ml dibutyl phthalate). The next day they were transferred to a change of araldite, prior to being placed in a plastic mould of fresh araldite for setting overnight in an oven (60 °C). Once the araldite blocks were set ultrathin sections were cut and viewed under the transmission electron microscope. Post-staining of the grids with uranyl acetate and lead citrate was not deemed necessary with the ultrastructure clearly defined in unstained grids.

RESULTS.

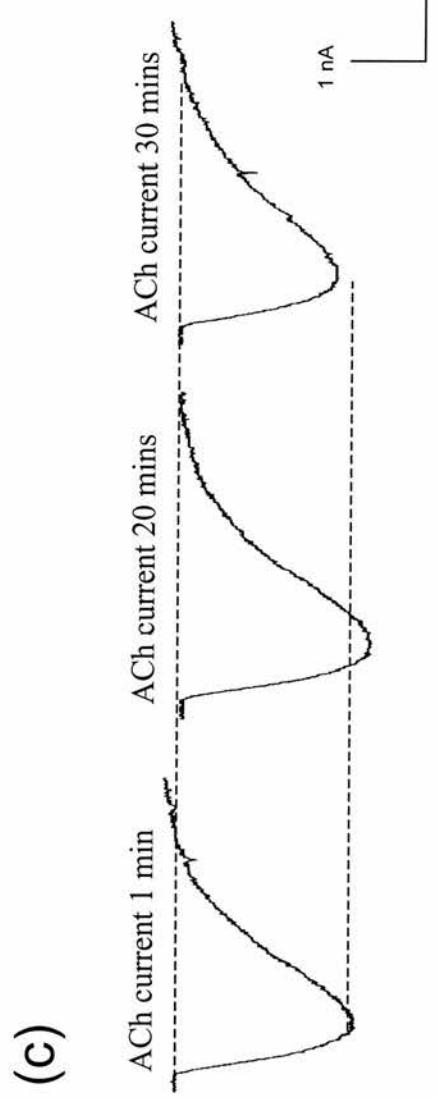
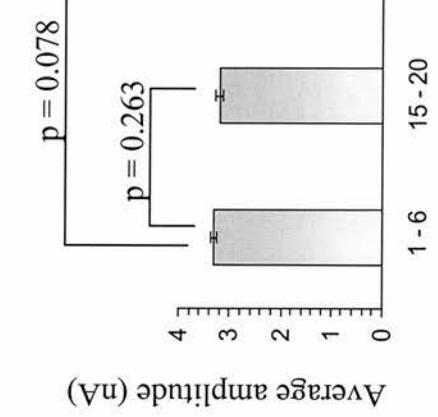
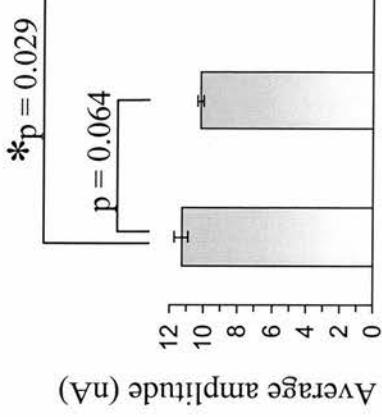
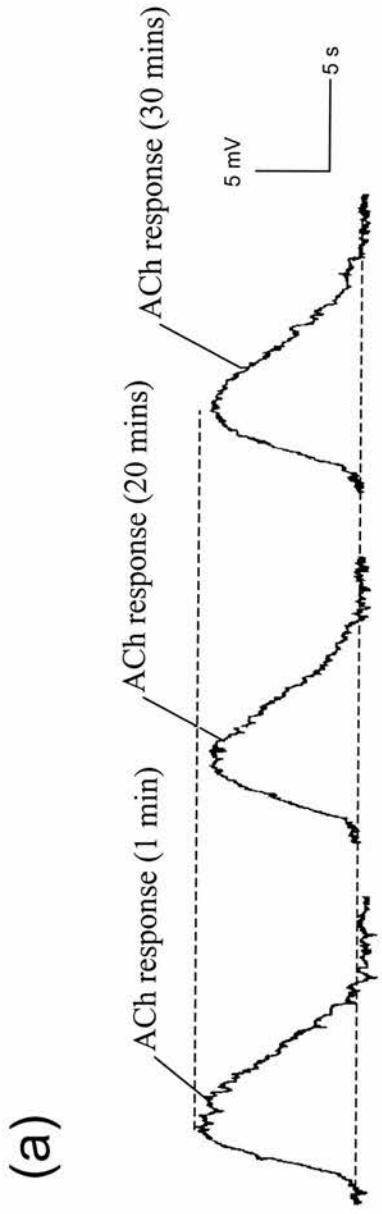
1 Suppression of nicotinic ACh responses by biogenic amines.

Brief pressure pulses of acetylcholine (ACh) applied locally to the soma of D_f motoneurone produce transient depolarising responses when recorded under the single electrode set-up. The duration of the picospritzer pulses (20 – 500 ms) was varied from one preparation to another in order to produce control membrane depolarisations of approximately 10 – 15 mV. Responses of this amplitude were typically of 8 to 20 seconds duration and could be applied at a frequency of 1 per minute without producing significant run-down over a half hour period. Figure 12a shows expanded individual ACh responses taken at different time intervals from a preparation to which ACh had been applied regularly every minute for a 30 minute period. There was no difference between the mean amplitude of the first six responses and the six responses recorded after 15 to 20 minutes ($p = 0.06$; t-test), although there was a gradual run down that resulted in a significant difference between the initial response and the six recorded after a half hour ($p = 0.03$; figure 12b). ACh inward currents recorded using the two electrode voltage-clamp set-up did not vary significantly over a half hour period ($p = 0.08$)(figure 12c and 12d).

The three monoamines dopamine (DA), octopamine (OA) and serotonin (5-HT) are present in the thoracic nerve cord of the cockroach *Periplaneta americana* (Evans, 1980). Application of these amines at concentrations of 10^{-4} M and greater have been demonstrated to differentially modulate excitatory postsynaptic potentials (EPSPs) recorded from thoracic interneurons (Casagrand and Ritzmann, 1992b) as well as motor output from nerve 5 that carries the axon of

Figure 12. Acetylcholine (10^{-1} M) pressure applied (1.5 Bar; 500 ms) to the fast coxal depressor motoneurone (D_f) motoneurone every minute over a half hour period does not produce desensitisation of the response.

ACh pulses resulted in transient depolarisations of 9 – 12 mV (a) or inward current of approximately 2 - 3 nA (c). No dramatic decrease was observed in the amplitude of ACh responses recorded under either recording technique. The size of the responses was measured and calibrated using a Gould Digital Oscilloscope (DSO)160A (cal. (a): vertical = 5 mV, horizontal = 5 seconds; cal (c): vertical = 1 nA, horizontal = 4 seconds), the standard error of the mean was calculated for the first six responses, the six responses recorded after 15 minutes and after a half hour. The pooled data from both controls are shown in (b) (single electrode current clamp recording set-up) and (d) (two electrode voltage-clamp). Under both conditions there was no significant change in the amplitude of the ACh response over a period of 20 minutes, although there was a significant difference ($p = 0.029$) in the depolarisation recorded after 30 minutes.



D_f (Pearson and Iles, 1971; Goldstein and Camhi, 1991). Application of 10^{-4} M of each of the amines caused prolonged reduction in the amplitude of ACh responses, seen as the vertical deflections of figure 13a, recorded from a single neurone. Maximum suppression of nicotinic responses was achieved after lengthy exposure to the amines; for all experiments the time to maximal effect was typically 5 – 7 minutes for dopamine and octopamine and 3 – 5 minutes for serotonin. After exposure of preparations to each amine for ten minutes, a 10 minutes wash with normal saline at a flow rate of $7 - 9 \text{ ml min}^{-1}$ restored the amplitude of the ACh response to control values. Figure 13a also shows another, probably related effect of amines that is observed in those D_f preparations exhibiting spontaneous synaptic activity. All three amines reduced this spontaneous activity of D_f (seen as the membrane fluctuations between successive ACh responses). This is particularly noticeable after the addition of serotonin (figure 13a); over the ten minute period of serotonin application the activity gradually reduces until the membrane potential becomes quiescent. The synaptic potentials do not return to control levels until 14 minutes after wash had been initiated.

As previously mentioned in the Methods section, perfusion of saline during the wash can cause artefacts in the recording of responses from the motoneurone. This is noticeable in figure 13a as fluctuation in the membrane potential during and after wash, principally during the washout to reverse the effects of dopamine and serotonin.

Figure 13b, 13c and 13d show individual responses to octopamine, dopamine and serotonin respectively, taken from figure 13a (marked with asterisks). Traces shown superimposed are of a typical control response, the response at which the maximal effect of amine was observed and a response recorded after the preparation

Figure 13a. The modulatory action of all three amines upon responses recorded from a single D_f motoneurone (resting potential \approx -83 mV) under current clamp conditions. Pressure application of ACh from microelectrodes (resistance 6 – 10 M Ω) resulted in control depolarisations of approximately 13 mV seen as the regular large vertical deflections from the baseline. Stock solutions of 10⁻² M amine were made up in normal saline and adjusted to pH 7.4 with NaOH. 20 μ l aliquots of the amines, final concentration 10⁻⁴ M, were bath applied for ten minutes under conditions of circulating saline, at the end of which washing with fresh saline was commenced, often resulting in a small artefact in the baseline (the period of wash marked with a thin red line). The rapid flow of saline through the bath (7 – 9 ml min⁻¹) often disrupted the resting potential of the cell, seen in the trace shown as the downward deflections after wash had commenced. All three amines gradually reduced the amplitude of ACh depolarisations. All three amines also reduced spontaneous synaptic activity exhibited by the motoneurone – particularly noticeable in the example shown as the reduction in the fluctuations in membrane potential between successive ACh responses during the application of dopamine and serotonin. Washing for a duration of 10 minutes resulted in a gradual reversal of the aminergic suppression to near control levels. (cal.: vertical = 5 mV, horizontal = 2 minutes).

The traces marked with the asterisks represent a typical control response (single asterisk), the maximal effect of the amine (double asterisks) and a recovery in ACh response amplitude after washout with fresh saline (triple asterisks). The marked traces are shown superimposed on an expanded time scale in (b), (c) and (d).

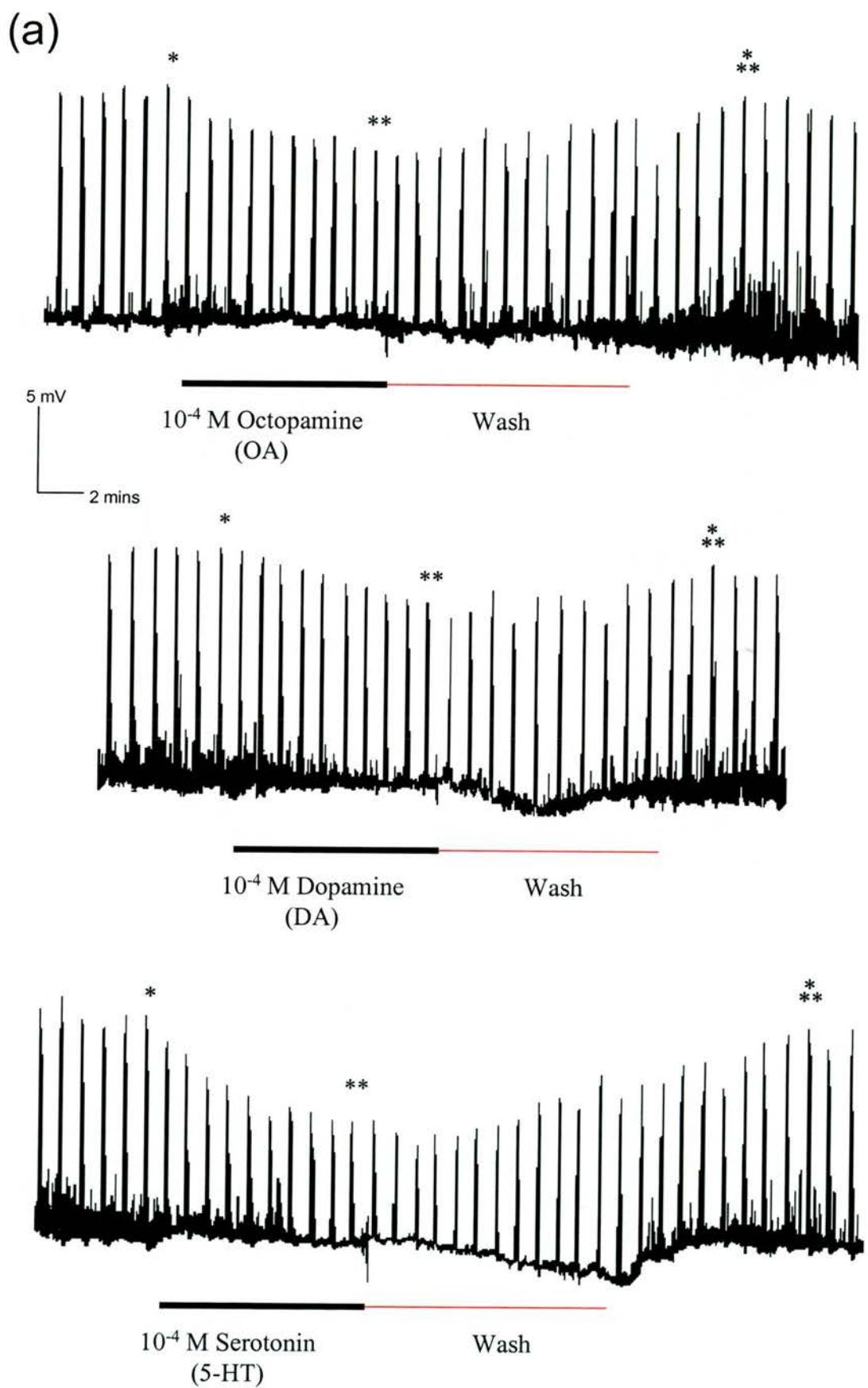
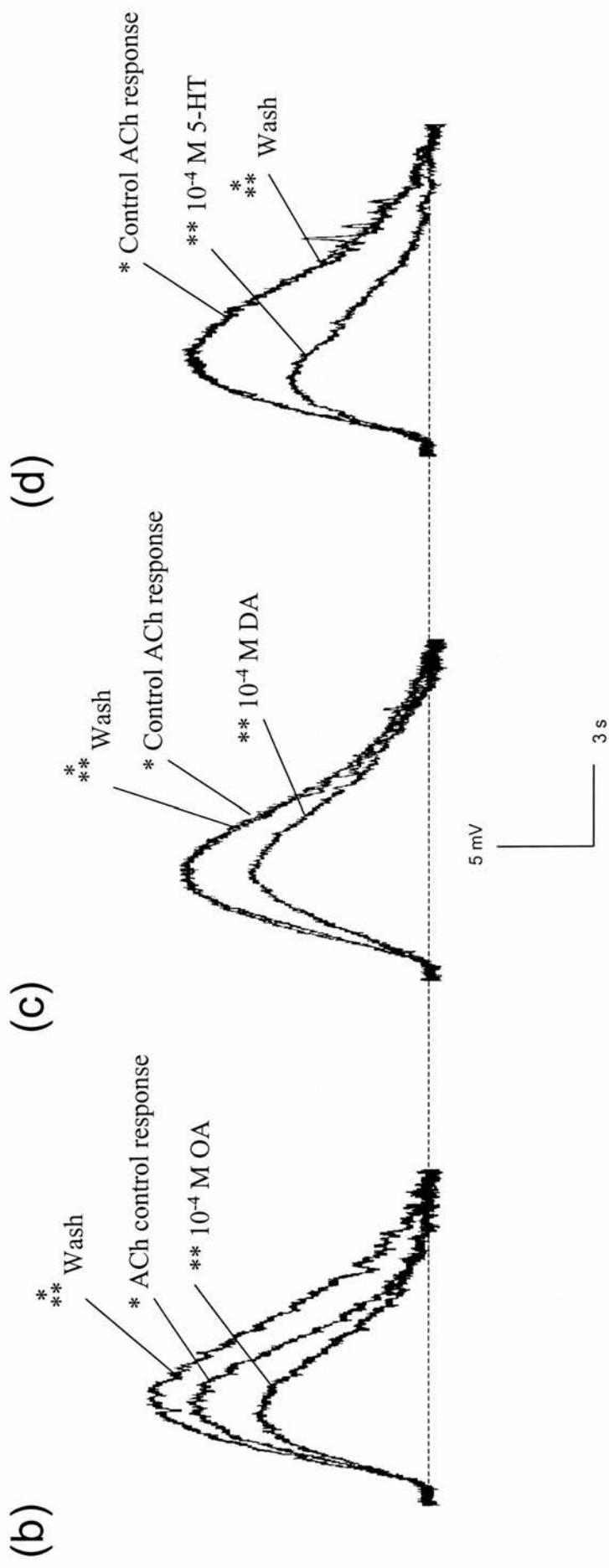


Figure 13b, 13c and 13d. For analysis of the results the amplitude of the control ACh responses were measured using the calibration performed at the end of the experiment, along with the responses recorded after application of 10^{-4} M octopamine (b), 10^{-4} M dopamine (c) and 10^{-4} M serotonin (d). The mean of the standard error was calculated for the control ACh amplitudes and all the responses expressed as percentage of that. The maximal effect of the amine was determined and marked with two asterisks as shown in the traces exhibited in (a). The three panels show individual traces on an expanded time scale representing a typical control response (single asterisk), maximal effect of the amine (double asterisk) and a response recorded after wash reversal (triple asterisk)(cal.: vertical = 5 mV, horizontal = 3 seconds).



had recovered after washing in normal saline. It is clear from figure 13 that serotonin produced a more profound suppression of ACh responses than did either octopamine or dopamine in this particular preparation. Figure 14a shows that 10^{-4} M serotonin was consistently more effective than either octopamine or dopamine, which had similar potencies to each other. After 10 minutes application, serotonin (10^{-4} M) reduced the ACh response to 49.6 % of the control (+s.e.m. 4.1, -s.e.m. 4.1; n = 7; arcsine transformed data), while dopamine and octopamine reduced the ACh response to 77.4 % (+s.e.m. 4.1, -s.e.m. 4.4; n = 5) and 74.2 (+s.e.m. 2.6, -s.e.m. 2.7; n = 5) of the control respectively.

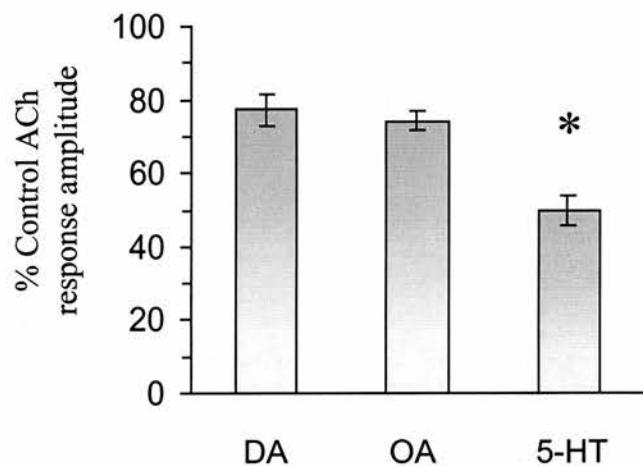
The actions of all three amines were dose-dependent (Figure 14b) with serotonin approximately 10-fold more effective ($IC_{50} \sim 50 \mu M$) than dopamine ($IC_{50} \sim 450 \mu M$). A full dose response curve was not compiled for octopamine, but at the range of concentrations tested (10^{-5} M to 10^{-3} M) its effectiveness at suppressing ACh responses was not significantly different from that of dopamine. Figure 14c shows representative traces illustrating the effect of serotonin at a range of concentrations. At concentrations above 10^{-2} M it was hard to determine the effect of serotonin on ACh responses since it produced significant changes in membrane potential (figure 14c, bottom left-hand panel). The same was also true for dopamine at concentrations in excess of 10^{-2} M. None of the amines completely inhibited ACh responses, suggesting a number of plausible hypotheses: firstly that, if they exert their action directly or allosterically on nAChRs, there are populations of amine-insensitive and amine-sensitive nicotinic receptors. Secondly, that the amines exert their action indirectly via a saturable signalling pathway or, thirdly, that they exert their effects via a system that is in some form of dynamic equilibrium (e.g. phosphorylation ↔ dephosphorylation).

Figure 14. Relative potencies of octopamine, dopamine, and serotonin in suppressing nicotinic ACh responses.

(a) Shows pooled data from a number of experiments showing the degree to which 10^{-4} M of each amine depressed nicotinic ACh responses. Serotonin ($n = 7$) was consistently more potent than the other two amines ($n = 5$ respectively) when tested at this concentration. This observation was born out when the effect of the amines was tested over a range of concentrations illustrated in (b). Neither dopamine nor serotonin which were tested at a wide range of concentrations completely abolished ACh responses recorded from D_f , exerting a maximal suppression of the ACh response of approximately 30% of the control at concentrations of 10^{-3} M for serotonin and 10^{-2} M for dopamine. Data points represent the arc-sine transformed mean suppression from at least four preparations except for the octopamine data ($n = 2$ or 3) and the highest concentration of dopamine tested (5×10^{-2} M, $n = 2$). The IC_{50} values for these two amines were calculated from the graph as indicated by the dotted line, with a value of $\sim 5 \times 10^{-5}$ M for serotonin and $\sim 4.5 \times 10^{-4}$ M for dopamine.

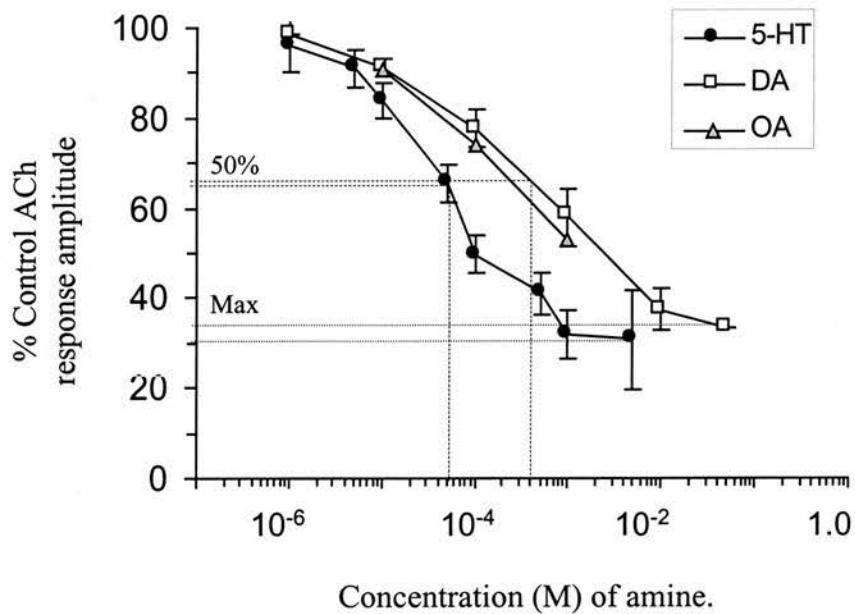
(c) Shows typical traces recorded under current clamp, demonstrating the effect of increasing concentrations of serotonin. The maximal effect of the amine often took 3 – 5 minutes to develop. Also noticeable is the fact that serotonin at a concentration of 10^{-2} M resulted in a significant, prolonged depolarisation of the resting potential, but does not completely abolish the ACh response. All the preparations used had resting potentials in range of -70 to -90 mV and exhibited robust control ACh responses, which reversed to within 10% of the mean control amplitude after washout with fresh saline (cal.: vertical = 5 mV, horizontal = 2 minutes).

(a)

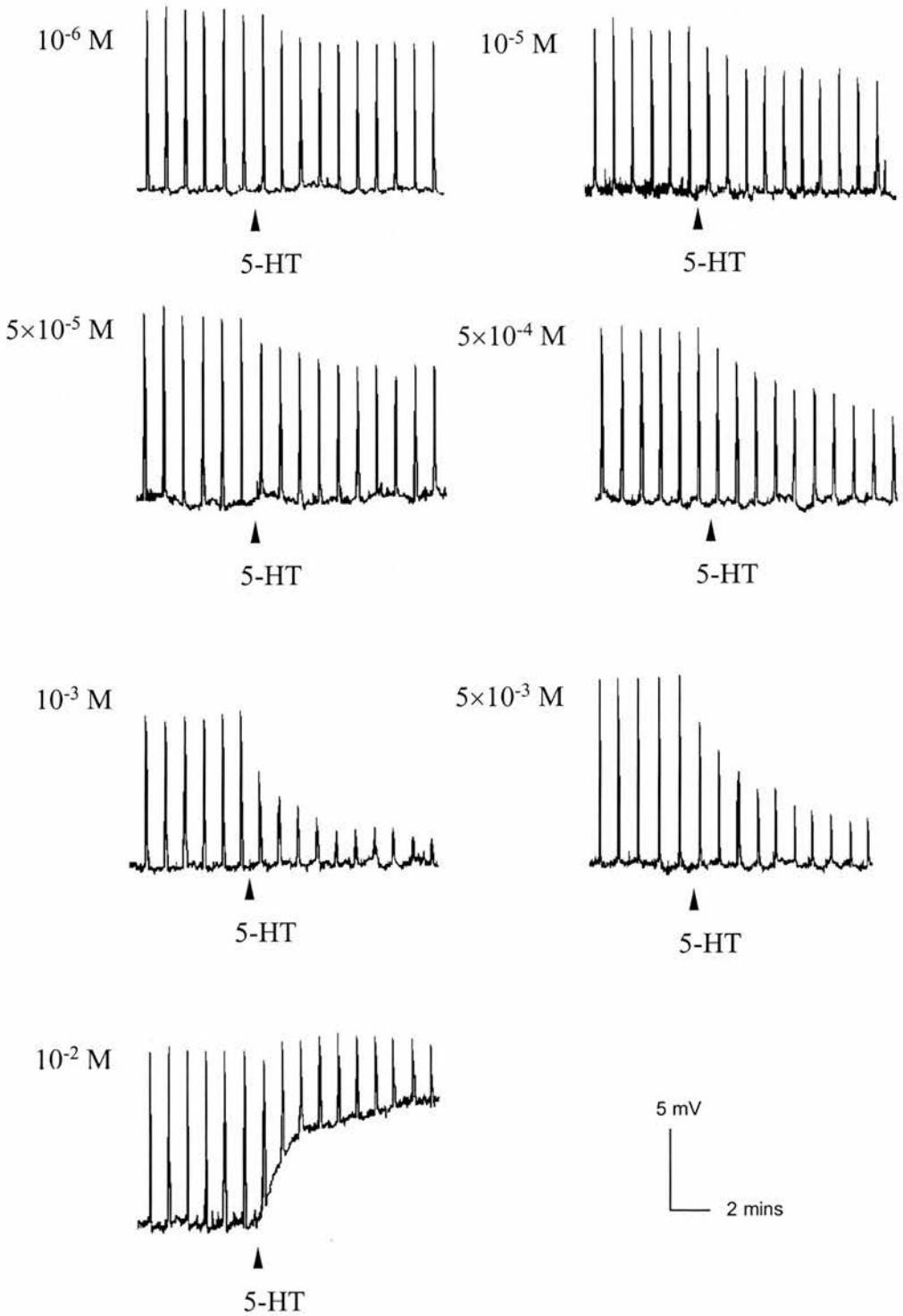


Maximal effect of 10 minute
amine application (10^{-4} M)

(b)



(c)



1.1 Amines act directly on the D_f motoneurone to attenuate nicotinic acetylcholine responses, and achieve this independently of acetylcholinesterase activity and muscarinic receptor activation.

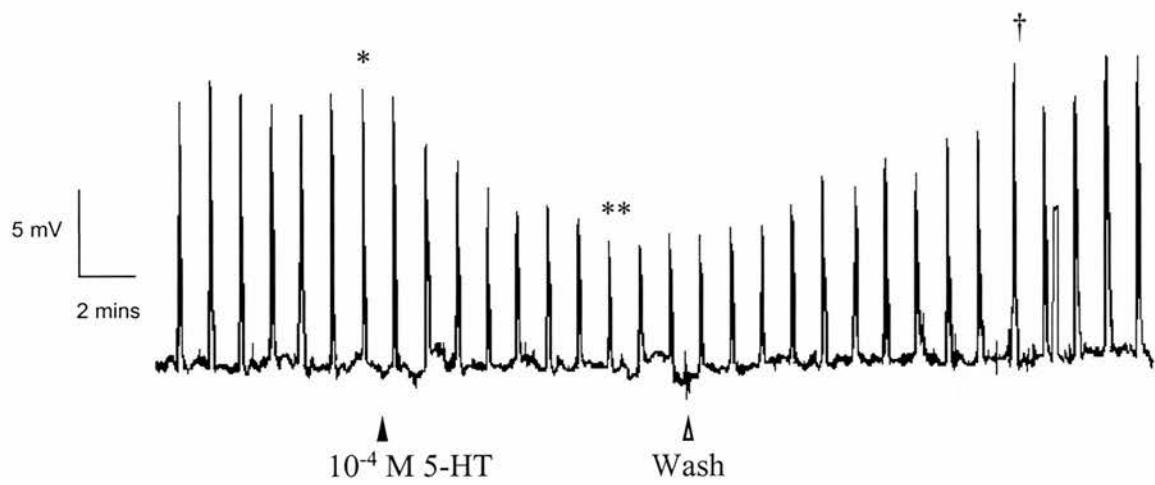
D_f motoneurone somata were surgically isolated to establish whether amines act indirectly via other neurones or act directly on the D_f motoneurone. This was important to demonstrate as the three amines tested have been found previously to modulate postsynaptic potentials exhibited by interneurones. Secondly, it would provide information as to whether aminergic suppression is mediated by sites local to the soma membrane or on more distal regions of the neurone. Undercutting of the soma did not result in deterioration of the normal properties of D_f; the resting potential of the cell shown in figure 15 was approximately -72 mV and the neurone exhibited stable control ACh responses. This procedure did not attenuate aminergic suppression of the ACh responses (figure 15a) or prevent reversal of the amine's action when washed with fresh saline. However, there was a discernible prolongation in the duration of ACh responses after washing (figure 15b).

Activation of muscarinic receptors produces a reduction in the amplitude of nACh responses similar to that seen in the presence of the three amines. To eliminate the possibility that the action of the amines is mediated through muscarinic AChRs, experiments were conducted in saline containing 10⁻⁶ M of the muscarinic antagonist pirenzepine, a concentration previously shown to selectively block activation of the muscarinic ACh receptors on the D_f motoneurone without affecting signal transduction at nAChRs (*personal communication Dr. R. Pitman*). Pirenzepine marginally enhanced the amplitude of ACh responses figure 16. Pooled data from nine experiments showed, however, that pirenzepine had no significant effect on

Figure 15. Aminergic suppression is mediated by sites local to the soma membrane.

Surgical isolation of the D_f soma shown in (a) did not alter the ability of the motoneurone to exhibit ACh responses nor remove their sensitivity to the modulatory action of the monoamine serotonin. The individual ACh responses marked are expanded in (b) and more clearly demonstrated the prolongation of the ACh response observed after a saline wash. This was occasionally seen in non-undercut preparations after an hours impalement or more and could represent a reduction in the ability of the soma to recover from the ACh depolarisation. (cal (a).: vertical = 5 mV, horizontal 2 minutes; cal (b).: Vertical 5 mV, horizontal 4 seconds).

(a)



(b)

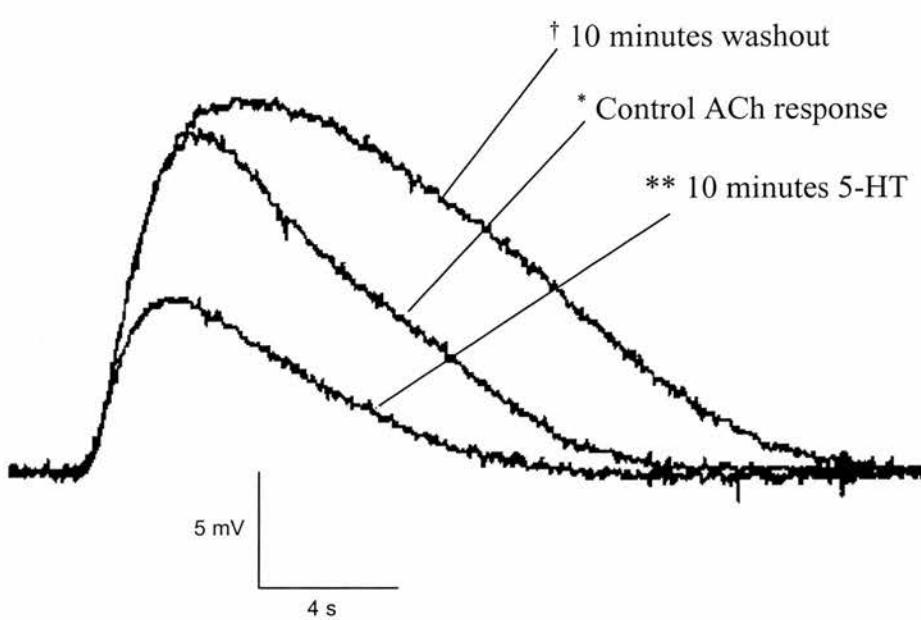
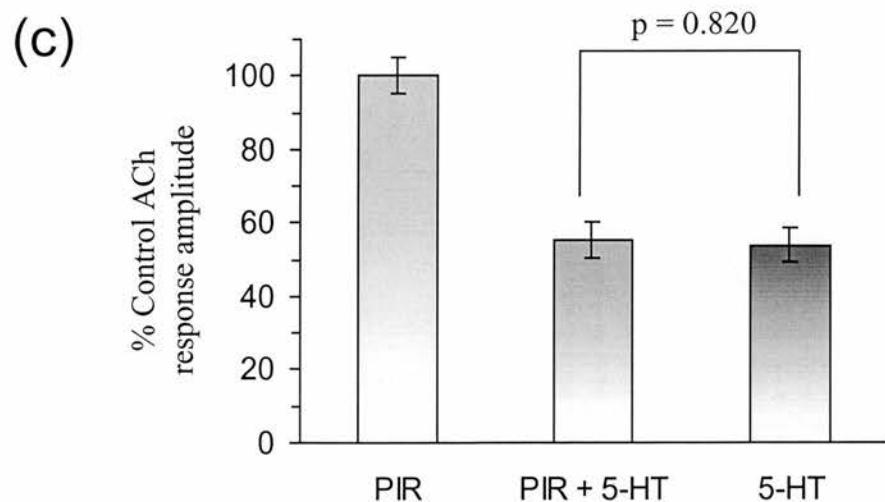
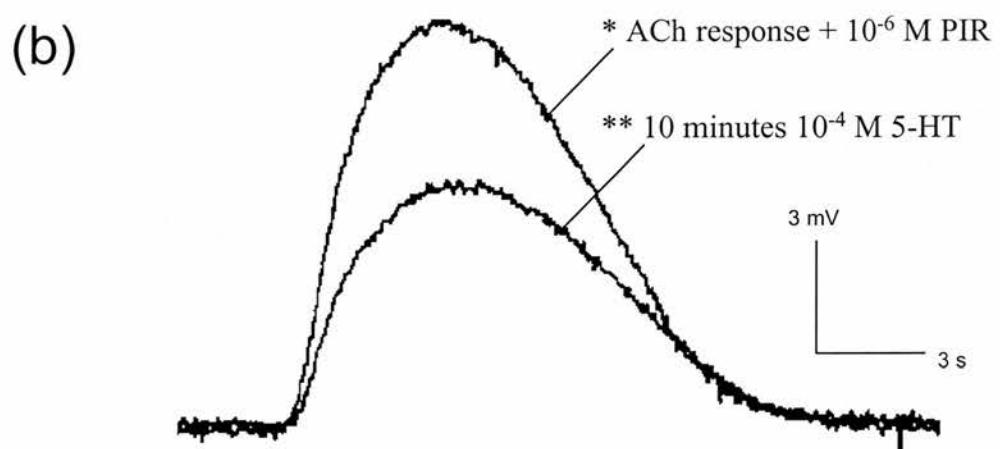
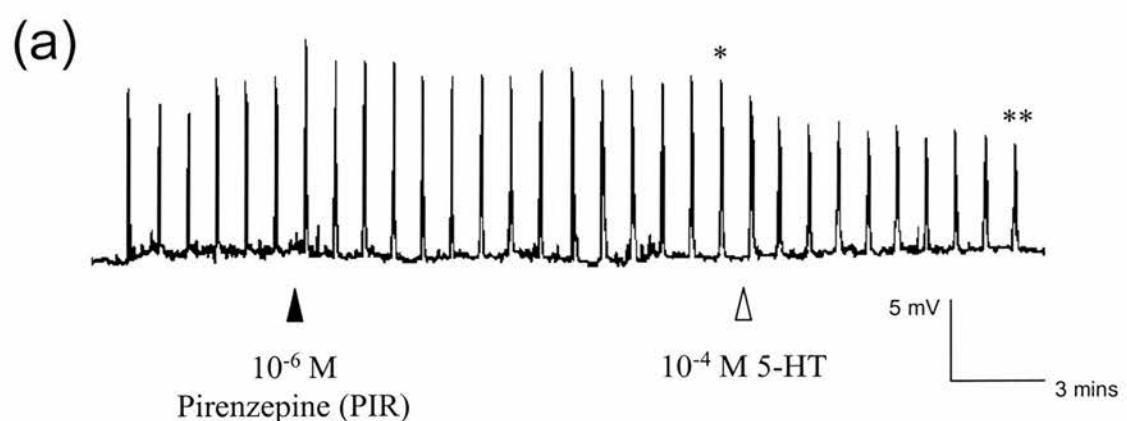


Figure 16. Amines modulate nicotinic ACh responses.

To eliminate the possibility that the amines exert their action via muscarinic receptors (which have previously been demonstrated to mediate suppression), the ability of the amines to suppress ACh responses was tested in the presence of the muscarinic antagonist pirenzepine (a). Preparations were incubated for 15 minutes in 10^{-6} M pirenzepine prior to application of the monoamine serotonin. Pirenzepine appeared to cause a small increase in the amplitude of the ACh response in the example shown. The muscarinic antagonist did not block the effect of 10^{-4} M serotonin. This was further confirmed by comparison of the amplitude of the ACh responses just prior to and after 10 minutes of monoamine addition (b). The pooled data from nine such experiments is illustrated in figure 16c; incubation with 10^{-6} M pirenzepine did not significantly alter the ability of serotonin to modulate nicotinic ACh responses.



either control ACh responses (amplitude 100.1 % ± s.e.m. 4.9 of control after 15 minutes incubation; n = 9) or on the modulatory action of the most potent monoamine serotonin. Serotonin in the absence of pirenzepine reduced the ACh response to 53.8% (+s.e.m. 4.7, -s.e.m. 4.7, n = 8) as opposed to serotonin in the presence of the muscarinic antagonist which suppressed the control ACh response to 55.4% (+s.e.m. 4.8, -s.e.m. 4.9, n = 9; p = 0.820).

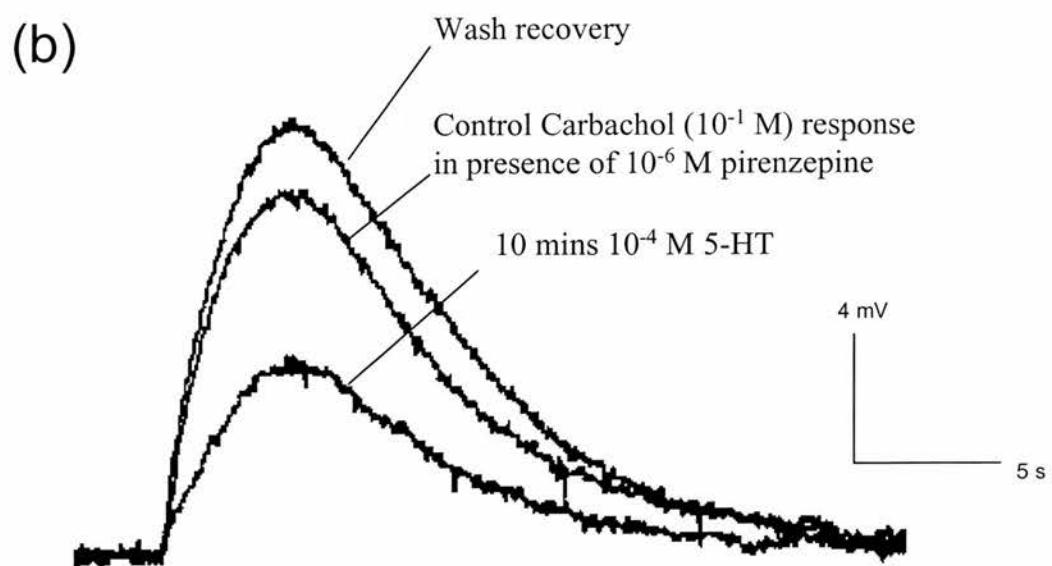
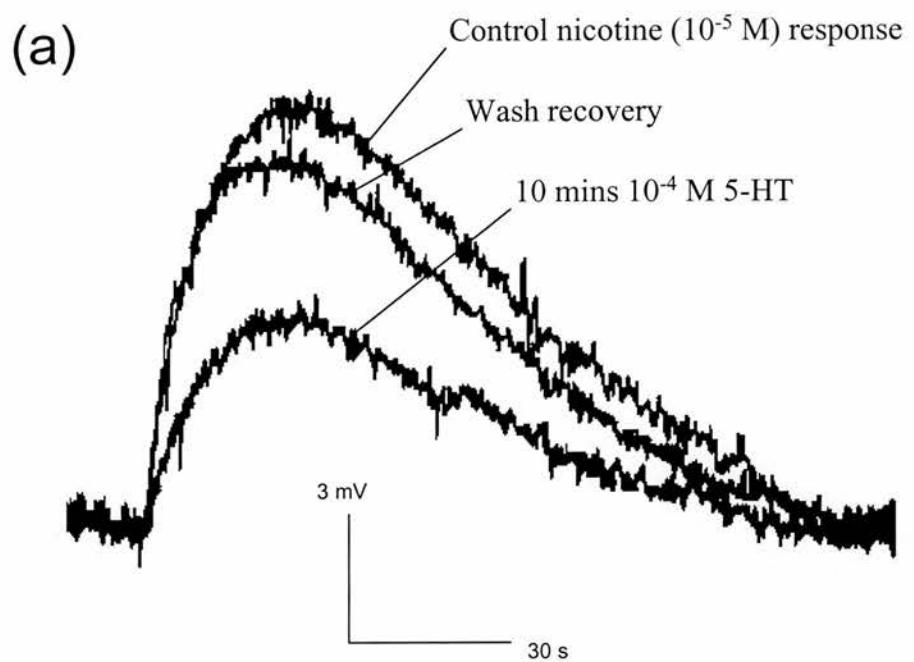
To rule out the possibility that the effects of the amines resulted from a change in the rate of ACh degradation, the action of amines upon nicotinic responses evoked by nicotine or carbachol were studied. These compounds are resistant to degradation by acetylcholinesterase, an enzyme known to be highly active in the cockroach (see Pitman, 1971). Brief pulses of nicotine (10^{-5} M) were pressure applied locally to D_f once every two minutes to minimise desensitisation of the control response. Nicotine elicited long duration depolarisations that were reversibly attenuated by bath application of 10^{-4} M serotonin (49.7% of control +s.e.m. 3.2, -s.e.m. 3.2; n = 3) (figure 17a). Depolarisations resulting from pressure application of 10^{-1} M carbachol (in the presence of 10^{-6} M pirenzepine) were also reduced by serotonin to 51.9% of control (+s.e.m. 3.9, -s.e.m. 3.9; n = 2) (figure 17b).

1.2 Amines modulate inward currents mediated by nAChRs.

The results provided so far indicate that the monoamines dopamine, octopamine and serotonin each reduce the amplitude of membrane potential changes evoked by activation of nicotinic ACh receptors present on the D_f motoneurone. However these recordings do not indicate whether the amines exert their primary action through an increase in membrane conductance (which merely short-circuits the ion conductance

Figure 17. Amines suppress nicotinic ACh responses mediated by nicotine and carbachol.

The ability of serotonin (10^{-4} M) to attenuate nicotinic responses mediated by either nicotine (a) or by carbachol in the presence of pirenzepine (b) was tested under current clamp conditions. Local pressure application of the agonists from microelectrodes containing either 10^{-5} M nicotinic or 10^{-1} M carbachol to the soma of the D_f motoneurone resulted in relatively long duration depolarisations. In both cases responses were elicited once every two minutes. The superimposed traces in figures (a) and (b) are typical of the suppression mediated by serotonin. Application of the monoamine resulted in rapid suppression of the control amplitude to levels similar to that produced when ACh responses were initiated by local application of acetylcholine chloride. These results indicate that the suppression of ACh-evoked depolarisations is independent of acetylcholinesterase activity.



mediated by nAChRs), or whether they actually reduce the current travelling through nAChRs. To establish which was the case, the actions of the amines were studied under two electrode voltage-clamp conditions, in which the amplitude of ACh-activated currents is independent of membrane resistance.

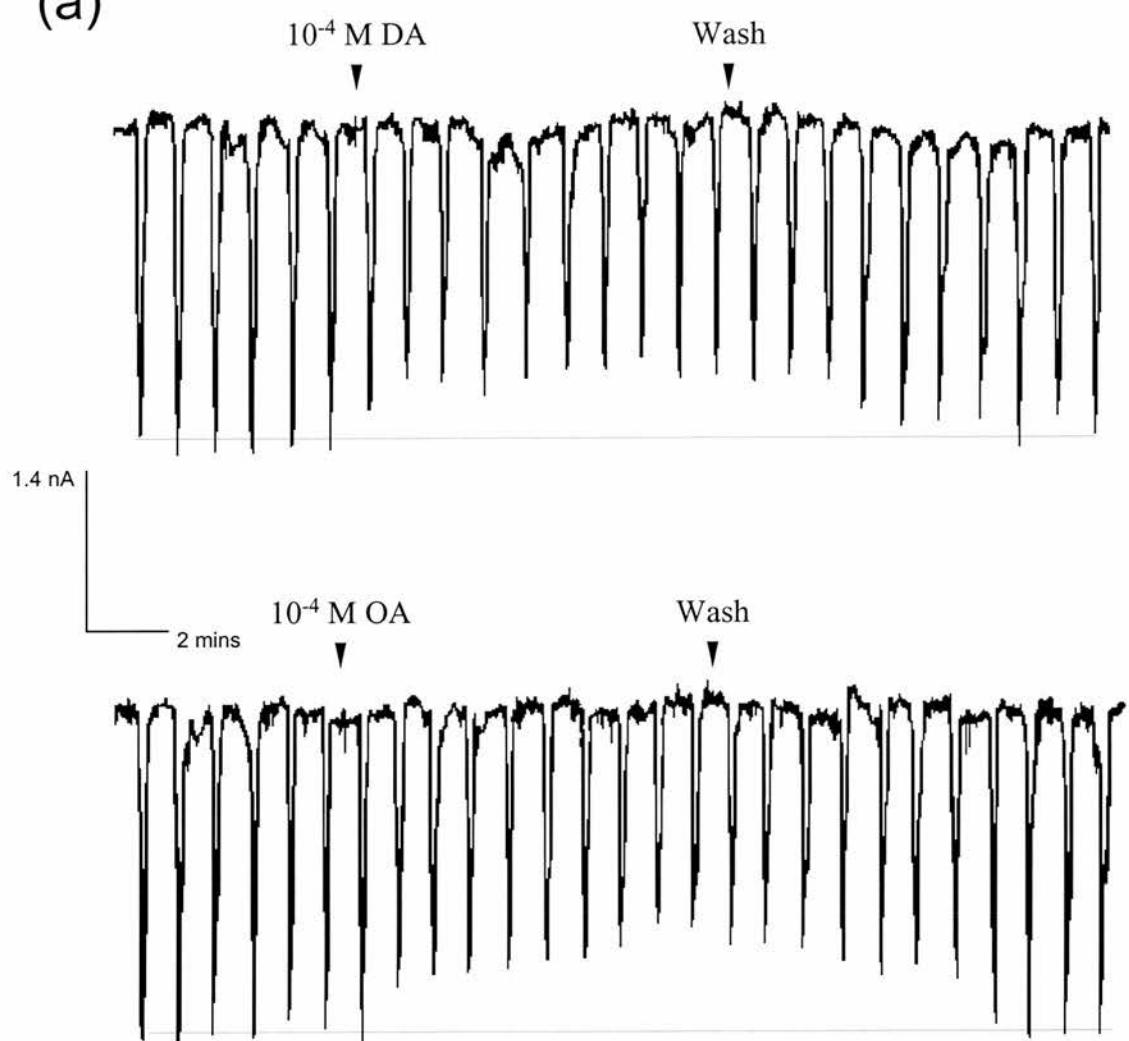
Under voltage-clamp all three monoamines reversibly suppressed ACh inward currents (Figures 18a, 18b and 18c), indicating that they exert their action by reducing the flow of ions through nAChRs. On average 10^{-4} M dopamine reduced the amplitude of the nicotinic inward current to 75.1% (+s.e.m. 2.1, -s.e.m. 2.2; n = 7), 10^{-4} M octopamine to 76.4% (+s.e.m. 3.8, -s.e.m. 4.0; n = 5), and 10^{-4} M serotonin to 50.9% (+s.e.m. 4.6, -s.e.m. 4.7; n = 8) of the control (figure 7d). These percentage reductions are similar to those for the decrease in amplitude of membrane potential responses obtained using the single electrode recording technique.

Figure 18. Suppression by amines of nicotinic currents measured under voltage-clamp conditions.

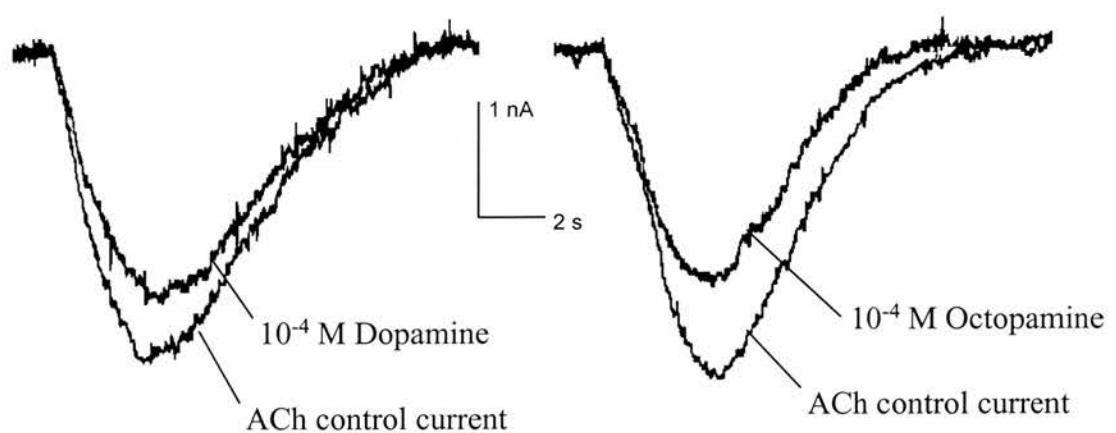
The traces in (a) show the effects of the amines dopamine and octopamine respectively on ACh currents recorded from the same D_f motoneurone in the presence of 10⁻⁶ M pirenzepine. Nicotinic inward currents are seen as the regular downward deflections in the traces in (a). 20 µl aliquots of the amines (final bath concentration 10⁻⁴ M) were added after six constant control responses and washed out after 10 minutes application, resulting in a gradual reversal of the induced suppression. The maximum effect of 10⁻⁴ M dopamine (left-hand panel (b)) and 10⁻⁴ M octopamine (right-hand panel) are shown in the expanded traces below. The individual traces in (b) are the final ACh control current and the response exhibiting the maximal effect of the amine. (cal. (a): vertical 1.4 nA, horizontal 2 minutes; (b): vertical 1 nA, horizontal 2 seconds).

(c) Shows the effect of the monoamine serotonin on nicotinic ACh currents. The top trace exhibits the typical effect of 10⁻⁴ M serotonin when applied to a preparation for ten minutes in circulating saline. Serotonin, in line with the observations under current-clamp, was considerably more potent than either dopamine or octopamine as revealed by the pooled data shown in (d). Serotonin reduced the nicotinic inward current to 50.9% (+s.e.m. 4.6, -s.e.m. 4.7; n = 7) of the control, as opposed to dopamine, which reduce the ACh current to 75.1 (+s.e.m. 2.2, -s.e.m. 2.3; n = 7) of control, and octopamine, which reduce the ACh current to 76.4% (+s.e.m. 3.8, -s.e.m. 4.0; n = 7) of the control.

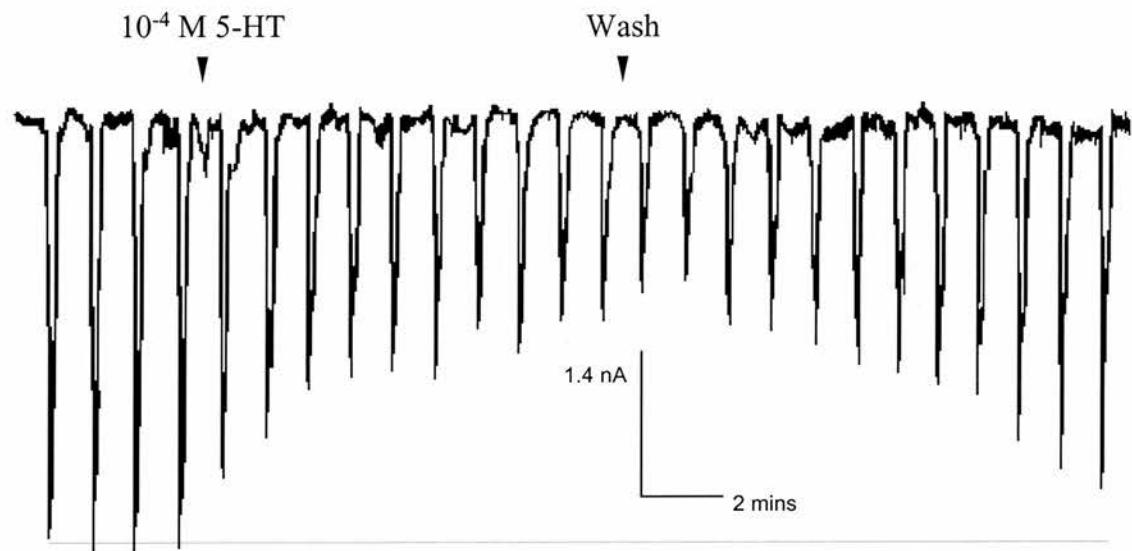
(a)



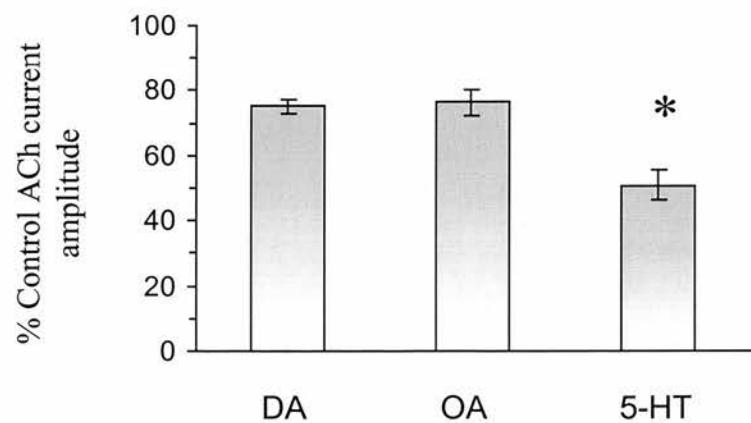
(b)



(c)



(d)



Maximal effect of 10 minute amine application
(10^{-4} M), recorded under voltage clamp conditions

2 Identification of aminergic processes local to the D_f motoneurone.

Exogenous application of the monoamines dopamine, octopamine and serotonin reversibly modulates nicotinic ACh responses of the D_f motoneurone. To determine whether such modulation could occur *in vivo* an immunohistochemistry study was performed in order to characterise putative aminergic afferents onto D_f. A total of 8 metathoracic ganglia were labelled with immunofluorescent antibodies and viewed under the laser scanning confocal microscope to confirm the labelling of aminergic varicosities in the ganglia. A further 21 ganglia were prepared for electron microscopy and ultrastructural analysis.

2.1 Laser scanning confocal microscopy.

The ramification of aminergic terminals throughout the metathoracic ganglion of *Periplaneta americana*, along with the presence of cell bodies that stain positively for amines have already been described in the cockroach (Dymond and Evans, 1979; Bishop and O'Shea, 1983; Baker and Pitman, 1989). Laser scanning confocal microscopy was used to confirm successful staining of immuno-positive aminergic profiles. Best results were achieved with rhodamine labelling, since the wavelength of light required to observe staining with fluorescein conjugated antibodies resulted in a significant degree of background tissue autofluorescence.

Specificity of rhodamine labelling of aminergic profiles was checked by using two control conditions: (1) omission of the primary amine antibody and, (2) pre-absorption of the primary antibody with BSA/antigen. Apart from the primary dopamine and serotonin antibodies tested, a GABA antibody previously proven to label insect GABAergic neurones was also tested to ensure that the fixation and staining protocol worked effectively. The location of immuno-positive cells was

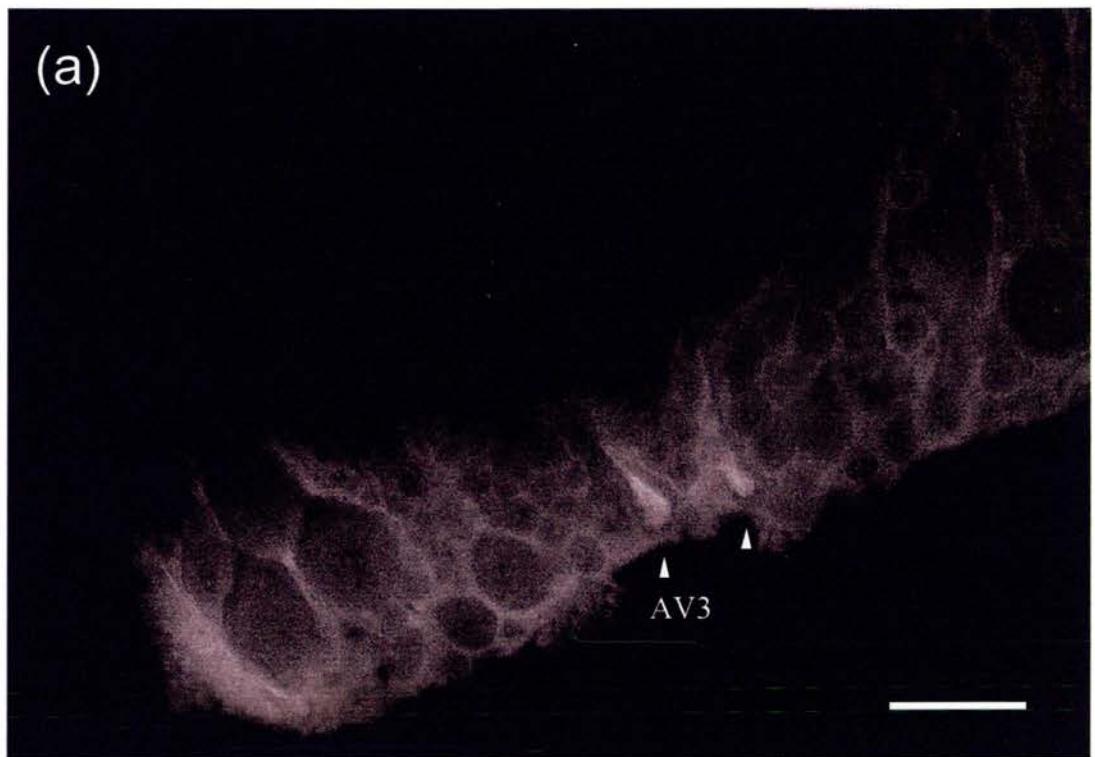
assigned according to the guides to the neuroanatomy of cockroach mesothoracic ganglion published by Gregory (1974, 1984; see also Tyrer and Gregory, 1982).

All three antibodies labelled immunoreactive profiles throughout the neuropile and a number of cell bodies at the periphery of the metathoracic ganglion. Figures 19a and 19b show consecutive confocal images of dopamine immunoreactive cell bodies detected in transverse vibratome sections 300 – 400 µm from the anterior end of the ganglion, at the level of nerve 3. The ventral midline cells (figure 19a, white arrowheads) are approximately 20 µm in diameter and are similar in position to biogenic amine containing cells identified by Baker and Pitman (1989) in the prothoracic ganglion of *Periplaneta americana*. The fluorescent product of the glyoxylic acid technique observed by Baker and Pitman (1989) was blue-green in colour, typical of catecholamine-containing neurones. This study would suggest that similar midline dopamine-immunoreactive neurones are present in the metathoracic ganglion. Secondary neurites of the midline neurones were visible either side of the cells corresponding in position to the midline ventral median (MVM) group of cell bodies identified by Gregory (1984). These neurites could be traced until they reached the edge of the neuropilar region. An additional dopamine-immunoreactive lateral ventral neurone of 12 µm diameter (figure 19b, yellow asterisk) was also evident in the same view under the confocal microscope. Lateral somata exhibiting dopamine-like immunoreactivity have previously been reported by Watson (1992) in the prothoracic ganglion of the locust *Schistocerca gregaria*, although more anterior and dorsal than those detected in the cockroach in this study. Extensive ramifications of dopamine-immunoreactive neurones were clearly detectable throughout the dorsal medial neuropilar regions in the same 100 µm vibratome slice (figure 20), although it

Figure 19. Rhodamine labelling of dopamine-immunoreactive cell bodies on the ventral surface of the metathoracic ganglion. Consecutive images from a slice 300 – 400 μm from the rostral end of the ganglion.

(a) Shows two dopamine immuno-positive cell bodies on the midline of the ventral surface of the metathoracic ganglion (as indicated by the white arrows). Their position, adjacent to the ventral neural sheath is approximately the same as the paired AV3 cells of the mesothoracic ganglion as described by Gregory (1984). Also discernible between the two cell bodies, although less distinct, is a bifurcating branch of a more medial neurone. This medial cell is more clearly visible in the subsequent scan (b) and is immediately ventral to cells approximate to the MVM mesothoracic cells (indicated by the white arrow). This second image also revealed the presence of a ventrolateral dopaminergic neurone of 12 μm diameter (labelled with a yellow asterisks). Scale bar: 60 μm .

(a)



(b)

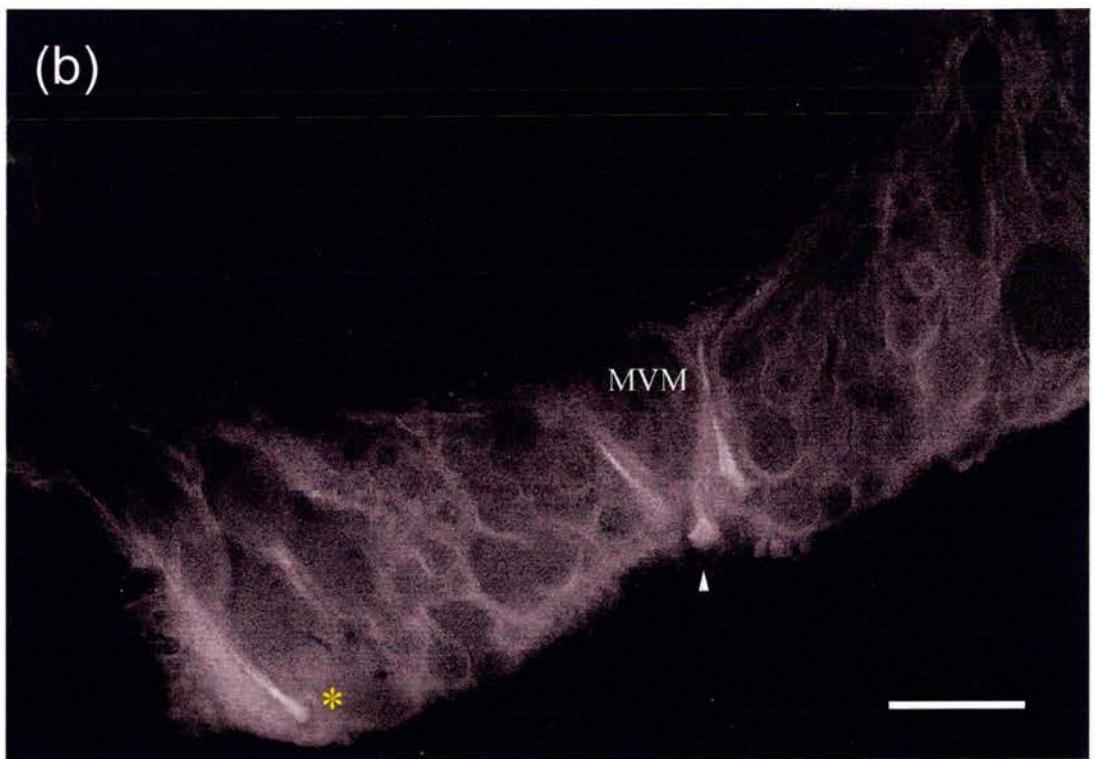
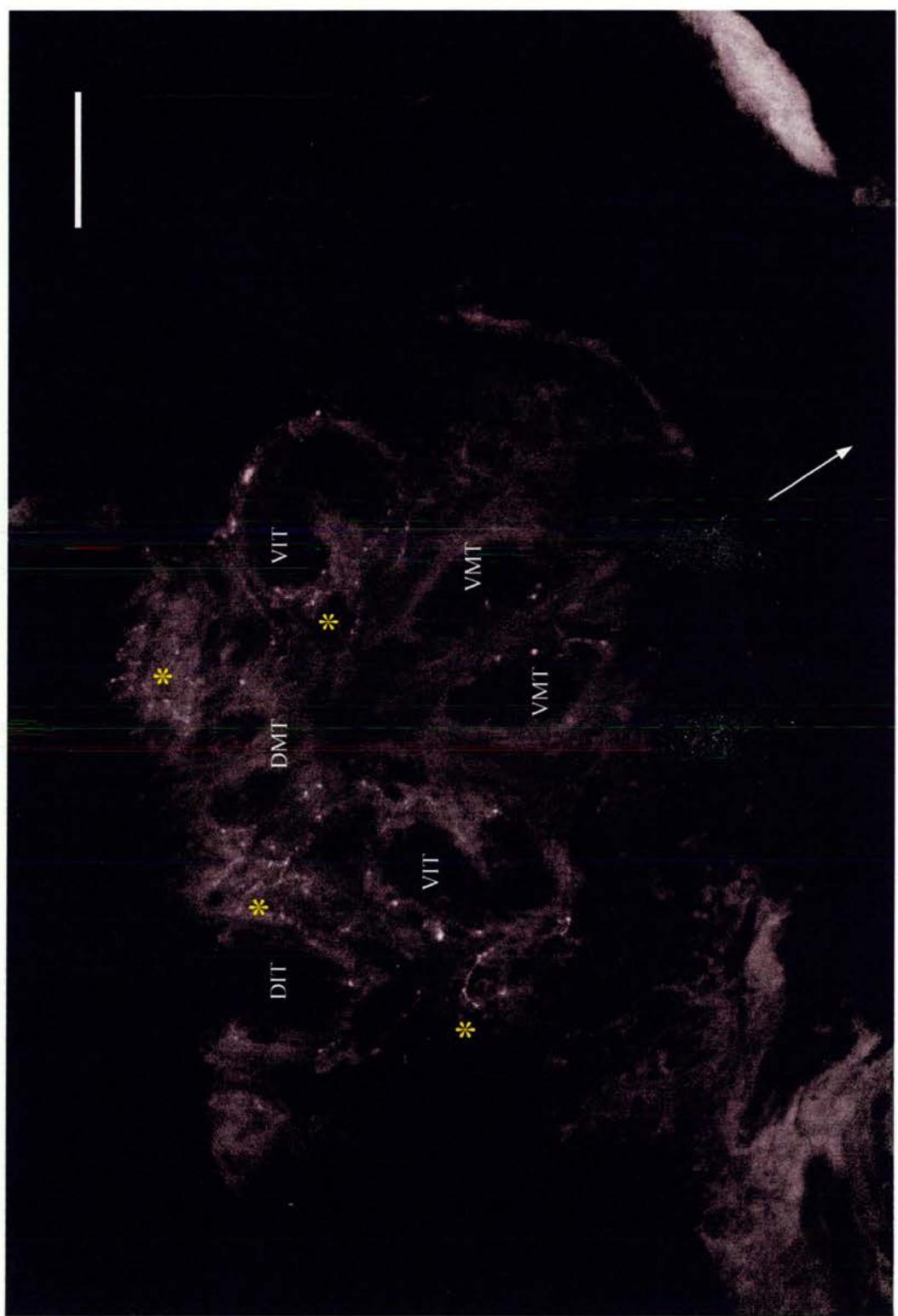


Figure 20. A section showing dopamine immuno-labelling of processes throughout the neuropile of the metathoracic ganglion, 300-400 µm from the rostral end of the ganglion.

The presence of dopamine-containing ramifications throughout the neuropile were evident in all the sections observed under the confocal microscope. The image shown in figure 20 was taken from the same 100 µm section as the cell bodies in figure 19, the alignment of the ventral-dorsal midline and position of the immuno-positive cell bodies indicated by the white arrow. The areas containing extensive arborisation of dopamine neurones (labelled with yellow asterisks) are bilaterally symmetrical and are concentrated around the dorsal medial tract (DMT; nomenclature from Tyrer and Gregory, 1982) and dorsal intermediate tract (DIT). Large processes are also detectable immediately surrounding the ventral intermediate tracts (VIT) and to a lesser extent in the midline between the ventral median tracts (VMT). Rhodamine labelling was specific to dopamine antibody-labelled cells. Auto-fluorescence was minimal with the exception of that exhibited by the neural sheath, clearly evident as the fluorescence in the bottom right-hand corner. Scale bar: 60 µm, white arrow indicates dorsal-ventral axis.



was not possible to ascertain whether these represented arborisations of the cell bodies identified in figures 19a and 19b.

Serotonin immuno-positive profiles were detected throughout the neuropile of the thoracic ganglia. The transverse section through the metathoracic ganglion shown in figure 21a and 21b was at the level of nerve 5, approximately 400 – 500 µm from the anterior end of the ganglion. A neurite (marked with a yellow asterisk, figure 21a) is visible toward the ventral surface. The position of such a ventrolateral process is in agreement with previous studies that have identified serotonin immunoreactive somata (termed LThBP and LThBP2) in the caudal section of the metathoracic ganglion of *Periplaneta americana* (Bishop and O'Shea, 1983; reviewed by Nässel, 1988). The longitudinal tracts shown in the images displayed in figures 21 are not as distinct as those illustrated in figure 20, although they were clearly identifiable under the confocal microscope; to assist identification they have been ringed with a pale dashed line. Also noticeable were the extensive arborisations of serotonin-immunoreactive neurones in the dorsal area of the neuropile, predominantly around the dorsal median tract (DMT)(figure 21b). The arborisation and varicosities of a serotonin-immunoreactive neurone highlighted by the yellow rectangle in figure 21b are shown under higher magnification in figure 22a –22c, which shows images of the same region at different optical planes. In more medial sections (approximately 200 – 400 µm) bilaterally symmetrical serotonin-like tracts were detected close to and parallel to the edge of the dorsal surface of the neuropile. These serotonin-immunoreactive profiles originated near the midline and were clearly seen to course for 80 – 150 µm along the dorsal edge, prior to branching laterally throughout the neuropile. Although no cell bodies were detectable on the dorsal surface, the tracts

Figure 21. Two sequential images of serotonin-immunoreactive labelled slices in the metathoracic ganglion.

(a) Although no cell bodies were discernible in the 4 slices viewed to determine successful specific labelling of serotonin processes, a large neurite was identified in the section 400 –500 µm from the anterior end of the ganglion. The process, labelled with a yellow asterisks, penetrated from a ventrolateral cluster of cell bodies into the neuropile lateral to the ventral median tract (VMT). The border of the neuropilar region is delineated by the dashed blue line. There is some auto-fluorescence of the sheath evident at the edge of the ganglion marked by the green dashed line. Faint labelling of serotonin-immunoreactive processes was evident in the neuropile dorsal to the median ventral tract (MVT). This arborisation of the serotonin-like immunoreactivity is considerably more obvious in the subsequent section shown in (b), highlighted by the yellow rectangle. Scale bar 110 µm.

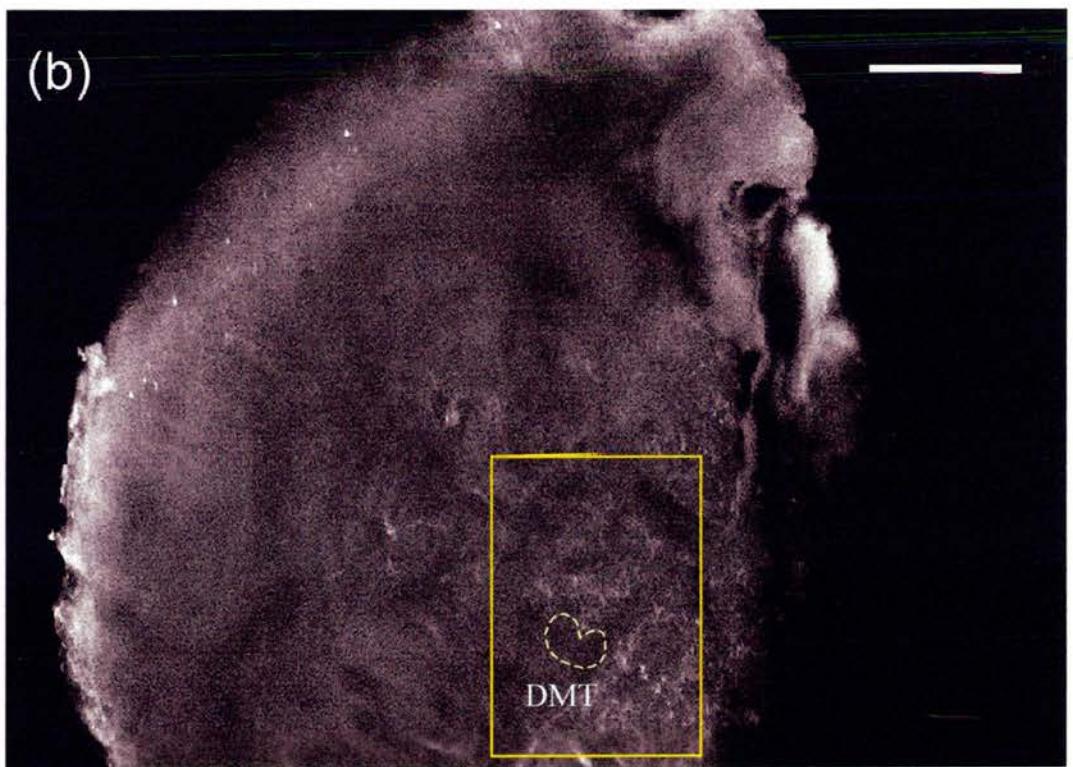
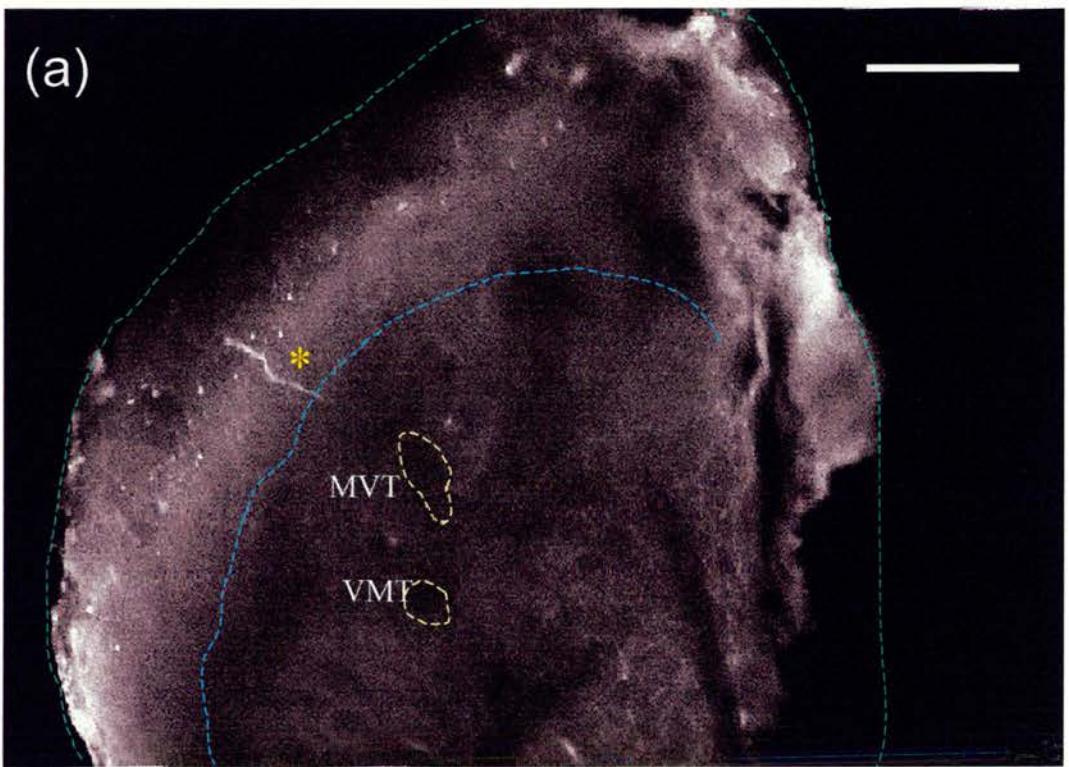
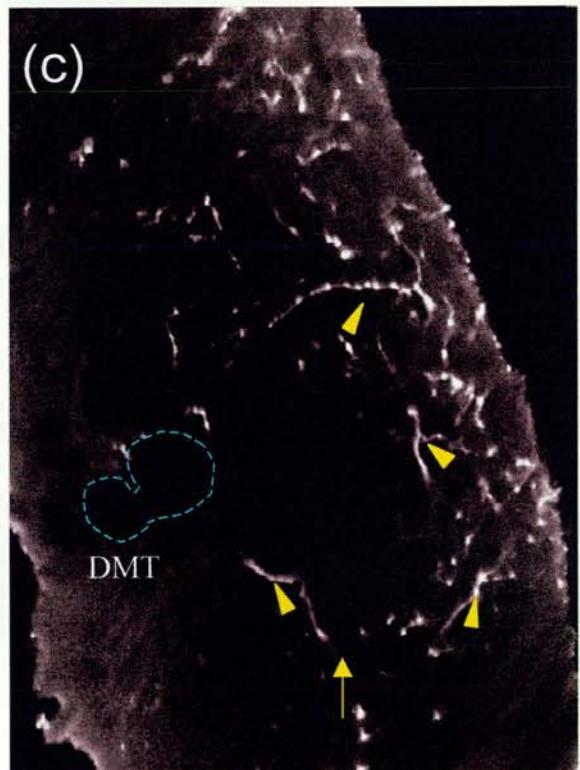
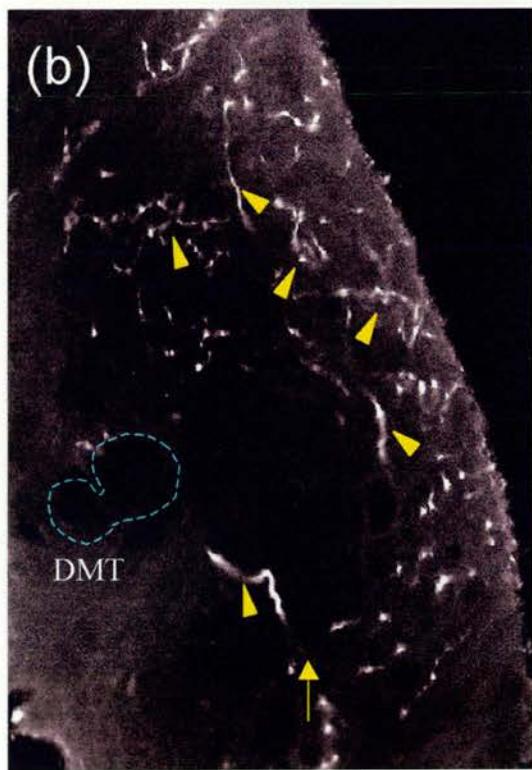
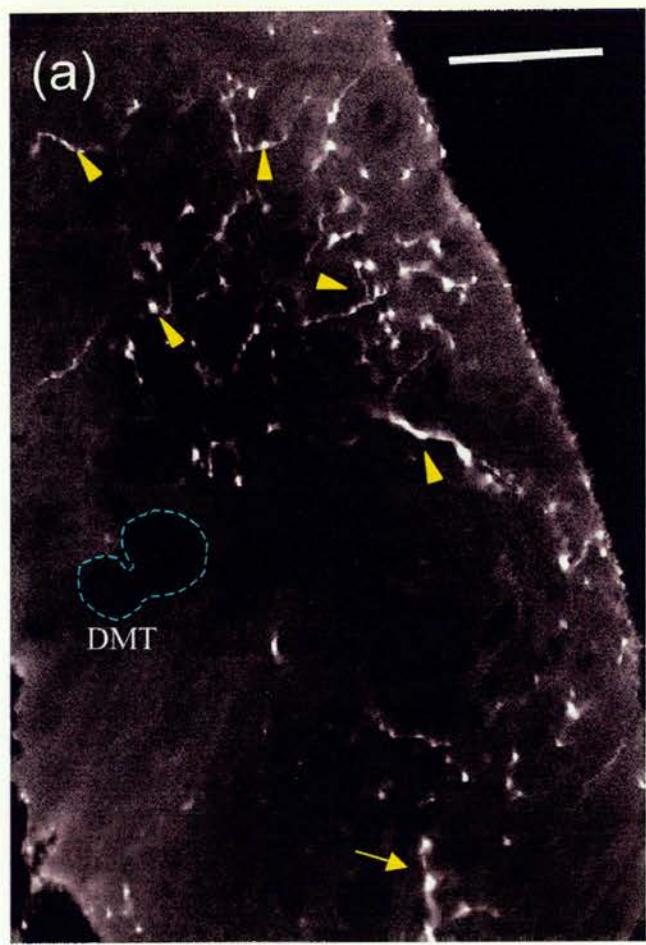


Figure 22. High-magnification view of serotonergic fibres in the dorsal area neuropile.

Three sequential images of the area of neuropile highlighted in figure 21b, illustrating the arborisation of a single serotonergic fibre local to the dorsal medial tract (DMT). The extensive arborisation, the principle branches marked by the arrowheads, stems from the large fibre lateral and dorsal to the DMT (yellow arrow). Varicosities are identifiable along a number of branches. Scale bar: 40 μm .



and branching pattern of the profiles are similar to those observed for dorsal unpaired median (DUM) neurones (*personal communication* Dr. R. Pitman), which have previously been shown to contain the amine octopamine (Dymond and Evans, 1979).

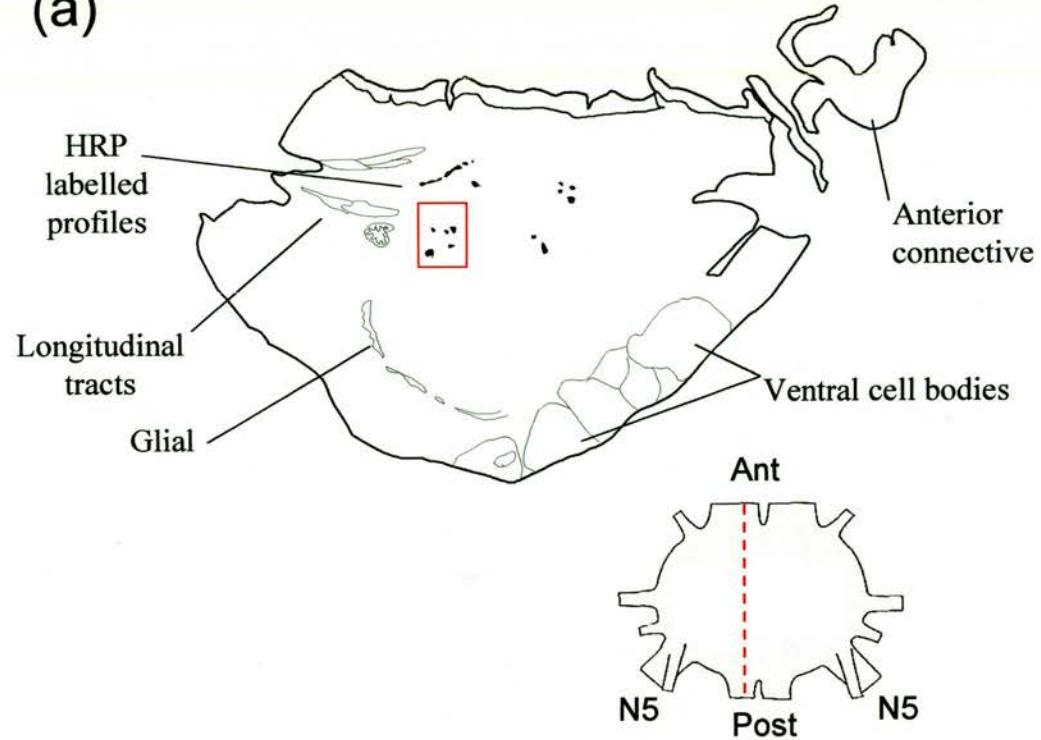
2.2 HRP labelling of the D_f motoneurone.

To ensure that the protocol for the peroxidase reaction worked correctly, 4 ganglia were injected with HRP and prepared as if for study under the electron microscope, without treatment with antibodies. The advantage of the peroxidase procedure is that the development protocol in which peroxidase is generated enzymatically by the action of glucose oxidase on D-β-glucose does not disrupt or obscure the fine structure of the tissue. Typical profiles containing the electron dense product of the diaminobenzidine (DAB) reaction are shown in figure 23. The profile highlighted by the white rectangle in figure 23b is shown enlarged in figure 23c. The distinction between labelled and unlabelled tissue is unequivocal, although not as distinct as that published previously by researchers using a similar protocol (see for example Watson, Burrow and Leitch, 1993; Leitch and Laurent, 1993). Uneven staining has previously been reported by Watson and Burrows (1981); the crystallisation of the DAB reaction being attributable to deficient cobalt-induced polymerisation. The only difference in the protocols for HRP labelling was that 0.5% cobalt chloride was used in this study, as opposed to 0.5 M quoted in previous publications for the polymerisation process. At the time, the HRP-labelling was considered distinctive enough to enable identification of D_f motoneurone processes.

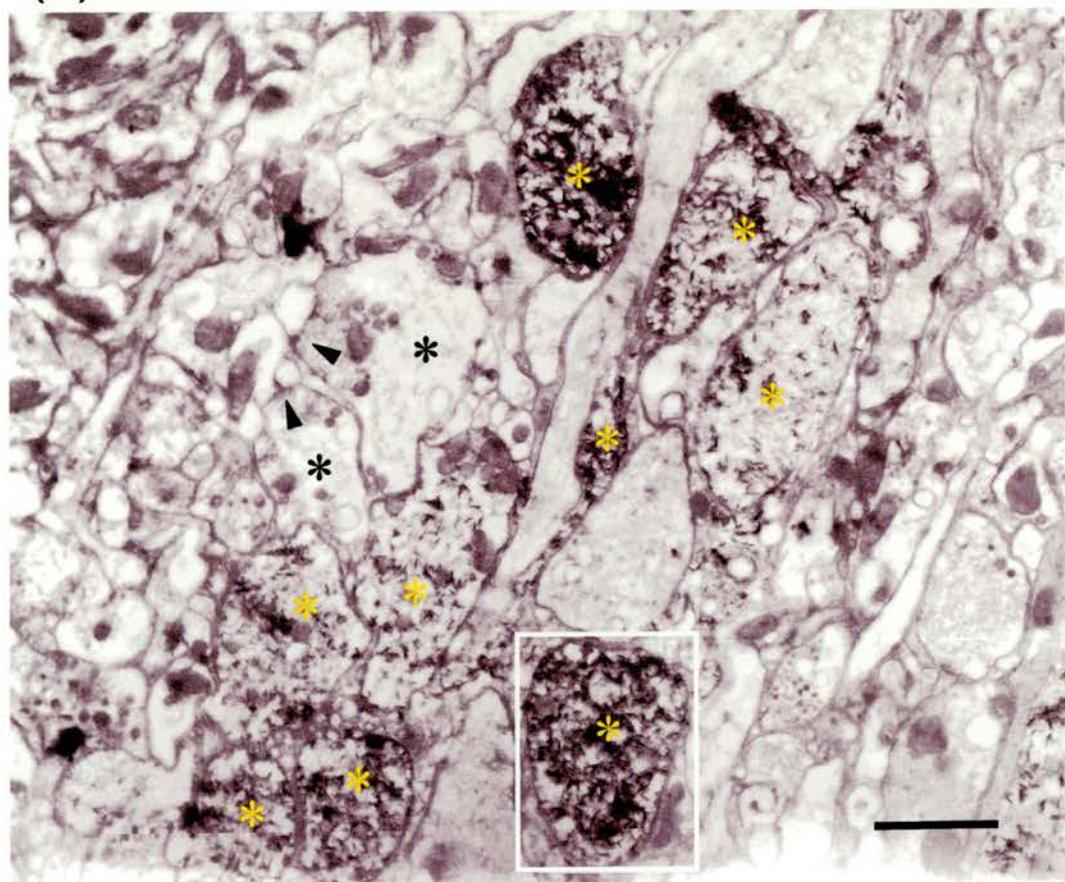
Figure 23. Horseradish peroxidase (HRP) labelling of the D_f motoneurone.

(a) A diagrammatic representation of a longitudinal section through the metathoracic ganglion, ventral surface downward. The principle landmarks were the anterior connectives (that were trimmed but not removed) and the large cell bodies proximal to the ventral surface. The sections shown are 76 µm from the cut midline of the ganglion, at which point HRP was detectable at the LM level toward the dorsal surface and located in distinct clusters. The area highlighted by the red rectangle in (a) is shown viewed under the electron microscope ×5900 in (b)(scale bar: 1.5 µm). A number of processes are labelled with electron dense peroxidase reaction product (yellow asterisks). The dense profile at the bottom of the picture, wrapped in a glial process and highlighted by the white rectangle, is shown under ×28000 magnification in (c)(scale bar: 0.75 µm). In general HRP labelling was very distinctive due to the electron dense product and only observed within membrane delimited profiles, the distinctive crystalline appearance is probably due to the lack of cobalt polymerisation. In the section shown in (b) and (c) there were no obvious synaptic connections onto the HRP filled D_f profiles. A number of dyadic synapses – where a single presynaptic terminal connects onto two postsynaptic profiles, are formed by the processes marked with the black asterisks (b). The synapses were identified by (1) the presence of vesicles, (2) presynaptic bar and (3) postsynaptic bar. Often presynaptic terminals also exhibited a large number of mitochondria.

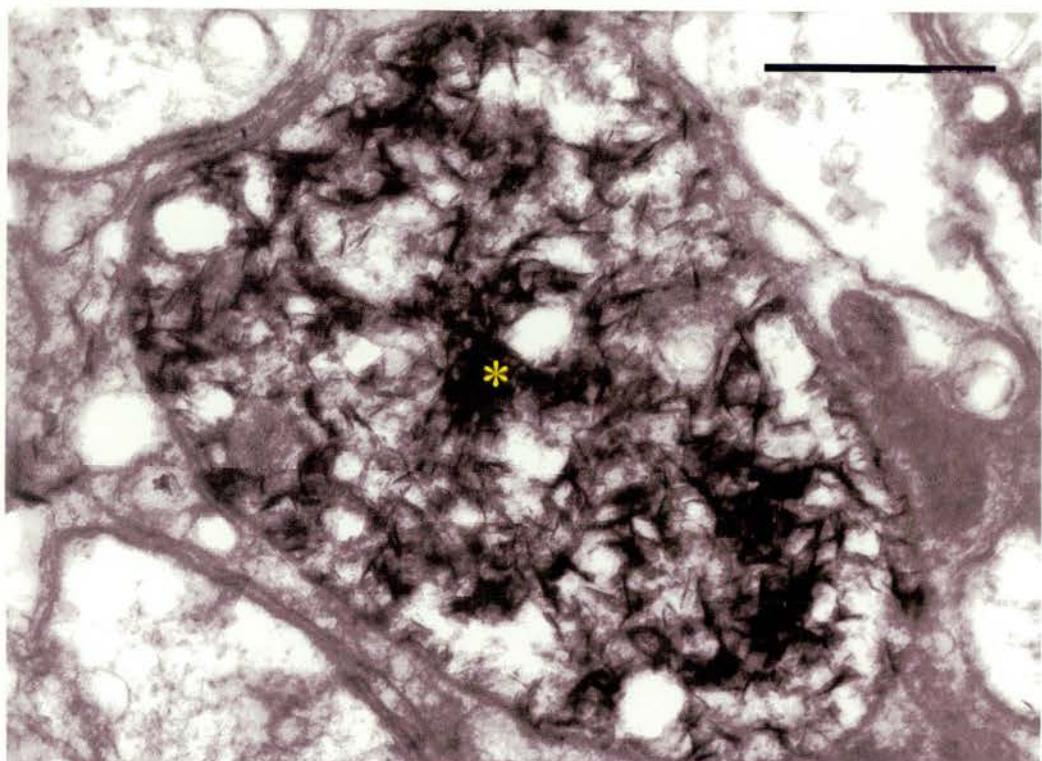
(a)



(b)



(c)



2.3 Ultrastructure of amine immuno-positive profiles.

The post-embedding gold labelling technique was effective at locating GABAergic neurones throughout the neuropile (figure 24a – 24c). Gold labelling was concentrated specifically over sections exhibiting characteristic associated with GABAergic immuno-positive neurones as previously identified in insect preparations (for example see Leitch and Laurent, 1993), in that they contained a high density of agranular pleomorphic vesicles along with a few larger granular vesicles.

Unfortunately the post-embedding technique was less effective for the dopamine antibody and failed to produce any staining with primary serotonin antibodies. Figure 25 shows typical dopaminergic profiles visible using the post-embedding antibody protocol. 10 nm gold particles labelled, at low density, specific tracts in the posterior dorsal region of the neuropile. Figure 25a shows this in the form of a diagram of a toluidine blue stained section. These ribbon-like tracts were in close proximity to and surrounded by a number of smaller neuronal processes, some which were stained with HRP (figure 25b). However no obvious signs of synaptic contact i.e. presynaptic densities, postsynaptic membrane thickening or vesicles, were evident.

Since immuno-positive profiles are clearly visible and abundant throughout the ganglion when viewed under the confocal microscope, tissue was labelled with antibodies prior to embedding in araldite. One explanation for the poor labelling seen using the post-embedding protocol could be that the antigenicity of profiles could be masked by the fixation process and preparation of the tissue for electron microscopy. Incubation with the antibodies prior to osmification might help to increase labelling. This approach resulted in a increase in the number of processes labelled with both amine antibodies. By grouping of vibratome slices into distinct areas of the ganglion (termed anterior, medial and posterior), the distribution of profiles could be

Figure 24. GABA immuno-positive profiles labelled using the post-embedding antibody protocol.

The post-embedding protocol for applying neurotransmitter antibodies was successful for labelling GABAergic processes. (a) Shows two adjacent GABAergic profiles marked with yellow asterisks adjacent to and ringed by a number of lightly HRP-stained D_f motoneurone profiles (black asterisks; scale bar 1 µm). (b) Enlargement ($\times 36000$; scale bar 0.5 µm) of area shown in the black rectangle in (a) to illustrate more clearly the 10 nm gold labelling. A putative monodic synapse – where a single presynaptic element synapses onto single post-synaptic profile, with an HRP-stained D_f process is marked with a white arrow. The majority of GABAergic central synapses previously identified in insects involve a postsynaptic dyad (see for example Leitch and Laurent, 1996) similar in structure to those visible in figure 23b. However monodic synapses similar to that identified here are not uncommon (Watson, Burrows and Leitch, 1993). The GABAergic profiles were of a distinctive appearance containing a high density of agranular pleomorphic vesicles and a smaller number of larger (100 nm) granular vesicles. This is more clearly seen in the profile from another section shown in (c). Both types of vesicle are clearly visible in the GABAergic process (yellow asterisk; scale bar: 0.5 µm). An example of the large dense vesicles is indicated by the white arrowhead, whilst a cluster of the smaller agranular vesicles highlighted by the black arrowhead. Similar identifying features were observed in all GABAergic terminals.

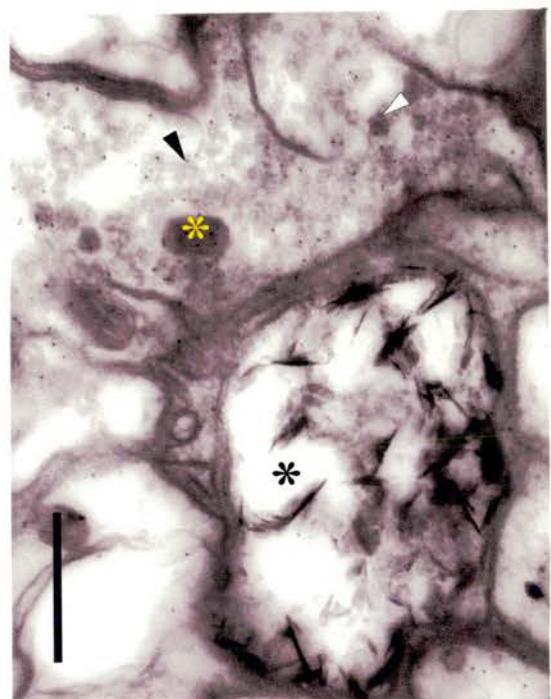
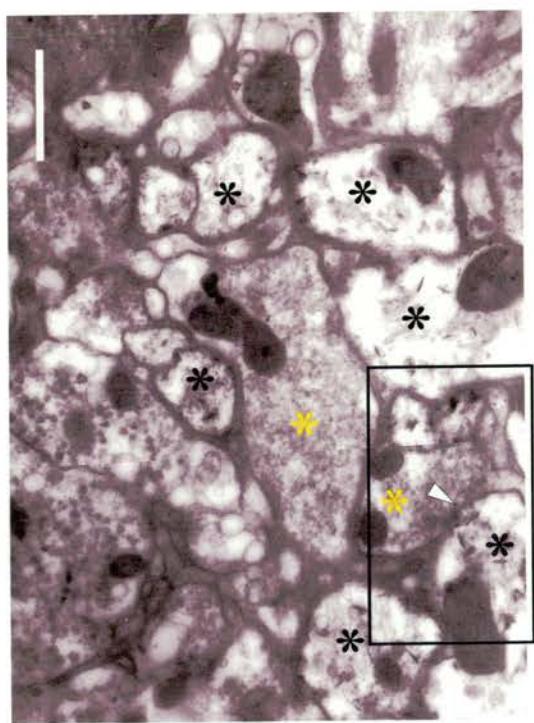
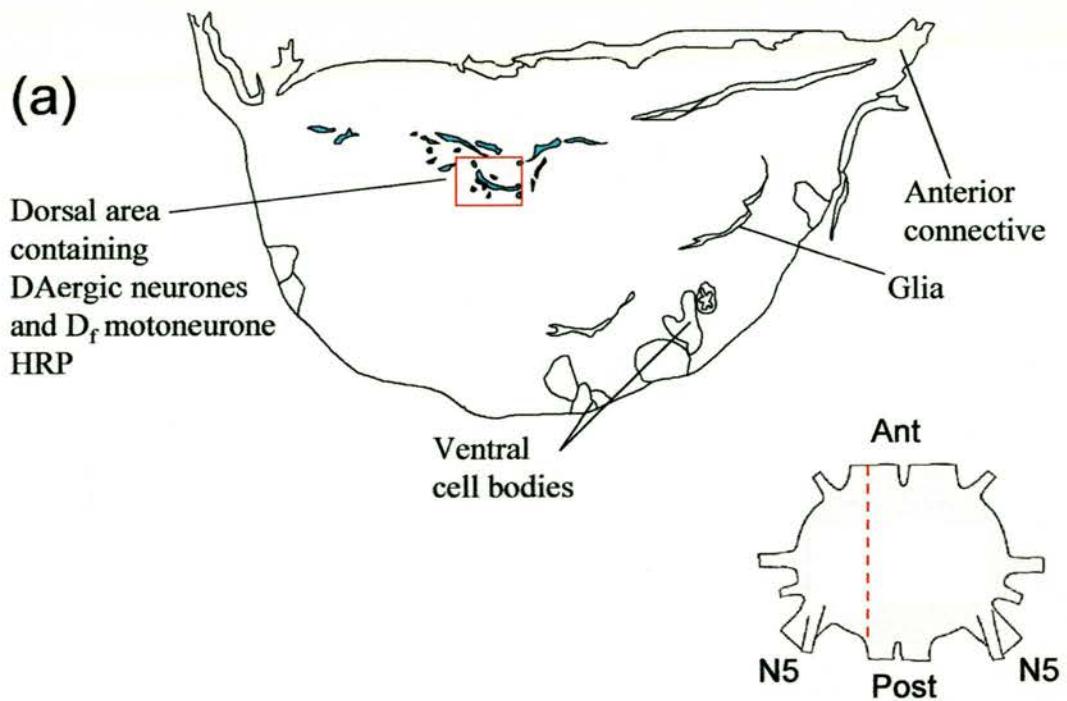


Figure 25. Identification of dopaminergic profiles close to HRP labelled D_f motoneurone processes.

Antibody labelling of aminergic profiles using the post-embedding protocol was not as successful as that observed for GABAergic neurones. (a) A diagram of a toluidine blue stained 0.5 µm microtome section showing the location of the dopamine labelled process shown in (b) marked with a red rectangle.

(b) A typical dopamine immunoreactive processes labelled using the post-embedding protocol (black asterisk). Gold-labelling was less dense than that seen for GABAergic processes, and was localised to a number of large ribbon-like dopaminergic tracts. These did not form any recognisable synaptic contact with the neuronal processes that they surrounded, a number of which included HRP stained D_f profiles one of which is labelled with the yellow asterisk ($\times 59000$; scale bar: 0.1 µm).



(b)



established, and the extent of their arborisation reconstructed. In the serotonin labelled sections the immuno-reactive profiles were found in specific areas of the neuropile; the main bulk were grouped around the dorsal tracts with a second cluster of immuno-positive profiles adjacent to the glial bordering the neuropile near the ventral surface of the ganglion (figure 26a). What was immediately noticeable was the considerable number of large serotonin immuno-positive processes in the medial and posterior (caudal) transverse vibratome slices (see figure 26b). These large processes did not form many recognisable output synapses onto neural processes nor receive a large number of inputs. Smaller serotonergic profiles and putative presynaptic terminals were located principally throughout the medial vibratome sections (figure 26c and 26d). Histologically they were similar to the larger tracts previously identified, exhibiting clear cytoplasm with distinct localisations of mitochondria and vesicles of dense content (70 – 90 nm) as well as numerous smaller (30 – 40 nm) clear vesicles. The gold immunoreaction, although concentrated in the areas containing vesicles (see for example 26b and 26d), were not preferentially associated with either vesicle type. Similar serotonergic profiles, exhibiting more than one type of vesicle, have been reported for serotonin process in the blowfly *Calliphora erythrocephala* (Nässel and Elekes, 1984), cricket (Elekes, Hustert and Geffard, 1987), and moth *Manduca sexta* (Sun, Tolbert and Hildebrand, 1993). Putative presynaptic sites were identified according to the presence of vesicles, a presynaptic bar and postsynaptic thickening of the membrane.

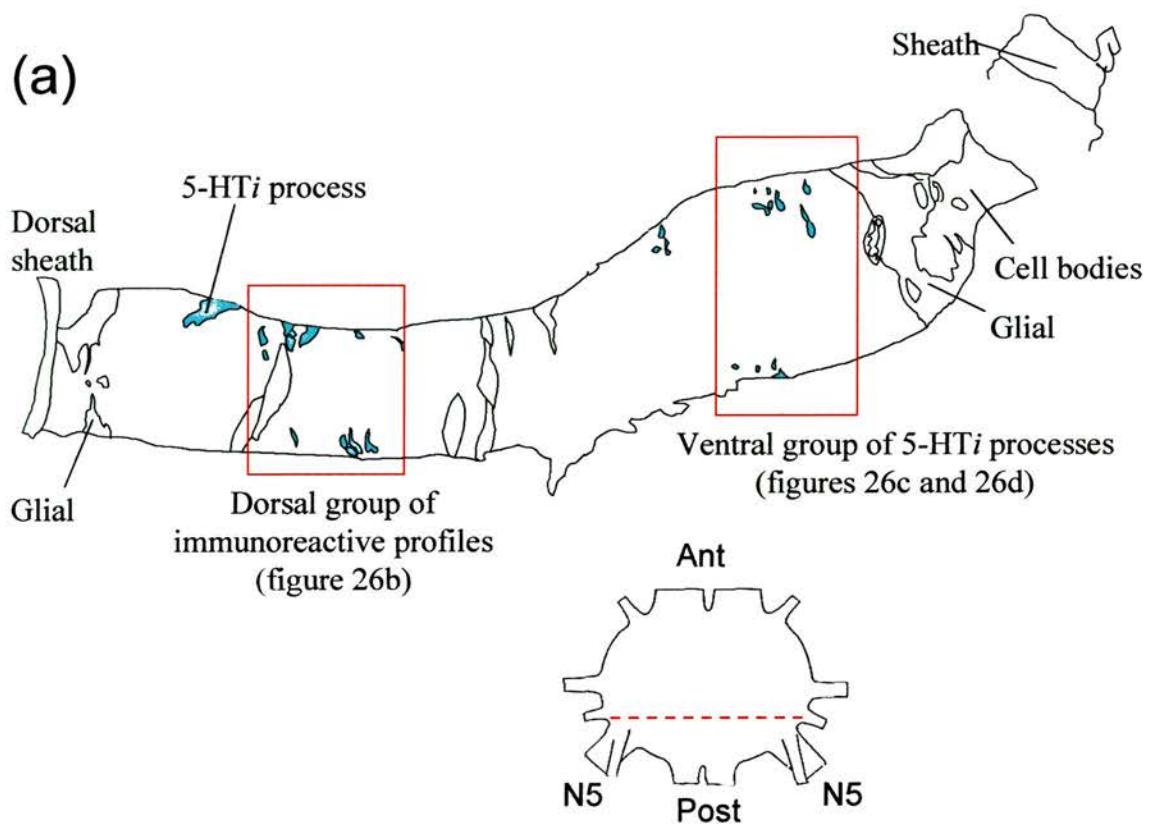
Of the slices labelled with the dopamine conjugated antibodies using the pre-embedding technique, only medial vibratome sections were cut on the microtome for viewing under the electron microscope, as these exhibited extensive dopamine-like arborisation detected using the secondary rhodamine antibody. Gold labelling of

Figure 26a and 26b. Identification of large diameter serotonin profiles in the posterior dorsal sections using the pre-embedding antibody protocol.

(a) A diagram of a toluidine blue-stained section from a vibratome slice approximately 450 - 500 μm from the rostral end of the ganglion. Due to the poor penetration of the antibody into the tissue only the superficial $\sim 8 \mu\text{M}$ were labelled with gold-conjugated antibody (see also Sun, Tolbert and Hildebrand, 1993). The dorsal areas of the more posterior vibratome slice contained an extensive network of large ($2 - 4 \mu\text{m}$ width) serotonin immuno-positive fibres, one of which is shown under the EM in (b).

The profile exhibited in (b) is heavily stained with 1 nm gold antibodies silver enhanced for 20 minutes. The process of enhancement often resulted in electron dense particles of varying size and shape. Serotonin fibres usually contained distinct vesicle clustering associated mitochondria (marked with the yellow asterisks). Two smaller neuronal processes (black arrowheads) formed synaptic contacts onto the serotonergic profile adjacent to a putative output synapse (indicated by the white arrowhead)($\times 7500$; scale bar $1 \mu\text{m}$).

(a)



(b)

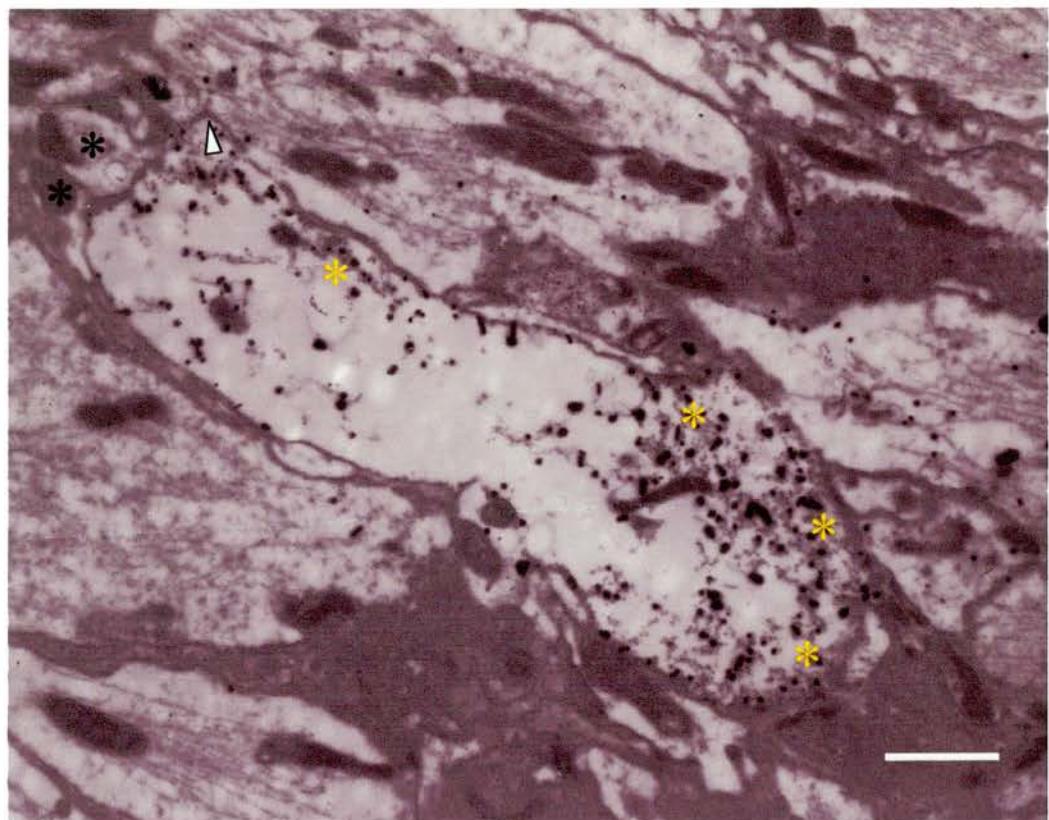


Figure 26c and 26d. Smaller serotonin-immunoreactive profiles are evident in the more medial sections of the neuropile.

Serotonin immunoreactive profiles in the medial (both dorsal and ventral) sections of the neuropile were of smaller diameter than the larger profiles detected in the more posterior and dorsal sections. The smaller serotonergic profiles, such as those shown in figure (c) and (d)(yellow asterisks), form a large number of output synapses (indicated by white arrowheads) predominantly onto postsynaptic profiles with clear cytoplasm. The presynaptic sites contain numerous mitochondria, a large number of small vesicles (approximately 30 - 40 nm in diameter) as well as a few larger granular vesicles. The immuno-positive cell marked with a single yellow asterisk is shown in both (c) and (d) ($\times 18000$; scale bar 0.5 μm). The silver enhancement process did not uniformly increase the size of the 1 nm gold particles, the majority not enlarged after 20 minutes in the silver processing.

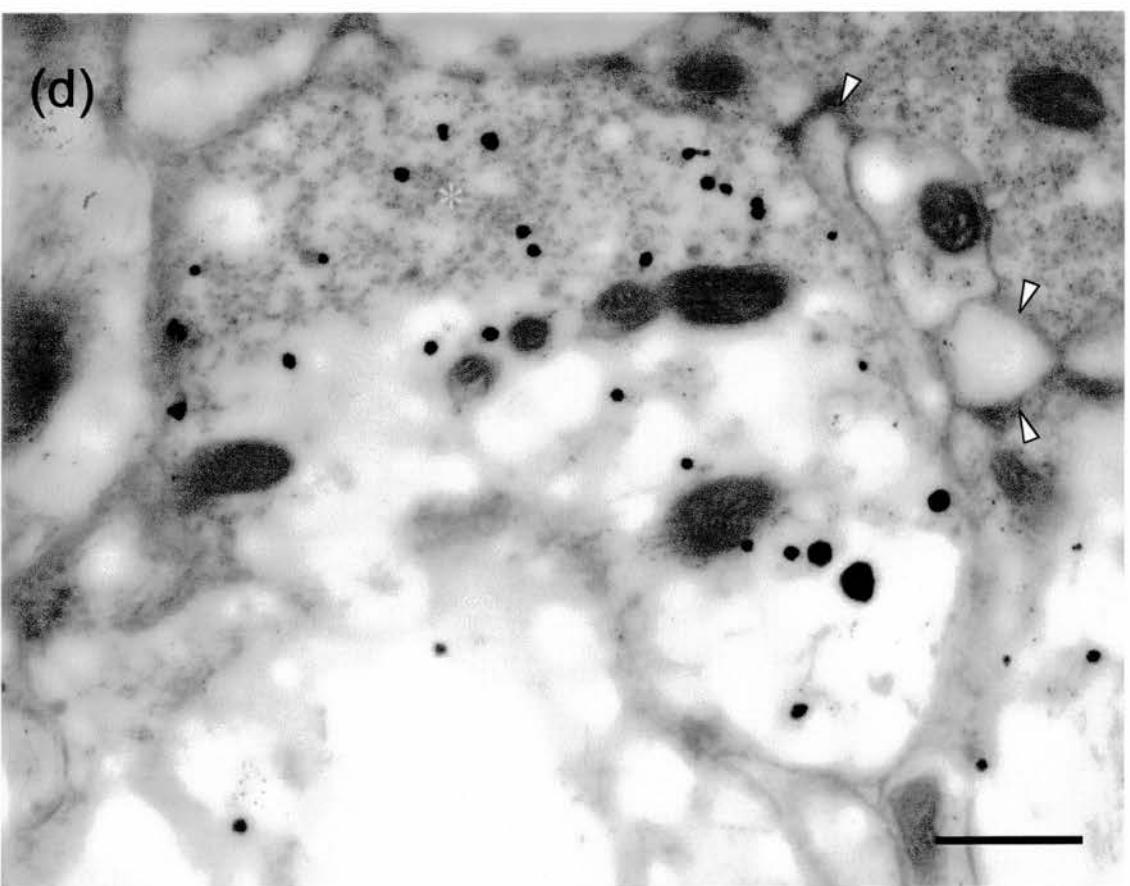
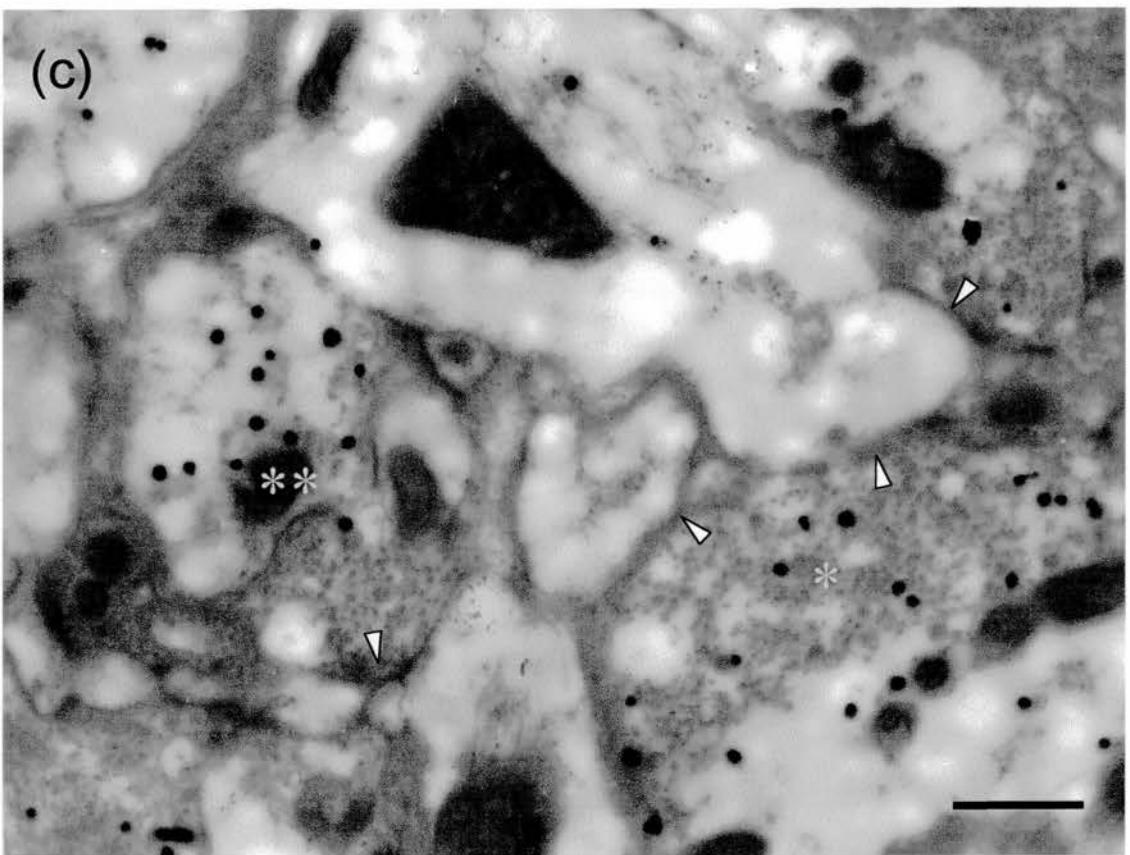
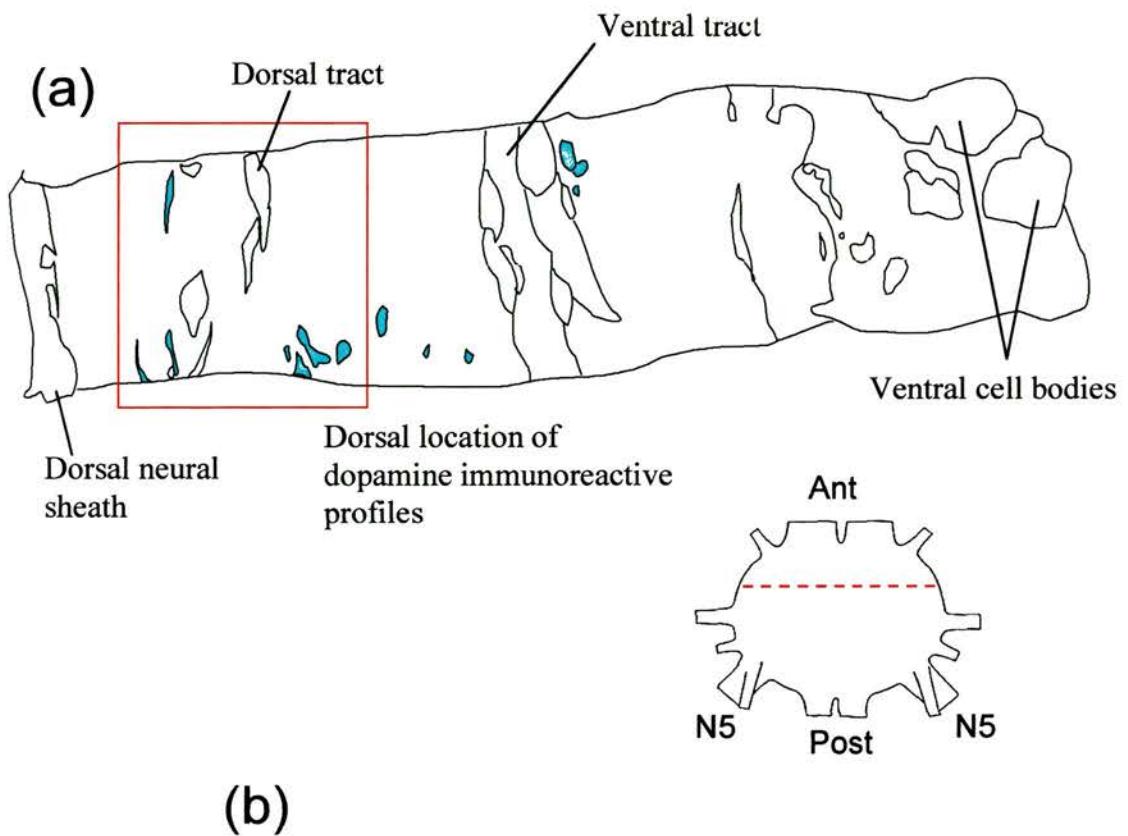
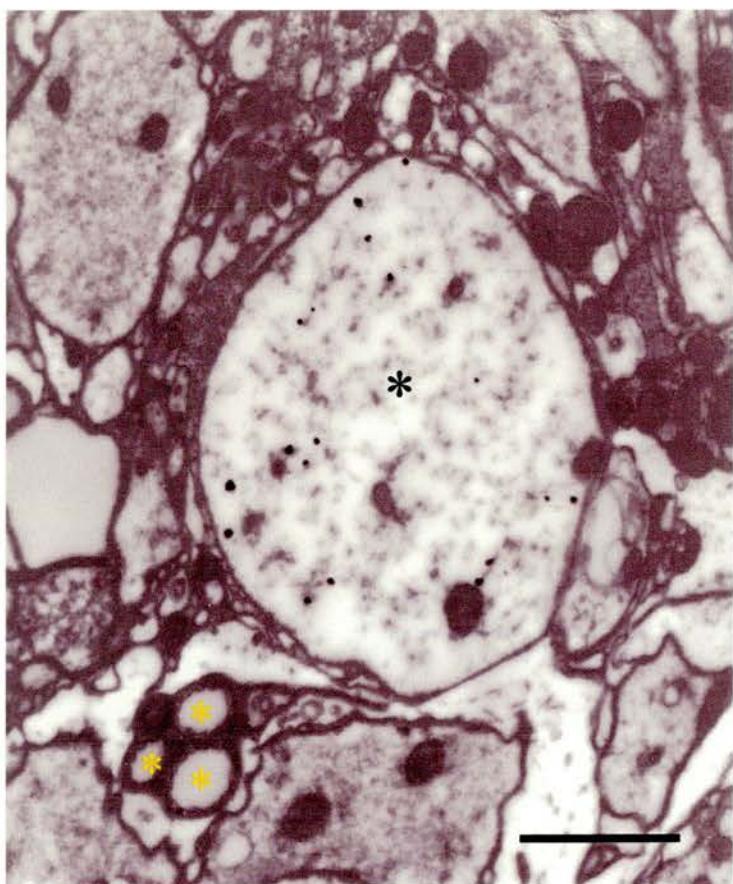


Figure 27. Dopamine-like immunoreactive processes, labelled using the pre-embedding technique, in the medial section of the thoracic ganglion.

Dopamine processes were detected at the ultrastructural level in the medial transverse section viewed (approximately 200 – 300 µm from the anterior end of the ganglion). The toluidine-stained LM section in (a) shows a predominantly dorsal distribution of dopamine-like profiles, the main areas highlighted by the rectangles. Viewed under the electron microscope a number of large profiles of approximately 2 – 5 µm can be seen, an example of which is shown in (b) (black asterisk; ×9800, scale bar 1.5 µm). The mitochondria and vesicles were not associated with any specialisations unlike those observed for serotonin immuno-positive profiles. A number of tracheoles are labelled with yellow asterisks.



(b)



dopamine profiles using the pre-embedding technique was not as obvious as that for the serotonin-labelled sections. The process resulted in the identification of a number of immuno-positive profiles near the dorsal tracts (figure 27) that were similar in morphology to those previously detected using the post-embedding protocol shown in figure 25b. Although the profiles contain a number of dense vesicles (30 – 40 nm in diameter) and mitochondria these were not obviously associated with synaptic specialisations.

2.4 Immunoreactive profiles are present local to the D_f motoneurone.

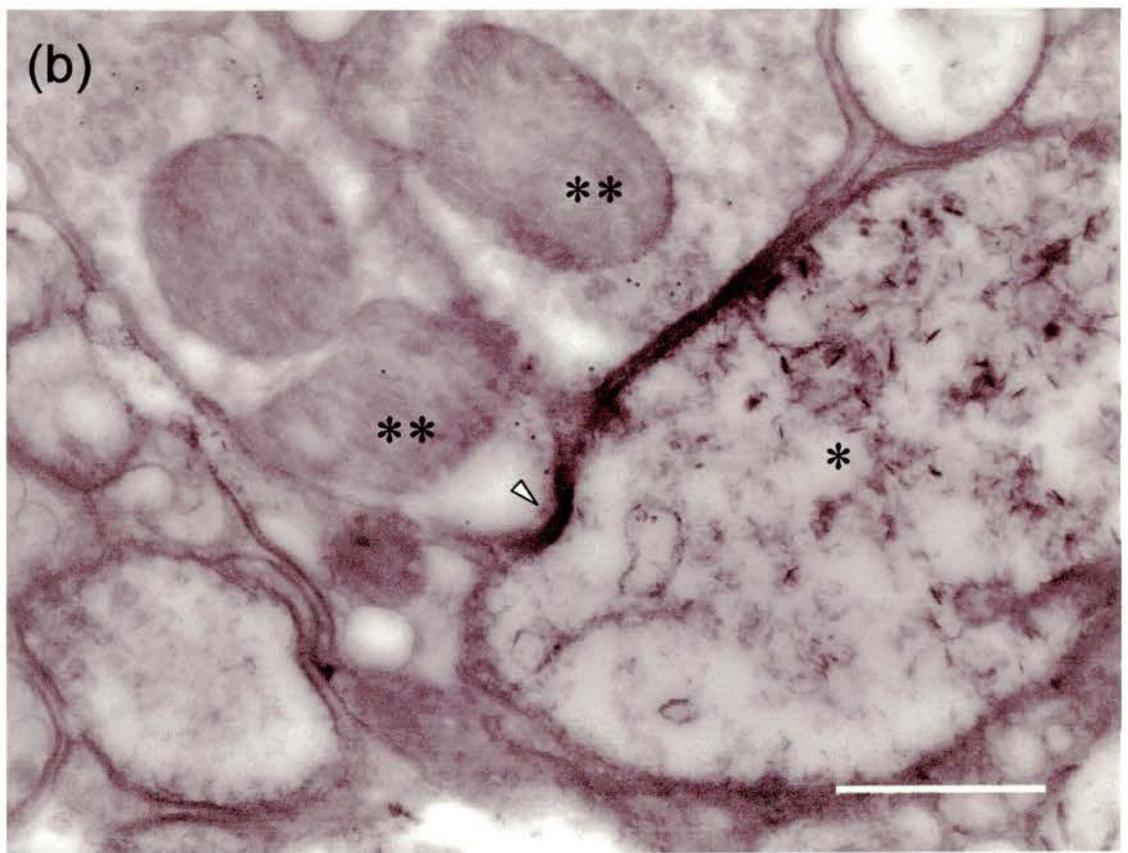
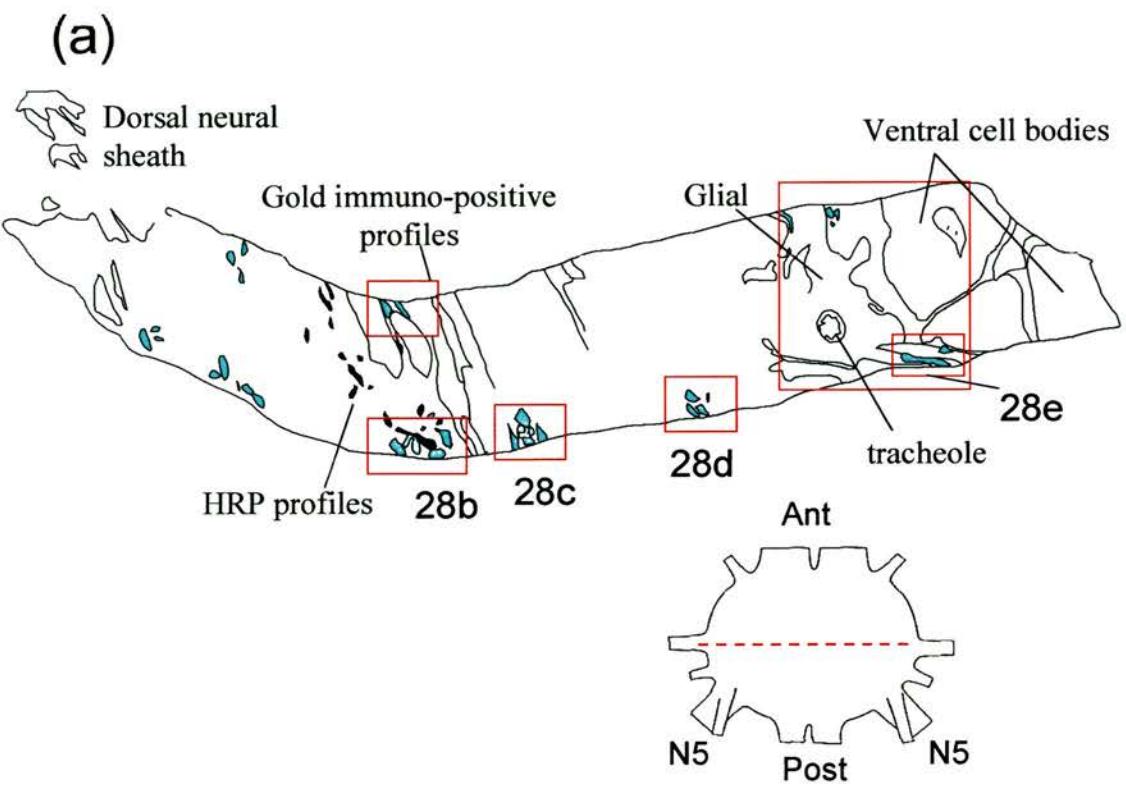
Having established that the protocols for the peroxidase reaction and the antibodies both effectively labelled tissue in the cockroach, the processes were combined to identify whether aminergic terminals form synapses directly onto the D_f motoneurone or on other neurones in its immediate vicinity. The location of HRP staining and amine antibody labelling in the dorsal neuropile in medial and posterior vibratome sections makes this a strong likelihood. Serotonergic processes were detected throughout the neuropile in the transverse vibratome slices 350 – 400 µM from the anterior end of the ganglion (as shown in the diagram in figure 28a). Figure 28b shows two serotonin immunoreactive processes (double asterisks) local to an HRP filled D_f profile. The putative serotonergic presynaptic sites contained the two types of vesicle types previously identified (see figure 26): large granular vesicles (~70 nm in diameter) and a large number of smaller clear vesicles (~30 nm in diameter), in close association with the membrane bordering onto the D_f profile. There was also a discernible thickening of the postsynaptic membrane associated with synaptic sites. In a number of the ultrathin sections there was an obvious association of the immuno-gold particles with the membranes of large pleomorphic vesicles, approximately 200

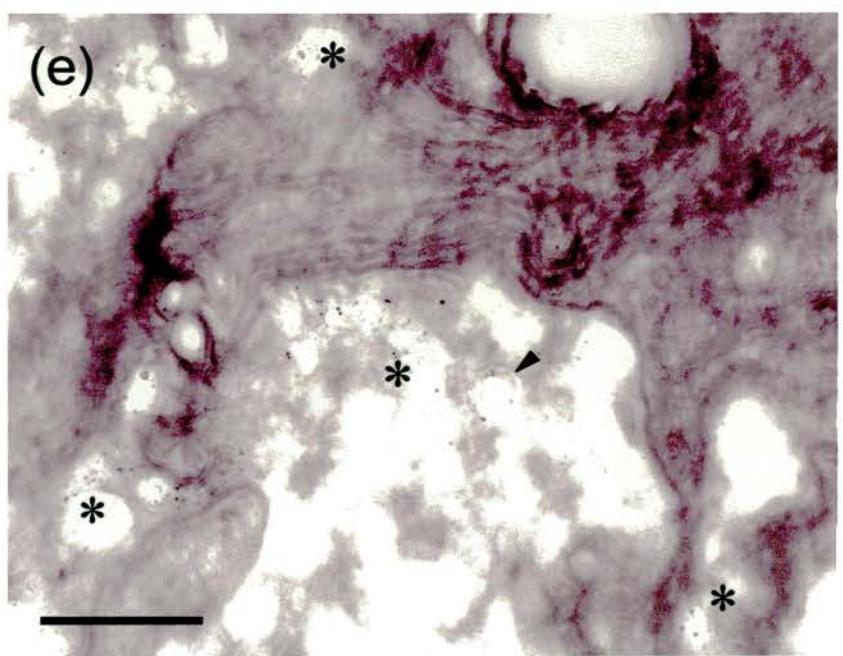
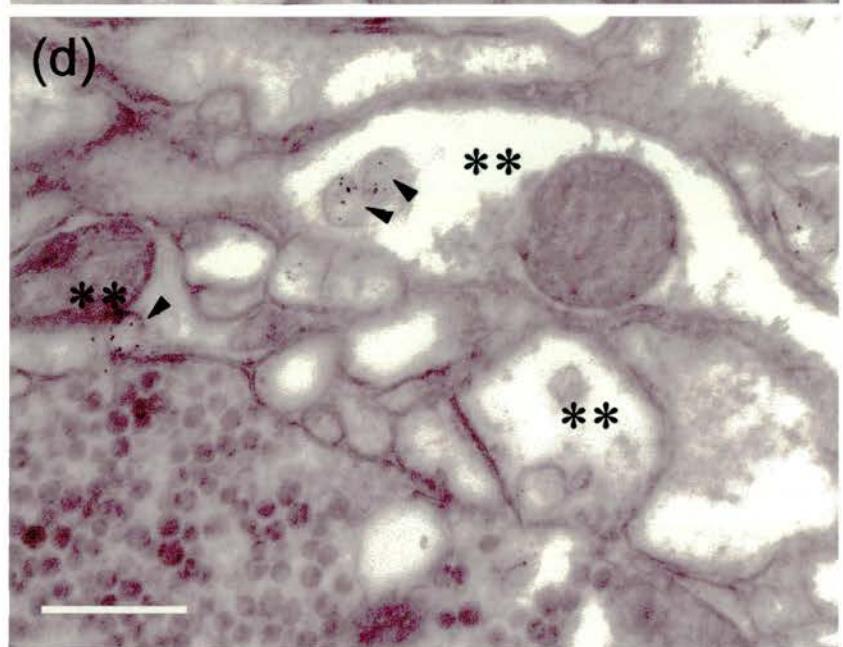
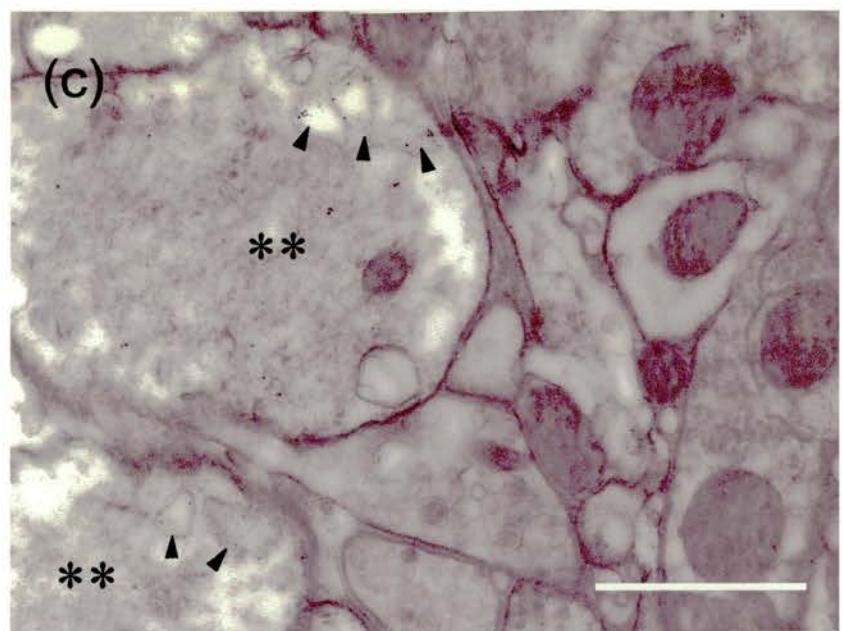
Figure 28. Serotonin immunoreactive profiles local to HRP stained D_f motoneurone profiles.

(a) Serotonin-immunoreactive profiles (turquoise profiles) were found extensively in the vibratome section 350 – 400 µm from the rostral end of the section. HRP staining (black profiles) of the D_f motoneurone and were confined to the dorsal section. The gold labelled profiles close to HRP-stained processes (for example (b), double black asterisks) were similar in appearance to those previously determined using immuno-gold labelling at the ultrastructural level (see figure 26c and 26d). They contained a mix of clear and granular vesicles in clusters often associated with a number of mitochondria. A monodic synapse onto the HRP filled profile is indicated by the white arrow (figure 28b: scale bar = 0.5 µm).

(c) (scale bar: 1 µm) and (d) (scale bar: 0.5 µm) are representative of serotonin immunoreactive profiles in the vibratome section viewed at the EM level. Both contained specific 1nm gold labelling, often concentrated around the profiles of large clear vesicles (~ 200 nm in diameter) as highlighted by the black arrowheads. Granular and clear pleomorphic vesicles were also evident in the immuno-positive profiles.

(e) A number of profiles were gold labelled for serotonin immunoreactivity in the ventral and dorsal areas of the neuropile immediately adjacent to the border of the neuropilar region (scale bar: 0.5 µm). This area (labelled by the red rectangles toward the ventral surface in (a) was characterised by large numbers of glial cells. The glial were clearly distinguished from neural process due to the electron dense nature of their cytoplasms, which contained extensive smooth endoplasmic reticulum. The serotonergic profiles did not form recognisable synapses onto glial or neural processes in this region but did contain both large diameter clear vesicles (as indicated by the black arrow) such as those identified in figures (c) and (d) and a number of smaller (~70 nm) granular vesicles.





nm in diameter (see figure 28c and 28d, highlighted by the black arrows). These large vesicles were not associated with presynaptic sites.

The evidence suggests that dopaminergic and serotonergic neuronal processes ramify extensively throughout the neuropile of the thoracic ganglion of *Periplaneta americana*. Furthermore neurones immuno-positive for both amines have concentrated arborisations in the dorsal areas of the neuropile, lateral to the dorsal medial tract in the region occupied by the arborisations of the D_f motoneurone. At the ultrastructural level it is evident that serotonergic neurones exhibit putative synaptic sites onto neural processes in this region. Dopamine immunoreactive cells did not exhibit such specialisations, ribbon-like tracts instead ramifying through the neuropile. There is no direct evidence that dopamine-containing neurones form connections onto the D_f motoneurone, however the close proximity of their arborisations would suggest that such connections are likely to occur. A number of putative serotonergic synapses were identified adjacent to D_f processes at sites in the dorsal neuropile, providing evidence to suggest that this motoneurone could receive serotonergic inputs *in vivo*. Alternatively the monoamines could be released extra-synaptically and exert more global effects on the surrounding neurones similar to that postulated by Descarries and Umbriaco (1995) to occur in the rat CNS.

3 Identification of the receptor site involved in amine-mediated modulation.

The fact that all three amines exert the same modulatory action on the nicotinic acetylcholine response of D_f is contrary to previous reports of their action in *Periplaneta americana*. Therefore it was key to establish whether each was capable of suppressing ACh responses by acting upon their own separate site or whether they act upon a common receptor target. To determine this, amines were co-applied to the preparation; if the effect of individual amines upon ACh responses are additive, this would imply that independent receptors are involved. On the other hand, if the action of one amine occludes that of another, the implication is that the two amines must either act upon the same receptor or share signalling pathways that interact.

Co-application experiments were recorded using the voltage clamp technique (holding potential of -80 mV). 20 µl aliquots of the amines were applied to the bath in rapid succession. Only experiments that successfully reversed on washout were used to compile the data (for example see figure 29). To ensure that the cells exhibited ‘typical’ aminergic responses in line with the action of the amines previously determined (see figure 14a), a control application of one of the amines was conducted after the initial co-application test.

Figure 29a and 29b show the effect of applying either dopamine (10^{-4} M) or octopamine (10^{-4} M) at the same time as serotonin (10^{-4} M). Co-application of these amines produced a suppression of nicotinic ACh currents (54.0% +s.e.m. 4.2, -s.e.m. 4.1; n = 4 and 60.1% +s.e.m. 2.8, -s.e.m. 3.0; n = 5 respectively) that was not significantly different from that observed when serotonin was applied alone. Exposure of D_f to all three amines simultaneously produced no significant additional suppression of ACh currents over that of observed when serotonin alone was applied, reducing the ACh response to 45.6% (+s.e.m. 6.0, -s.e.m. 5.9; n = 4). Expanded

Figure 29. Co-application of monoamines under voltage clamp conditions.

Co-application of 10^{-4} M dopamine (a) or 10^{-4} M octopamine (b) with serotonin to cells voltage clamped at a holding potential of -80 mV resulted in a suppression of nicotinic ACh currents that was not significantly different to the effect of serotonin alone. Both traces are taken from the same preparation, in which the inward currents were elicited by 500 ms duration pressure pulses of 10^{-1} M ACh. The effects of the monoamines were reversed by 10 – 15 minute periods of perfusion with fresh saline.

20 μ l aliquots of all three amines were applied at the same time to another preparation under identical voltage clamp conditions (c). There was a rapid reduction in the amplitude of the nAChR-mediated inward currents that reached a maximum suppression after 7 minutes. Commencement of washout with fresh saline resulted in perturbation of the baseline which gradually stabilised. The preparation was washed for 15 minutes, after which the amplitude of the currents had reversed to control levels (as shown in the right-hand panel). The nicotinic inward currents marked with asterisks are shown expanded in figure 30.

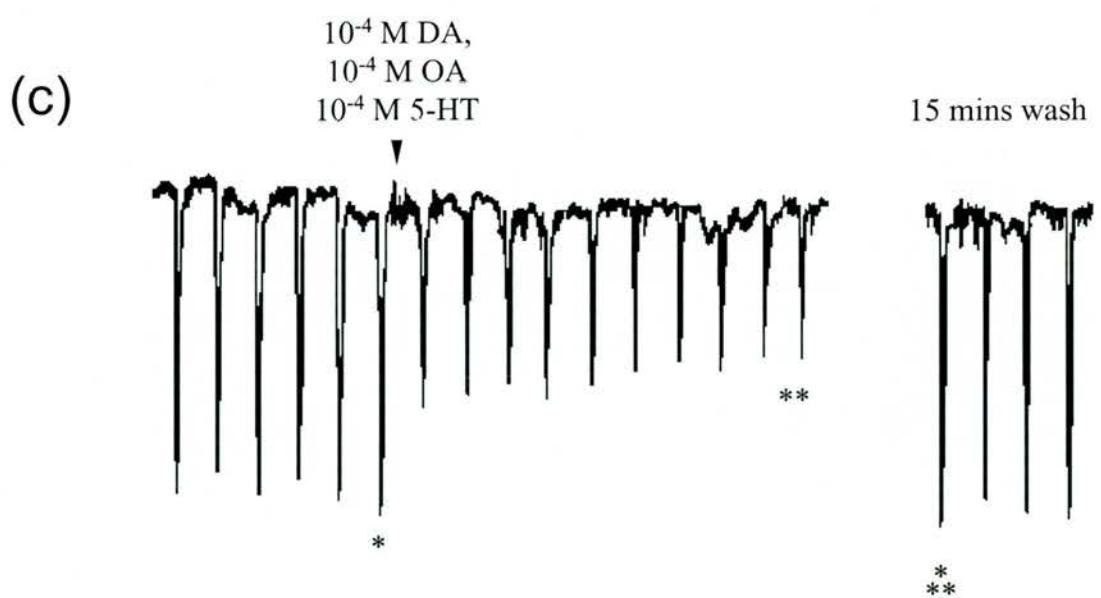
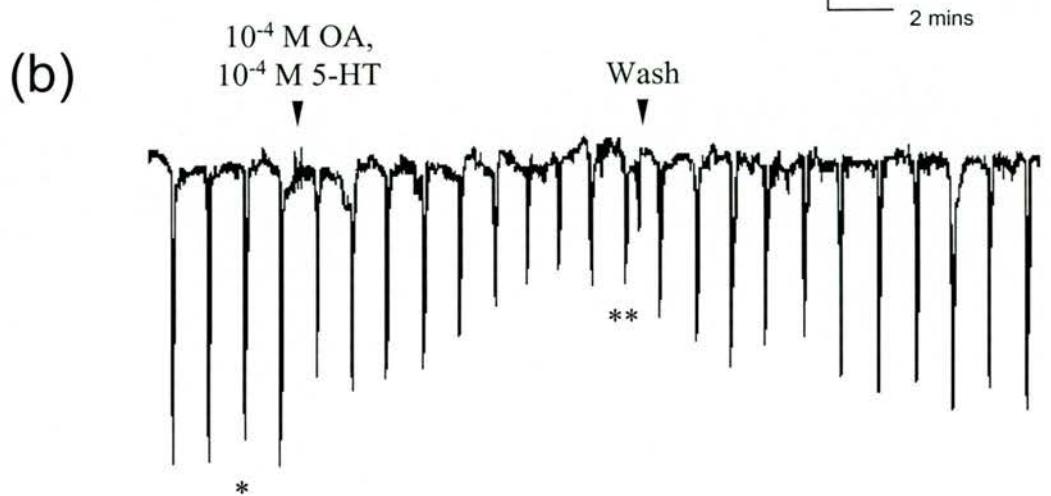
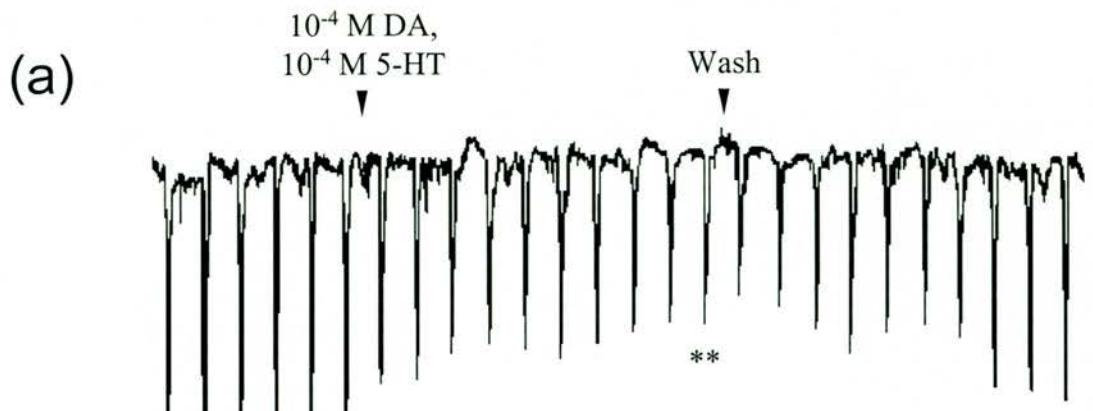
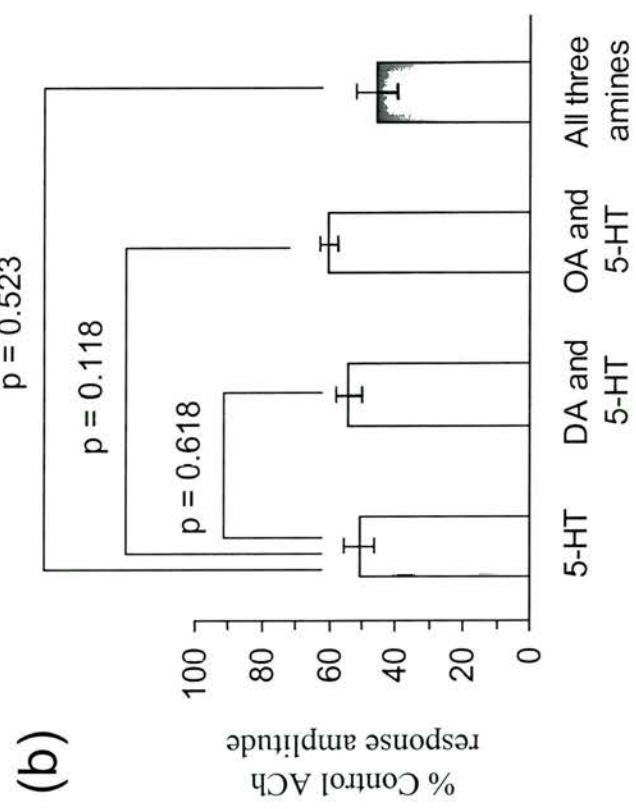
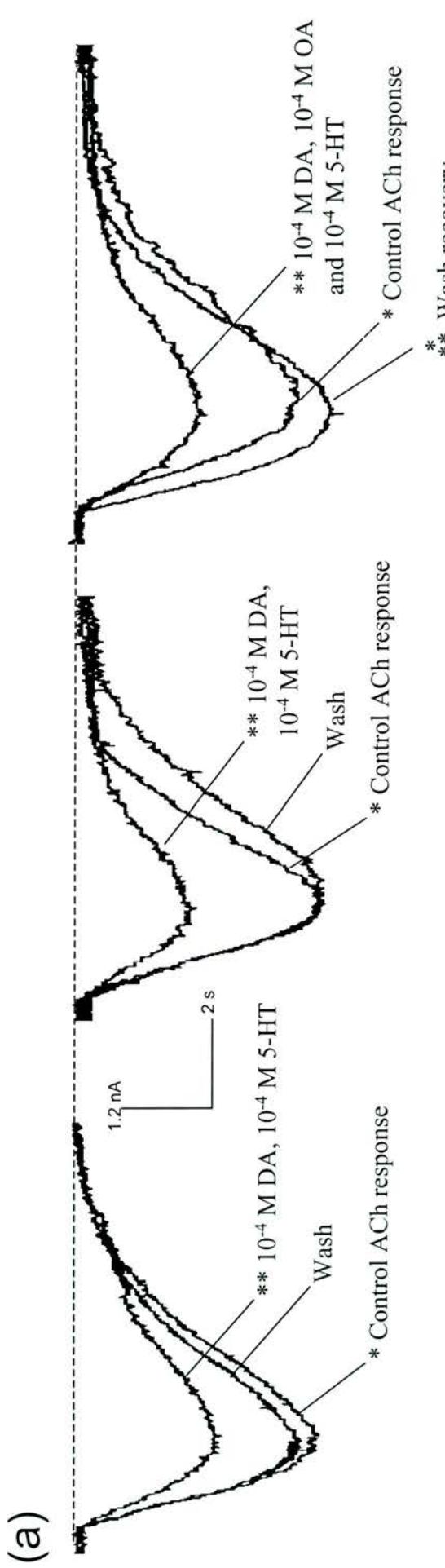


Figure 30. Monoamines do not modulate nACh currents in an additive manner.

The three panels (a) show the expanded individual ACh currents marked with asterisks from figures 29a, 29b and 29c. Pooled data from co-application experiments (b) reveal that no combination of the amines significantly reduces ACh currents when compared to the action of serotonin alone.



traces from the traces in figure 29 are shown in figure 30a and a histogram of the pooled data exhibited in figure 30b. It must be noted that concentrations of 10^{-4} M amine do not represent saturating doses of the agonists (see figure 14b), and therefore, although these observations are consistent with a conclusion that all three amine act upon the same receptor site or via the same second messenger pathway, their interaction is unlikely to be straight forward. This could involve either variability in the efficacy and selectivity of ligand binding such as that determined for vertebrate 5-HT₂ receptors (Wang, Gallaher and Shih, 1993; Almaula *et al.*, 1996) and, or signal transduction (for example, see Reale *et al.*, 1997). This issue will be covered in more depth in the discussion section. Co-application of amines at bath concentrations of 10^{-3} M or higher resulted in prolonged inward currents independent of the transient ACh responses, this change in the resting membrane current distorted analysis of the results.

3.1 Amines do not competitively inhibit binding of ACh to nicotinic AChRs.

The amines could act at variety of sites to modulate ACh transmission at nicotinic AChRs. Firstly they could compete directly for the ACh binding site of AChRs, the amines acting as classical nicotinic antagonists. Alternatively, they could act at extracellular sites on the nAChR independent of the ACh binding pocket and allosterically inhibit signal transduction. Thirdly, they could enter the channel mouth and block ion flow through the nicotinic AChR ion pore. Fourthly, amines could exert their effect indirectly via intracellular second messenger pathways stimulated by aminergic G-protein receptors, the site of amine binding being distinct from the nAChR macromolecule.

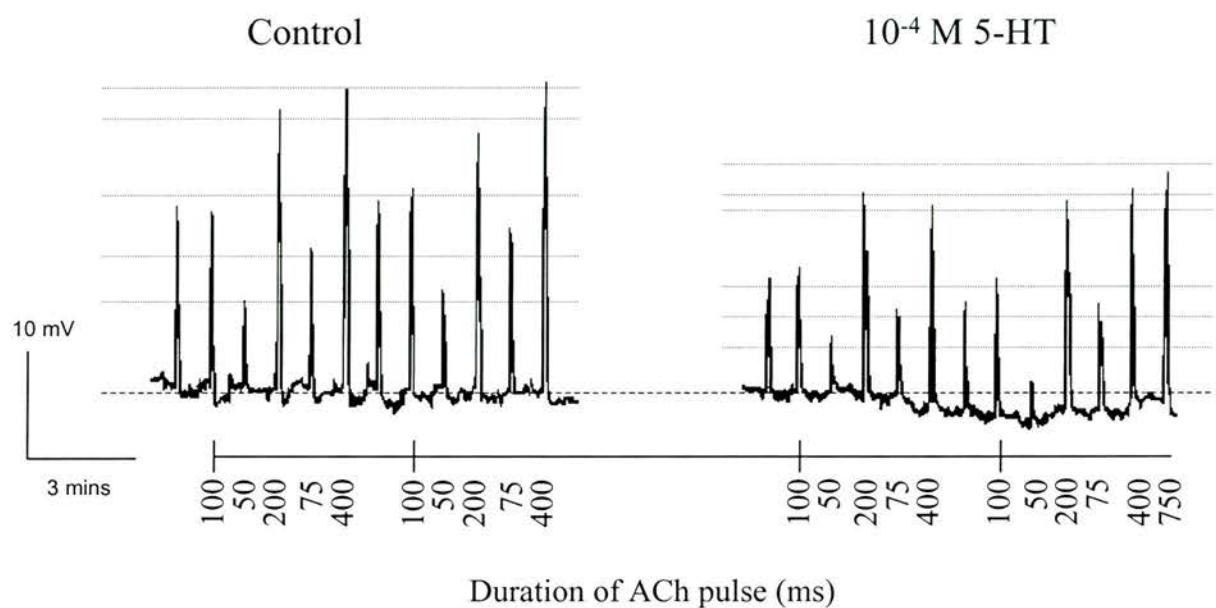
To investigate whether the amines compete with ACh for the same binding site, a dose-response curve for ACh was plotted in the presence and absence of the amines. The duration of the pulse was varied cyclically (25, 50, 75, 100, 200, 400, 600, and 750 ms; see figure 31a), to elicit a range of membrane depolarisations from the same cell corresponding to depolarisations evoked by specific concentrations of ACh (Sattelle, 1985). The cell was exposed to 4 control cycles of ACh depolarisation, prior to addition of either dopamine or serotonin. The same number of responses was recorded in the presence of the amine prior to ten minutes of washout. Figure 31b shows the graph compiled from the experiment, the ACh data expressed as mV depolarisations in response to concentration of ACh applied. The dose-response relationship shows that increasing concentrations of ACh results in a maximum saturable membrane depolarisation of the D_r motoneurone at approximately 10^{-3} M. After addition of the amines, saturating doses of ACh could not overcome aminergic suppression. This indicates that the amines act in a non-competitive manner. This is confirmed by Hill plot analysis of the data. Hill plots of the three dose-response curves were constructed according to the methods of Palma, Mileo, Eusebi and Miledi (1996) and Garcia-Colunga and Miledi (1999)(figure 31c). The calculated Hill coefficients (n_H) are summarised in figure 31d, along with the maximal responses and EC₅₀ values (parameters estimated by eye). The derived data indicates that although the amines non-competitively reduce ACh responses, they do not drastically alter the ability of the ACh ligand to bind to nAChRs. This would suggest that amines do not compete with ACh but rather modulate the gating properties of nAChRs. The analysis does not distinguish between direct block of the ion channel or allosteric changes due to phosphorylation of the intracellular domains of nAChR subunits or via binding of the amine to secondary sites on the nAChR complex.

Figure 31. Amines do not compete with ACh for the ligand binding site of nAChRs.

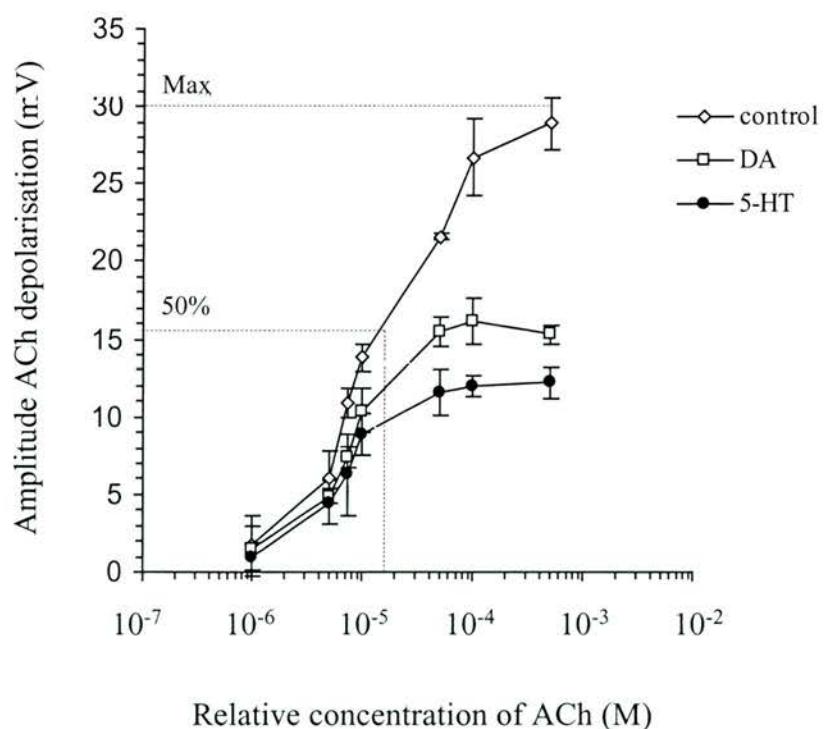
Varying the duration of the ACh pressure pulse applied locally to the D_f soma resulted in a range of membrane depolarisations (a), the amplitudes of which corresponded to the concentration of ACh to which the neurone was briefly exposed. D_f motoneurones exhibited relatively constant responses to the range of ACh pulses as shown in the left-hand panel. Application of either dopamine (10^{-4} M) or serotonin (10^{-4} M) results in suppression of the ACh depolarisations and could not be overcome by increasing the duration of ACh response further, typically to 600 ms or 750ms as in the right-hand panel.

The amplitude of the control and amine-suppressed ACh responses are plotted as a dose response curve shown in (b). From the graph it is evident that increasing ACh pulses result in a saturating control depolarisation response. Incubation with either of the amines suppressed the ACh response at the range of concentrations tested and could not be surmounted by increasing the concentration of ACh. This data was used to derive Hill coefficients, plotted in (c) and summarised in the table (d).

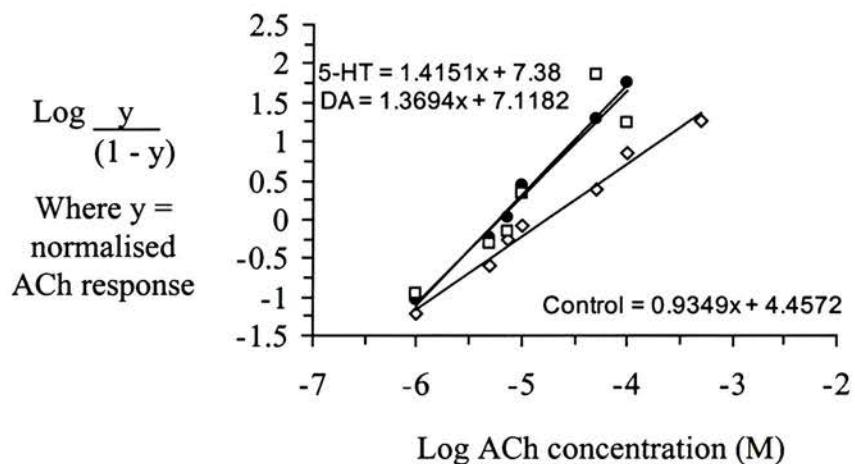
(a)



(b)



(c)



(d)

	Max depolarisation (mV)	$\text{EC}_{50} (\mu\text{M})$	n_H
Control	30.5	12	0.93
Dopamine	16	8	1.37
Serotonin	12.5	8	1.42

3.2 Pharmacology of the aminergic receptor site.

It is apparent from the data presented so far is that all three amines reversibly suppress nicotinic ACh currents. Furthermore having ascertained from the occlusion experiments that the three amines tested exert their action via a shared mode of transduction it was of interest to establish whether or not the initial site of action - the 'amine' receptor site, conforms to the pharmacology of any classical vertebrate aminergic G-protein-linked receptors.

A number of vertebrate dopamine receptor (DAR) agonists were tested for their ability to suppress ACh responses recorded from the D_r motoneurone. All agents were tested at a concentration of 10^{-4} M unless otherwise stated. The dopaminergic agonist causing the most potent suppression of ACh responses was the D1 subtype-specific SKF-38393 (figure 32a). The broad range agonist apomorphine was of similar potency to dopamine, whereas the D2 agents LY-163504 and N-0434 although more potent than dopamine were not as effective as SKF-38393 (figure 32b).

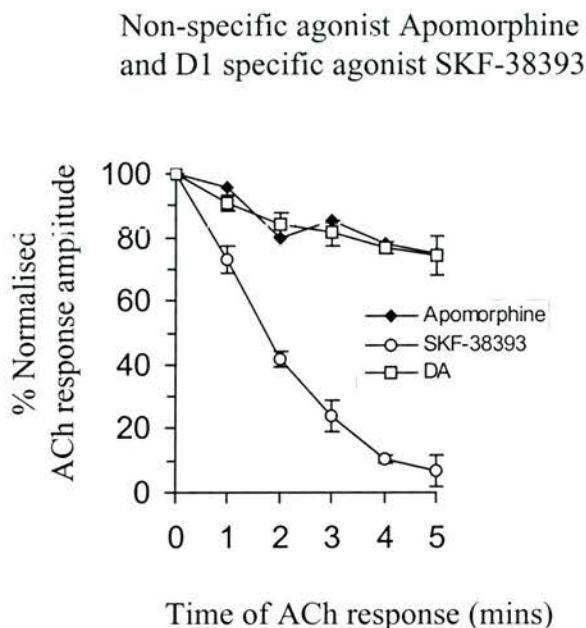
The ability of the DAR-specific agonists to mimic the suppressive effect of the amines on nicotinic transmission was also exhibited by a number of DAR antagonists. Application of the global dopaminergic antagonists fluphenazine to the preparation mimicked the effects of the amines on nicotinic responses but did not block the action of 10^{-4} M dopamine (see figure 33). The D1 receptor antagonists SCH23390 and SKF-83566 exhibited similar actions to fluphenazine when applied at a concentration of 10^{-4} M. Both rapidly and reversibly suppressed ACh depolarisations (figure 32c) to such an extent that it was not feasible to determine any attenuation of suppression mediated by dopamine. These actions of the antagonists were in contrast to the

Figure 32. The action of dopaminergic antagonists and agonists on the nicotinic ACh response of the D_f motoneurone.

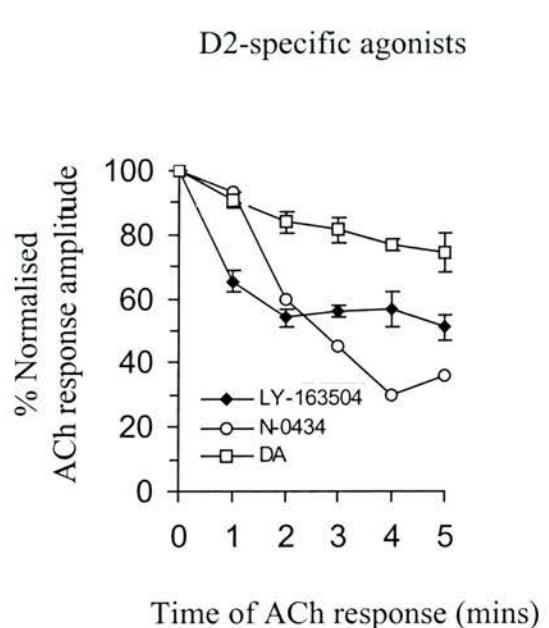
A range of vertebrate dopaminergic agents were examined for their ability either in the case of antagonists to block the action of dopamine, or in the case of agonists to mimic it's effect on the ACh response of the D_f motoneurone. All agents were initially tested at concentrations of 10^{-4} M, and all, apart from the D2 receptor agents spiperone and sulpiride, mimicked the effects of the amines on ACh responses, rapidly suppressing the control ACh response similar to that seen with fluphenazine. Most of the agents were only applied for 5 minutes such was the extent of their action before being perfused with fresh saline. The results for the antagonists are represented graphically in (a) (D1-specific) and (b) (D2-specific antagonists). SKF-83566 (n = 1), spiperone (n = 3) and sulpiride (n = 3) were initially dissolved in ethanol (95%) and then diluted to stock solutions of 10^{-2} M containing a final concentration of 0.01% ethanol. This concentration of ethanol had previously been determined to not modulate nicotinic ACh responses nor the resting potential of the cell.

(c) and (d) show the effects of D1 and D2-specific agonists as compared to the action of dopamine (open squares). Apomorphine (n = 2), like dopamine, was prepared in saline containing ascorbic acid, whereas N-0434 was dissolved in DMSO prior to dilution to stock concentration of 10^{-2} M. All other chemicals were prepared in normal saline.

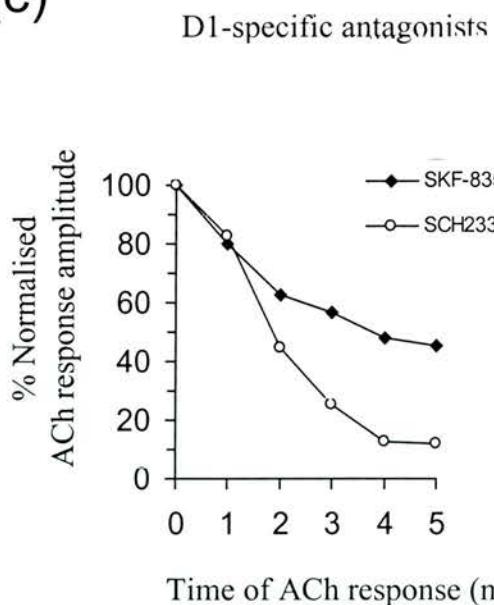
(a)



(b)



(c)



(d)

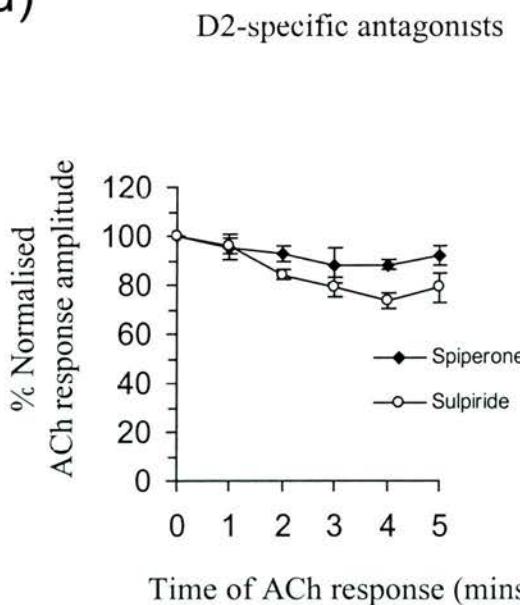
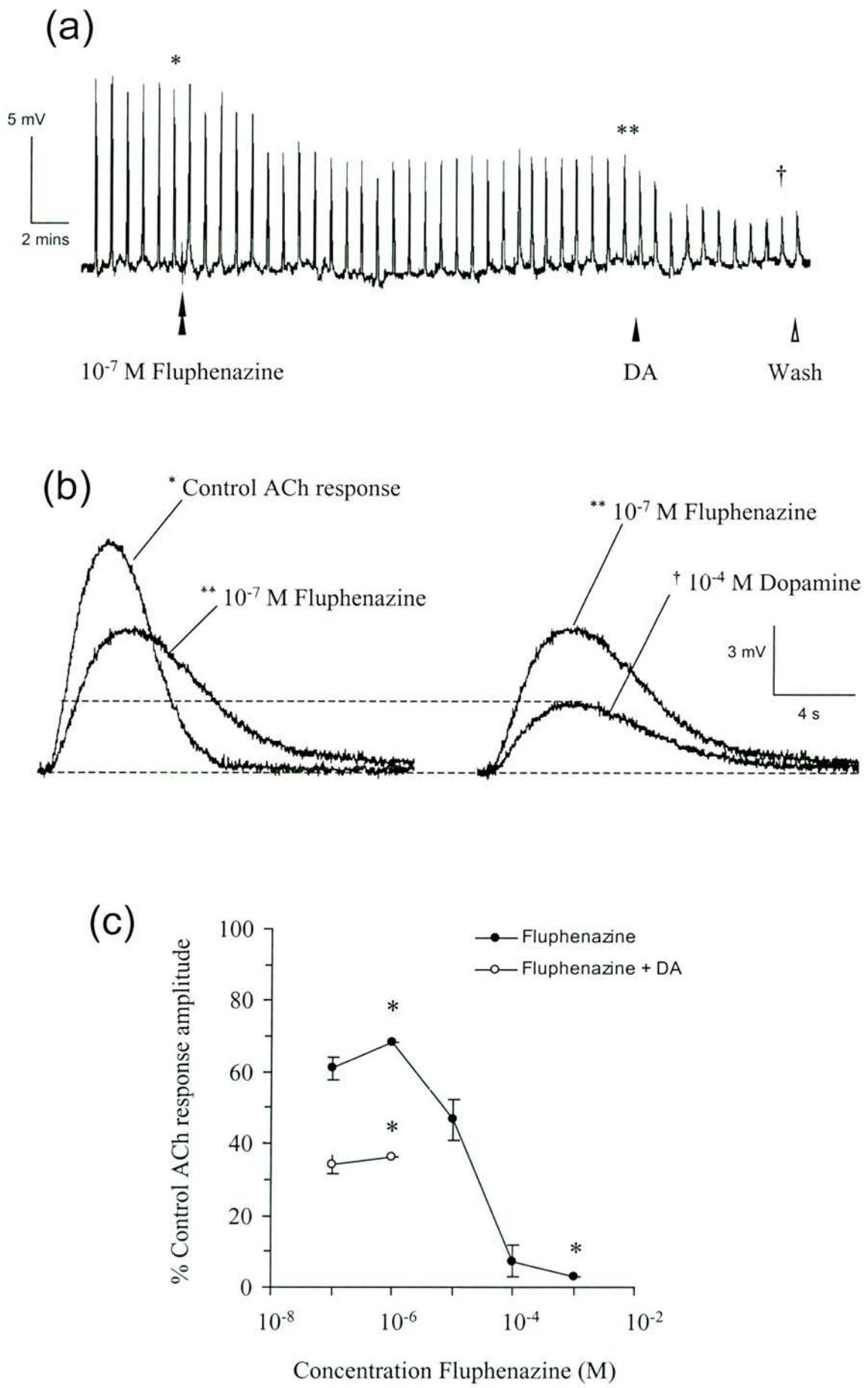


Figure 33. The broad specificity dopaminergic antagonist fluphenazine does not antagonise the action of dopamine.

(a) A typical trace from an experiment in which the ability of the dopamine antagonist fluphenazine to block aminergic suppression was tested. The antagonist was made up in normal saline and adjusted to pH 7.4. Application of a 20 μ l aliquot of 10^{-5} M fluphenazine reduced the amplitude of control ACh responses, exerting its maximal effect after approximately 8 minutes. Subsequent addition of 10^{-4} M dopamine to the bath resulted in an additional drop in the size of the ACh depolarisation. The action of both agents could be reversed to control levels after prolonged wash out with normal saline (not shown). Individual ACh responses marked are illustrated on an expanded time scale and amplitude in (b). The effect of the antagonist was to mimic the action of the amines by reducing the amplitude of the ACh responses. Furthermore it clearly prolonged the duration of the ACh depolarisation. It did not however attenuate or occlude suppression of nicotinic transmission mediated by dopamine as shown in the right-hand panel of (b).

Increasing the concentration of fluphenazine reduced the control ACh response independently of the action of dopamine such that, at a concentration of 10^{-4} M, the antagonist had almost completely abolished the observed ACh depolarisation (c). Such a dramatic effect was totally out of line with the saturating effects of the monoamines (see figure 14b) and could be rapidly reversed by perfusion with saline (typically 5 – 7 mins). The graph data points indicated with asterisks are single experiments otherwise they represent the mean and arc-sine standard error of 4 results. The ability of low concentrations of fluphenazine (10^{-7} M and 10^{-6} M) to antagonise dopaminergic suppression was also examined. In none of the experiments was any block of the action of dopamine recorded.



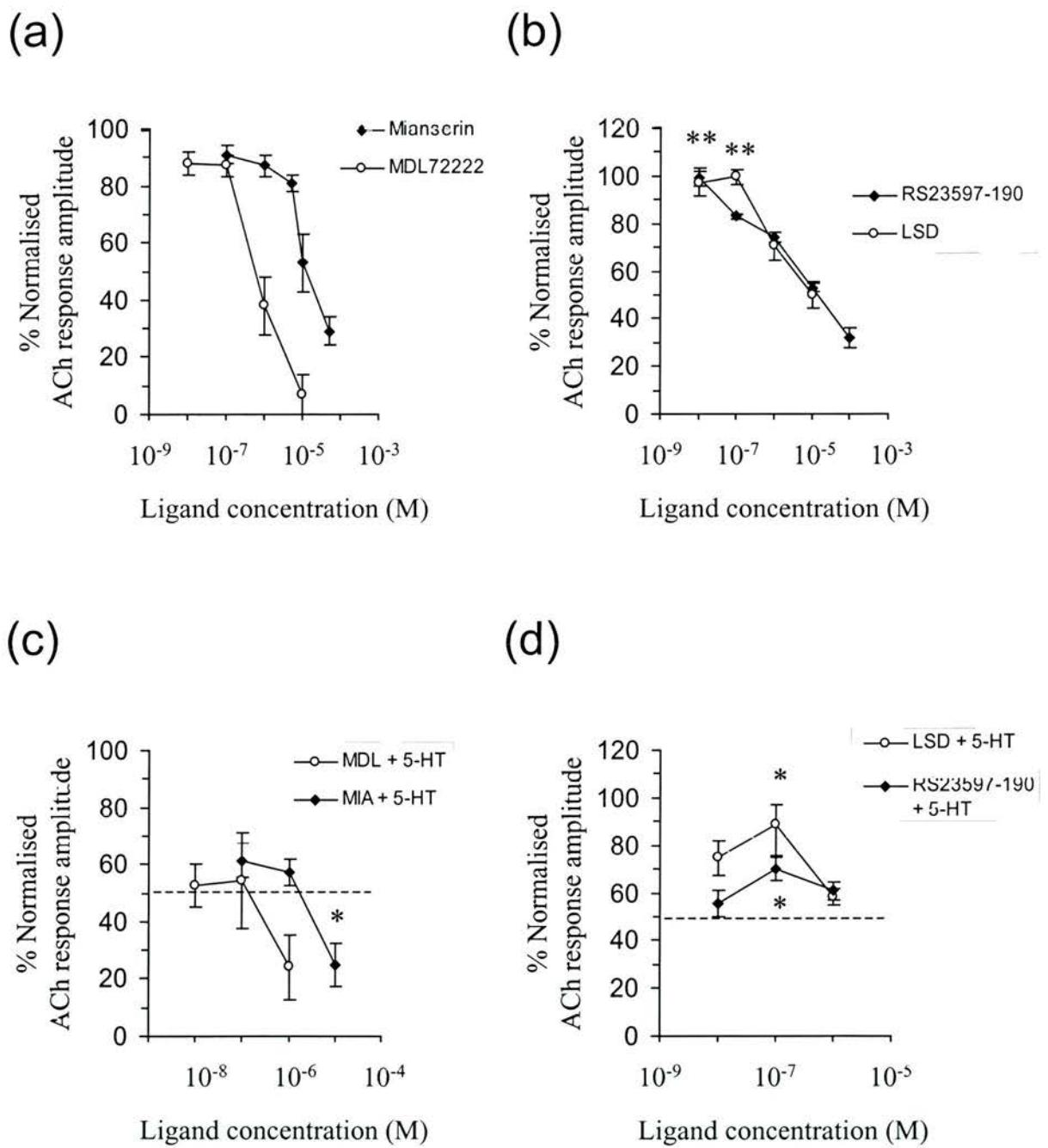
effects of the D₂ receptor antagonists sulpiride and spiperone (shown in figure 32d), neither of which displayed either significant agonistic or antagonistic actions when applied at a concentration of 10⁻⁴ M.

The only agent studied in detail was the broad range antagonist fluphenazine because, as reported by some other investigators, such global vertebrate antagonists have proved more effective than receptor-specific antagonists on invertebrate preparations (for example Wedemeyer, Roeder, Gewecke, 1992). The fact that other agents were only tested at one concentration (10⁻⁴ M) makes comparisons between them difficult. However a number of interesting phenomena are evident. Firstly, agents specific for vertebrate D₁ receptors are considerably more effective than dopamine itself or D₂-specific agonists at suppressing ACh responses, while D₂ antagonists have no significant effect. A number of the agonists and antagonists rapidly and completely inhibit ACh responses, raising the possibility that their mode of action is distinct from that of the natural amines, none of which completely abolished nicotinic ACh responses exhibited by the D_f motoneurone.

A number of serotonergic antagonists were used in an attempt to classify the receptor. However none exhibited a purely antagonistic action on the serotonin-mediated modulation of nicotinic ACh responses, instead having mixed agonistic and antagonistic actions. The most potently agonistic of the compounds were the 5-HT₃ – specific antagonists MDL72222 (figure 34a) and mctoclopramide (results not shown), which exerted similar effects over a range of concentrations (10⁻⁸ M to 10⁻⁵ M). The 5-HT₂ receptor antagonist, mianserin, exhibited a solely agonistic action on the ACh response. Spiperone, which acts as a 5-HT₂ antagonist as well as acting at D₂ receptor sites, has already been discussed in its context as D₂ receptor antagonist. Two agents tested exhibited a mixed agonist-antagonist action. These were the broad

Figure 34. Serotonergic antagonists exhibit mixed effects on aminergic suppression mediated by serotonin.

The vertebrate subtype-specific antagonists mianserin (5-HT₂), MDL72222 (5-HT₃) and RS23597-190 (5-HT₄) and the broad specificity antagonist lysergic acid diethylamide (LSD) were tested at a range of concentrations for their ability to attenuate serotonin-mediated suppression under current clamp conditions. Graphs (a) and (b) show the direct suppressive effects of the agents on ACh responses. MDL72222 and LSD were dissolved in DMSO prior to dilution to stock solution concentrations; all other chemicals were soluble in saline. The ‘agonist’ action of all the ligands was reversible on washing with fresh normal saline. Neither LSD nor RS23597-190 has a significant effect on ACh responses when applied at concentrations of 10⁻⁸ M and 10⁻⁷ M as indicated by the double asterisks. (c) and (d) show the resultant suppression observed after incubation with the antagonists and after 10 minutes exposure to 10⁻⁴ M serotonin. The dashed line indicates the mean value for the effect of serotonin alone. Results significantly different from the mean serotonin value are marked with a single asterisk.



specificity serotonergic antagonist lysergic acid diethylamide (LSD) and the 5-HT₄ agent RS23597-190 (figures 34b and 34d). At low concentrations (between 10⁻⁸ M and 10⁻⁷ M) both exhibited a purely antagonistic action, such that, when applied in the absence of serotonin, they had no effect upon the amplitude of nicotinic responses. However at these concentrations, they did reduce the efficacy of serotonin in suppressing ACh responses. At higher concentrations, however, these antagonists themselves actually depressed the amplitude of ACh responses in addition to blocking the effect of serotonin. The dual effect of LSD is illustrated in figure 35. To determine whether the monoamines dopamine and octopamine exert their action via a similar LSD-sensitive site, they were also applied to cells incubated with 10⁻⁷ M LSD (figure 36a). As shown in figure 36b LSD significantly inhibited modulation mediated by dopamine (n = 4) and octopamine (n = 3).

The observation that low concentrations of antagonists can effectively block aminergic suppression indicates that serotonin, dopamine and octopamine are likely to down-regulate nicotinic ACh responses in the preparation by a receptor-mediated process. Although the actions of the range of amine receptor antagonists tested did not enable the receptors to be classified unequivocally, the susceptibility of all three amines to block by the serotonin-selective ligand LSD, taken together with the fact that serotonin has a higher potency than the others and can occlude their effects, would suggest that it is the most likely candidate for the natural ligand mediating the modulation of nicotinic ACh receptor. In the experiments described below pharmacological agents were only tested on modulation of ACh responses mediated by serotonin.

Figure 35. LSD, when applied at low concentrations, is a potent antagonist of the effect of serotonin on ACh responses exhibited by D_f motoneurones.

Although LSD itself exhibited agonistic actions at relatively high concentrations, it did attenuate the ability of serotonin to suppress nicotinic ACh responses. (a) Shows three experiments in which D_f motoneurones were exposed to 10⁻⁸ M LSD (top panel), 10⁻⁷ M LSD (middle panel) and 10⁻⁶ M LSD (lower panel). 10⁻⁸ M and 10⁻⁶ M LSD alone reduced the ACh depolarisation observed under current clamp. Addition of 10⁻⁴ M serotonin after 10 minutes incubation with the antagonists resulted in a further suppression of the ACh response in the top panel (10⁻⁸ M LSD), but had no additional effect to that of LSD at antagonist concentrations of 10⁻⁷ M and 10⁻⁶ M. ((a) cal.: vertical 6 mV, horizontal 150 seconds).
(b) Expanded individual ACh response from the traces shown in (a), as indicated by asterisks and hash marks. The top panel shows the direct effect of LSD, clearly illustrating the ‘agonist’ effect of the ligand at 10⁻⁶ M. The lower panel demonstrated the ability of LSD to block suppression mediated by serotonin at all concentrations tested (cal.: vertical 5 mV, horizontal 4 seconds). The effect of LSD was reversible on perfusion with normal saline (not shown).

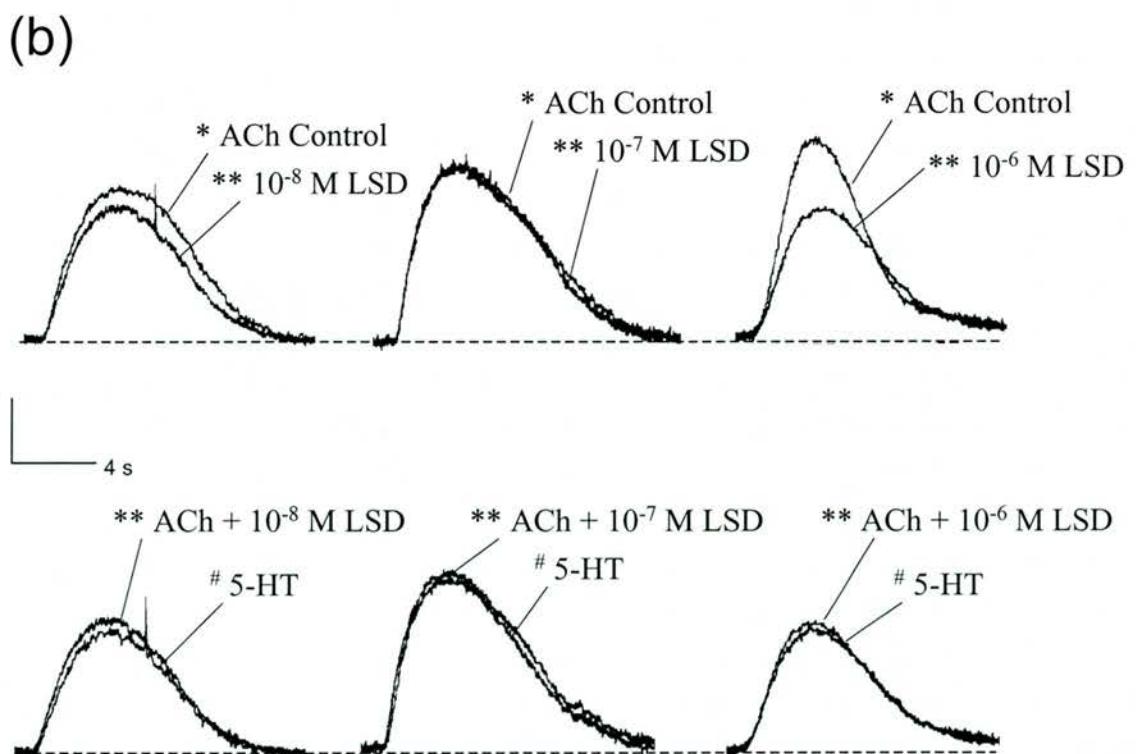
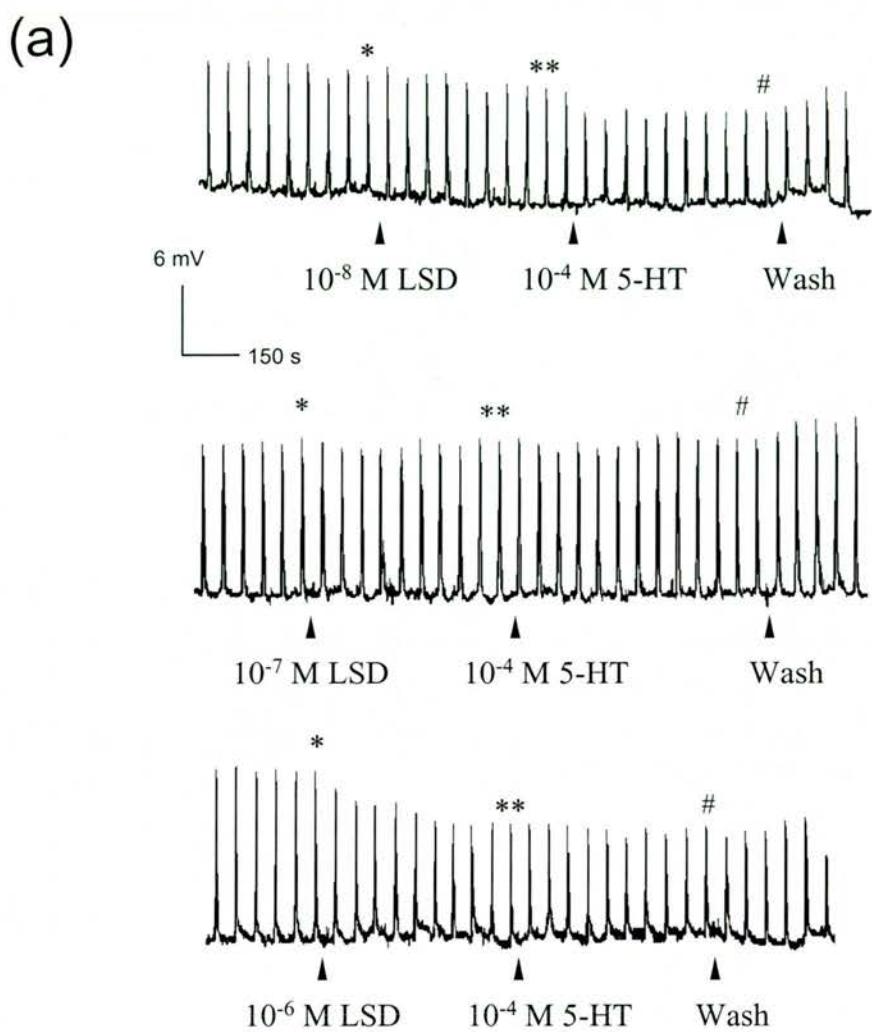
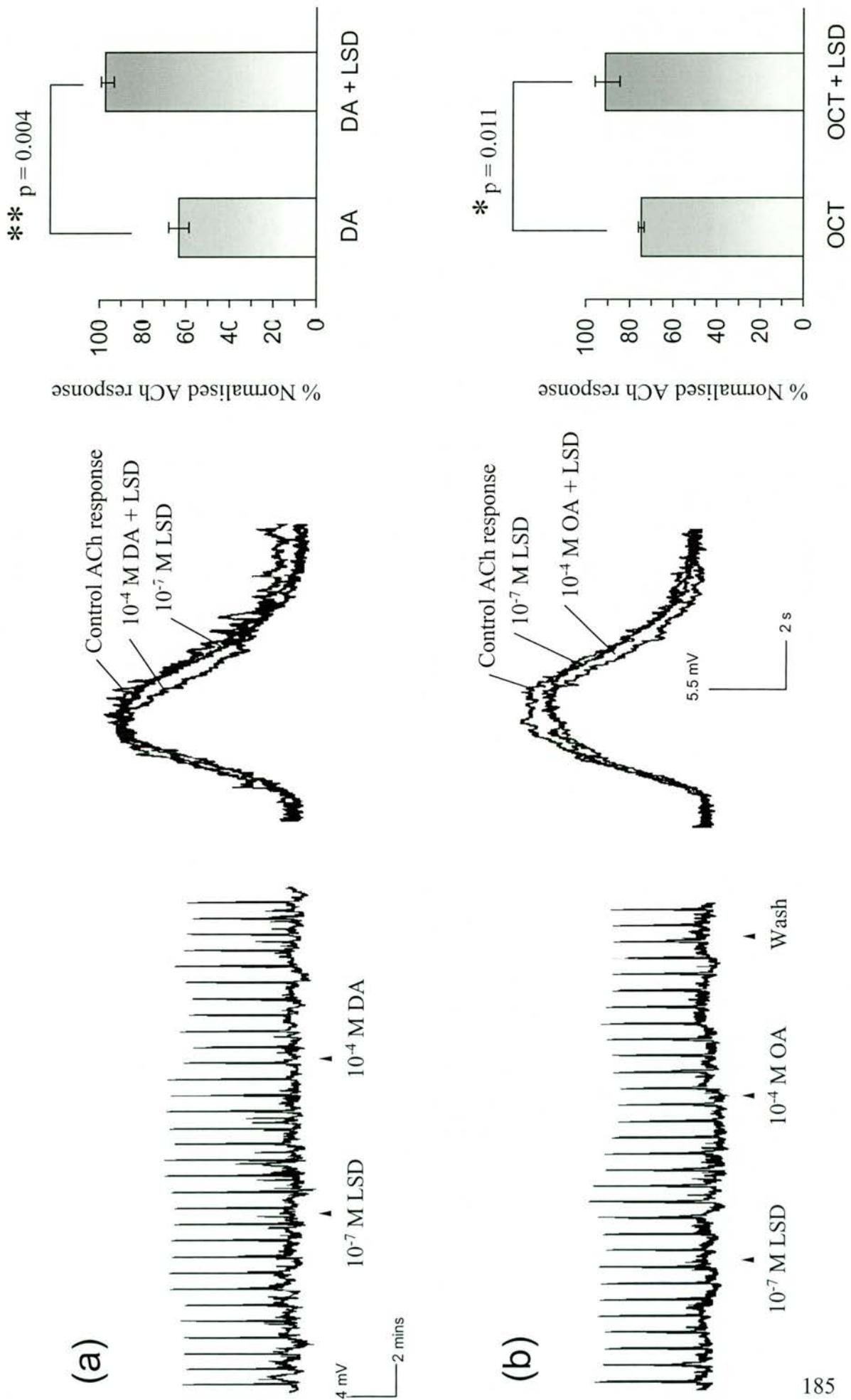


Figure 36. LSD (10^{-7} M) blocks the suppressive action of octopamine and dopamine on ACh responses.

The ability of LSD to attenuate the effect of dopamine (a) and octopamine (b) was tested. The far left-hand panels show the full traces of the experiments conducted on the same preparation under current clamp (resting potential approximately -78 mV). Superimposed individual responses are enlarged in the middle panels, responses are taken from just prior to antagonist application, the one proceeding amine application and just before washout was commenced as indicated by the asterisks. The traces for the control application of dopamine and octopamine are not shown but the data is included in the histograms in the right-hand panels. The pooled data from all the experiments shows that LSD significantly attenuates the action of both dopamine (n = 3) and octopamine (n = 4).



3.3 Involvement of G-proteins in the modulatory action of serotonin

The experimental data indicate that the amines are most likely to modulate nicotinic ACh responses via an indirect mechanism involving a receptor site sensitive to the serotonergic ligand LSD. With the exception of the 5-HT₃ ligand-gated ion channel, transduction of the actions of serotonin are mediated by G-protein-linked (metabotropic) receptors.

The most unequivocal method of demonstrating indirect modulation of nicotinic membrane channels is to utilise the patch-clamping technique, which would exclude from the membrane under the patch electrode any amines applied to the bathing solution. Therefore if amines applied in this way influence the activity of any nicotinic channels within the patch this would have to be mediated by an indirect mechanism. Unfortunately attempts to patch ACh-gated ion channels of the D_f motoneurone proved unsuccessful. Although gigaohm seals could be obtained, these were too unstable to provide information on this question. As a consequence, all experiments had to be based upon intracellular recordings made with sharp electrodes in which the intracellular signalling pathways were manipulated pharmacologically.

Neuromodulators that activate intracellular signalling pathways normally do so via trimeric G-proteins. To determine whether amines down-regulate nicotinic ACh currents via such a process, the D_f motoneurone was injected with the GDP analogue GDP-β-S, which prevents G-protein activation by competing with endogenous GTP for the guanine nucleotide binding site. A protocol was used similar to that of Matsushima, Tegner, Hill, and Grillner (1993).

To determine whether the injection of GDP-β-S affected the properties of the D_f motoneurone in the absence of applied amines the data from experiments using voltage clamp ($n = 7$) were pooled, particularly as to whether or not GDP-β-S

injection altered the amplitude or duration of the ACh currents. The data revealed that following GDP- β -S injection the amplitude of ACh response was not significantly altered (95.8% of the control amplitude +s.e.m. 3.0, -s.e.m. 4.6; n = 7). However in 3 out of the 7 experiments there was an observable increase in the duration of the ACh response after injection of GDP- β -S. The reasons for this is unclear. Injection of GDP- β -S significantly attenuated the ability of serotonin to modulate the ACh response under voltage clamp recording conditions (figure 37). This implies GDP interferes with modulation of ACh responses mediated by serotonin, because this process involves one or more G-proteins. The effect of GDP- β -S was also tested under current-clamp conditions as shown in figure 38. The stability of ACh responses under single electrode recording meant that the effect of GDP- β -S could be analysed over a longer time course. GDP- β -S was actively injected at the start of the experiment after a control application of serotonin had been successfully reversed; there was no further injection of GDP- β -S. Initially GDP- β -S blocked the serotonin-mediated suppression of ACh responses in a manner similar to that observed under voltage clamp. However the ability of the GDP analogue to attenuate aminergic suppression gradually declined with time, such that after 90 minutes the ability of the amine to reduce ACh responses had returned to initial, pre-GDP injection levels. GDP- β -S is non-hydrolyzable and therefore the decrease in effectiveness can not be attributed to breakdown of the molecule to GMP. Therefore the decline in the action of GDP- β -S is likely due to gradual diffusion of the injected analogue throughout the neurone, thereby decreasing the concentration of GDP- β -S at soma and proximal dendrite sites to which ACh is being applied.

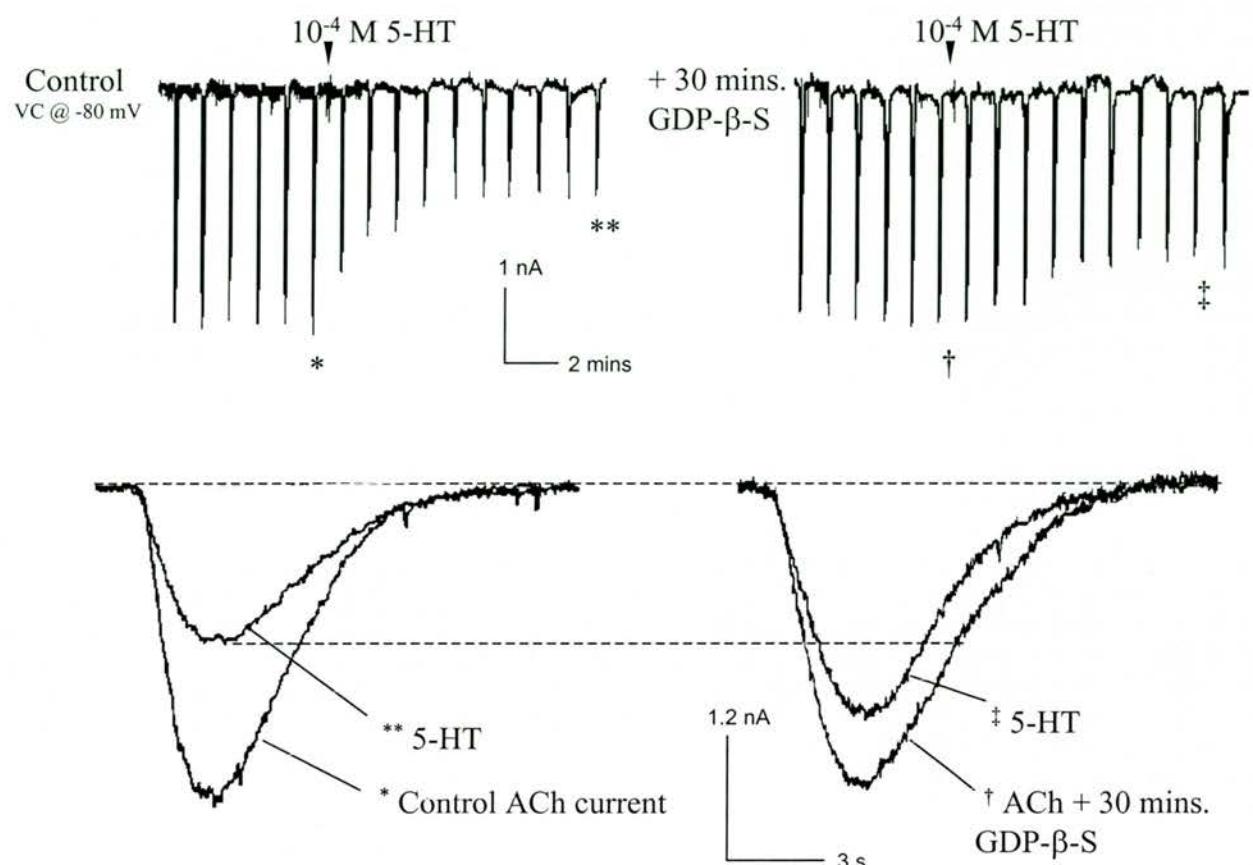
The purified bacterial toxins pertussis toxin (PTX) and cholera toxin (CTX) have been found to alter G-protein coupling of receptors to intracellular signalling

Figure 37. Injection of the GDP analogue GDP- β -S attenuates serotonergic suppression.

(a) Traces taken from the same preparation are displayed in the top panels. On the left-hand side is a pre- GDP- β -S injection application of 10^{-4} M serotonin. Addition of the monoamine to the bath resulted in suppression of the ACh responses, shown on an expanded time scale in the panel below the trace. After GDP- β -S injection (right panel) the preparation was allowed to stabilise for a half hour prior to a second application of serotonin. The efficacy of serotonin in reducing the ACh response was severely curtailed by injection of the GDP analogue (lower panel). (trace calibration: vertical 1 nA, horizontal 2 mins; expanded traces vertical 1.2 nA; horizontal 3 seconds).

(b) Pooled data from the seven experiments conducted under voltage clamp. There was a significant reduction in suppression mediated by serotonin when the soma of the D_f motoneurone was injected with GDP- β -S.

(a)



(b)

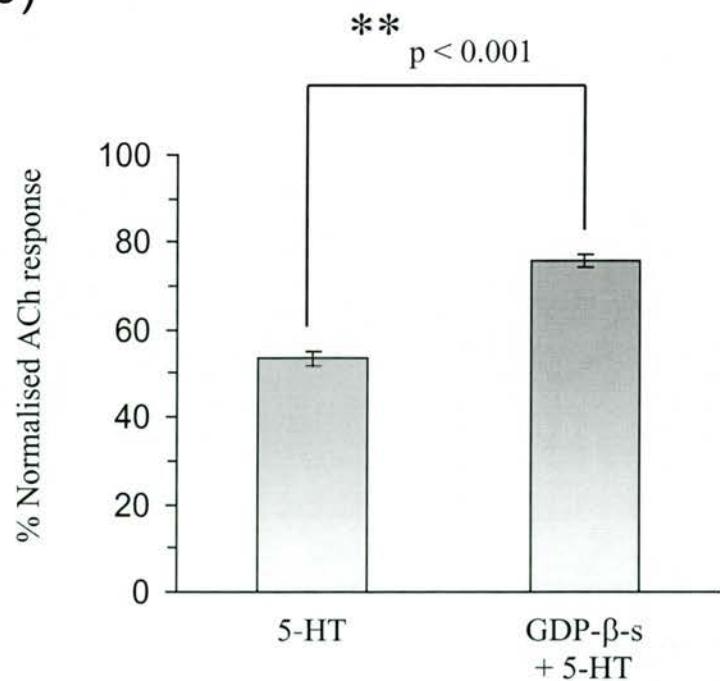
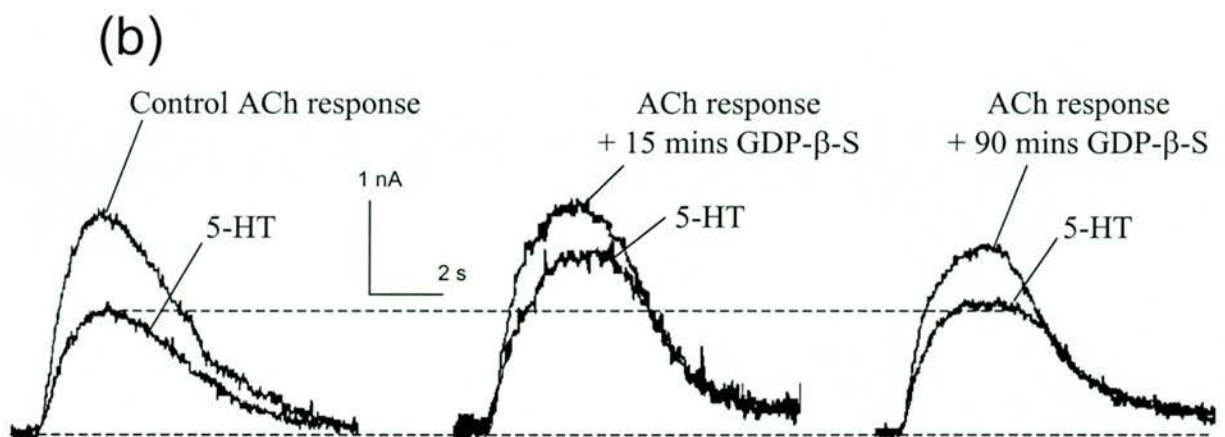
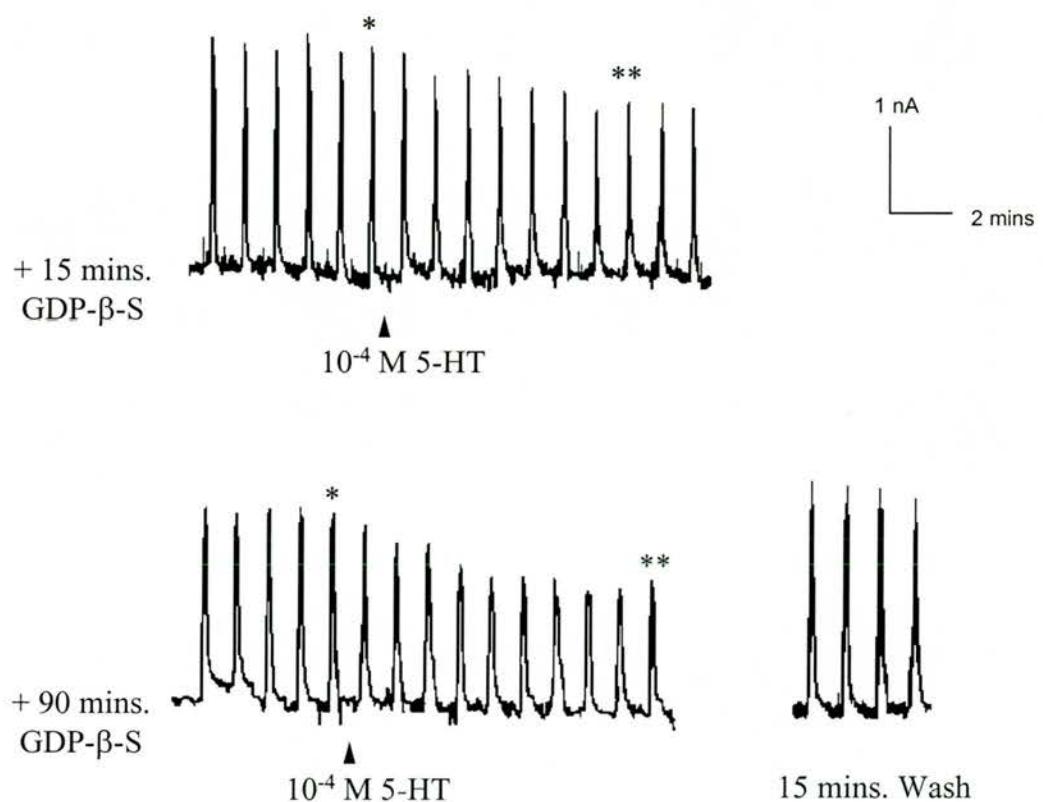
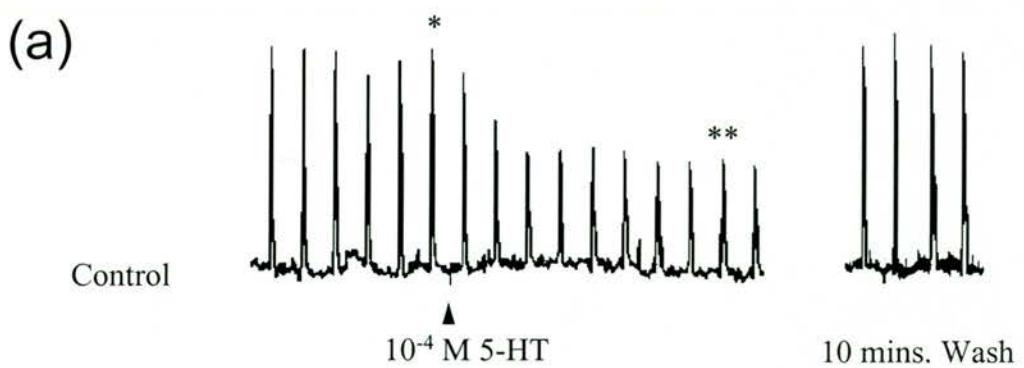


Figure 38. GDP- β -S reduces the ability of serotonin to modulate ACh depolarisations observed under current clamp.

(a) Shows the effect of serotonin over a 90 minute period. In the absence of GDP- β -S, serotonin suppresses ACh responses. Similarly, as seen under voltage clamp conditions, injection of the GDP analogue significantly reduced the ability of the amine to modulate nicotinic transmission. After 90 minutes there was a slight recovery in the ability of serotonin to exert an effect. The individual responses marked are shown expanded in (b). (cal. (a): vertical 1 nA, horizontal 2 mins; (b): vertical 1 nA, horizontal 2 seconds).



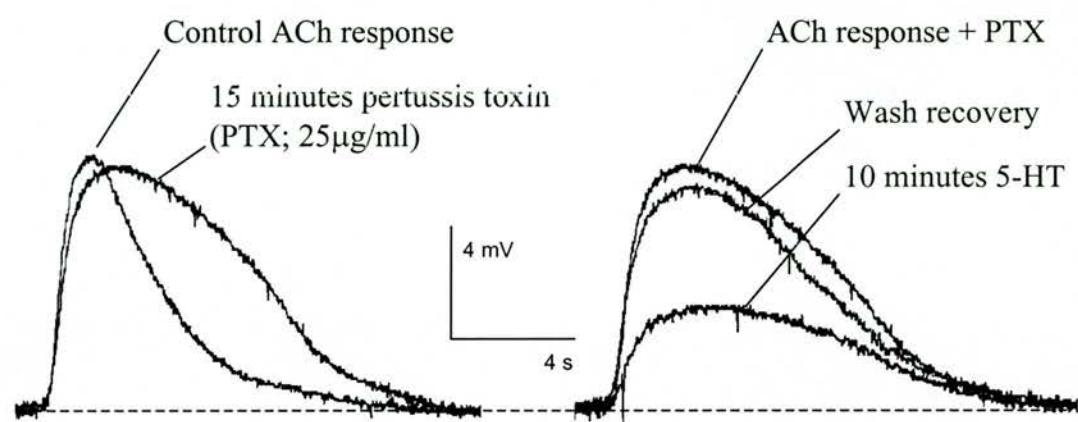
pathways. PTX prevents receptor activation of $G_{i/o}$ proteins, whereas CTX reduces the inherent GTPase activity of G_s proteins and hence prolongs their activity. Both toxins were applied at high concentrations relative to that reported in the literature (typically 0.1 – 10 µg/ml) to compensate for the reduced incubation times, typically periods of 15 minutes to 1 hour required in these experiments as compared to 1 – 3 days used in a number of other studies (for example see Blier *et al.*, 1993; Babila and Klein, 1994; Sidhu *et al.*, 1998;). Pertussis toxin (25 µg/ml) exerted an observable effect within 5 – 15 minutes, significantly prolonging the duration of the ACh response (figure 39a, left-hand panel). It did not, however, directly modulate the amplitude of the ACh response nor significantly attenuate aminergic suppression initiated by application of 10^{-4} M serotonin (Figure 39b). The $G_{s\alpha}$ activator cholera toxin (CTX; 50 µg/ml) when applied alone does not significantly alter the amplitude of the ACh responses after incubation for a half hour (figure 40a, left-hand panel), reducing the size of the response to 82.1% (+s.e.m. 2.6, -s.e.m. 2.7; n = 3) of the control (p = 0.082, compared to a control application of 20 µl saline). G_s proteins positively couple receptor activation to adenylyl cyclase enzymes, which when stimulated lead to an increase in cAMP concentration. In a number of other studies the rise in cAMP stimulated by CTX was shown to be masked by endogenous phosphodiesterase (PDE) activity. Therefore, and also partly due to the time limit of the experiments, it was decided to apply CTX to cells previously exposed to the PDE inhibitor theophylline in order to ascertain whether CTX exerts an effect on the ACh response of the D_f motoneurone. Application of CTX to cells in the presence of theophylline (10^{-4} M) results in a significant reduction in the ACh response over a shorter time course (figure 40a, right-hand panel). Typically CTX reduced the ACh

Figure 39. Pertussis toxin prolongs ACh-induced depolarisation but does not block the ability of serotonin to suppress the ACh response.

The effect of incubation for 15 – 20 minutes with pertussis toxin (25 µg/ml) was to prolong the control ACh response as shown in (a), left-hand panel, although it did not attenuate serotonergic suppression of the ACh response (right panel).

(b) The pooled data from 4 experiments shows that incubation with 25 µg/ml pertussis toxin does not significantly alter the amplitude of the ACh responses, nor does it attenuate the ability of the amine serotonin to suppress ACh responses. Serotonin in the presence of pertussis toxin reduced the ACh response to 51.5% (+s.e.m. 4.0, -s.e.m. 4.1; n = 4) of the control.

(a)



(b)

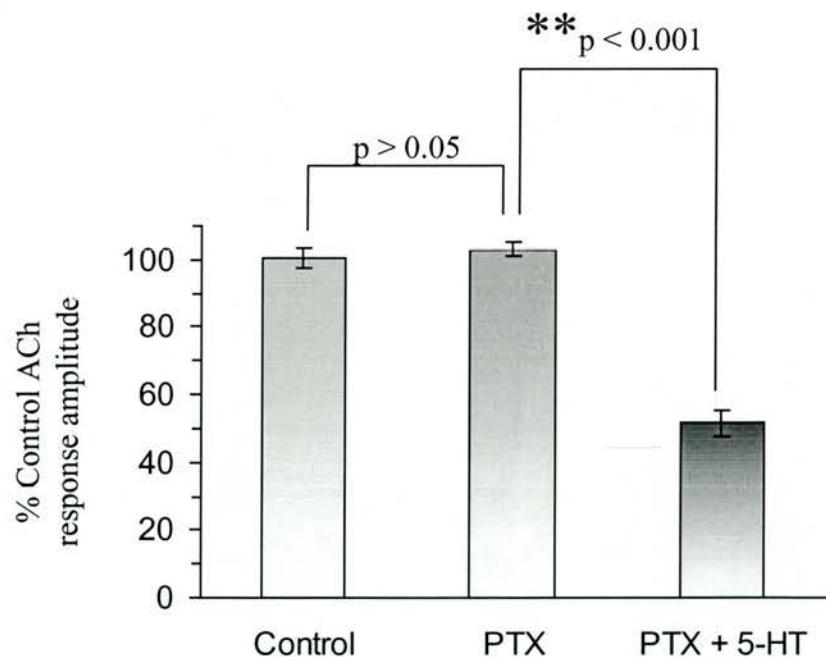
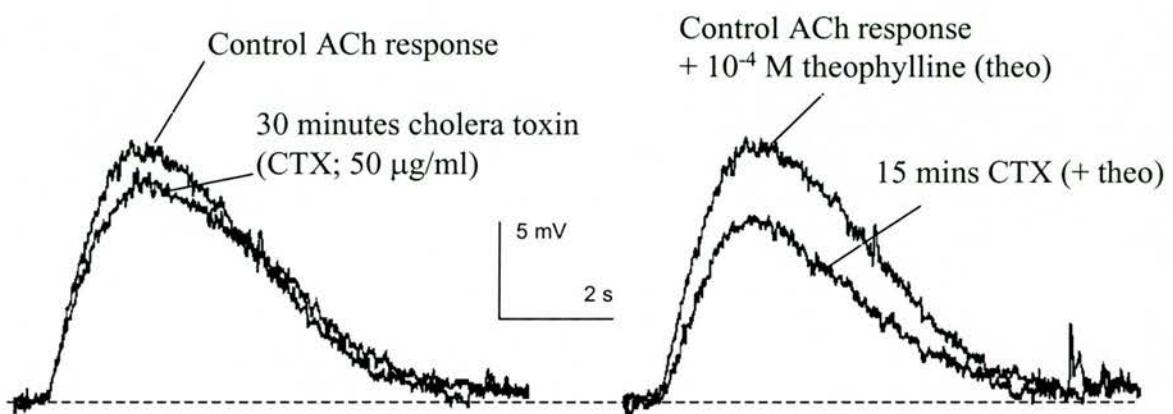


Figure 40. Cholera toxin (50 µg/ml) in the presence of the phosphodiesterase inhibitor theophylline significantly reduces the control ACh response.

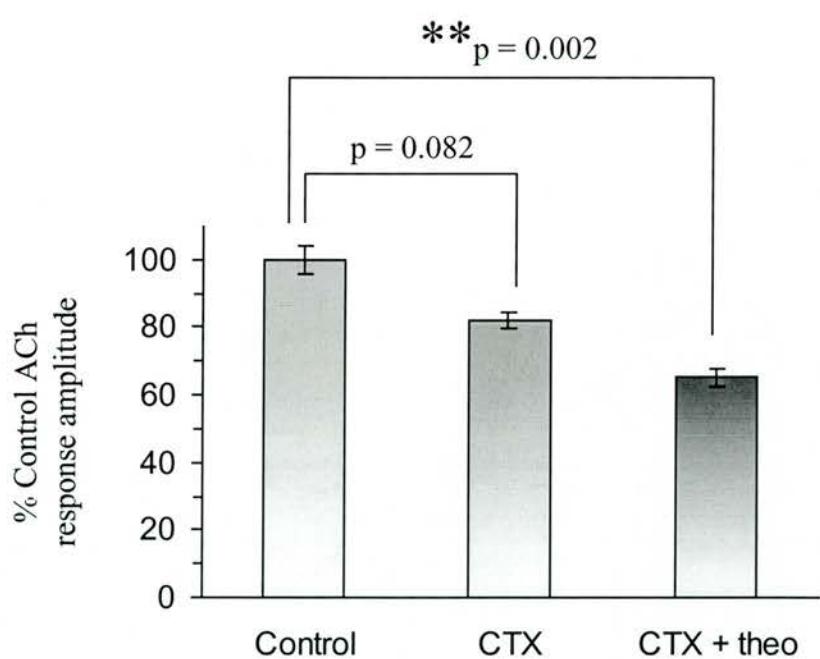
(a) Incubation with the G_{as} activator cholera toxin marginally reduces the control ACh response when compared to a control application of 20 µl saline. This effect is potentiated when the cell is pre-incubated for 10 – 15 minutes with theophylline (10^{-4} M).

(b) Pooled data from three experiments shows that the action of cholera toxin is significantly different to that of a control application of 20 µl saline when the cell is incubated with the phosphodiesterase (PDE) inhibitor theophylline. Theophylline potentiates the ability of cholera toxin to elevate cAMP levels in the D_f motoneurone by inhibiting the PDE enzyme responsible for degradation of cAMP.

(a)



(b)



response to 65.2% (+s.e.m. 2.8, -s.e.m. 2.9; n = 3) of the control within fifteen minutes of application (figure 40b).

3.4 Summary of the mechanism by which serotonin modulates nACh responses.

The experimental data favours the hypothesis that the amines exert their modulatory action on the nicotinic ACh response of the D_f motoneurone by an indirect mechanism. Certainly the amines dopamine and serotonin do not directly compete with ACh for the nicotinic receptor ACh binding site, as increasing the concentration of ACh does not overcome aminergic suppression. The presence of such an aminergic receptor site independent of the nicotinic ACh receptor is supported by the antagonistic actions of LSD and RS23597-190. Both significantly block the ability of serotonin to suppress ACh responses, whilst the former also inhibits the effects of octopamine and dopamine. The action of these two ligands is inconsistent with a direct action of the amines on nAChRs. LSD has been demonstrated to selectively inhibit a serotonin-sensitive adenylyl cyclase in *Periplaneta americana* (Nathanson and Greengard , 1974) at similar concentrations to those reported here. These data are consistent with the presence of a receptor site selective for serotonin as opposed to dopamine and octopamine.

The fact that the GDP analogue GDP-β-S attenuates serotonergic suppression of nicotinic ACh responses provides strong evidence for the involvement of G-proteins in the transduction mechanism and hence that the amines exert their effect via a G-protein-linked receptor. Furthermore it is unlikely that the effects of serotonin are mediated by a pertussis toxin-sensitive G-protein, discounting the involvement of the G_{i/o} protein family. The reduction in amplitude of ACh response after application

of cholera toxin in the presence of the PDE inhibitor theophylline is indicative of the presence of endogenous G_s protein activity.

4 Influence of calcium-dependent intracellular pathways on aminergic modulation

Since muscarinic agonists modulate nicotinic ACh responses by a process involving an increase in intracellular calcium ($[Ca^{2+}]_i$), (David and Pitman, 1996b), a number of experiments were performed to establish whether serotonin exerts its modulatory effects via a similar mechanism. Experiments investigated the possible dependency of aminergic suppression on a rise in $[Ca^{2+}]_i$. Additionally a number of pharmacological agents known to interfere with the downstream Ca^{2+} -pathways were tested to determine whether they blocked the action of the monoamine serotonin.

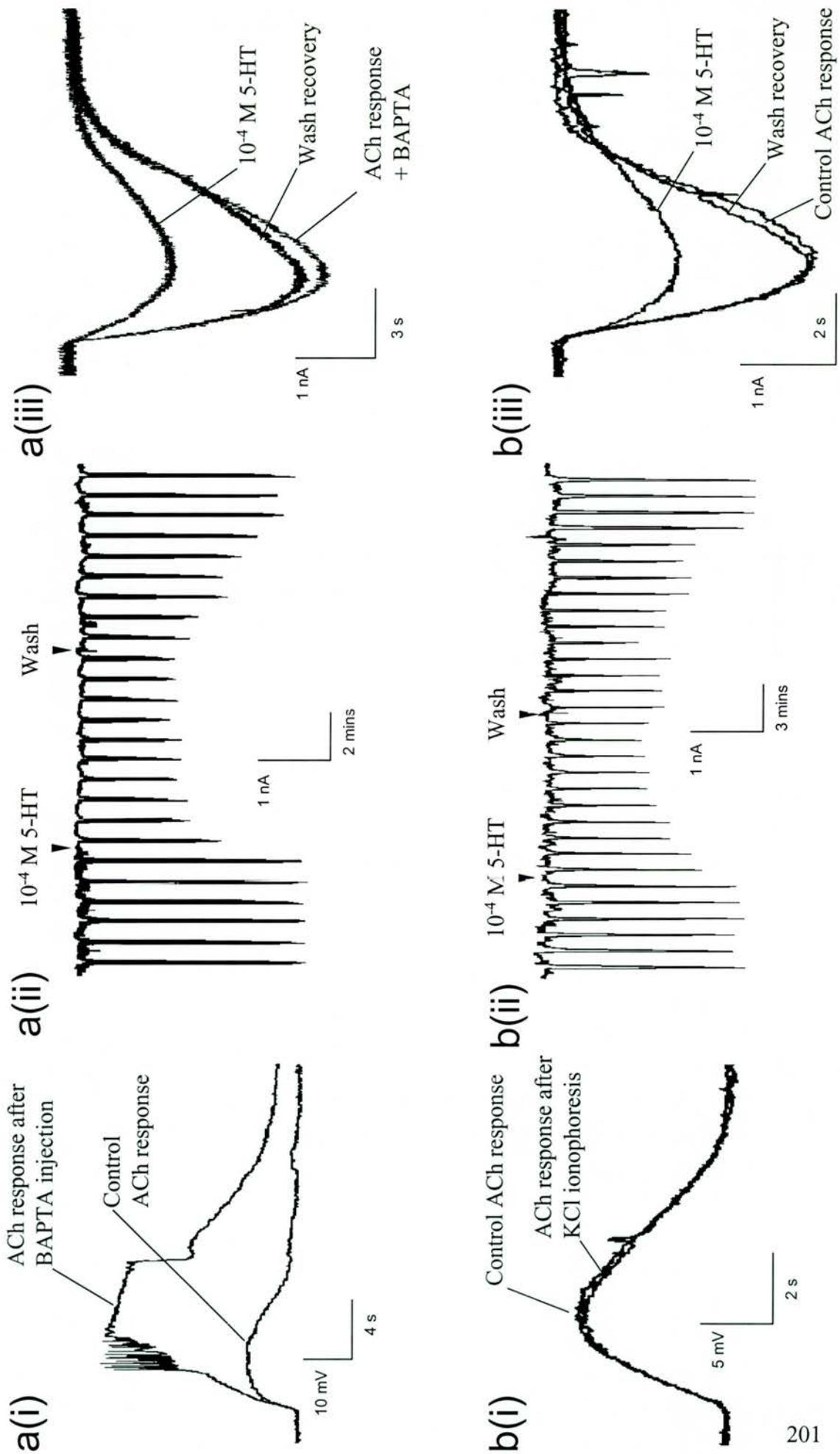
4.1 Injection of the calcium chelator BAPTA does not inhibit the modulatory effect of serotonin.

If the amine exerts its action via the same mechanism as muscarinic agonists, intracellular injection of calcium chelating agents should limit any fluctuations in $[Ca^{2+}]_i$ and thus attenuate the serotonin-mediated suppression of nicotinic responses. The calcium chelator BAPTA was injected intracellularly into the soma of the D_f motoneurone by ionophoretic pulses of 2 – 10 nA and 200 ms in duration, at 0.2 Hz for at least 20 minutes, using electrodes containing a solution of 10^{-1} M BAPTA and 10^{-1} M KCl. Control experiments were also conducted where a similar protocol was followed but BAPTA was excluded from the electrode solution. It was apparent when sufficient BAPTA had been injected to significantly lower $[Ca^{2+}]_i$ since neurones began to give action potentials of approximately 40 – 60 mV in amplitude (c.f. Pitman, 1979). The amplitude of ACh responses was also enhanced to the extent that in many preparations they could trigger plateau potentials (figure 41a(i)). The effect of BAPTA was prolonged, since the amplitude of ACh responses remained at

Figure 41a and 41b. Injection of BAPTA does not inhibit the modulatory action of serotonin.

Panels (i) show the effect of the current injection protocol in the presence and absence of BAPTA on the amplitude of ACh responses observed under current clamp. The central panels (ii) illustrate the suppressive effect of serotonin on ACh inward currents on a long time scale and the right-hand panels (iii) the expanded traces taken from the central traces.

It is evident from a(i) that BAPTA injection enhances the ACh depolarisation recorded under current clamp (cal.: 10 mV, 4s). However, the presence of the calcium chelator does not attenuate the action of serotonin recorded under voltage clamp conditions. In panels a(ii) and a(iii) the rapid effect of serotonin in the presence of BAPTA is clearly visible (cal. a(ii) trace: vertical 1 nA, horizontal 2 mins; a(iii): 1 nA, 3 seconds). b(i) KCl ionophoresis does not enhance ACh responses under current clamp, nor modulate the efficacy of serotonin to suppress ACh currents under voltage clamp (b(ii) and b(iii))(cal. figure b(i) effect of KCl injection vertical 5 mV, vertical 2 s; b(ii) trace 1 nA, 3 mins.; expanded responses b(iii): 1 nA, 2s).

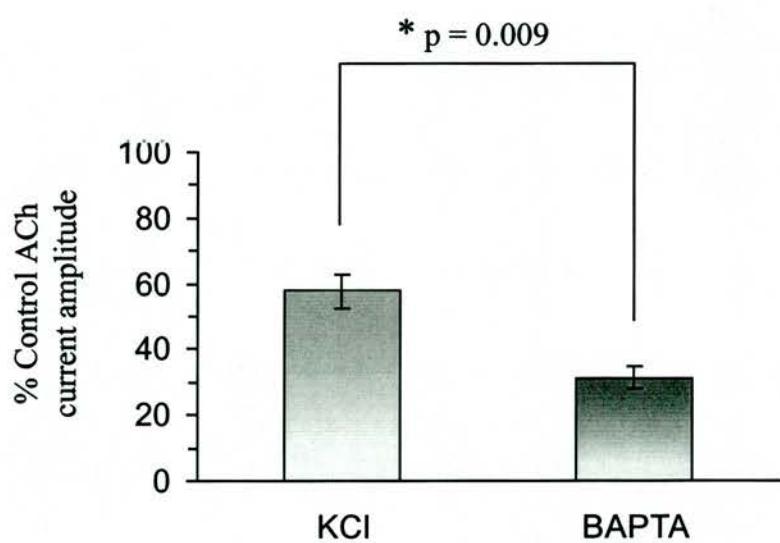


Figures 41c and 41d. Statistical analysis of BAPTA data.

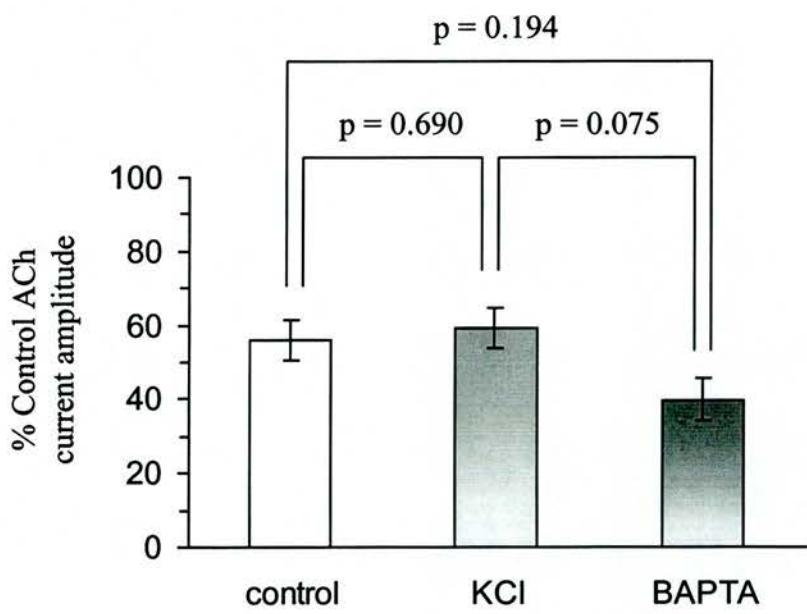
(c) and (d) Pooled data from the BAPTA ionophoresis experiments and the KCl controls. In (c) the suppressive effect of serotonin is expressed as a percentage of the ACh current amplitude observed after control current injection (KCl) or BAPTA injection by ionophoresis. Serotonin suppressed the ACh response after KCl injection by 57.7% (+s.e.m.5.2, -s.e.m. 5.4; n = 5) as opposed to after BAPTA injection in which addition of serotonin to the bath reduced the observed response to 31.4% (+s.e.m.3.4, -s.e.m. 3.3; n = 5) of the post-BAPTA injection ACh response. The significant enhancement of the action of serotonin could be explained by the increase in amplitude of the control ACh response caused by BAPTA injection.

The data shown in (d) is the serotonin-induced change in amplitude of ACh responses observed under normal conditions (control), and after KCl or BAPTA injection, expressed as a percentage of the initial control ACh response. Neither of the injection protocols caused a significant change in the action of serotonin when compared to the effect of serotonin tested prior to intracellular injection.

(c)



(d)



the enhanced level for the duration of all experiments. The augmentation of ACh responses after injection of BAPTA could not be attributed to the injection protocol itself as no enhancement was observed in the experiments using microelectrodes filled with 10^{-1} M KCl only (figure 41b(i)). Because reduction in $[Ca^{2+}]_i$ could both increase the amplitude of ACh responses and increase neuronal excitability, interpretation of results obtained during voltage recording might have been compromised. For this reason, the effect of BAPTA upon serotonin-mediated suppression of ACh responses was studied using the two electrode voltage clamp recording set-up. Figures 41a(ii) and 41b(ii) show typical traces firstly from a control experiment where KCl alone was injected and secondly when BAPTA was ionophoretically injected.

Statistical analysis (shown in figure 41c) of the data revealed that after BAPTA injection serotonin suppressed ACh currents by a greater percentage than it does in the absence of BAPTA. This observation, however, may result from the fact that BAPTA causes an increase in the ACh current amplitude. To test this possibility, the amplitude of the ACh currents observed in the presence of serotonin and BAPTA was expressed as a percentage of control ACh currents recorded before BAPTA injection (figure 41d). This revealed that the percentage suppression in ACh currents induced by serotonin was not significantly ($p = 0.075$) increased in the presence of the Ca^{2+} chelator BAPTA. This indicates that the extent to which serotonin modulates nACh currents is not decreased by BAPTA which would reduce any changes in $[Ca^{2+}]_i$. Thus the modulatory action of serotonin is not primarily dependent on a change in $[Ca^{2+}]_i$.

4.2 A role for calmodulin kinase II (CaMK II) in the regulation of ACh responses.

Although the BAPTA data indicate that the monoamine serotonin is unlikely to exert its effect on ACh responses primarily via an increase in $[Ca^{2+}]_i$, a number of pharmacological tools were applied to the preparation to interfere with Ca^{2+} handling in D_f in order to ascertain whether Ca^{2+} -dependent pathways, particular those reliant on calmodulin (CaM) influence the aminergic suppression of ACh responses. Three agents were tested; the blocker of inositol triphosphate-sensitive calcium release from intracellular stores 8-[diethylamino]octyl-3,4,5-trimethoxybenzoate HCl (TMB-8), the calmodulin antagonist N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide HCl (W-7), and the calmodulin kinase inhibitor KN-62.

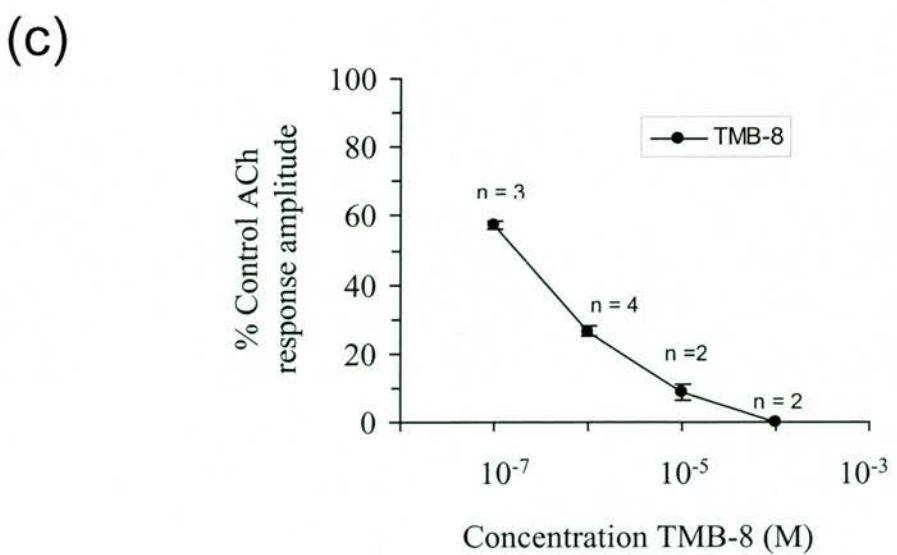
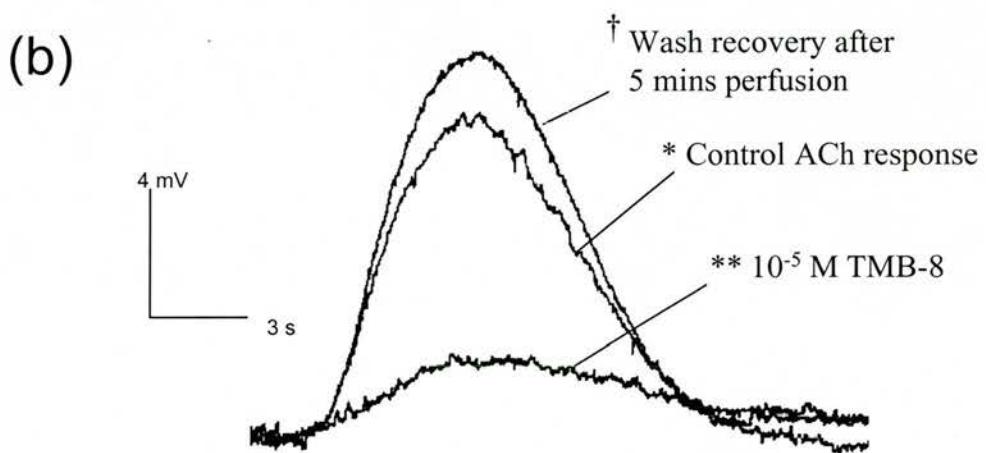
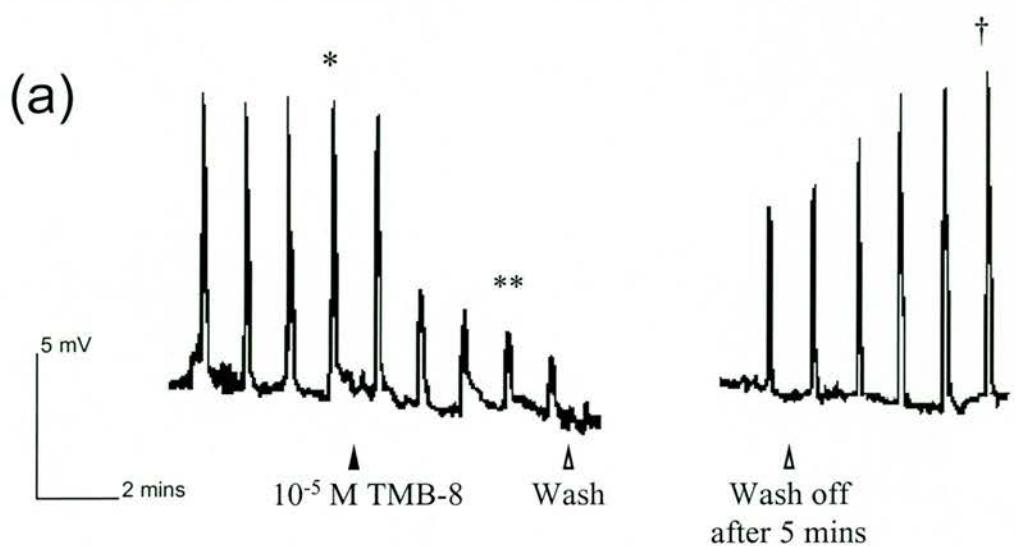
TMB-8 is a useful tool for distinguishing between dependence of cellular events on Ca^{2+} release from intracellular stores or influx of Ca^{2+} from the extracellular medium. Extracellular application of low concentrations of TMB-8 resulted in a rapid suppression of the nicotinic ACh response, that was fully reversed after only 5 minutes wash (see figure 42a). Such was the effect of increasing concentrations of TMB-8, shown in figure 42b, that at concentrations of 10^{-4} M application of the drug completely abolished nicotinic transmission. The dramatic effect, completely blocking ACh responses, and rapid time course of TMB-8 is not consistent with an intracellular site of action for the agent, and is more compatible with the reported direct blocking effect of TMB-8 on nicotinic AChRs (Bencherif, Eisenhour, Prince, Lippiello and Lukas, 1995).

The phenothiazine, W-7, antagonises the action of calmodulin (CaM) by inhibiting the ability of CaM to interact with target proteins including CaM-dependent PDEs and CaM-kinase II. A range of concentrations of W-7 were tested and all

Figure 42. TMB-8 rapidly inhibits nicotinic acetylcholine responses.

TMB-8 was applied to the D_f motoneurone to determine if intracellular Ca²⁺ release from IP₃ stores was important for aminergic suppression of the ACh response. However TMB-8 itself rapidly reduced control ACh responses as shown in figures (a) and on an expanded time scale in (b). TMB-8 was readily soluble in normal Ringer solution and applied in 20 µl aliquots to the bath solution. After a delay of only 1 minute there was a rapid and dramatic reduction in the amplitude of the ACh depolarisation. Washout with fresh saline was commenced, upon which ACh responses were restored. The effect of 10⁻⁵ M TMB-8 was fully reversed by only 5 minutes wash.

The effect of TMB-8 was concentration-dependent as shown in the graph in (c); each data point represents the mean and standard error of two or more experiments. ACh responses were completely inhibited by concentration of 10⁻⁴ M TMB-8 and greater.



suppressed the ACh response (see figure 43b). The maximum suppression observed when the drug was applied at a concentration of 10^{-5} M was 44.4% (+s.e.m. 7.8, -s.e.m. 7.7; n = 5) of the control. The effect of W-7 was irreversible; after a half hour of washout the effect of W-7 was not significantly reversed ($p = 0.587$) to 55.3% (+s.e.m. 14.7, -s.e.m. 15.3; n = 5) of the control ACh amplitude. Although W-7 reduced the amplitude of ACh responses, it did not block the effect of 10^{-4} M serotonin at the concentrations tested. This suggests that CaM is not involved in the main signalling pathway by which serotonin suppresses of ACh responses. However the irreversibility of the effects of serotonin in the presence of W-7, suggests that CaM might have a regulatory role involved with the processes that reverse the actions of the amine.

KN-62 selectively binds to the CaM site of CaMK-II and therefore, unlike W-7, selectively prevents CaM from activating of its target kinase. Concentrations of KN-62 of 10^{-5} M and above resulted in a prolonged inward current independent of ACh application. Therefore to avoid this problem, experiments were performed using KN-62 at a concentration of 10^{-6} M to test its effects on suppression of nicotinic ACh transmission. Exposure to KN-62 (10^{-6} M) resulted in a reduction in the amplitude of the ACh current to 81.5% (+s.e.m. 8.5, -s.e.m. 10.3; n = 6), of control. This was not significantly different ($p = 0.07$) from the effect of a control application of 20 μ l saline (97.2% +s.e.m. 1.7, -s.e.m. 1.6; n = 6). Incubation with the calmodulin kinase inhibitor for 10 minutes did not block the suppressive effect of serotonin (10^{-4} M) and, unlike W-7, the effect of KN-62 was reversible after 10 – 20 minutes wash (for example see figure 44a). An interesting feature of KN-62 was that it produced a rapid and reversible increase in the number of synaptic potentials (seen most clearly in figure 44a).

Figure 43. The calmodulin-agent W-7 suppresses ACh responses but does not block or occlude aminergic modulation.

The three panels of (a) represent the three major effects of W-7. The left-hand panel shows the direct action of 10^{-5} M W-7, which was to reduce the amplitude of the control ACh current. W-7 was readily dissolved in normal saline and applied in 20 μ l aliquots to the bath. Subsequent addition of serotonin (10^{-4} M) further reduced the amplitude of the ACh current, the maximal effect of the amine observed after 10 minutes application being shown in the central panel. Wash with normal saline did not reverse the effect of the two agents added to the bath as can be seen in the third panel. The effect of both drugs was compared to the amplitude of the initial control ACh response in all three panels. This was because it was impossible to determine whether the action of W-7, seen in the left-hand panel of (a), is independent of the action of the amines on nicotinic responses. The continued suppression of ACh responses after prolonged washing would suggest that W-7 somehow inhibits the processes involved in reversing aminergic suppression. The effect of W-7 increased with higher concentrations as shown in (b), but no inhibition of aminergic suppression of ACh responses was observed. Data shown represents three or more results for each point apart from that for 10^{-7} M W-7 which represents a single observation.

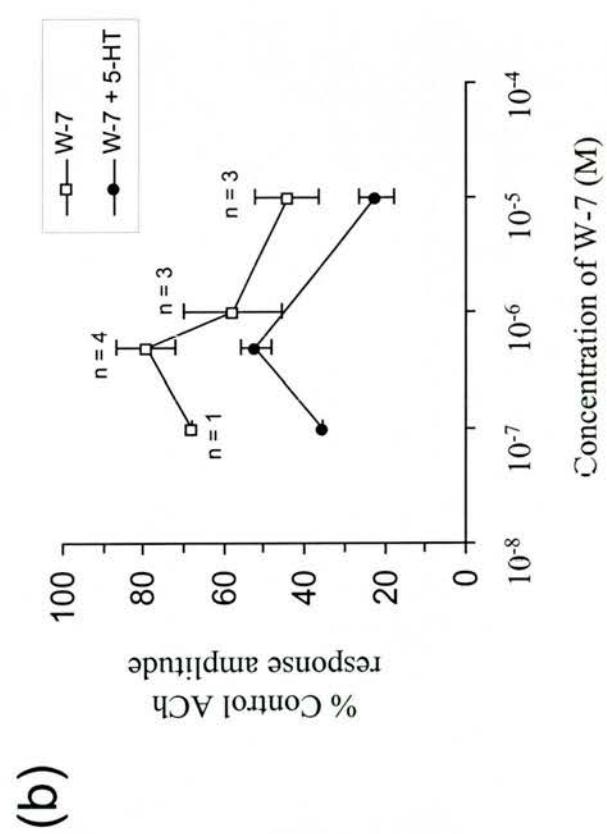
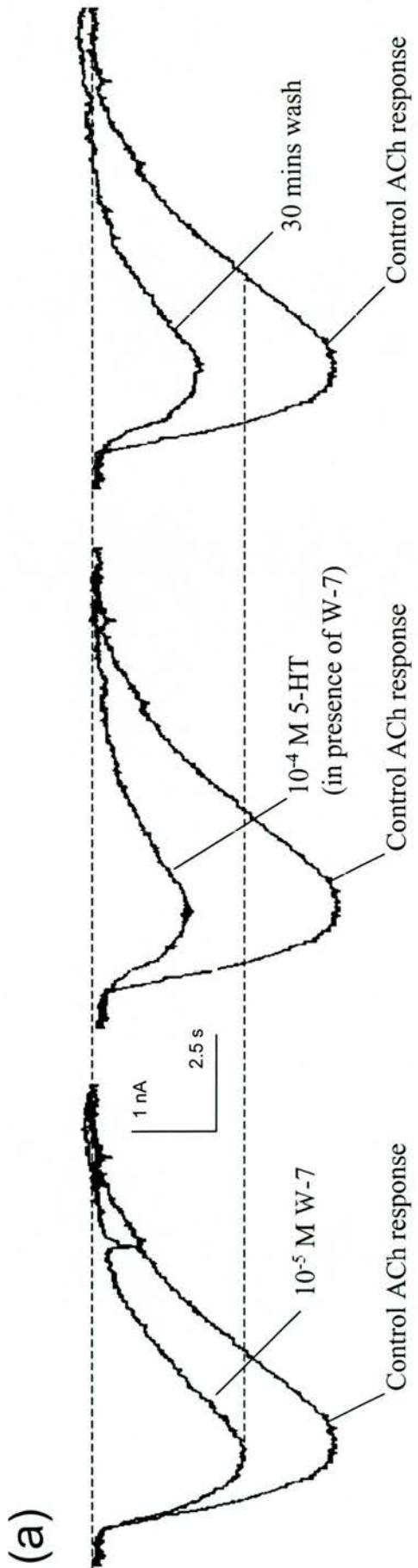
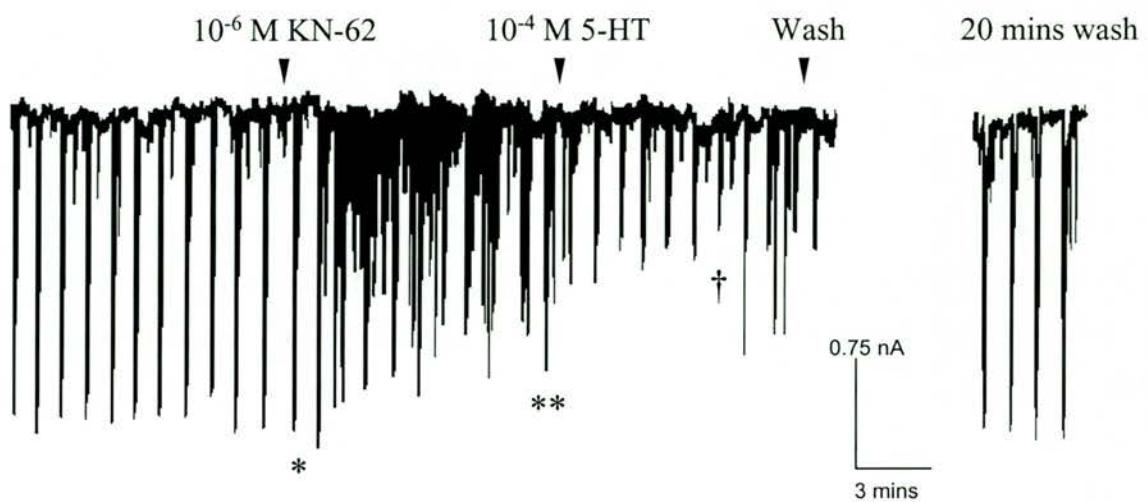


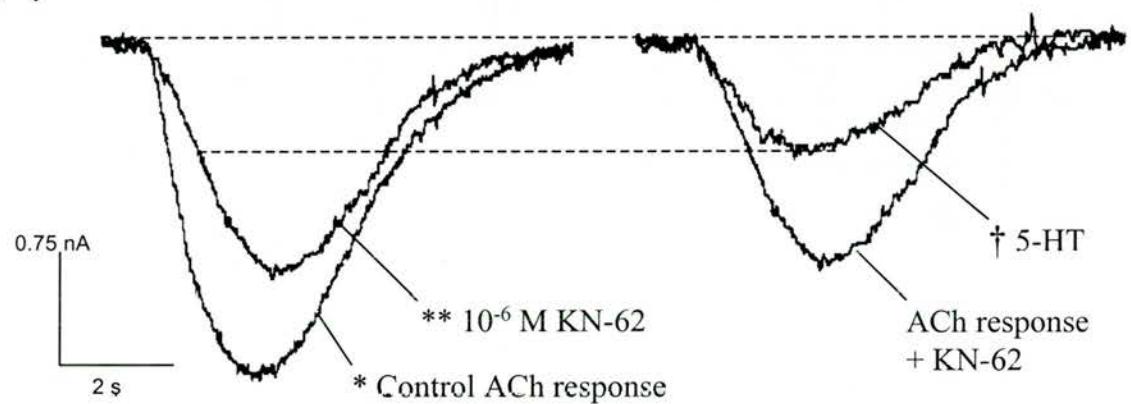
Figure 44. KN-62 does not prevent aminergic modulation of nicotinic ACh responses.

(a) Application of the CaM kinase inhibitor, KN-62, had a direct suppressive effect on the ACh pressure pulses, more clearly seen in the expanded traces shown in (b) (cal. (a): vertical 0.75 nA, horizontal 3 mins. Cal. (b): vertical 0.75 nA, horizontal 2 seconds). Although KN-62 was prepared in DMSO(0.05%), this concentration was not sufficient to significantly affect ACh responses. KN-62 also resulted in a rapid and dramatic increase in the observable synaptic potentials as shown in (a). Bath application of serotonin (10^{-4} M) after 10 minutes of exposure to KN-62 resulted in a further decrease in the amplitude of the ACh inward current. There was also a discernible decrease in the synaptic potentials after serotonin application. Pooled data from six experiments is shown in the histogram below (c). The action of 10^{-6} M KN-62 was not significantly different from a control application of 20 μ l normal saline, nor was the combined effect of KN-62 and serotonin different to the effect of serotonin alone.

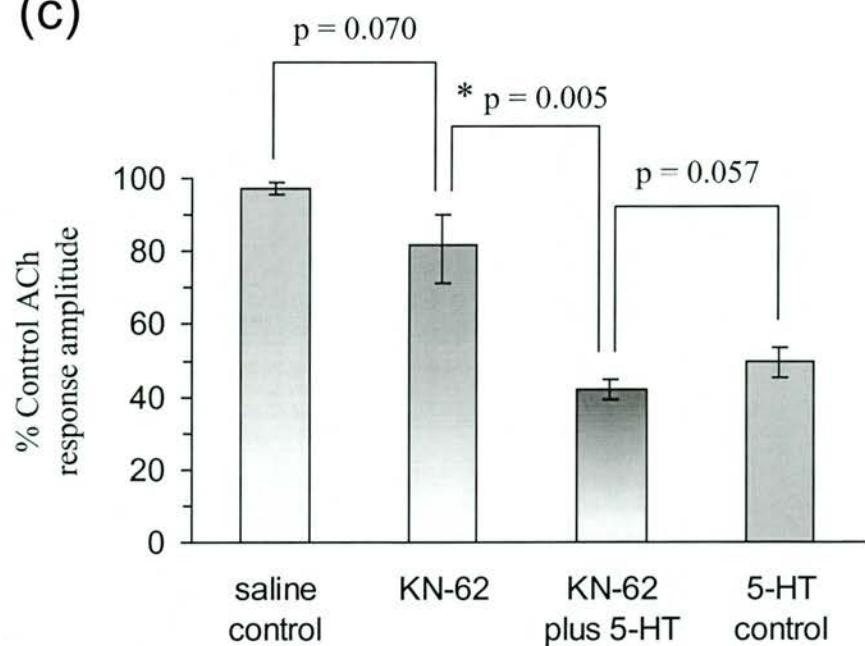
(a)



(b)



(c)



The evidence from the three pharmacological agents used to investigate calcium metabolic pathways (TMB-8, W-7 and KN-62) taken with the results obtained with BAPTA indicate that calcium does not play a fundamental role in aminergic modulation, but that it may exert a regulatory influence either directly on nAChRs of the D_f motoneurone or on the aminergic modulatory pathway. These issues will be considered further in the discussion.

5 A role for cyclic nucleotides in the regulation of nicotinic ACh responses.

Cyclic nucleotides are important intracellular second messengers, exerting their actions via stimulation of specific kinases (Scott, 1991). Enzymes responsible both for the synthesis and degradation of cyclic nucleotides are present in insect nervous systems. These include a number of amine-sensitive adenylyl cyclases that have been characterised in the metathoracic ganglion of *Periplaneta americana* (Nathanson and Greengard, 1973 and 1974).

To determine whether elevations in either adenosine 3', 5'-monophosphate (cAMP) or guanosine 3', 5'-monophosphate (cGMP) modulate nicotinic transmission, a range of membrane-soluble analogues were applied to the preparation. Application of the cAMP analogues dibutyryl cyclic AMP (Db-cAMP), and chlorophenylthio-cyclic AMP (CPT-cAMP) as well the cGMP analogue 8- bromo cyclic GMP (Br-cGMP), all mimicked the effect of the amines by attenuating the ACh responses (figures 45 and 46). The effect of 15 minutes incubation with CPT-cAMP recorded using current clamp was to reduce the ACh response to 59.7% (+s.e.m. 8.0, -s.e.m. 8.3; n = 7) of the control, while Db-cAMP and Br-cGMP reduced it to 64.4% (+s.e.m. 5.6, -s.e.m. 5.8; n = 6), and 49.2% (+s.e.m. 12.2, -s.e.m. 12.1; n = 5) of the control respectively. All three cyclic nucleotide analogues had a similar action on ACh currents recorded under voltage clamp conditions.

The time course for the suppression of nicotinic responses mediated by the cyclic nucleotide analogues was not as rapid as that observed after addition of the amines, the delay being most likely attributable to the fact that the analogues have to permeate through the cell membrane to exert their action. All three achieved maximal suppression of the ACh response only after 10 – 15 minutes exposure.

Figure 45. Cyclic nucleotide analogues mimic the action of the amines on ACh response of the D_f motoneurone.

The modulatory action of the cyclic nucleotide analogues CTP-cAMP (a), Db-cAMP cAMP (b) and Br-cGMP cAMP (c) are shown under current clamp conditions. All three analogues were soluble in Ringer solution and made up as stock solutions of 10^{-2} M. 20 μ l aliquots were applied to the preparation, resulting in a final bath concentration of the analogue of 10^{-4} M. Fifteen minute incubation with all three resulted in a significant suppression of the control response, particularly evident in the Db-cAMP trace (b). The effect of the cyclic nucleotide analogues could be partially reversed after prolonged (10 - 20 minute) washout as shown in the right-hand panels. (cal. vertical 4 mV, horizontal 2 mins).

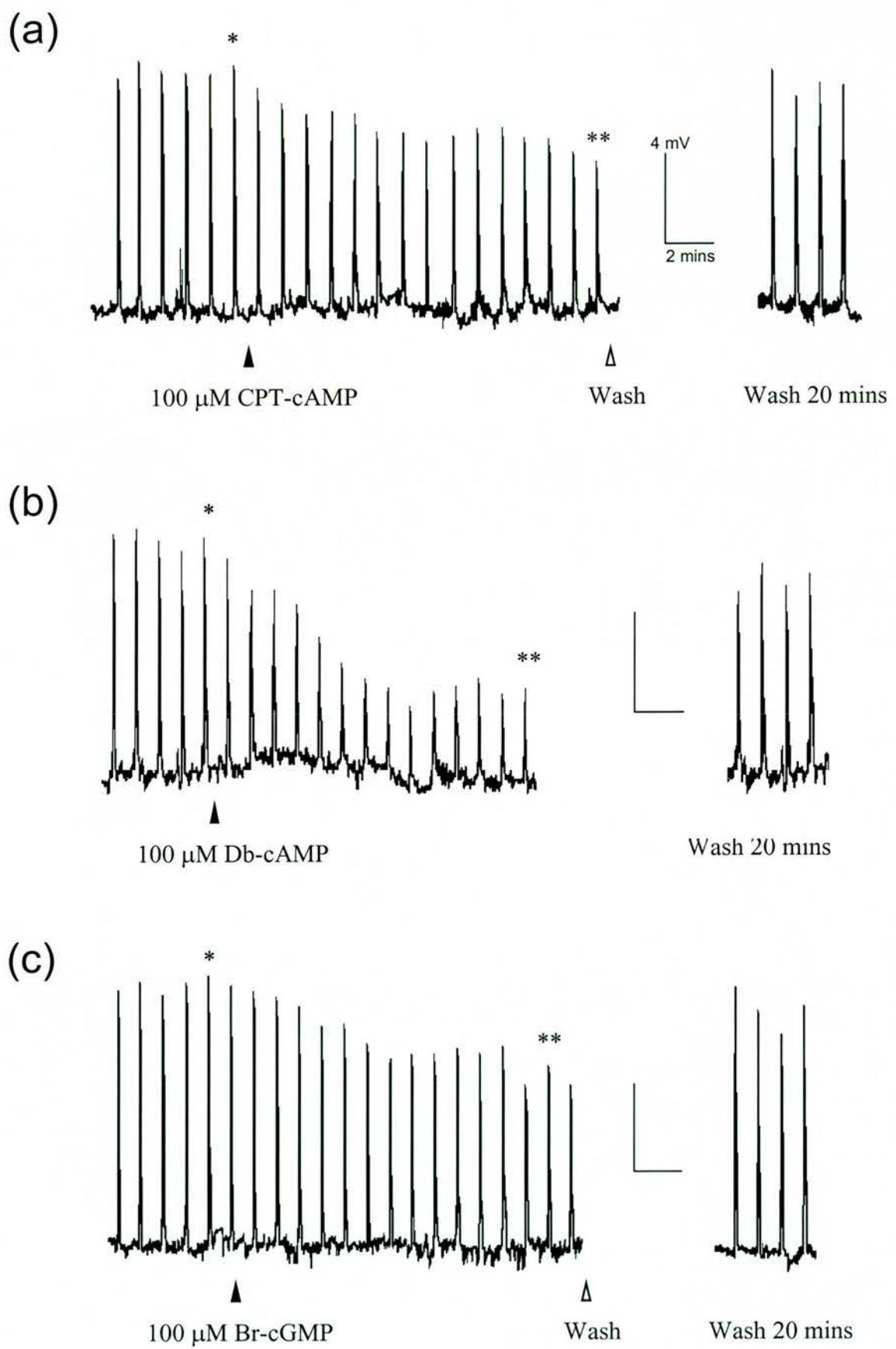
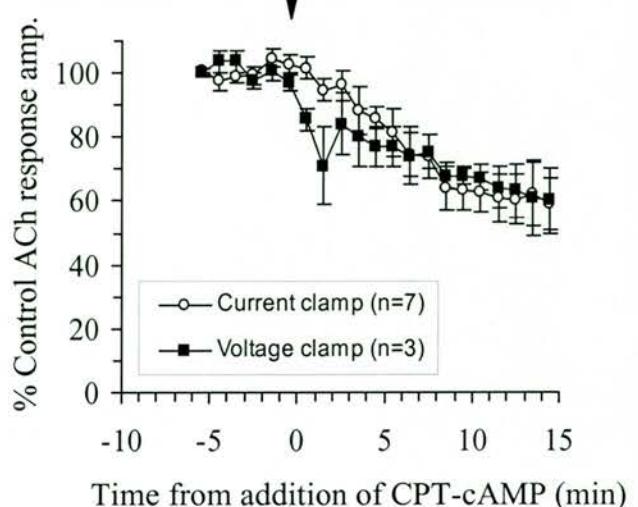
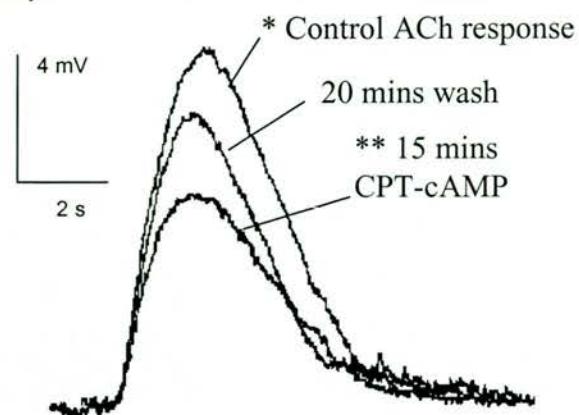


Figure 46. Cyclic nucleotide analogues suppress ACh responses recorded from the D_f motoneurone.

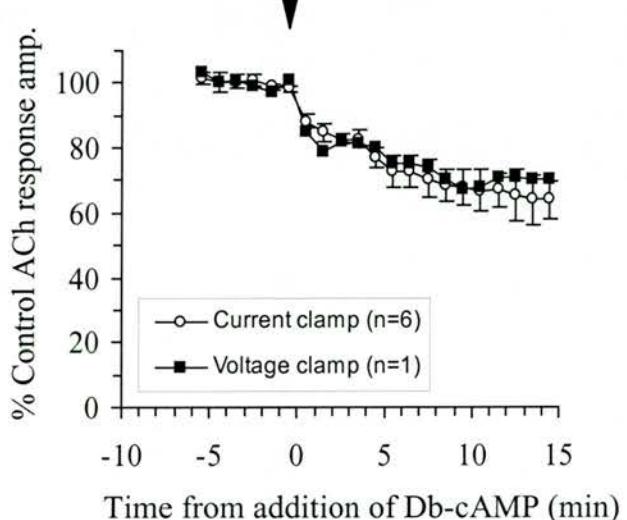
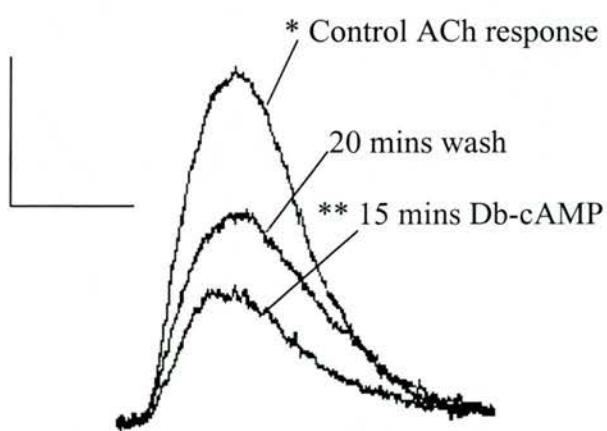
The left-hand panels contains superimposed individual responses (marked with asterisks) from the traces shown in figure 45. On the right side is displayed the pooled data for each of the amines, each data point representing the standard error of the mean for three or more results with the exception of Db-cAMP which was only tested once under voltage clamp conditions. The point at which the analogues were added to the bath is indicated by the back arrow heads.

It is evident from the graphs that the effectiveness of the cyclic nucleotide analogues in suppressing ACh responses of D_f did not differ according to the recording protocol used. All three gradually suppressed the nicotinic ACh response, exerting maximal effects after 10 –15 minutes. (calibration: vertical 4 mV, horizontal 2 seconds).

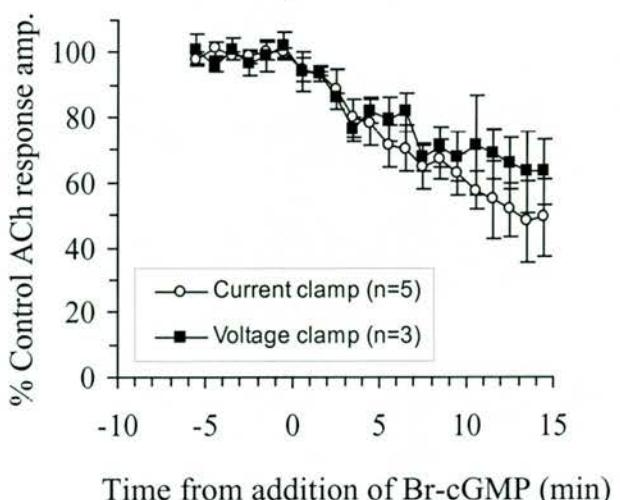
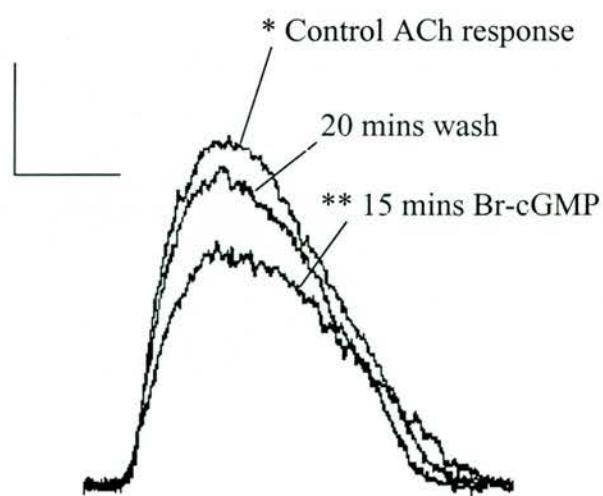
(a)



(b)



(c)



5.1 Cyclic nucleotide analogues partially occlude the effects of monoamines.

The ability of cyclic nucleotide analogues to mimic the effect of monoamines suggests that the latter could exert their action via an elevation in intracellular concentrations of the second messengers cAMP or cGMP or both. If this were the case, prior incubation with the analogues should reduce the sensitivity of the neurone to amine application by saturating the cyclic nucleotide intracellular pathways.

Experiments to determine if the suppression mediated by the cyclic nucleotides occludes the effect of the monoamine serotonin were conducted under voltage clamp conditions. Control applications of serotonin were conducted prior to incubation with either 10^{-4} M CPT-cAMP ($n = 6$) or 10^{-4} M Br-cGMP ($n = 7$). In all experiments the cyclic nucleotides and serotonin individually reduced the amplitude of ACh responses. When 10^{-4} M serotonin was applied to cells incubated with either CPT-cAMP (figure 47) or Br-cGMP (figure 48) the sum of the suppression due to both agents was not significant greater than the suppression of ACh observed when serotonin alone was applied to the D_f soma ($p = 0.099$ and 0.382 respectively; figure 47). As both agents exhibit similar suppressive actions on the nicotinic ACh response, it can reasonably be assumed that elevating intracellular cyclic nucleotide concentrations by incubation with the analogues in effect saturates the cyclic nucleotide-dependent second messenger pathway and occludes the action of the monoamines. This supports the notion that serotonin exerts its effect on ACh responses via an elevation in intracellular cyclic nucleotide concentrations. However it does not allow any conclusion to be reached as to which cyclic nucleotide pathway, be it cAMP or cGMP or indeed both, that mediates the effect of the amine.

Figure 47. CPT-cAMP partially occludes the action of 10^{-4} M serotonin.

The ability of the cyclic nucleotides to occlude the effects of serotonin was tested under voltage clamp. The three sets of ACh inward currents shown in (a) illustrate on the left side the effect of serotonin, tested prior to incubation with the nucleotide analogue. In the middle panel, the effect of 10^{-4} M CPT-cAMP once the suppression mediated by serotonin has been reversed. The response shown typical of the suppression mediated by the analogue observed after 15 minutes exposure. Subsequent addition of serotonin further reduced the ACh response as shown on the far-right. Pooled data from 6 experiments is shown in figure (b); statistical analysis revealing that the combined effect of CPT-cAMP and serotonin (58.9% of control +s.e.m. 3.4, -s.e.m. 3.5; n = 6) was not significantly different from that of the amine alone (51.0% of control +s.e.m. 2.0, -s.e.m. 2.0; n = 6).

Figure 47a calibration: vertical = 1 nA, horizontal = 8 seconds.

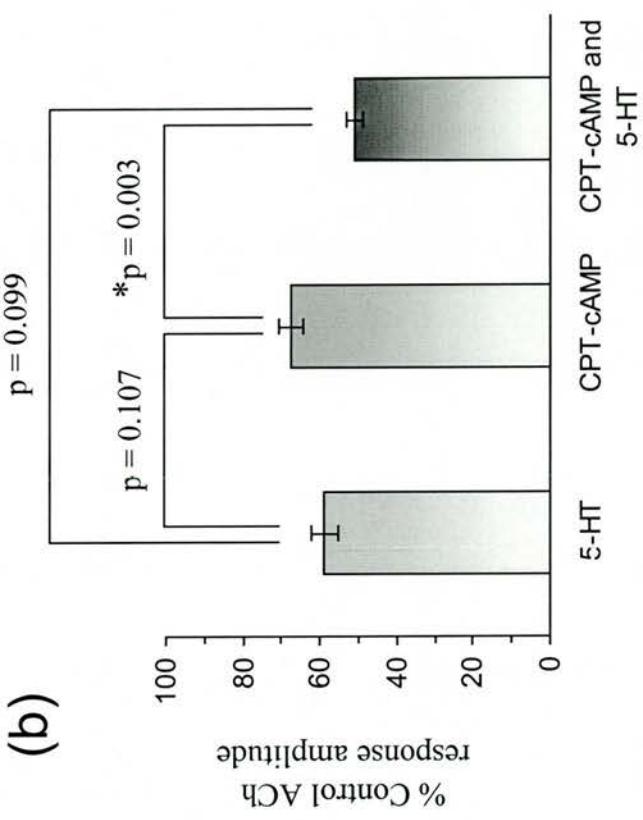
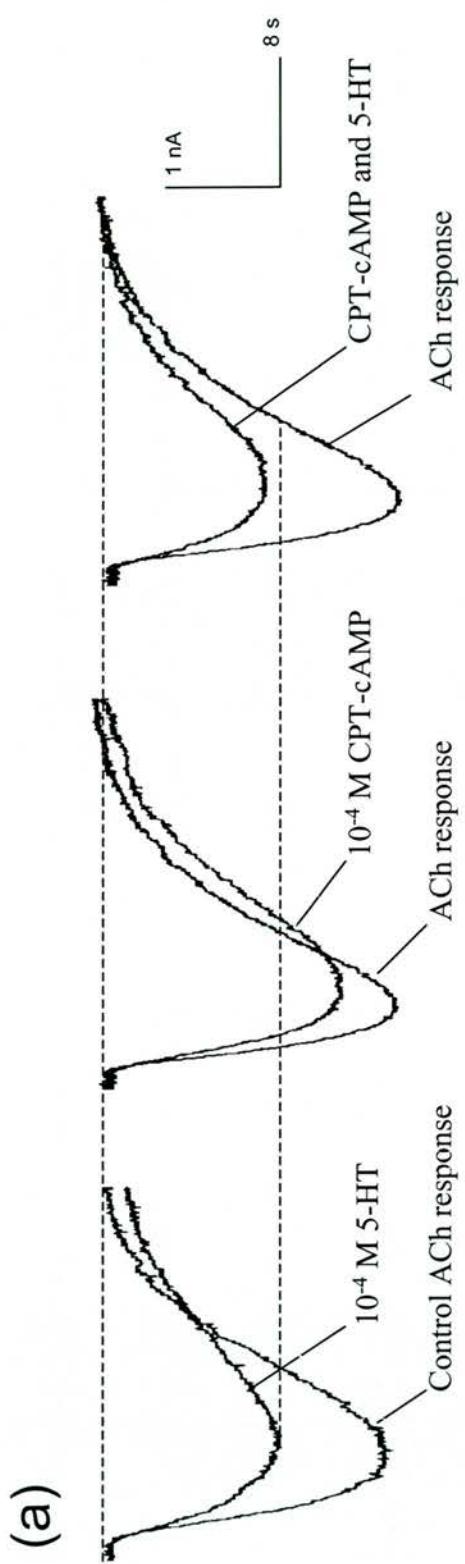
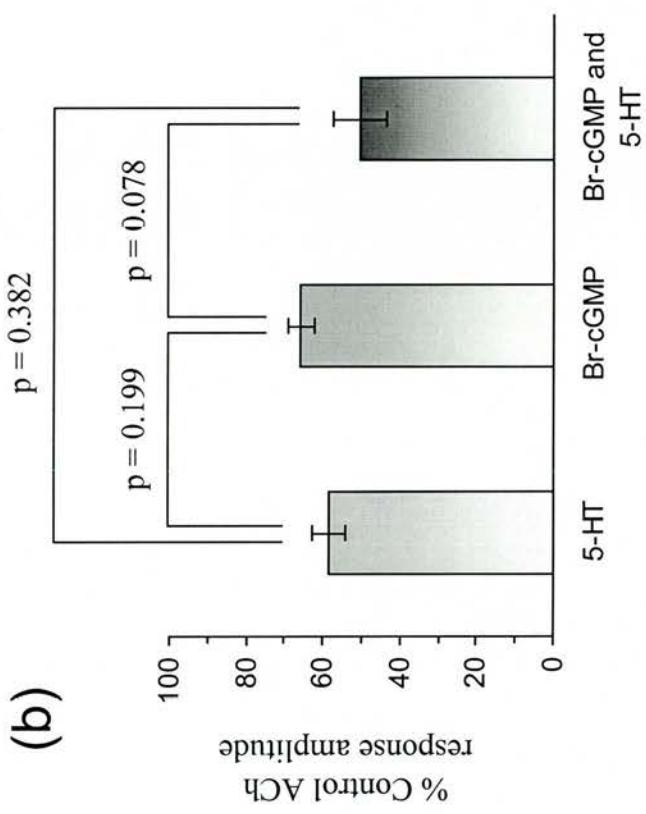
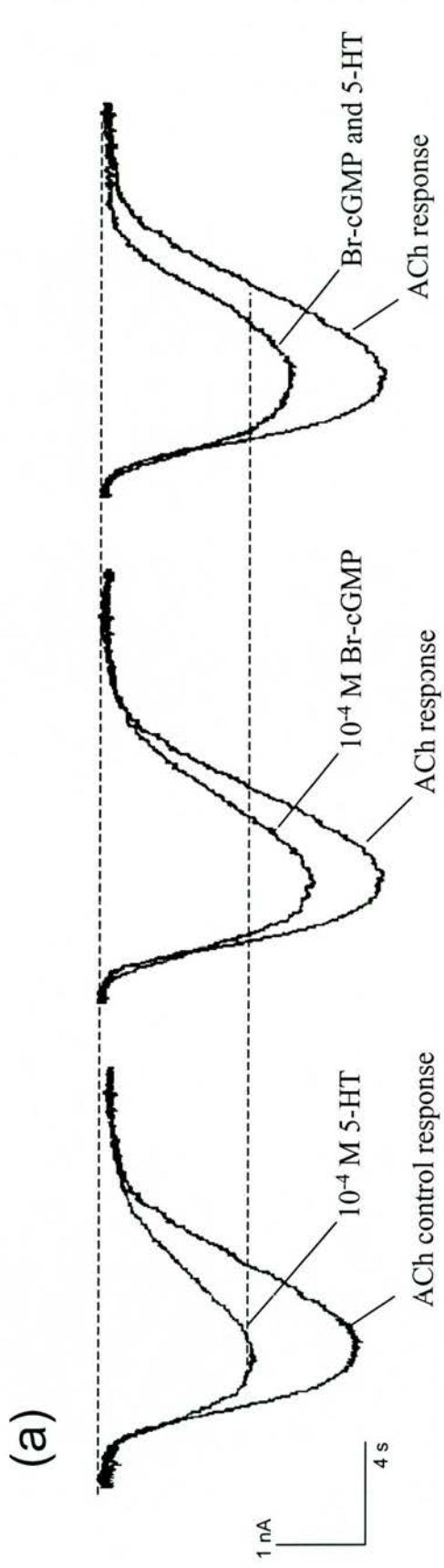


Figure 48. Br-cGMP partially occludes modulation of ACh responses mediated by serotonin.

The results for Br-cGMP are presented in the same format as those for CPT-cAMP in figure 47. (a) A test application of 10^{-4} M serotonin was applied to the preparation to ascertain that the preparation exhibited aminergic suppression of the ACh response (left hand panel). The effect of serotonin was reversed by washing with normal saline and then the preparation incubated for 15 minutes with 10^{-4} M Br-cGMP (central panel) prior to a second application of serotonin (right panel). Pooled data from the seven experiments conducted are illustrated in the histogram below (b). The combined effects of serotonin and Br-cGMP were not significantly different ($n = 0.382$; $n = 7$) than that of serotonin alone.

Calibrations for (a) is vertical = 1 nA, horizontal = 4s.



5.2 Action of adenylyl and guanylyl cyclase inhibitors on amine-mediated suppression of nAChR responses.

In order to further clarify the role of cyclic nucleotides in transducing aminergic suppression, inhibitors of cyclic-nucleotide-generating enzymes were applied to the preparation to determine whether they would selectively attenuate the modulation.

2',5'-Dideoxyadenosine (DDA) is a potent and selective inhibitor of adenylyl cyclases, the intracellular enzymes responsible for generation of cAMP. Figure 49 demonstrates the observed effect of DDA on nACh voltage responses recorded from D_f and the subsequent effect of serotonin. In 3 out of 5 preparations 10⁻⁵ M DDA enhanced control ACh responses, whilst in the other two it exhibited no observable effect. The average effect of DDA for all 5 experiments was to increase the ACh response to 106.2 % (\pm s.e.m. 8.3; n=5) of the baseline response. This would suggest that there is a small degree of endogenous adenylyl cyclase activity that causes a slight tonic suppression the ACh response. In all preparations tested there was a significant reduction in the suppressive effects of serotonin on ACh responses compared with controls. This provides additional evidence to support the hypothesis that serotonin exerts its action via stimulation of adenylyl cyclase and an elevation in intracellular cAMP levels .

To further investigate the role of cGMP in aminergic modulation, the ability of the guanylyl cyclase (GC) inhibitor methylene blue to attenuate suppression of ACh responses was tested. These initial experiments were conducted using 10⁻⁴ M dopamine as the test amine, as the actions of all three amines had not been fully characterised at the time they were performed. Incubation with 10⁻⁴ M methylene blue did not significantly alter the amplitude of the control ACh response (figure 50a, left-hand panel) nor did it prevent the ability of dopamine to suppress nicotinic

Figure 49a. 2',5'-Dideoxyadenosine (DDA) attenuates the suppressive action of serotonin on ACh response recorded from D_f.

All the traces in (a) are taken from the same preparation. The top trace illustrates the effect of 10^{-4} M serotonin on ACh depolarisations. Addition of the amine to the bath produced a reversible reduction in the amplitude of responses. Incubation with the adenylyl cyclase inhibitor DDA (10^{-5} M) had no overall effect on the amplitude of ACh responses but subsequent addition of serotonin did not attenuate the ACh response as much as previously observed. To ensure that the ability of the cell to respond to the amine had not been compromised by some other factor other than the action of DDA, the preparation was washed for 20 minutes in an attempt to reverse the AC inhibitor action. Wash reversed the block of serotonergic suppression. Indeed it was marginally enhanced.

(a)

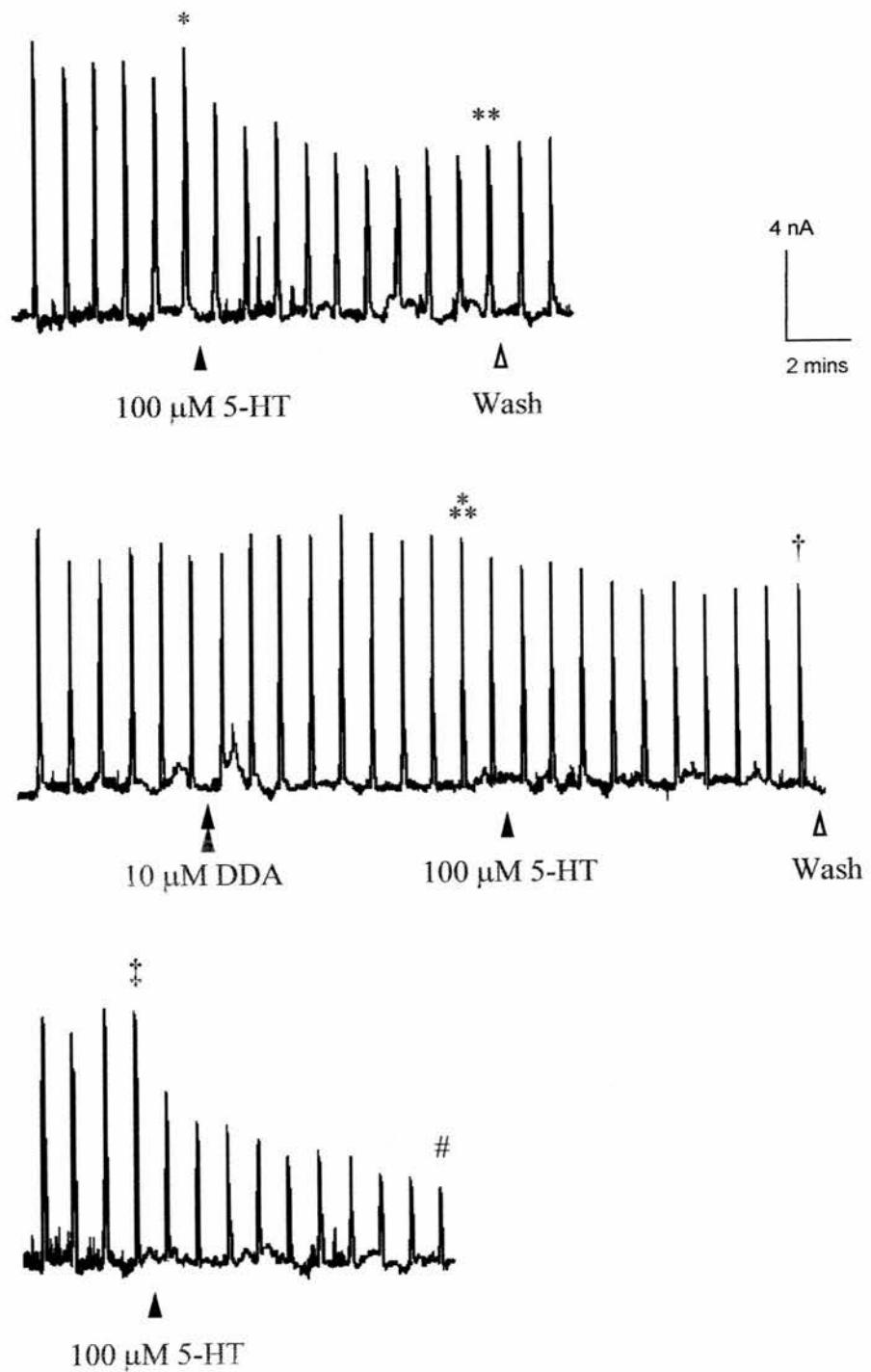
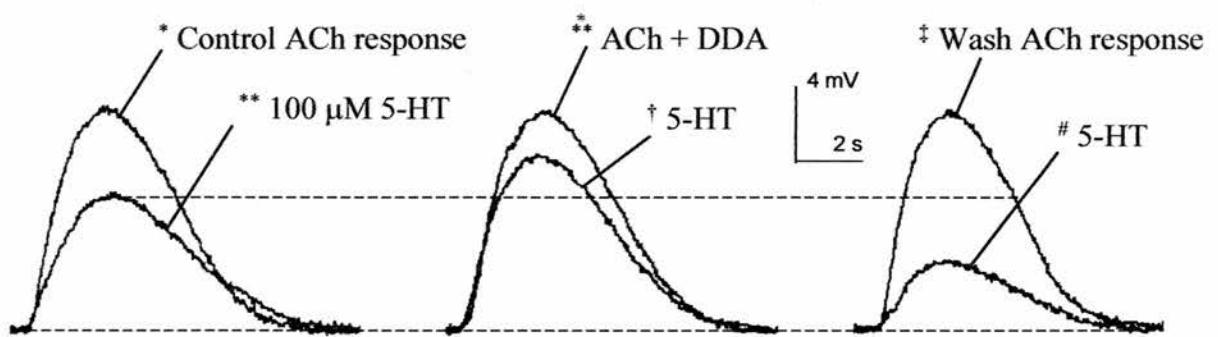
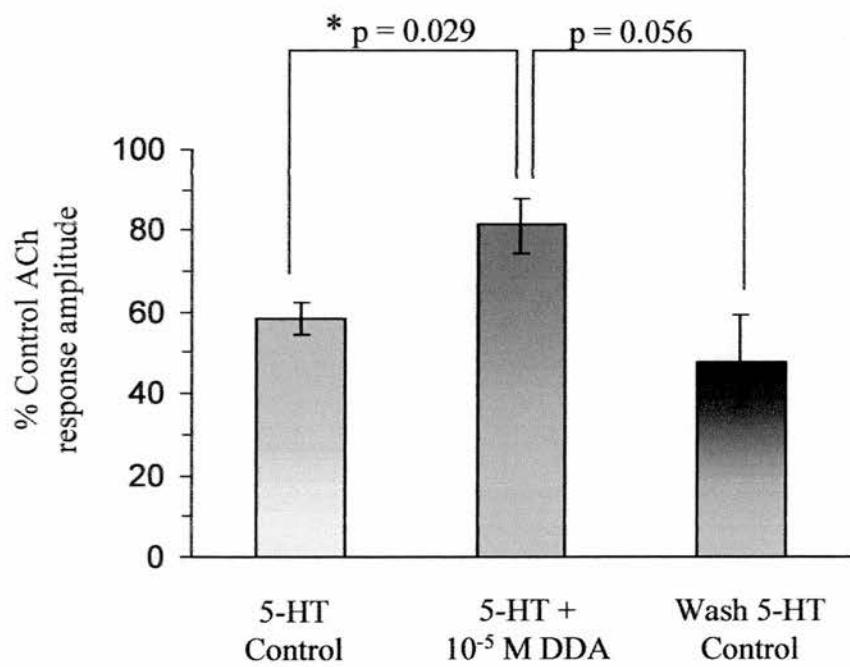


Figure 49b and 49c. The ability of DDA to attenuate the action of serotonin in suppressing ACh responses is shown more clearly in the expanded ACh responses taken from figure (a) (those responses which were selected are indicated by the asterisks and symbols). It is also noticeable in the traces shown, that after washout of DDA, the action of serotonin was enhanced. Pooled data from five experiments is shown in the histogram below (c). The effect of DDA (10^{-5} M) was to significantly reduce the ability of serotonin to modulate ACh responses recorded from D_f . The action of DDA in attenuating aminergic suppression of ACh responses was partially reversed after 20 minutes washout (cal. vertical 4 mV, horizontal 4 seconds).

(b)



(c)



transmission (figure 50a, right-hand panel). Pooled data from 3 current clamp experiments are shown in figure 50b. Dopamine in the presence of 10^{-4} M methylene blue reduced the ACh response to 68.4% (+s.e.m. 4.7, -s.e.m. 4.9, n = 3) of control, which was not significantly different from the action of dopamine in the absence of this GC antagonist (70.2% +s.e.m. 3.2, -s.e.m. 3.3, n = 3). Therefore it can be reasonably assumed that the suppression of ACh responses due to the amines is not mediated by a methylene blue-sensitive guanylyl cyclase.

Since some guanylyl cyclases are sensitive to the intracellular signalling molecule nitric oxide, SNAP (S-nitroso-N-acetylpenicillamine), the nitric oxide donor, was tested to determine if a nitric oxide-sensitive guanylyl cyclase was involved in the regulation of nicotinic responses exhibited by the D_f motoneurone. SNAP was dissolved in DMSO and applied at a final bath concentration of 10^{-4} M. Application of the chemical to the preparation, under conditions of darkness (since this compound is photolabile), did not directly modulate ACh responses recorded from D_f. In one of the three preparations tested there was a discernible increase in the number of EPSPs observed.

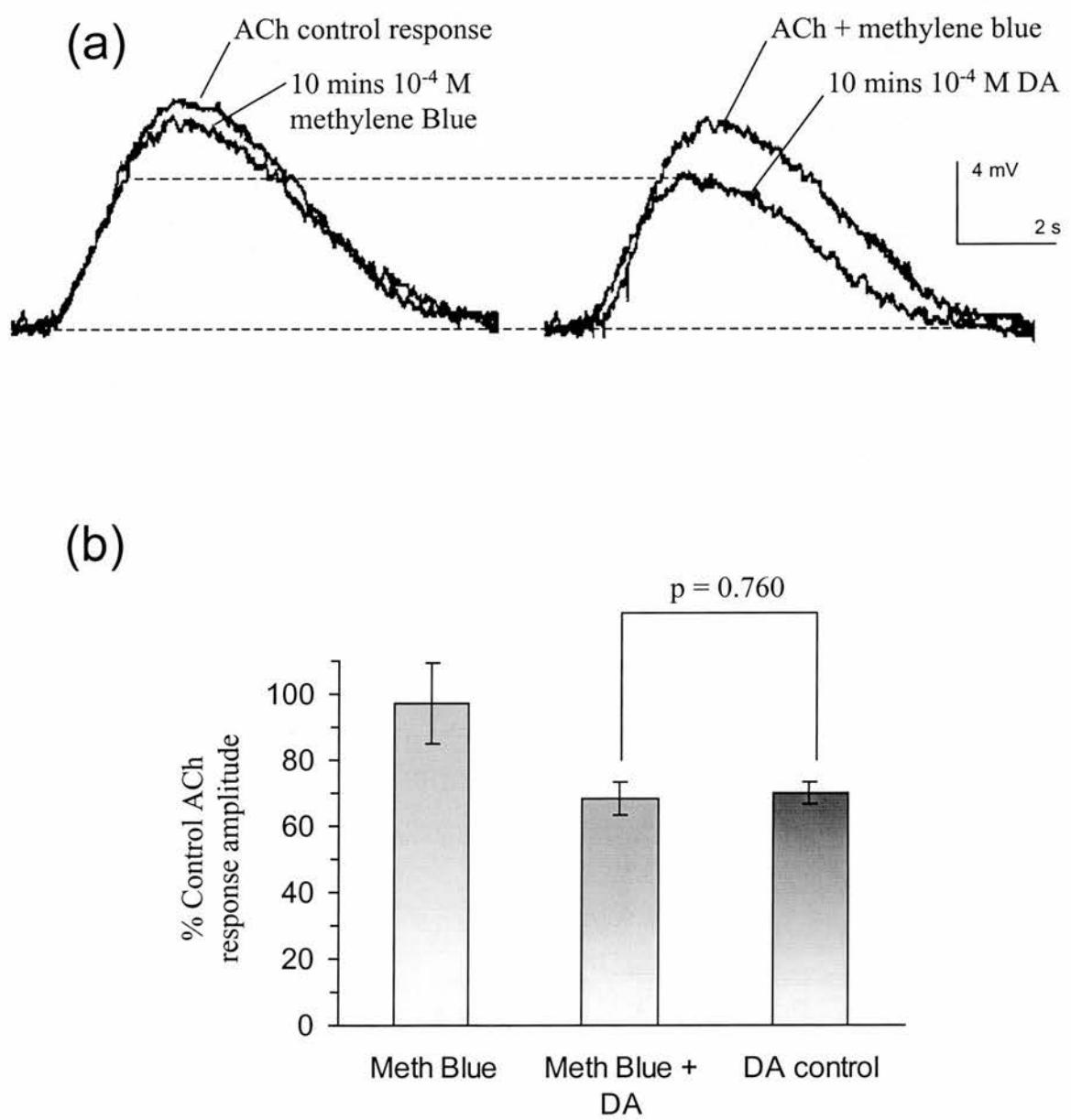
5.3 Inhibition of phosphodiesterases enhances aminergic suppression of nicotinic ACh responses.

Receptor-stimulated rises in cyclic nucleotides are normally brief, because phosphodiesterases (PDEs) rapidly hydrolyse these second messengers. Therefore, inhibitors of PDE activity, such as xanthines, are potentially useful tools in the study of receptor coupling to cyclic nucleotide cascades, since they will cause any rise in concentration of the second messenger to be augmented and prolonged. To investigate the action of PDEs in D_f the xanthine, theophylline, was applied to the

Figure 50. The ability of the soluble guanylyl cyclase inhibitor methylene blue to block suppression of nACh responses mediated by dopamine was tested under current clamp.

(a)The left-hand panel shows the typical direct effect of incubation with 10^{-4} M methylene blue; on average addition of a 20 μ l aliquot of methylene blue did not significantly affect the amplitude of ACh response, reducing it to 97.4% of control (+s.e.m. 12.2, n = 3; (b)). Methylene blue did not block suppression mediated by 10^{-4} M dopamine as shown in the right-hand panel (a).

The pooled data from three experiments is shown in (b); dopamine in the presence of methylene blue reduced the ACh response amplitude to 68.4% of the control ACh response (+s.e.m. 4.7; - s.e.m. 4.9; n = 3). This action was significantly different ($p = 0.760$) to the effect of dopamine itself, which suppressed the ACh response to 70.2% (+s.e.m. 3.2; - s.e.m. 3.3; n = 3) of control.



preparation to determine whether PDE activity influences either the duration or extent of aminergic suppression of ACh response. For the latter, preparations were exposed to 10^{-4} M theophylline for 15 minutes in the closed circulating bath set-up (see Methods) before application of 10^{-5} M serotonin (figure 51a). Applications of 10^{-5} M serotonin in the absence of theophylline reduced ACh responses to 78.1% (+s.e.m. 3.4, -s.e.m. 3.6, n = 4) of control (figure 51b). After addition of the PDE inhibitor the effectiveness of the same concentration of serotonin in suppressing ACh responses was significantly enhanced, so that the ACh response was reduced to 51.0% of control (+s.e.m. 8.3, -s.e.m. 8.4, n = 4; p = 0.040).

The ability of theophylline to prolong serotonergic suppression of the ACh response was tested under conditions of continual perfusion of fresh saline through the bath, as this method of saline flow provides better information as to the time course of modulation. After a control application of 10^{-3} M serotonin in normal saline, the reservoir of fresh saline was switched to a solution containing 10^{-4} M theophylline. The effect of theophylline was to prolong the suppression mediated by a brief exposure to serotonin (figure 52). The amplitude of the ACh responses remained suppressed for the duration of perfusion with saline containing theophylline. Only once the inward flow of saline was switched back to the control saline solution did the amplitude of the ACh response begin to recover back to control levels.

Figure 51. 10^{-4} M Theophylline (THEO) causes a decrease in amplitude of ACh responses and enhances the maximal suppression mediated by 10^{-5} M serotonin. Individual ACh responses shown on an expanded time scale reveal the effectiveness of 10^{-5} M serotonin in reducing the control ACh amplitude response (left-hand panel (a)). Application of theophylline for a period of 15 minutes had a suppressive effect, reducing the amplitude of the ACh response recorded after the action of 10^{-5} M serotonin had been reversed. Incubation with the PDE inhibitor did significantly enhance the action of the low dose of serotonin as shown in the right-hand panel.

(b) The histogram shows the mean and standard error of the reduction in the ACh response observed after 10 minutes 10^{-5} M serotonin expressed as a percentage of the initial control ACh amplitude. Incubation with 10^{-4} M theophylline significantly ($p = 0.040$) enhanced the action of serotonin.

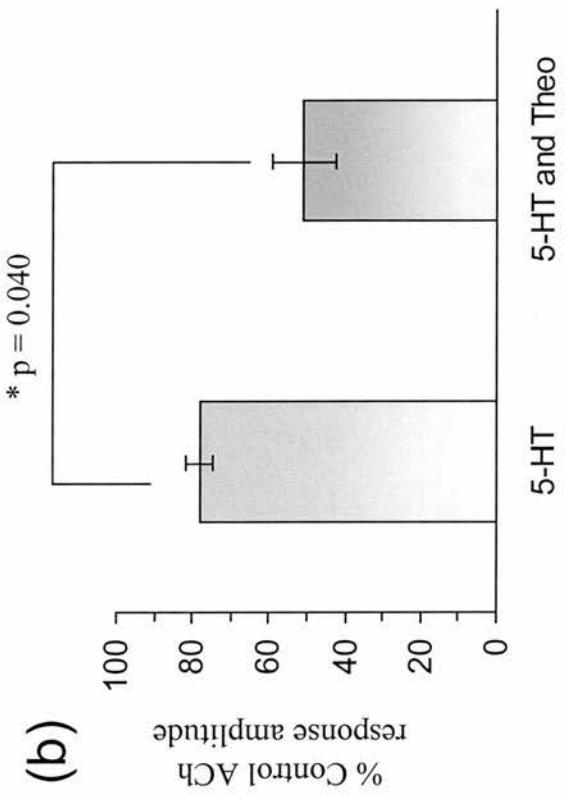
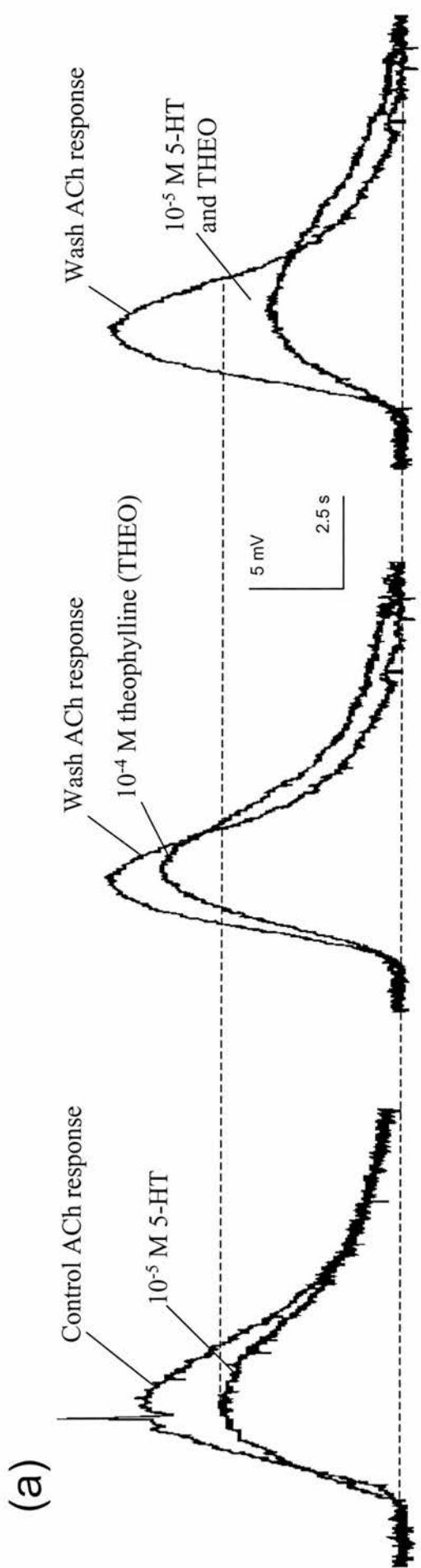
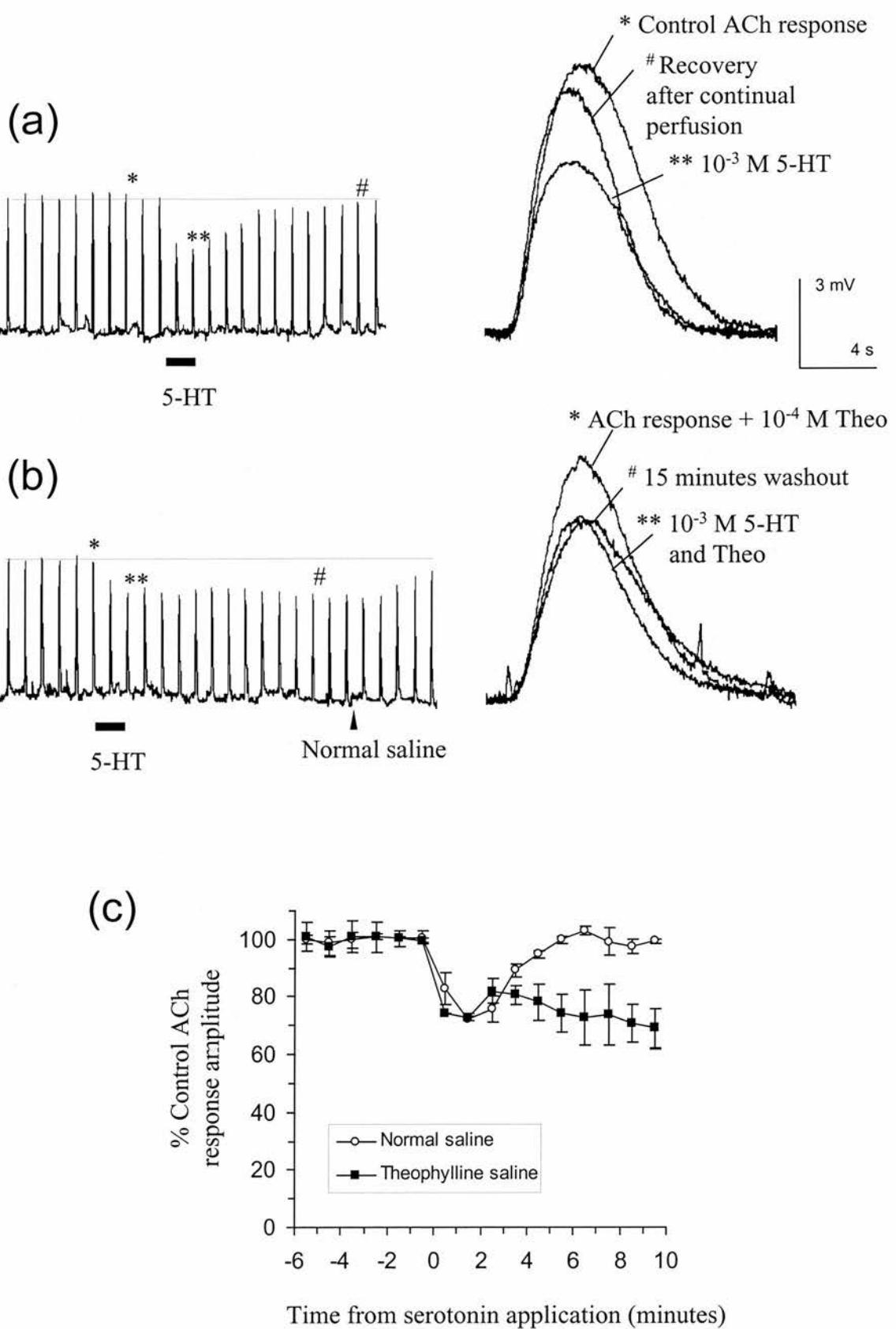


Figure 52. The PDE inhibitor, theophylline, prolongs the duration of suppression mediated by serotonin.

(a) Application of 10^{-3} M serotonin under continual perfusion resulted in a transient suppression of the ACh response amplitude. The amine was present for approximately 90 seconds as indicated by the bar below the trace. Example responses, as indicated by the asterisks, are shown expanded on the right.

(b) Perfusion of the preparation with saline containing 10^{-4} M theophylline caused a dramatic prolongation in the effect of serotonin such that suppression of ACh responses was only reversed when theophylline was washed from the bath. The averaged data from 4 experiments is presented in (c).

(c) Averaged data from 4 experiments in which the action of theophylline was studied. The plot shows clearly that in the presence of theophylline (filled squares) the time course of aminergic suppression was prolonged as opposed to a control application (open circles).



6 Aminergic suppression of ACh responses is dependent on phosphorylation.

Elevations in intracellular concentrations of the cyclic nucleotides cAMP and cGMP stimulate the activity of their respective kinases, protein kinase A and protein kinase G respectively, which in turn promote phosphorylation of consensus sequences on target peptides or proteins. Proteins that constitute such targets have been shown to include vertebrate nAChRs, so it was of interest to determine if phosphorylation was necessary for aminergic modulation.

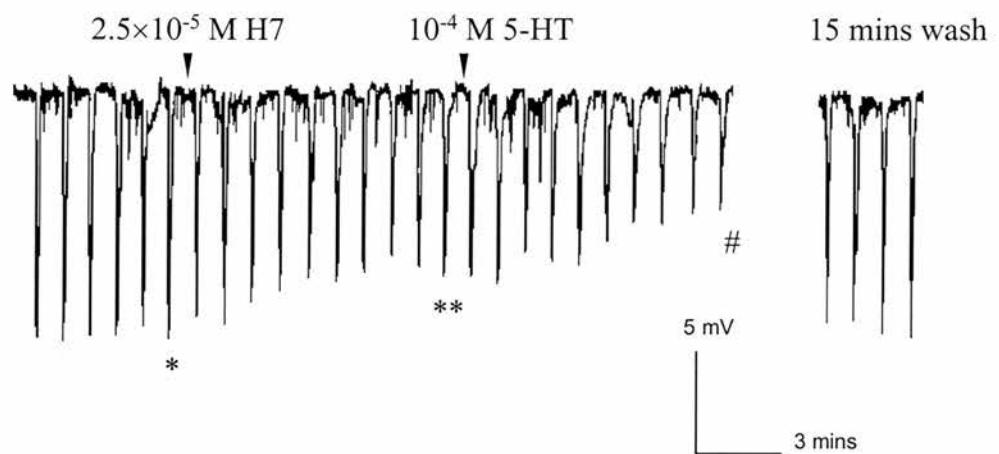
Initially two broad specificity protein kinase inhibitors were tested. These were the isoquinolinesulphonamide, H7, which has the following K_i values for inhibition of different kinases: (PKA (K_i 3 μM) > PKG (5.8 μM) = PKC (6 μM); Hidaka, Inagaki, Kawamoto and Sasaki, 1984) and the potent inhibitor staurosporine (PKA (K_i 0.007 μM) = PKC > PKG (0.013 μM) > CaMK (0.02 μM); Hidaka and Kobayashi, 1993). H7 is structurally related to the CaMK inhibitor W-7, and has a similar direct effect on the amplitude of ACh responses when applied alone to the preparation (figure 53a and 53b); at a concentrations of 10^{-4} M and greater H7 dramatically suppressed ACh responses. Therefore to test the ability of this kinase inhibitor to attenuate aminergic suppression, it was applied at a concentration of 2.5×10^{-5} M. At this concentration the reduction of the ACh response caused by H7 alone was only 80.2% of the control (+s.e.m. 3.4, -s.e.m. 3.7, $n = 4$). Subsequent addition of 10^{-4} M serotonin resulted in a further reduction of the ACh response to 59.8% of the control (+s.e.m. 4.0, -s.e.m. 4.1, $n = 4$), which was not significantly different to the effects of an application of serotonin in the absence of H7 (figure 53c; $p = 0.216$). It is hard to judge whether H7 attenuates to any degree the action of serotonin, certainly it is not possible to prise apart the direct suppression of ACh responses mediated by H7 from its effect upon the modulation mediated by

Figure 53. Effect of the broad range protein kinase, H7, on aminergic suppression of the ACh response.

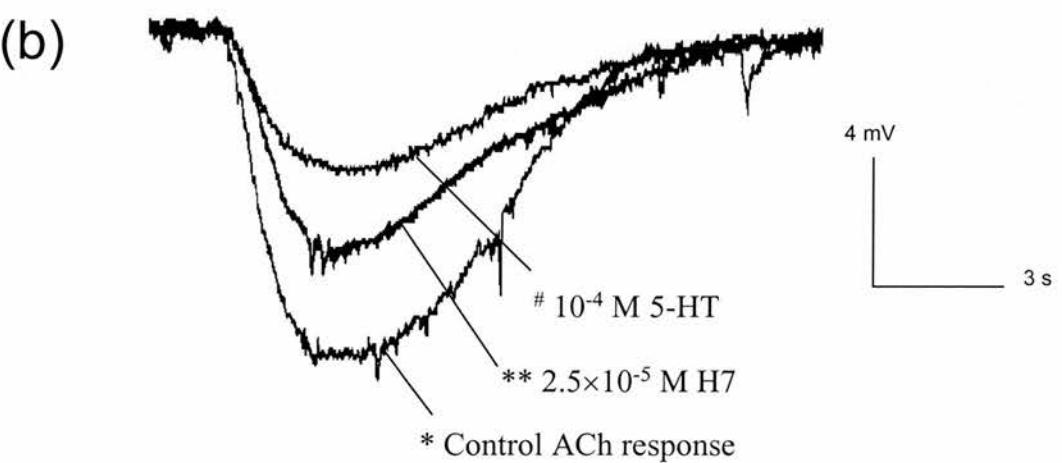
(a) The broad range protein kinase antagonist H7 when applied to a preparation voltage clamped at a holding potential of -80 mV reduced the amplitude of the control ACh current over a period of 10 minutes. Subsequent addition of 10^{-4} M serotonin to the preparation resulted in a further decrease in the size of the inward current. The action of both agents was fully reversed after 15 minutes washout with fresh saline.

(b) The individual ACh current responses marked with the asterisks and hash-mark from (a) are shown on an expanded scale. The currents superimposed include the one just prior to addition of H7, serotonin and after 10 minutes exposure to the amine, and show the accumulative actions of H7 and serotonin on the ACh-mediated inward current. Comparison of the actions of the agents is shown in the histogram at the bottom (c), the combined effect of H7 and serotonin was not significantly different from the effect of serotonin alone.

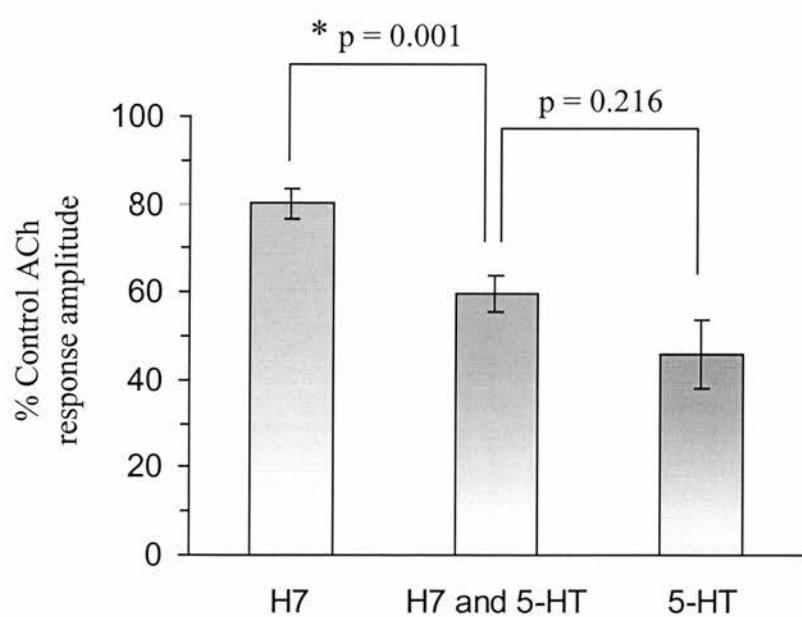
(a)



(b)



(c)



serotonin.

Staurosporine is a highly potent kinase inhibitor that, unlike W-7 and H7, did not suppress nicotinic ACh responses. Although this agent appeared to cause an enhancement of the control ACh response (116.5% of the control after 15 minutes \pm 9.0, n = 6; for example see figure 54a), this was not significant compared to a control application of saline ($p = 0.066$). Staurosporine attenuated suppression of ACh responses mediated by serotonin when recorded under conditions of current clamp. There was a discernible reduction in the amplitude of the ACh response after addition of serotonin, however it did not fall below the level of the control responses recorded prior to application of the protein kinase inhibitor. The pooled amplitude of the ACh responses after 10 minutes application of serotonin was 100.6% \pm 7.5 of the control (n = 6). The action of staurosporine was reversible after prolonged washing (typically 20 – 30 minutes); a subsequent application of serotonin suppressed the ACh response to 58.0% of control (+s.e.m. 7.6, -s.e.m. 7.4; n = 4)(figure 54).

The ability of the protein kinase inhibitor staurosporine to so effectively attenuate aminergic suppression of ACh responses is compelling evidence to suggest that kinase activity, and the resultant phosphorylation is central to the process underlying aminergic modulation of ACh responses exhibited by D_f. However the broad specificity of the inhibitor does not allow any conclusions to be made about the specific kinases involved.

6.1 Rp-8-Br- analogues of cAMP and cGMP effectively attenuate aminergic suppression

The dependence of nAChR modulation on kinase activity, added to the evidence that the amines appear to mediate their effect via elevations in intracellular concentrations

Figure 54a. Three traces taken from an experiment in which the preparation was exposed to the highly potent and broad spectrum protein kinase inhibitor staurosporine. The top trace shows the effect of 10^{-4} M serotonin on ACh responses recorded from the cell under control conditions. Reversal of the amines effect was achieved after 12 minutes washout with normal saline (not shown), after which six constant responses were recorded prior to application of 5×10^{-6} M staurosporine. The second trace shows that the protein kinase inhibitor augmented the ACh response. There was also a discernible increase in spontaneous synaptic activity, similar to that seen in the presence of the CaM kinase inhibitor KN-62. In the presence of staurosporine, serotonin did suppress ACh responses. However the amplitude of the ACh responses was not reduced below that of the pre-staurosporine control responses. The effect of staurosporine was reversible since after 30 minutes washout, a clear response to serotonin was observed (bottom panel). Staurosporine was prepared by dissolving it in DMSO. This stock solution was kept frozen until required, when it was thawed and diluted with normal saline prior to injection into the side chamber of the bath.

(a)

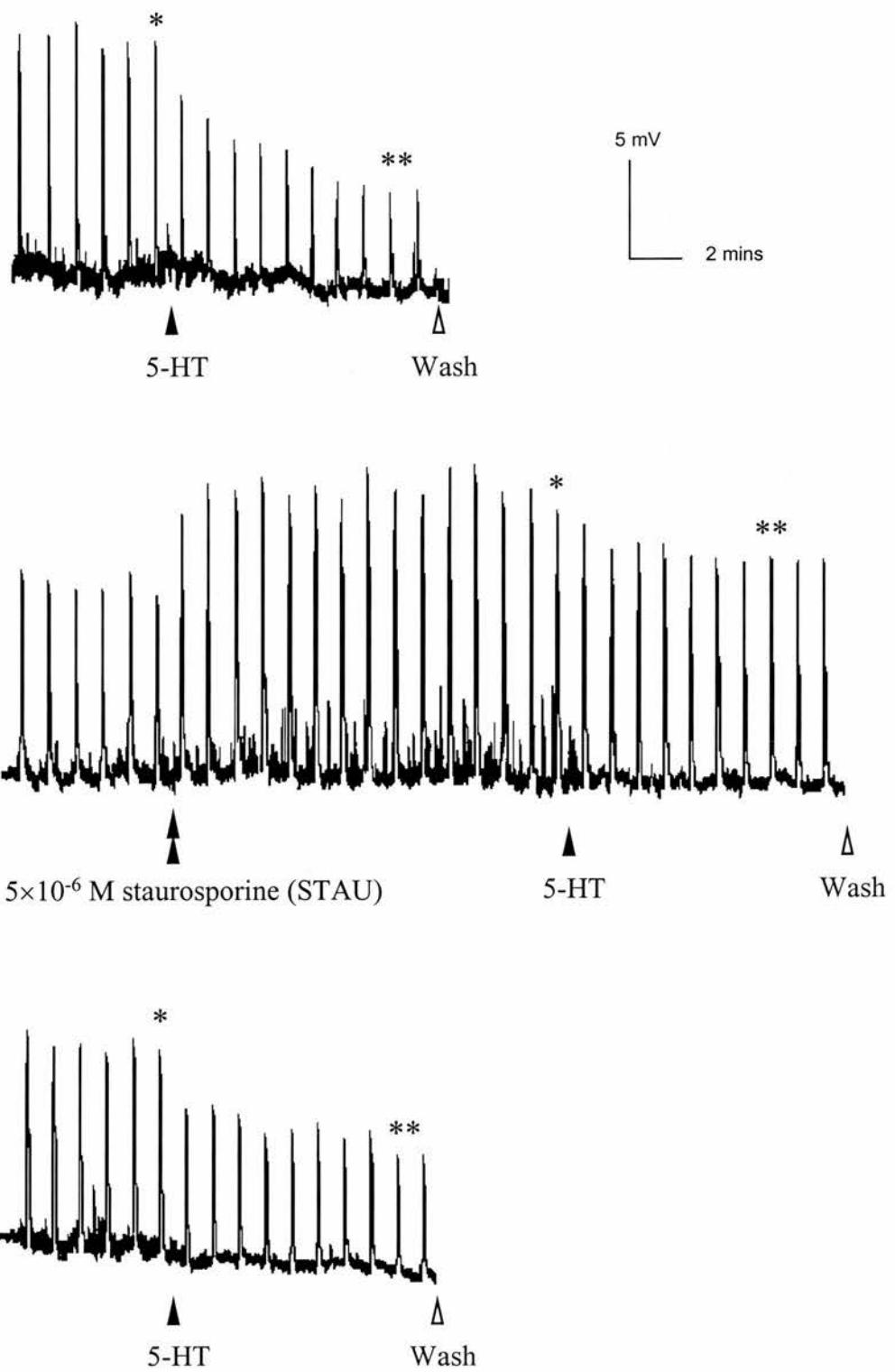
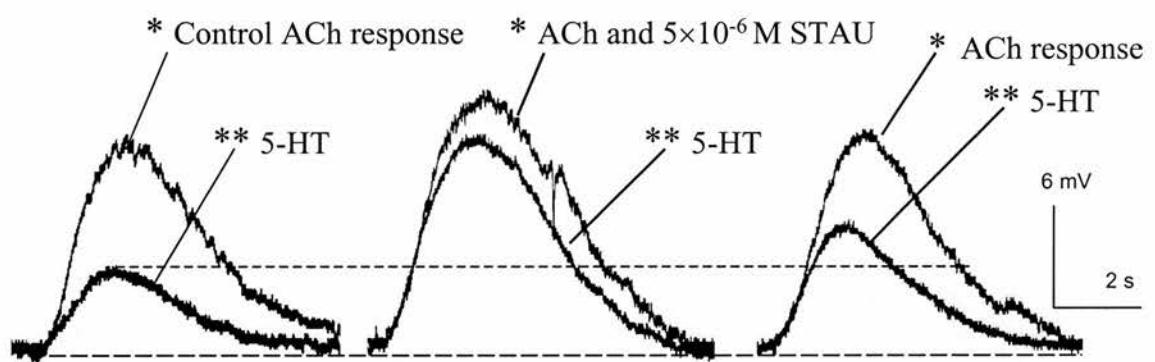


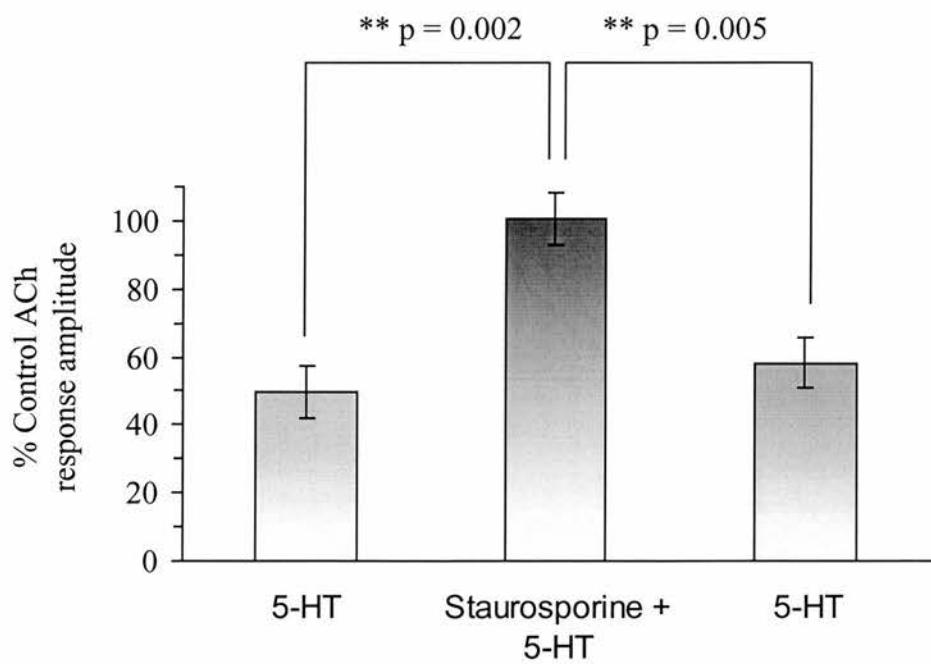
Figure 54b and 54c.

(b) Expanded traces from (a) are illustrated in the top trace. Comparison of the responses prior to, during and after staurosporine incubation confirm the ability of the protein kinase inhibitor to attenuate modulation of ACh responses mediated by serotonin (cal. vertical 6 mV, horizontal 2 seconds). (c) This result was substantiated by statistical analysis of the pooled data from 6 experiments, shown in the histogram below.

(b)



(c)



of cyclic nucleotides would suggest that the cyclic nucleotide-dependent kinases, PKA and PKG, represent prime candidates as the specific kinases involved. To test this reasoning, specific inhibitors of cyclic nucleotide-dependent kinases were examined for their effectiveness in blocking aminergic modulation of nAChR function. The Rp-8-Br- analogues of cAMP and cGMP are membrane permeable, metabolically stable inhibitors of PKA and PKG respectively.

Bath application of Rp-8-Br-cAMPS reduced the control ACh response to 88.5% (+s.e.m. 2.7, -s.e.m. 3.0; n = 10) (Figure 55b(ii)), whereas the cGMP analogue had no discernible effect (98.7% ± 8.6; n = 6) (Figure 56b(ii)). Both the analogues significantly attenuated the ability of serotonin to reduce nicotinic responses (for examples see figures 55 and 56 respectively). Serotonin in the presence of Rp-8-Br-cAMPS only reduced the ACh response to 84.1% (+s.e.m. 2.9, -s.e.m. 3.3, n = 10) of control, whereas when incubated with Rp-8-Br-cGMPS 10^{-4} M serotonin reduced the ACh response to 70.9% of control (+s.e.m. 4.9, -s.e.m. 5.1, n = 10). The actions of the kinase inhibitors in blocking aminergic suppression of ACh responses were partially reversed after prolonged wash-out.

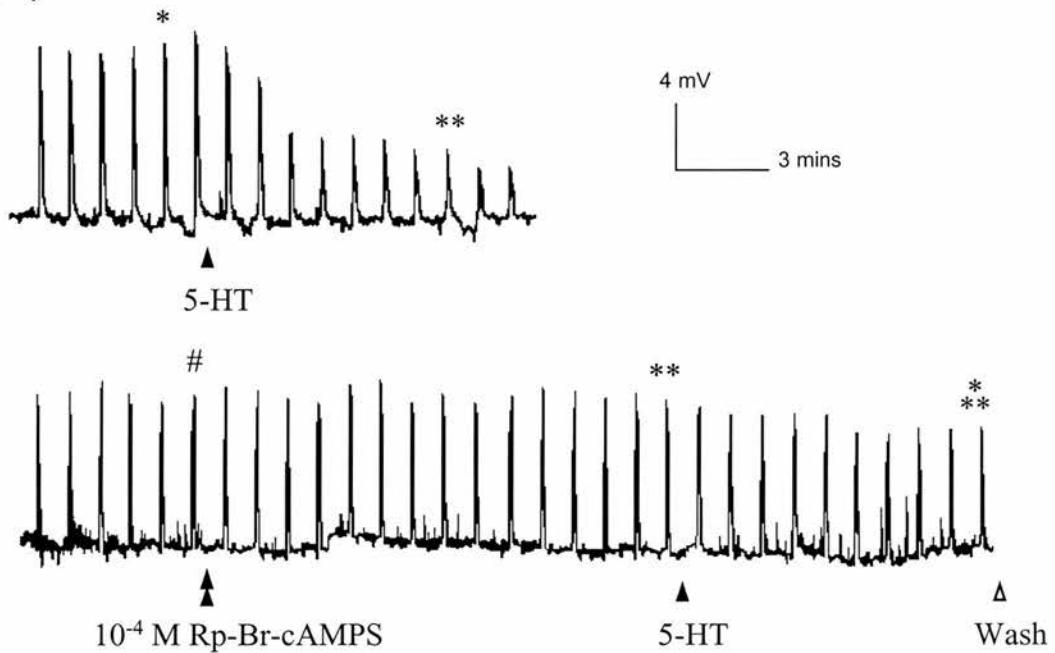
The approximate order of potency for the kinase inhibitors, based on their effectiveness in reducing aminergic suppression of ACh responses mediated by serotonin at the concentrations tested is staurosporine > Rp-8-Br-cAMPS > Rp-8-Br-cAMPS > H7 >> KN-62 = W-7, the last three all exerting significant direct effects on the amplitude of ACh responses. The ability of a number of inhibitors to significantly attenuate aminergic suppression provides good evidence that the effect of serotonin is mediated by elevations in levels of cyclic nucleotides, most probably cAMP, and that this, in turn leads to protein phosphorylation.

Figure 55. To further characterise the protein kinase involved in aminergic suppression the PKA inhibitor Rp-Br-cAMPS was applied to a number of preparations and its effect recorded under current clamp.

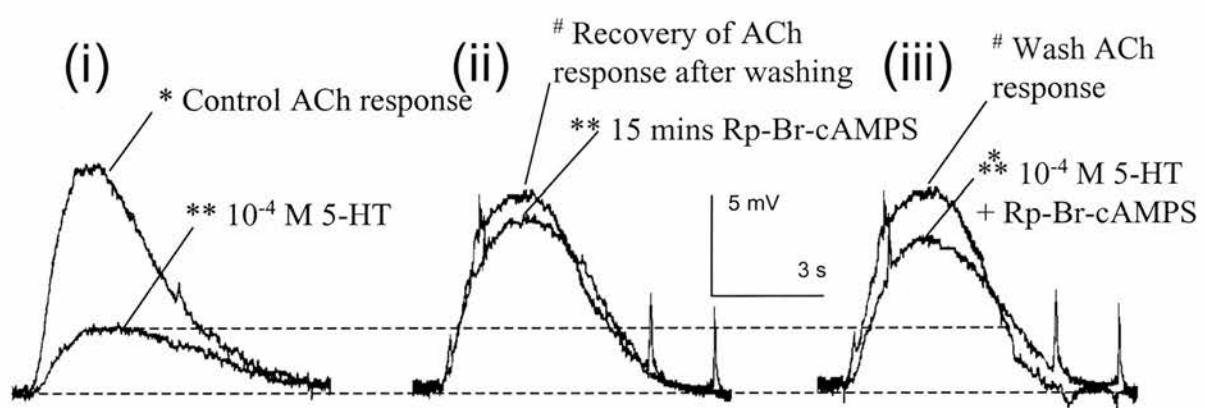
The traces (a) show that the inhibitor attenuates suppression of ACh responses mediated by 10^{-4} M serotonin. A trace showing an application of serotonin in the absence of the kinase inhibitor is included for reference. (b) Individual response on an expanded time scale.

The effect of Rp-Br-cAMPS, and the action of serotonin in the presence of the inhibitor were expressed as a percentage of the ACh response amplitude recorded prior to application of the kinase inhibitor. (c) Pooled data on the effect of Rp-Br-cAMPS including the effect of serotonin after washing the kinase antagonist from the preparation (which was not shown in figure (a) or (b)). The purpose of this final run was to establish that the effect attributable to Rp-Br-cAMPS was not in fact due to a spontaneous decline in magnitude of suppression mediated by serotonin.

(a)



(b)



(c)

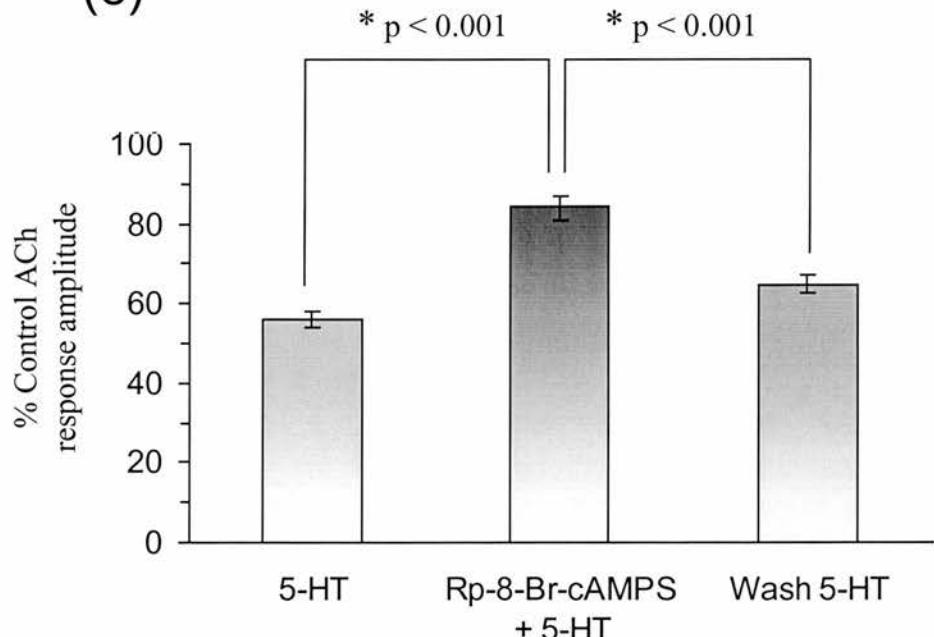
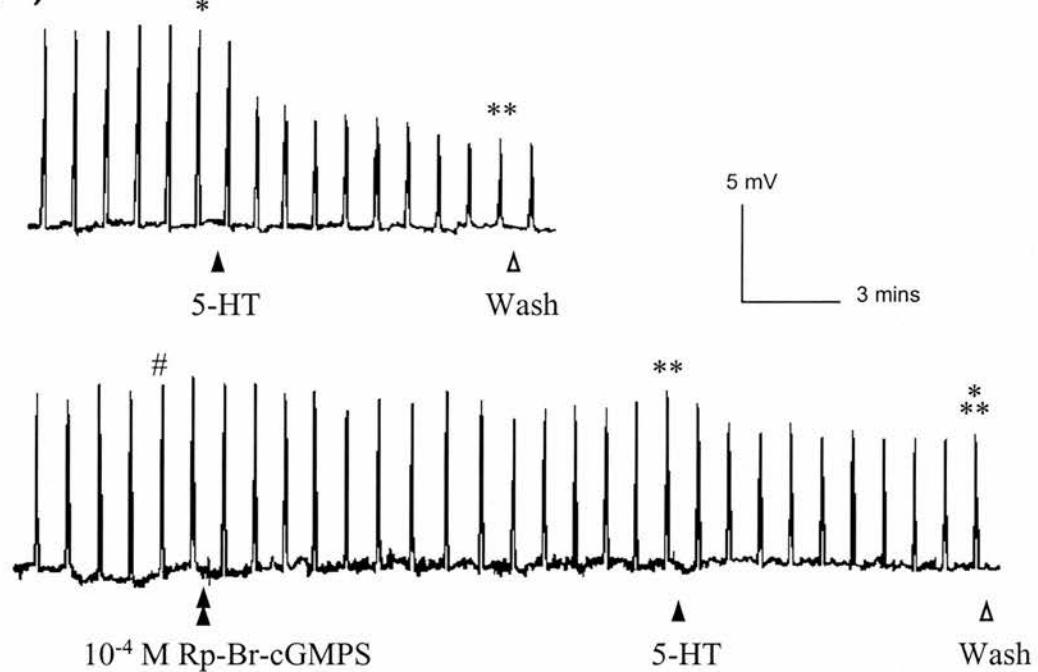


Figure 56. The guanosine analogue Rp-Br-cGMPS was similarly effective in attenuating suppression of ACh responses due to application of 10^{-4} M serotonin to the preparation. The figures shown present the data in the same format as for Rp-Br-cAMPS.

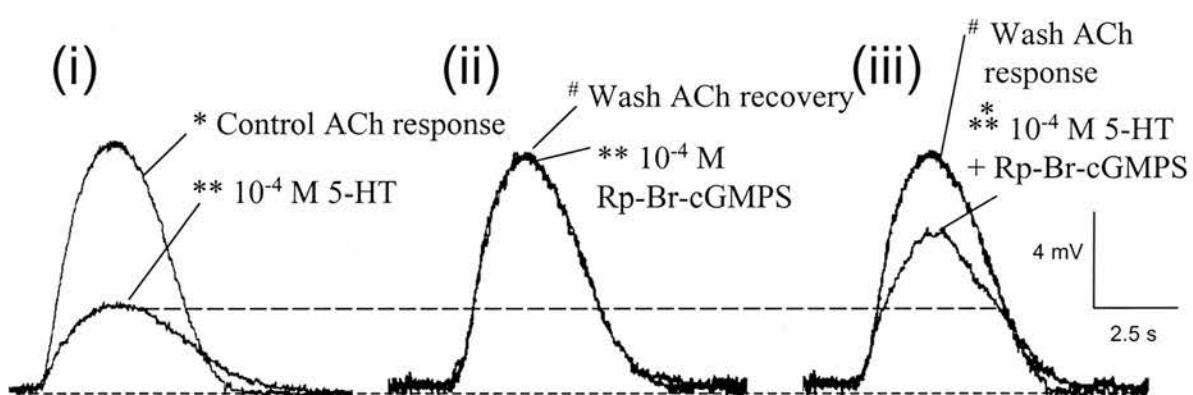
(a) The top trace is of a control application of serotonin in the absence of protein kinase inhibitor. The lower trace is from the same preparation and shows the effect of incubating the preparation with 10^{-4} M Rp-Br-cGMPS before adding serotonin. The marked traces are illustrated expanded in (b) and show the ability of Rp-Br-cGMPS to antagonise serotonergic modulation of ACh responses (central panel). Washing out of the protein kinase inhibitor restored the ability of serotonin to suppress ACh response (right-hand panel).

(c) The pooled data from 10 experiments. Rp-Br-cGMPS significantly ($p=0.020$) reduced the ability of serotonin to suppress ACh responses.

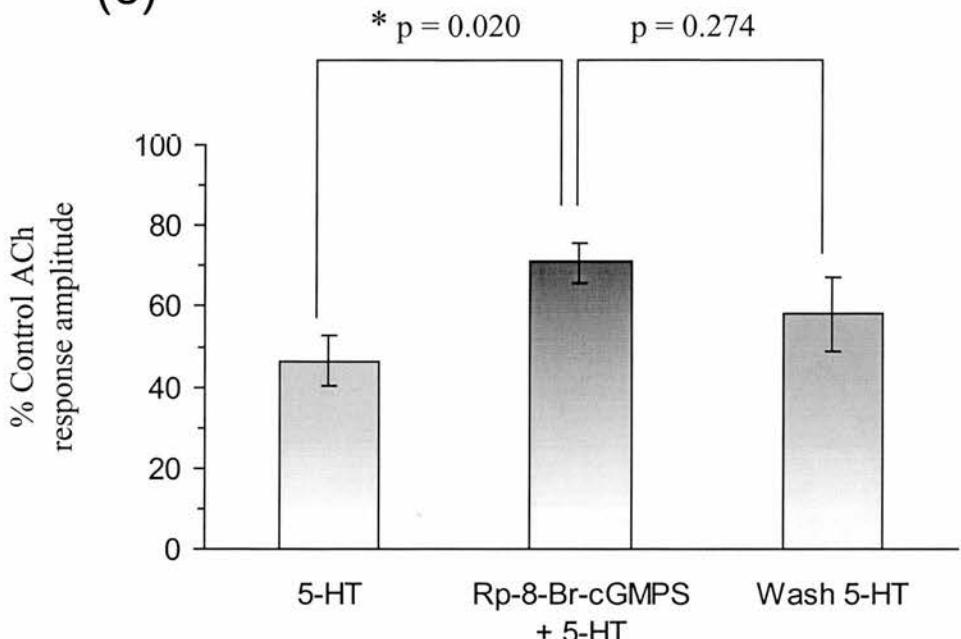
(a)



(b)



(c)



6.2 Phosphatase inhibitors prolong aminergic suppression.

If phosphorylation is essential to the action of amines, recovery of nAChR modulation will probably require dephosphorylation by a protein phosphatase. To substantiate a role for phosphorylation in the mechanism of aminergic suppression, phosphatase inhibitors were tested to determine if these enzymes are involved in nAChR modulation exhibited by D_f. If phosphatases do reverse the effects of kinases during modulation, protein phosphatase inhibitors should extend the period of modulation.

Two membrane permeable phosphatase inhibitor were tested, okadaic acid (PP2A (IC_{50} 0.1 nM) > PP1 (10-15 nM) >> PP2B (5 μ M)) and cantharidin (PP2A (IC_{50} 40 nM) > PP1 (473 nM)). The potent broad range phosphatase inhibitor okadaic acid was tested under conditions of circulating saline. Okadaic acid at concentrations of 10^{-4} M and above attenuated the control ACh response over a time course of 5 minutes. This direct action, in suppressing the ACh response, was less marked when the phosphatase inhibitor was applied at 10^{-5} M (figure 57a). The phosphatase inhibitor also dramatically enhanced the action of monoamine dopamine (10^{-4} M), reducing the ACh response to 48.3% of the pre-okadaic acid control (+s.e.m. 6.5, -s.e.m. 6.4, n = 3). Prolonged washout (30 – 40 mins) with normal saline, only partially reversed the effect of dopamine, the ACh response recovering to 75.8% of the control (+s.e.m. 7.3, -s.e.m. 8.3, n = 3).

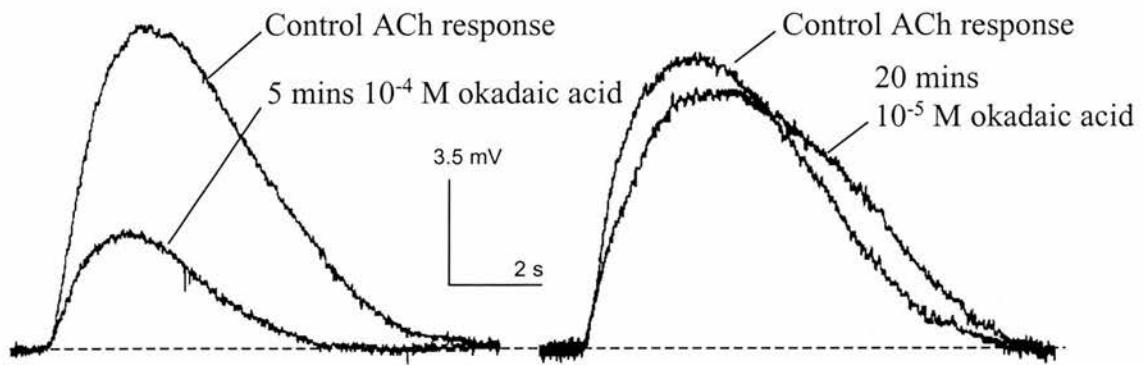
Experiments to test the action of the protein phosphatase inhibitor cantharidin were performed under conditions of continual perfusion. Controls were also conducted before the test runs perfusing with normal saline to allow direct comparison of the duration of serotonin modulation in the presence and absence of the phosphatase inhibitor. Serotonin was applied at a concentration of 10^{-3} M, and under continual perfusion a maximal suppression in the ACh response amplitude of 66.5%

Figure 57. The ability of the phosphatase inhibitor, okadaic acid, to potentiate and prolong aminergic suppression was tested using dopamine under conditions of current clamp.

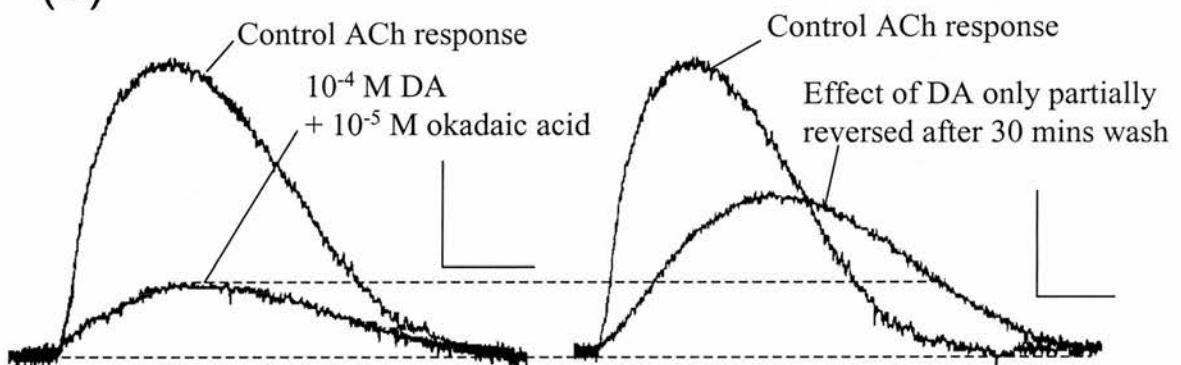
(a)(left-hand panel) Application of 10^{-4} M okadaic acid alone resulted in a significant reduction in the amplitude of the ACh response when compared to the control level. (right-hand panel) At the lower concentration of 10^{-5} M, okadaic acid has a smaller effect on ACh responses, slightly increasing the duration of the response. (b) The phosphatase inhibitor increased the effectiveness of 10^{-4} M dopamine in modulating ACh responses, furthermore the effect of the amine was not fully reversed even after 30 minutes wash. In the absence of any phosphatase inhibitor suppression of ACh responses mediated by dopamine is normally fully reversible within 10 minutes.

(c) Histogram comparing the maximal effect of 10^{-4} M dopamine in suppressing ACh responses in the absence and presence of the phosphatase inhibitor, okadaic acid. The pooled data from three experiments revealed that okadaic acid significantly ($p = 0.037$) enhances suppression of D_f ACh responses mediated by dopamine.

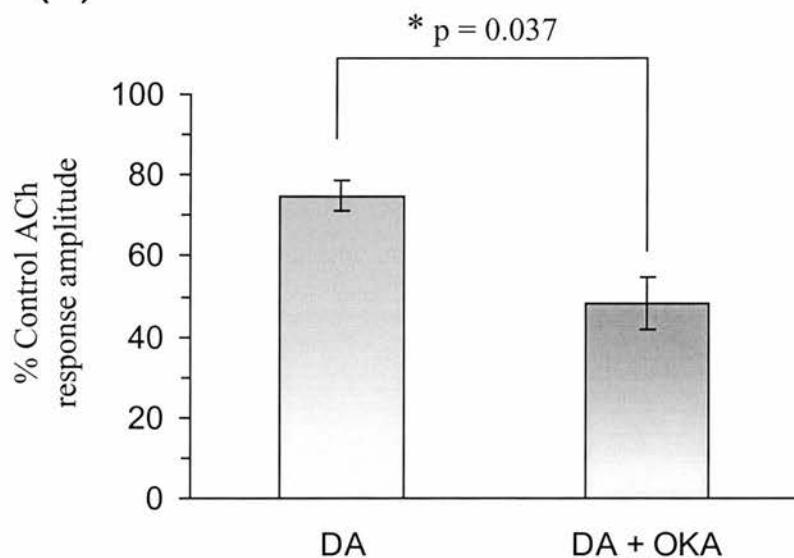
(a)



(b)



(c)



(+s.e.m. 6.5, -s.e.m. 6.9) was observed 90 seconds after application of the amine to the bath. Although the amine was only present for on average 2 minutes, the ACh responses remained depressed for at least 10 minutes (figure 58a). The effect of the brief exposure to serotonin did not reverse when perfused with saline containing 10^{-4} M cantharidin (figure 58b). Indeed, not only did it persist but increased to an amplitude of 47.8% of control (+s.e.m. 9.5, -s.e.m. 9.3) 15 minutes after injection of serotonin (figure 58c). Preparations were then washed in normal saline but there was no observable reversal in aminergic suppression after a half hour of washing (figure 58b,c).

The ability of both cantharidin and okadaic acid to prolong the action of serotonin in suppressing ACh responses provides evidence that protein phosphatases regulate recovery from the modulatory effects of the amine. This is consistent with the involvement of phosphorylation as a means the means of signal transduction.

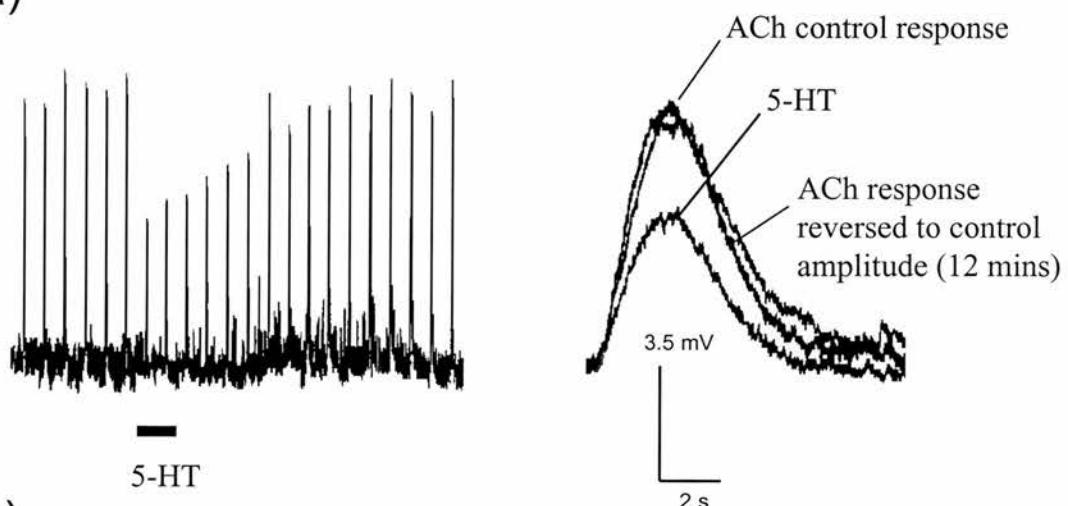
Figure 58. A second, less potent phosphatase inhibitor cantharidin was also tested for its ability to influence serotonergic suppression of ACh responses, this time under conditions of continual perfusion.

(a) A typical application of 10^{-3} M serotonin under rapid perfusion with fresh saline. The amine was estimated to have been present in the bath for approximately 1 minute, the duration of the suppression was 8 – 10 minutes by which time the amplitude of ACh responses had returned to control levels.

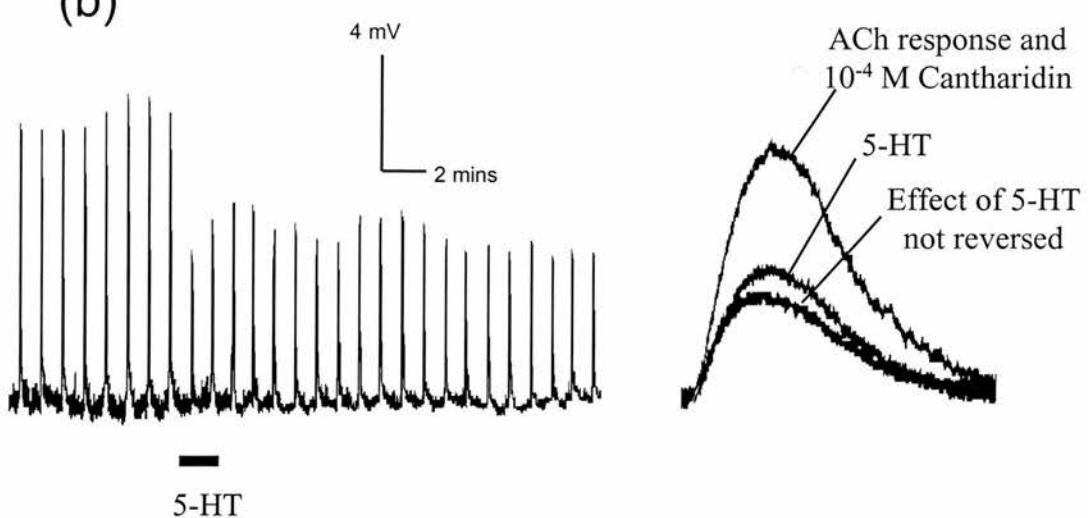
(b) When the bath was perfused with saline containing 10^{-4} M cantharidin for at least 10 minutes prior to the addition of serotonin, addition of the amine to bath for a brief a moment resulted in an irreversible suppression of the ACh response. Cantharidin was prepared in DMSO prior to dilution with saline.

(c) Illustrates graphically the ability of cantharidin (filled squares) to prolong the action of serotonin on ACh responses in the D_f motoneurone. Such was the effect of the phosphatase inhibitor that even on washing with normal saline there was no noticeable recovery in the ACh depolarisation. The time course of the control serotonin applications is indicated by the open circles.

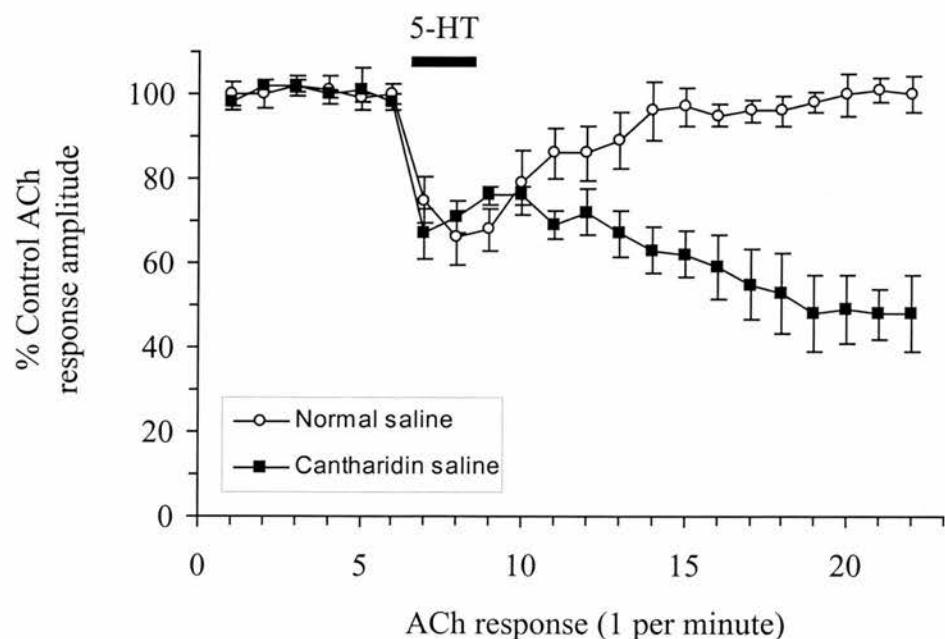
(a)



(b)



(c)



SUMMARY OF RESULTS

- The three monoamines dopamine, octopamine and serotonin all suppress the nicotinic ACh response of the D_f motoneurone in a concentration-dependent manner. Serotonin is the most potent of the three - with an approximate IC₅₀ of 50 µM as compared to dopamine with an IC₅₀ of ~ 450 µM. The action of the amines is mediated by receptors on or near the soma of the motoneurone and does not result from a change in the activity of acetylcholinesterase. The amines tested decrease inward currents mediated by nicotinic ligand-gated ion channels in a non-competitive manner.
- Neurones immuno-positive for dopamine and serotonin have ramifications throughout the metathoracic ganglion. Serotonin immuno-labelled processes are evident in the vicinity of the arborisation of the D_f motoneurone in the neuropile. This provides evidence in support of the proposal that amines physiologically modulate synaptic transmission onto the motoneurone.
- A pharmacological approach was used to further determine the site and mechanism of aminergic modulation. The suppressive action of the three amines studied upon ACh responses was not additive suggesting that each operates via a shared or interrelated mechanism. The observation that LSD blocks the actions of all three amines suggests that the amines act on the same receptor rather than merely sharing some down-stream mechanisms but does not discount the possibility that additional receptors are involved. The ability of low concentrations of LSD to effectively antagonise the action of the amines on ACh responses also adds credence to the proposal that amines act at a receptor site separate from the nAChR molecule. The pharmacology of the receptor site was

hard to define using selective vertebrate antagonists since, with the exception of the serotonergic antagonists LSD and RS23597-190, all the other antagonists tested themselves had amine-mimicking effects on ACh responses and did not block the action of the amines.

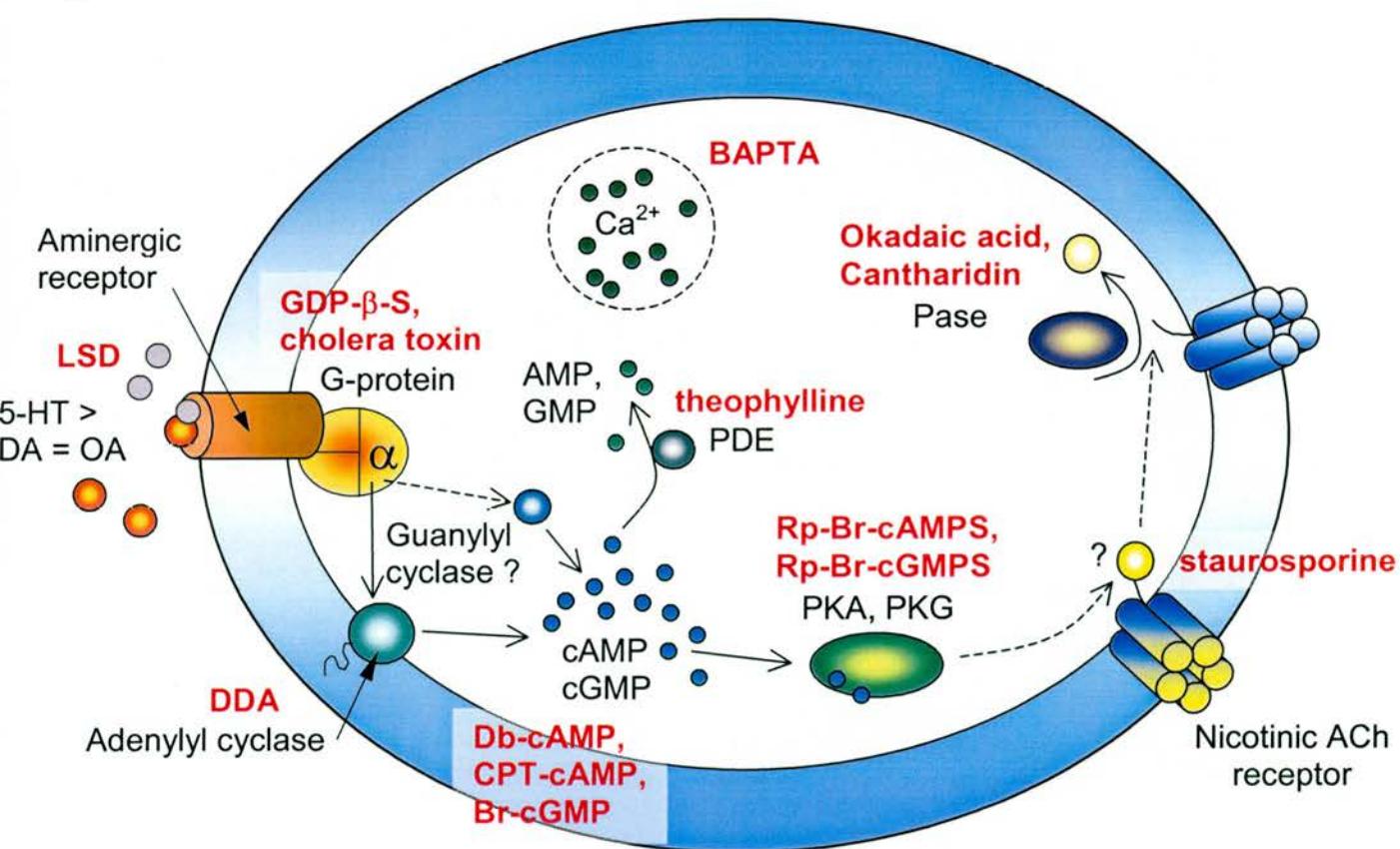
- Aminergic suppression is dependent on G-proteins, but is not attenuated by pertussis toxin the inhibitor of the $G_{i/o}$ protein family. The ability of cholera toxin to suppress of the ACh response indicates that a G_s protein pathway is actively involved in modulation of ACh transmission in the D_f.
- Aminergic modulation of ACh currents is independent of a rise in intracellular Ca^{2+} . The results from experiments using the Ca^{2+} chelator BAPTA discount the possibility that the amines operates via a similar mechanism to that of suppression mediated by muscarinic agonists, which is dependent on a rise in $[\text{Ca}^{2+}]_i$ and is blocked by intracellular EGTA.
- Calmodulin-stimulated intracellular events do not mediate the effect of amines in suppressing ACh transmission, however, application of agents that inhibit calmodulin and CaMK II function reduce the amplitude of ACh responses directly. This suggests that CaM might function to regulate other aspects of nicotinic ACh transmission.
- Analogues of both cAMP and cGMP mimic the action of amines on ACh responses. Incubation with the analogues partially occludes the effect of serotonin, whereas exposure to the PDE inhibitor theophylline both prolonged and enhanced modulation of ACh transmission mediated by serotonin. Inhibition of the cAMP-generating enzyme adenylyl cyclase significantly reduced the effect of serotonin. This suggest that enzymes responsible both the generation and

degradation of cyclic nucleotides function to mediate aminergic suppression of ACh responses.

- Suppression of ACh responses mediated by amines is dependent on phosphorylation. Inhibitors of protein kinases, specifically those of PKA and PKG, prevent aminergic suppression whereas phosphatase inhibitors prolong and enhance modulation.

The figure below (figure 59) is a diagrammatic representation of the signalling pathway by which the amine serotonin is proposed to modulate nAChR function. Red text indicates the principle pharmacological agents used to investigate each step. Dashed arrows indicate steps not fully elucidated, and the yellow spheres - phosphorylation.

Figure 59



DISCUSSION

Although there is now a considerable body of evidence indicating that vertebrate ligand-gated ion channels may be modulated in a number of ways, there is little direct evidence indicating that this situation is true of arthropod receptors. The results presented here conclusively demonstrate that the nicotinic acetylcholine response of the D_f motoneurone of *Periplaneta americana* can be down-regulated by the monoamines dopamine, octopamine and serotonin via a mechanism involving one or more G-proteins. Furthermore, evidence is presented that the modulation is mediated by a transduction pathway dependent on intracellular cyclic nucleotides and protein phosphorylation.

The effect of pharmacological agents was tested on responses elicited by local application of ACh to the soma of the D_f motoneurone. The advantages of using the D_f motoneurone are firstly that it has a large soma (approximately 80 µm in diameter) and easily identifiable from one preparation to another. Secondly that is robust, such that it can be impaled with two or more electrodes thus allowing two electrode voltage clamp and manipulation of cellular events by intracellular injection. Thirdly, the soma is separate from synaptic sites so that locally applied drugs are less likely to produce indirect effects. This was one of the reasons for pressure applying ACh locally to the soma, such a method avoiding to a large degree the actions on ACh on other neurones, that, themselves, release transmitters of undetermined type onto D_f. Additionally, pressure application meant that ACh was applied rapidly to the soma and that the cholinergic responses initiated could be controlled by the parameters of the pressure pulses and thus limited to prevent significant run-down over the experimental time period.

One consideration is whether the modulation observed by recording from the soma is representative of cellular mechanisms that occur in the neuropile, or whether it serves a unique function at sites near to the soma. Undercutting of the soma did not abolish or reduce the sensitivity of ACh response to aminergic suppression, so the assumption must be that the signal transduction mechanisms are present in either the membrane of the soma or in the proximal neurite. A role for modulation in the soma would not fit easily with the historical view that the cell bodies of insect neurones lack synaptic inputs are essentially inexcitable and only function to metabolically sustain transmission in the neuropile. There are a number of alternative hypotheses that might account for the presence of receptor molecules in the somal membrane that would not require them to have a physiological role at that location. They could be expressed there entirely because cellular mechanisms are unable to limit their distribution to those regions in which they normally operate. Alternatively, it could be that proteins are initially imbedded in the somal membrane and subsequently migrate to the sites at which they are required in the neuropile. The former hypothesis does not account for the pre- and postsynaptic specialisation observed at the ultrastructural level of insect synapses (see review by Keshishian, Broadie, Chiba and Bate, 1996; Susuki, Rose and Chiba, 2000). There is evidence of glutamate receptor clustering at embryonic neuromuscular postsynaptic sites of *Drosophila* (Broadie and Bate, 1993), although a mechanism of receptor clustering akin to that mediated by agrin in vertebrates has remained elusive in insect systems (see Keshishian, Broadie, Chiba Bate, 1996; for vertebrate examples see Wallace, 1995; Meier, Perez, and Wallace, 1995). Gerschenfeld and Stefani (1968) proposed a non-uniform distribution for cholinergic and serotonin receptors in the membrane of molluscan neurones that exhibit long duration inhibition - CILDA neurones. The

use of localised recording and drug application pipettes revealed a somatic cap devoid of ACh receptors. Cholinergic receptors were distributed over the rest of the soma but serotonin receptors only present 72 µm from the recording point, predominantly on the axon hillock. There is additional evidence to suggest that neurones can express distinct receptor populations at differing loci. The sensitivity to picrotoxin of GABA receptors expressed by cockroach DUM neurones varies according to whether they are located on the soma or in the neuropilar arborisation (Dubreil, Sinakevitch, Hue and Geffard, 1994). Similarly the pharmacology of glutamate neurotransmitter receptors in the neuropile of the locust *Schistocerca gregaria* is distinct from that observed in the extrasynaptic somal membrane (Parker, 1994). Furthermore nAChR genes cloned from *Locusta migratoria* are differentially expressed in regions of neurones from the optic lobe, ganglionic cells, and soma of mushroom body neurones (Hermsen *et al.*, 1998). These findings provide some support for the notion that the receptors expressed in the somal membrane are distinct from those present in the neuropile and therefore might exhibit a specific function. Certainly it is unlikely that the receptors are present on the soma as a result of random distribution.

Although the cell bodies of insect neurones are classified as extrasynaptic because they do not receive anatomically defined input synapses from afferents directly onto the somal membrane, there is evidence to suggest that they can contribute to neuronal processing. Some insect motoneurone soma exhibit 'active' electrical properties such as plateau potentials, which can often be observed on depolarisation. Under a number of experimental conditions the soma of the D_f motoneurone can support both Na⁺ and Ca²⁺-dependent spikes, the expression of such events under normal conditions being smothered by a rapidly activating,

rectifying Ca^{2+} -dependent K^+ conductance (Pitman, 1975; Pitman, 1979; Thomas, 1984; Hancox and Pitman, 1992; David and Pitman, 1995). In support for a physiological role for such events, D_f somal spikes and associated plateau potentials can be elicited by activation of presynaptic inputs to the neurone (Hancox and Pitman, 1993). The non-linear events exhibited by D_f would imply that the cell body assists in the shaping motor output and therefore could be subject to modulatory influences.

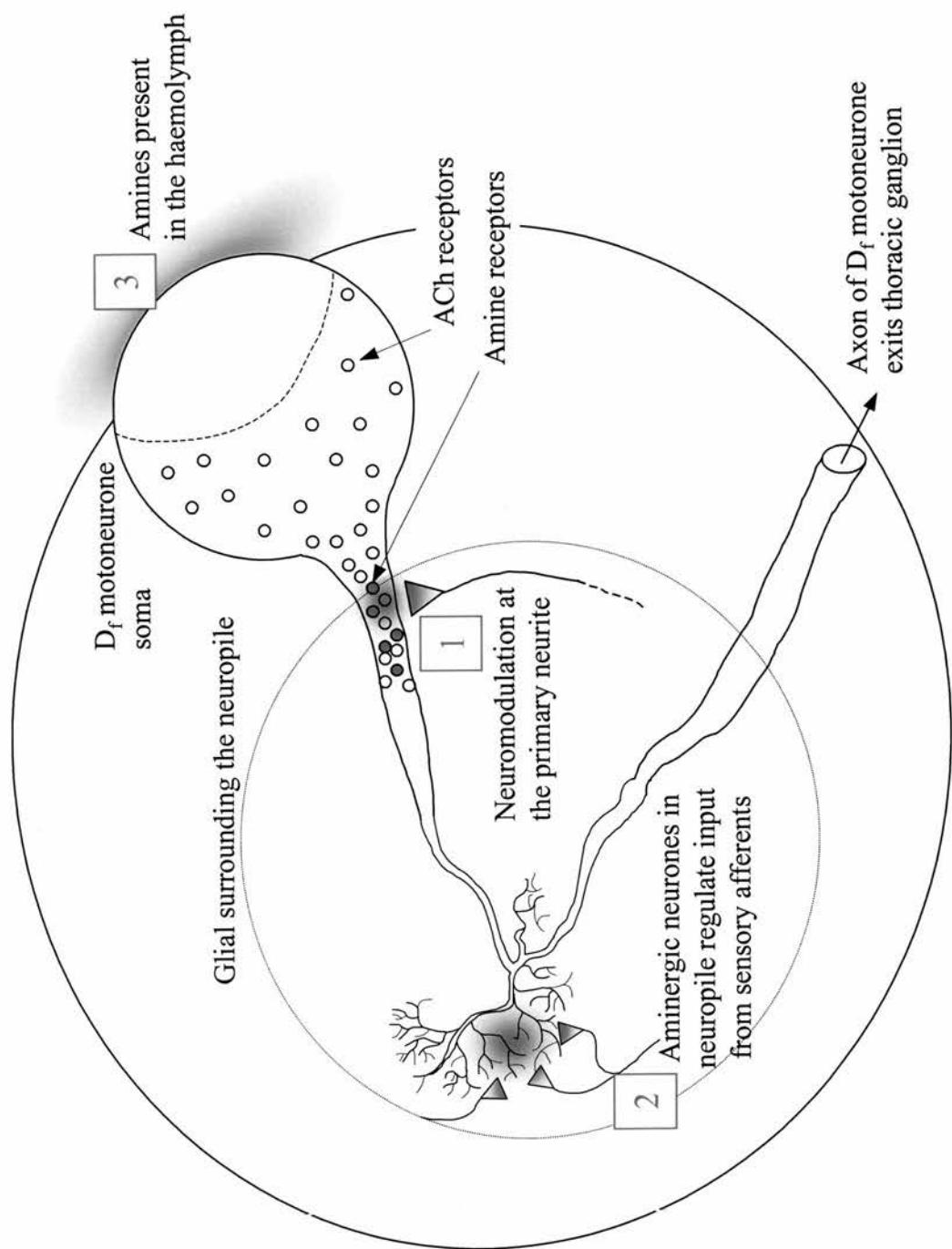
Aminergic transmission in arthropods is not exclusive to conventional chemical synapse sites. Certainly it is quite probable that soma receptors are exposed to neuromodulators circulating in the haemolymph rather than released from localised synapses in the neuropile (see Livingstone, Harris-Warrick and Kravitz, 1980; Elofsson *et al.*, 1982; Duch and Pflüger, 1999). The presence of these neuromodulators in the haemolymph provides simultaneous regulation of multiple neuronal, hormonal, muscular and metabolic processes. There is also evidence to suggest that amines might be secreted into the extracellular spaces below the neural sheath - immunoreactive terminals for serotonin have been detected in the neural sheath of peripheral nerves (Nässel and Elekes, 1984). Additionally, there are a number of reports that invertebrate glial cells similar to their vertebrate counterparts (see for review Verkhratsky, Orkand and Kettenmann, 1998) might contribute to neuronal signalling. Certainly invertebrate glial cells possess receptors for classical neurotransmitters (Leitch, Watkins and Burrows, 1993; Schlue and Hochstrate, 1995; Dierked, Hochstrate and Schlue, 1996) and glial cell cultures possess functional ion channels (for example see Keen, Amar, Beadle and Bermudez, 1994). Therefore, there are a number of potential sources for aminergic influence exterior to the neuropile. Sites local to the nerve sheath such as those identified by Nässel and

Elekes (1984) were unlikely to be detected in the present study since the neural sheath was removed from ganglia prepared for immunocytochemistry in order to assist rapid penetration of the paraformaldehyde and glutaraldehyde fixatives as well as for identification and impalement of the D_f motoneurone for HRP injection.

The primary neurites of insect neurones that penetrate the glial border of the neuropile often receive synaptic inputs where the glial sheath is lacking (see for example Watson and Burrows, 1982). The extensive branching of serotonin immunoreactive profiles at the border of the neuropile and the presence of profiles at ultrastructural level in the locality of the glial bordering the neuropile indicates that such areas might be subject to aminergic modulation in *Periplaneta americana* (see figure 60, page 264). The location of serotonin receptors in the neurite membrane proximal to the soma of neurones proposed by Gerschenfeld and Stefani (1968) supports this hypothesis. The primary neurite of the D_f motoneurone penetrates though to the dorsal areas of the neuropile where it arborises, its axon passing into the trunk of nerve 5. Analysis of serotonin and dopamine immuno-positive processes under the fluorescent and confocal microscope revealed that amine-containing neurones branch extensively throughout the dorsal tracts. The ability of serotonin immunoreactive profiles to form synaptic contacts with the arborisation of the D_f motoneurone was further confirmed at the ultrastructural level. Such a finding is consistent with the hypothesis that monoamines modulate transmission mediated by cholinergic sensory afferents as well as inter-neuronal input onto D_f within the neuropile.

If the site of aminergic signal transduction is primarily located on the dendritic spines of the D_f motoneurone deep within the dorsal areas of the neuropile or within the glial cells surrounding the primary neurite and bordering the neuropile,

Figure 60



Proposed sites of amine modulation:

- (1) On the primary neurite, local to the soma.
- (2) In the dorsal areas of the neuropile.
- (3) At the soma, in response to amines circulating in the haemolymph.

it could explain why the concentration of amine required to elicit modulation of ACh responses was comparatively high – a factor that is contrary to a role for amines in the haemolymph reaching neuronal target sites. Bath application of the pharmacological agents would, in such circumstances, result in their poor access to the amine receptor sites at the neuropile synapses. It must be noted that similar insensitivity to a range of physiological agonists is a general feature of insect central neurones. For example the effective agonistic doses of ACh can be as high as 10^{-2} M (Pitman, 1985). Amines were typically applied at a concentration of 10^{-4} M, slightly greater than the IC_{50} of serotonin, as at this level they have easily observable effects on the nicotinic response. This concentration is similar to that used to study the modulatory effects of the same amines on thoracic interneurones and motor output of the escape circuit of the cockroach (Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992b). It is also similar to the concentration range used to elicit modulation in the activity of cockroach DUM neurones (Washio and Tanaka, 1992) and flight initiation in the abdominal ganglia (Weisel-Eichler and Libersat, 1996). A similar concentration range has also been reported for the octopamine-stimulated rises in cAMP in a locust neuromuscular preparation (Evans, 1984).

The number of dopaminergic cell bodies and serotonergic neurites identified on and near the ventral surface of the metathoracic ganglion of *Periplaneta americana* represent good potential candidates for electrophysiological recording. Wholemount studies using the antibodies utilised in this study should allow their position to be accurately mapped, although from these initial studies it appears that they are in a locality similar to that of the AV3 cells identified by Gregory (1984) in the mesothoracic ganglion of the same animal. If the putative aminergic neurones could be reliably identified from one preparation to another and proved amenable to

impalement with microelectrodes, it would be of interest to determine whether, by stimulating them, one could drive modulation of the D_f motoneurone *in situ* and if that modulation corresponds to that elucidated using the pharmacological approach utilised in this study.

1.1 Time course of aminergic modulation.

The effect of bath applying amines was usually apparent after 1 minute but often they did not exert their maximum effect on the ACh response until after 3 minutes exposure. Circulation of the saline by bubbling oxygen through a side chamber ensured rapid mixing within 2 - 5 seconds of adding the drugs to the bath saline. Therefore, the gradual modulation mediated by the amines is most likely attributable to two factors: firstly, as previously mentioned it could result from the delay whilst chemicals penetrate through the tissue to access receptor sites. However, this is unlikely, since the time course of onset of modulation of ACh responses observed in isolated cells did not differ from that in intact preparations. Secondly, the delay could be due to the reliance on a G-protein-stimulated intracellular pathway. The time course of aminergic suppression of ACh responses observed in the D_f motoneurone of intact metathoracic ganglia is in line with evidence from a variety of preparations in which the action of modulators is mediated by G-protein dependent rise in cAMP (see below). Additionally it must be noted that suppression mediated by the muscarinic agonist Ca²⁺-dependent pathway in D_f operates with a latency of approximately 3 seconds (David and Pitman, 1996b). A delay in onset of 10 seconds and a lag of approximately 2 minutes to achieve the maximal effect was considered 'relatively slow' by Liu and Lasater (1994) when

investigating the cAMP-dependent action of dopamine on calcium currents in turtle retinal ganglion cells.

The use of fluorescence labelling allowed Hempel *et al.* (1996) to study the dynamics of cAMP signals in arthropod neurones, specifically those of the intact stomatogastric ganglion (STG) of lobster *Panulirus interruptus*. Perfusion of the monoamines dopamine, octopamine and serotonin onto the STG elicited rises in cAMP concentration throughout the preparation on a virtually instantaneous time scale. To further investigate the dynamics within individual neurones, rises in intracellular cAMP were triggered by stimulation of identified neuromodulatory afferents. This allowed analysis of the compartmentalised, directional response of neurones from the fine proximal neurites along to the soma. The postsynaptic neurites in the neuropile exhibited significant rises in cAMP after approximately 20 seconds as opposed to the soma which responded with a mean lag of 245 seconds, the latter attributable to the diffusion of the second messenger to the soma. Such dynamics are consistent with time course reported here. A number of studies have used molluscan systems to investigate the dynamics of cAMP signalling in neurones. An initial investigation by Hockberger and Yamane (1987) showed that cAMP elevation in response to the adenylyl cyclase stimulant forskolin were compartmentalised within the neuropilar region of molluscan cells supporting a localised role for cAMP-mediated modulation. Whereas Bacskai *et al.* (1993) reported that cAMP activity was not so spatially restricted and a response evident in the soma of the neurone when stimulated by higher concentrations of serotonin (5×10^{-5} M to 10^{-4} M). They concluded that the spatial spread of cAMP stimulated by the monoamine serotonin was not directionally restricted. The gradient in response was due to receptor localisation and non-uniform distribution of signal transduction

mechanism. It could be that a similar effect occurs in D_f; modulation observed at the soma only when high concentrations of monoamine are applied (typically between 10⁻⁵ M and 10⁻³ M), and the delay apparent due to firstly the penetration of the amine to the synaptic sites and secondly the spread of the diffusible messenger from the proximal dendrites to the soma.

It is of interest to determine whether the time course is consistent with the involvement of a G-protein transduction pathway. The responses of G-protein-linked receptors to amines have been investigated for a number of cloned receptors expressed in *Xenopus* oocytes. Reale *et al.* (1997) detected a dopamine-stimulated rise in intracellular Ca²⁺ within 20 seconds of adding the ligand to oocytes expressing the cloned *Drosophila* DopR99B G-protein-linked receptor. The rise in [Ca²⁺]_i was monitored indirectly as an inward current mediated by the endogenous *Xenopus* Ca²⁺-activated chloride channel. This probably accounts for the slight difference in the time course from that reported for the McN-A-343-mediated rise in [Ca²⁺]_i observed in D_f (David and Pitman, 1996b). David and Pitman (1996b) used the fluorescent Ca²⁺ indicator fluo-3 to directly record the rise in [Ca²⁺]_i, and reported a maximal effect in response to a brief application of McN-A-343 within 4 seconds. Both, however, reported a rise in intracellular Ca²⁺ within a time course of seconds. The same *Drosophila* DopR99B receptor (Reale *et al.*, 1997) was found to couple to multiple intracellular signalling pathways and stimulated an increase in cAMP concentration with a lag period of 3 minutes. Similar time scales have been reported for a number of G-protein receptors positively linked to cAMP production. The *Drosophila* octopamine OAMB receptor identified by Han, Millar and Davis (1998) is linked to both cAMP production and rise in [Ca²⁺]. When this protein was expressed in *Drosophila* S2 and human HEK

cells it operated on a time scale of seconds. Octopamine also stimulates a significant rise in the cAMP content of the locust extensor tibiae neuromuscular preparation within 3 minutes (Evans, 1984). Comparable time courses have been reported for molluscan preparations; injection of cAMP into *Aplysia* sensory neurones, mimicking the action of serotonin, maximally enhances Ca^{2+} current within 10 – 20 seconds (Braha *et al.*, 1993). In the R15 neurone, application of serotonin enhances an inwardly rectifying K^+ current and a voltage-gated Ca^{2+} current within 140 s (Kramer and Levitan, 1990). Specific stimulation of a cAMP-dependent chloride channel by dopamine has been studied in isolated leech (*Hirudo medicinalis*) neurones. The time course for increased activation of chloride channels was within 5 – 10 seconds after application of 10^{-4} M dopamine to the bath (Ali, Catarsi and Drapeau, 1998). Therefore, one would expect activation of G-protein receptors positively linked to cAMP production to exert an effect on a maximum time scale of seconds. The application of ACh at a rate of one pulse per minute does not allow an accurate resolution of the time course for the effect of the amine below a period of one minute. The use of cAMP fluorescent labelling would be of extreme interest as it would help determine the dynamic of the second messenger in this system and go some way to resolving the initial location of the amines action.

In the D_f motoneurone the onset of action observed for the amines was often within a minute but usually took 3 – 5 minutes to achieve maximal effect. The most likely explanation for the slight inconsistencies in time course is the location of the receptors. If the receptors responsive to the amines are located on the primary neurite or dendrites immediate to it one would expect the effect of adding serotonin to the bath to be slightly delayed whilst the drug penetrates to receptor location. However once there, serotonin initiates a rise in cAMP within a matter of

milliseconds to seconds, consistent with the data from other G-protein-linked receptor systems. This delay in observing modulation at the recording site in the soma is likely to be further accentuated by the diffusion of the second messenger from the ligand-receptor transduction sites to the soma akin to that reported by Hempel *et al.* (1996). The net result being that modulation observed at the soma exhibits a gradual onset over a period of minutes. The similarity in time course between intact preparations and the under-cut soma is most likely attributable to the fact that the undercutting procedure did not excise all the D_f dendritic arborisation and that some of the processes local to the soma express amine receptors.

1.2 Receptor site for aminergic modulation.

Evidence to support the hypothesis that the initial site of aminergic signal transduction in D_f is a specific G-protein linked receptor independent of the nicotinic ACh ligand-gated ion channel, and that this is linked to an adenylyl cyclase system is summarised below.

The amines do not interact competitively with the ACh binding site of nAChRs to reduce the sensitivity of the D_f motoneurone to cholinergic ligands; increasing the relative concentration of ACh applied to the soma did not overcome aminergic suppression. Furthermore the reduction in nicotinic responses cannot be attributed merely to classical short-circuiting effect of an increase in membrane conductance, but rather that they reduce conductance through nAChR channels, since amines depress ACh responses when observed under voltage clamp conditions. The action of all three amines can be blocked by low concentrations of LSD. The vertebrate receptor antagonist RS23597-190 also significantly inhibited the action of serotonin on ACh responses. These results are compatible with an event mediated

by receptors distinct from the nAChR. LSD binds aminergic receptor sites in a variety of tissues (for examples see Farrow and Vunakis, 1972; Drummond, Bucher and Levitan, 1978; Drummond, Bucher and Levitan, 1980; Blenau, May and Erber, 1995; Watts *et al.*, 1995) and has previously been reported to block serotonin stimulation of adenylyl cyclase activity of cockroach nerve cord homogenates (Nathanson and Greengard, 1974).

Experimental data are presented which supports the presence of the three components required for an adenylyl cyclase signal transduction mechanism as defined by Schramm and Selinger (1984): receptor, G-protein and adenylyl cyclase catalytic subunit. The putative receptor site was identified by the ability of the amines to be antagonised by low concentrations of LSD. Additionally, ligand transduction is dependent on G-proteins as evident by the action of the GDP analogue, GDP- β -S, in reducing the extent of suppression mediated by serotonin. Added to these lines of evidence is the fact that aminergic modulation is dependent on the functioning of an adenylyl cyclase enzyme sensitive to the inhibitor DDA. The sum of the evidence clearly supporting the involvement of an adenylyl cyclase system in transduction of the effect of the amines.

In a previous study by Nathanson and Greengard (1974) the effect of LSD was found to be specific to the serotonin adenylyl cyclase; low concentrations of LSD did not inhibit cAMP accumulation stimulated by either dopamine or octopamine acting at their specific cyclases. RS23597-190, which also exhibited antagonistic action at 10^{-7} M on amine mediated ACh modulation in Df, is a potent specific antagonist of vertebrate 5-HT₄ receptors, a receptor subtype positively coupled to adenylyl cyclase (Ouadid, Seguin, Frapier, Dumuis, Bockaert, and Nargeot, 1991; Eglen, Wong, Dumuis, and Bockaert, 1995; Markstein, Matsumoto,

Kohler, Togashi, Yoshioka, and Hoyer, 1999). It must also be noted that vertebrate 5-HT₆ and 5-HT₇ receptors which show high affinity LSD binding, also stimulate adenylyl cyclase, but are harder to characterise, since specific antagonists have yet to developed (Markstein *et al.*, 1999). The pharmacological profile of the cockroach receptor is too complex to enable the precise identity of the receptor to be established. The major problem observed in the present investigation was that those antagonists that were active on the preparation, including LSD and RS23597-190, all had some agonist action and mimicked the action of the amines. LSD has previously been reported to exert dual antagonist-agonist actions on the serotonin-sensitive adenylyl cyclase of *Periplaneta americana* reported (Nathanson and Greengard, 1974). Indeed, with the majority of compounds this agonistic effect was sufficiently powerful that it made it impossible to assess any antagonistic effect they may have had. In fact, the pharmacology of aminergic antagonists is often complex. For instance metoclopramide is a known agonist at vertebrate 5-HT₄ receptors but an antagonist at 5-HT₃ subtypes (Kawa, 1994; Rizzi, Mierau and Lidinsky, 1997). The complex pharmacology of these agents is further compounded when they are used on invertebrates preparations in which their actions may be even more unpredictable. Often cloned invertebrate receptors display pharmacological profiles distinct from vertebrate counterparts, for example, the *Drosophila* and *Lymnaea* serotonin receptors. The dopamine- and serotonin-sensitive adenylyl cyclases of *Helix aspersa* characterised by Deterre and colleagues (1986) show a number of non-specific, non-competitive responses to antagonists. The antagonist fluphenazine, often used as a broad range dopaminergic antagonist in vertebrates preparations, irreversibly reduced the sensitivity of the adenylyl cyclase responsive to serotonin in homogenates preparations only (Deterre, Paupardin-Tritsch, and Bockaert, 1986).

Perfusion with the antagonist over intact tissue did not exert any inhibitory action on cAMP accumulation. In this instance the researchers concluded that the site at which fluphenazine blocked adenylyl cyclase activation was not accessible from the extracellular side of the membrane. In the same preparation no antagonists were found to inhibit the adenylyl cyclase responsive to dopamine. Similar insensitivity of aminergic receptors to vertebrate dopaminergic agents was also reported by Sugamori *et al.* (1995); the cloned *Drosophila* adenylyl cyclase-linked *dDA1* receptor showed poor affinity for benzazepines such as SKF38393 and SKF-82526. This is not exclusive to dopamine sites; the putative serotonin receptor site of the honey bee *Apis mellifera* shows poor affinity for ligands selective for specific subtypes of vertebrate serotonin receptor (Blenau, May and Erber, 1995). One explanation could be that the tertiary structure of the amine binding pocket of invertebrate receptors is subtly different from those of vertebrate receptors. However structural variation in the amino acids that compose the binding pocket has not proved to be the case in the cloned receptors identified so far (Cox *et al.*, 1992), and where receptors contain elongated amino terminals such as that exhibited by the DmDop1 *Drosophila* subtype, the additional amino acids do not impart reduced sensitivity to benzazepines (Gotzes and Baumann, 1996). Certainly deletion-mutagenesis of the amino terminals of insect receptors does not interfere with ligand binding and the ability of the natural ligand dopamine to stimulate adenylyl cyclase.

The action of most of the vertebrate aminergic ligands was to rapidly and completely abolish nicotinic ACh responses mediated by the D_f motoneurone. Their actions were not compatible with the time course and saturable effect previously determined for the amines dopamine, octopamine and serotonin. One plausible explanation could be a direct interaction of the antagonists with the nicotinic ACh

receptor. Dopaminergic agents, including SKF38393, SCH23390 and sulpiride but interestingly not dopamine, have been reported to exert such a direct effect on nicotinic ACh receptors expressed in *Xenopus* oocytes (Nakazawa, Akiyama , and Inoue, 1994). Similarly, serotonergic agents are known to block the pore of mammalian $\alpha 7$ and muscle nAChRs expressed in *Xenopus* oocytes (Grassi *et al.*, 1993; Palma, Mileo, Eusebi, and Miledi, 1996; Garcia-Colunga and Miledi, 1999). In the latter investigation it was determined that the antagonists including spiperone acted at a site independent to that of serotonin, but that both loci were within the ion channel. The other alternative effect of the antagonists on the ACh response of D_f is that the reagents exhibit undetermined non-specific effects on the motoneurone akin to the intracellular site of action of fluphenazine reported by Deterre *et al.* (1986). For instance the membrane permeant dopaminergic agonist apomorphine along with the D1 receptor specific agonist SKF-38393 have been found to inhibit rat brain protein phosphatase PP2A with IC₅₀ values comparable to that of cantharidin (see Herzig and Neumann, 2000).

The general finding of studies using antagonists developed for use with vertebrate receptors to characterise receptors of invertebrate preparations is that the classical antagonists, such as LSD are often more effective than receptor subtype-specific ligands (Wedemeyer, Roeder, Gewecke, 1992). Furthermore the pharmacology exhibited by intact invertebrate preparations rarely conforms to that defined for vertebrate receptor subtypes.

The action of the three monoamines tested was to suppress the nicotinic ACh response of D_f, which contrasts with previous studies that showed serotonin to have effects that were opposite to those of dopamine and octopamine on synaptic transmission between ventral giant interneurones and thoracic interneurones of

Periplaneta americana (Goldstein and Camhi, 1991; Ritzmann and Casagrand, 1992b). This could be due to different pharmacological profiles for interneurones and motoneurones, reflecting innervation from different aminergic systems. Suppression of ACh responses in D_f mediated by each of the amines was not additive when they were co-applied, suggesting that either all three amines exert their effect via a common transduction mechanism or alternatively that they bind to separate receptor populations and stimulate inter-woven intracellular transduction pathways. The fact that non-maximal doses of the amine do not act in an additive manner would support the latter conclusion and raises interesting questions as to the potential role of octopamine and dopamine in the preparation. It is however inconsistent with the findings of Nathanson and Greengard (1973) who reported that the antagonist actions of LSD were specific to the serotonin-sensitive AC in cockroach nerve cord homogenates. Their finding would support the latter hypothesis, and suggest that a common point in the transduction pathway to all three amines could be the receptor expressed in the surface membrane. The poor ability of putative aminergic receptors to distinguish between natural ligands has been reported in a variety of other tissues and therefore, would not be a property exclusive to the amine receptor present in the D_f motoneurone. For example the dopamine receptor of the *Apis mellifera* brain characterised by Kokay and Mercer (1996) also binds, with less affinity, serotonin, octopamine, tyramine and histamine. Serotonin on the other hand, has been demonstrated to act as a partial agonist of the octopamine-mediated modulation of twitch tension relaxation in locusts (Evans, 1981). A number of studies have focused on the enzyme systems responsive to amine G-protein receptors, of which some receptors do not readily distinguish between natural ligands. Uzzan and Dudai (1982) identified adenylyl cyclases in *Drosophila*

homogenates responsive to dopamine, octopamine and serotonin. Octopamine and serotonin acted via the same receptor-adenylyl cyclase system, whereas dopamine stimulated cAMP accumulation via a signal transduction mechanism independent to that of octopamine. This is in contrast to the adenylyl cyclase system present in the nervous tissue of *Octopus vulgaris* (Capasso *et al.*, 1991) which is stimulated by octopamine and dopamine in a non-additive manner, akin to that determined for D_f here. A number of these studies suggest that invertebrate amine receptors are poor at distinguishing between natural ligands. Some work has been done to map the serotonin binding cleft of the vertebrate 5-HT₂ receptor in an attempt to elucidate the amino acids responsible for selective ligand binding (Wang, Gallaher and Shih, 1993; Almaula *et al.*, 1996). Similar to a number of other G-protein receptors, high affinity binding of the ligand is mediated via an aspartate residue (5-HT₂ : Asp155) situated in the third transmembrane loop (Wang, Gallaher and Shih, 1993). This negatively charged residue is believed to form a complex with the charged amine group of the ligand. The range of ligands that contain such a residue is considerable and includes the amines tested in this investigation as well as acetylcholine, and is believed to be a prime determinant for receptor activation. The specificity of the 5-HT₂ receptor for serotonin is further specified by a serine residue (Ser159) near to the aspartate residue (Almaula *et al.*, 1996). This residue is thought to orient the ligands in the binding pocket by forming a hydrogen bond with the same functional amino group of the ligand, imparting to the receptor sensitivity to the stereochemistry of ligands (see Blenau, May and Erber, 1995; Watts *et al.*, 1995). The structure of a number of ligands, for example LSD, allows them to interact with the aspartate residue Asp155 but not with serine Ser159 residue due to steric hindrance. This partial interaction could be in part responsible for the mixed

antagonist-agonist action of this drug. As for the three amines tested, it could well be that the cockroach amine receptor shows less selectivity for ligands than that of the vertebrate 5-HT₂ receptor, allowing the receptor to bind to varying degrees dopamine, octopamine and serotonin; the degree of ligand binding directly corresponding to the extent to which the receptor undergoes a conformational change (see Almaula *et al.*, 1996). To resolve such concerns it would be of interest to conduct molecular studies similar to those of Almaula *et al.* (1996), in order to resolve the determinants for successful ligand binding in insect amine receptors.

Trimeric G-proteins link neurotransmitter receptors to intracellular second messenger pathways (for reviews see Hille, 1994; Rens-Domiano and Hamm, 1995; Gudermann, Kalkbrenner and Schultz, 1996). The ability of the non-hydrolyzable GDP analogue, GDP-β-S, to oppose the receptor activation of G-proteins and has been demonstrated in a variety of neuronal systems (see for examples Deterre, Paupardin-Tritsch, and Bockaert, 1986; Eusebi, Grassi, Molinaro and Zani, 1987; Dolphin and Scott, 1987; Lee, Chung, Bang, Baek and Uhm, 1997; Lundquist and Nässel, 1997). Raising the intracellular concentration of GDP by injecting the analogue GDP-β-S, opposes G-protein activation by competing with GTP. The ability of GDP-β-S to significantly attenuate amine-mediated suppression of ACh responses recorded from the D_f motoneurone is a strong indication that G-proteins are not only involved in, but that their activation is key to, transduction of the aminergic ligand signal. To establish which G-proteins are involved, the two bacterial toxins, pertussis and cholera toxin were tested. The receptor is unlikely to be linked to a member of the G_{i/o} family since aminergic suppression of ACh responses was unaffected by pertussis toxin. However the action of cholera toxin in suppressing the ACh response, and the fact that this effect was potentiated by the

PDE inhibitor theophylline, implies that a G_s protein is present in the D_f motoneurone akin to those reported by Awad and Anctil (1993) in the cnidarian *Renilla koellikeri* and Sidhu *et al.* (1998) in transfected rat pituitary GH4C1 cells. Although the involvement of the G_s protein in aminergic suppression was not tested, it would be logical to assume that the LSD-sensitive receptor site is positively coupled to adenylyl cyclase via a trimeric G-protein of this family.

1.3 Second messenger pathways.

Buffering intracellular levels of calcium by injecting the Ca²⁺-chelator BAPTA does not attenuate the action of serotonin in suppressing ACh responses. This suggests that the suppression observed is mediated by a mechanism distinct from that by which muscarinic agonists suppress ACh responses in the same motoneurone (David and Pitman, 1996b). The success of the injection protocol in this study was judged by the ability of BAPTA to enhance ACh responses (David and Sattelle, 1990) and enable the production of action potentials (Pitman, 1979). The same protocol has previously been demonstrated to dampen Ca²⁺ kinetic in the D_f motoneurone (Mills and Pitman, 1997).

The marginal enhancement of modulation mediated by the amine in the presence of BAPTA is of interest as Ca²⁺-dependent and cyclic nucleotide systems are known to interact at multiple levels of the transduction process (see review by Cooper, Mons, and Karpen, 1995). Calcium-dependent events are known to modulate cyclic nucleotide cascades, regulating the ability of adenylyl cyclase enzymes to increase the cytosolic cAMP concentration (see table 8, page 76) as well as the activity of Ca²⁺ and CaM-regulated phosphodiesterases (for example see Kramer and Levitan, 1990; Kostyuk and Lukyanetz, 1993). The observed

enhancement and rapid onset of aminergic suppression of ACh responses in the presence of BAPTA is consistent with increased adenylyl cyclase activity when intracellular Ca^{2+} concentrations are reduced. Such an enhancement occurs with mammalian adenylyl cyclase AC5 and AC6 isoforms which are inhibited by $[\text{Ca}^{2+}]_i$. Additionally the AC5 isoform of adenylyl cyclase has been shown to positively couple to G_s protein-linked 5-HT₆ and 5-HT₇ receptors when co-expressed in HEK 293 cells (Baker *et al.*, 1998). It has been previously demonstrated by using confocal Ca^{2+} -fluo3 imaging that application of dopamine to the D_f motoneurone results in a rapid quenching of the $[\text{Ca}^{2+}]_i$ signal (Prothero, 1996). The purpose of such an action could be to further enhance and prolong adenylyl cyclase stimulation by the receptor-activated G_s protein. Such regulation is not implausible; dual signalling via divergent targets of the α and $\beta\gamma$ subunits is an apparent function of a number of G-protein linked receptors (see Gudermann, Kalkbrenner and Schultz (1996) for review). A number of studies have identified aminergic receptors that couple to both calcium and cyclic nucleotide pathways (Reale *et al.*, 1997; Baker *et al.*, 1998).

The principle finding of the research has been that cyclic nucleotides and their downstream kinases mediate aminergic suppression of ACh responses recorded from D_f. The experimental evidence from application of the nucleotide analogues and the specific PKA and PKG inhibitors does not distinguish between cAMP or cGMP. A role for the latter in invertebrate neurotransmission is well supported and has been implicated in the modulation of cholinergic transmission in a number of molluscan preparation (see for example Drozdova and Pivovarov, 1995; Mothet, Fossier, Tauc and Baux, 1996a and 1996b). However in insects the action of cGMP appears to be exerted predominantly at presynaptic sensory sites (for review see

Bicker, 1998; see also Bicker and Schmachtenberg, 1997; Wildemann and Bicker 1999). Of the monoamines tested on D_f only serotonin has been demonstrated to stimulate an increase in intracellular cGMP concentration in vertebrate systems. In the pig choroid plexus serotonin 5-HT_{2C} receptors are positively coupled to cGMP production via a Ca²⁺-dependent G-protein dependent pathway (Kaufmann, Hartig and Hoffman, 1995). Ca²⁺ influx via 5-HT₃ receptor ligand-gated ion channels is implicated in transduction of the 'fast' Ca²⁺-dependent cGMP response to serotonin in cultured NG108-15 (mouse neuroblastoma-rat glioma hybrid cells) (Tohda and Nomura, 1990). The inability of BAPTA injection to block suppression of ACh responses mediated by serotonin rules out the likelihood that either of these mechanisms operates in the D_f motoneurone. There is evidence in the literature to suggest that members of the guanylyl cyclase family can be differentially regulated by Ca²⁺ and that some guanylyl cyclases can be inhibited by Ca²⁺ as can AC5 and AC6 isoforms of adenylyl cyclase (Mayer, Klatt, Böhme and Schmidt, 1992). As yet there is no indication that these Ca²⁺-inhibited guanylyl cyclases are positively linked to serotonin receptors.

One explanation for why the analogues of both cAMP and cGMP exert an effect on nicotinic ACh responses of the D_f motoneurone could be poor selectivity of the analogues for their specific protein kinases. Certainly it is known that both cyclic nucleotides can activate either PKA or PKG, although they exhibit significantly lower affinities for the others kinase (see Scott, 1991; Wang and Robinson, 1997). It is entirely possible that at the concentrations of cyclic nucleotide analogue applied (10⁻⁴ M) there is some cross-activation. For example, CPT-cAMP, the more effective analogue of the two cAMP ones used, was found to be almost equipotent at stimulating PKA (K_a 0.05 μM) and PKG (K_a 0.11 μM;

Sandberg *et al.*, 1991). The same analogue also acts as a potent inhibitor of cyclic GMP-dependent PDE (Connolly *et al.*, 1992), thereby theoretically promoting the accumulation of cGMP in neurones. The multiple non-specific effects of the cyclic nucleotide analogues should therefore be borne in mind when considering the results using such reagents.

Application of cyclic nucleotide analogues does effectively mimic the action of amines in a number of neuronal systems (see for example Walsh and Byrne, 1989; Liu and Lasater, 1994). Although it has been proposed that cGMP and cAMP analogues mimic the effects of amines on nACh responses in D_f because the selectivity for PKG and PKA may be poor, no such non-selectivity was observed in studies of phosphorylation of vertebrate nAChRs (Huganir and Greengard (1983); Margiotta *et al.* (1987a); Vijayaraghavan *et al.* (1990)). All reported phosphorylation of nAChRs by cAMP-dependent protein kinase but not by the cGMP-dependent kinase when the preparations were exposed to the respective analogues.

The action of cAMP, specifically the analogue Db-cAMP, has previously been investigated to determine whether cyclic nucleotides mediate muscarinic modulation of the voltage-dependent Ca²⁺ currents (I_{Ca}) exhibited by the D_f motoneurone (David and Pitman, 1996a). Bath application of Db-cAMP mimicked the action of the muscarinic agonist McN-A-343, but not by reducing I_{Ca}, instead by attenuating the outward Ca²⁺-dependent K⁺ current (I_{K,Ca}). This would indicate that cAMP is not involved in muscarinic modulation, although it does suggest that cAMP-dependent cellular events modulate not only nAChRs but also other ion channels, including the predominant outward, rectifying current observed in D_f. It was observed in this study that, in a number of preparations, application of the

monoamines served to prolong ACh-induced depolarisations as well as suppress the amplitude, this ‘effect’ might in part reflect cAMP down-regulation of the rectifying currents. One other piece of circumstantial evidence perhaps of note is that addition of pertussis toxin dramatically prolonged the ACh response. Pertussis toxin inhibits the action of the G_{i/o} family of G-proteins, of which G_i protein α subunits are known to inhibit adenylyl cyclase activity. Increased adenylyl cyclase activity would lead to a rise in intracellular cAMP, which would in turn inhibit the rectifying currents thus prolong recovery from depolarisation. The fact that pertussis toxin prolongs the ACh response suggests that the toxin promotes adenylyl cyclase activity. The most logical way it could achieve this is by inhibiting a G-protein responsible for the down regulation of cyclase activity - a G_i protein. If a G_i protein is present in the D_f motoneurone, no ligand or receptor has yet been identified to stimulate its activity. Such a system would be of interest as it might serve to enhance or at least maintain nicotinic transmission exhibited by the motoneurone by counteracting aminergic modulation.

1.4 Target of protein kinase-mediated phosphorylation.

The involvement of phosphorylation in aminergic suppression of the nicotinic ACh response of the D_f motoneurone is supported by a number of lines of evidence. Protein kinase inhibitors significantly attenuate the modulation, whereas phosphatase inhibitors prolong the observed action of the amine serotonin. The ability of the Rp-Br-analogues of cAMP and cGMP, which selectively inhibit the cyclic nucleotide-dependent protein kinases, to reduce the ability of serotonin to suppress the ACh response further confirms the reliance of the modulation on a rise in intracellular cyclic nucleotide concentrations and activation of kinases. Cyclic

cAMP-dependent phosphorylation has already been demonstrated to modulate neurotransmission, for example that mediated by vertebrate glutamate receptors (for example see Cerne, Rusin and Randic, 1993; Blackstone *et al.*, 1994), and affect the phosphorylation state and activity of numerous neuronal proteins (Homayouni, Byrne and Eskin, 1995).

The ability of protein kinases to phosphorylate nAChRs was first demonstrated in the 1970s (Gordon, Davis and Diamond, 1977; Gordon, Davis, Milfay and Diamond, 1977; see also Teichberg and Changeux, 1977; Davis, Gordon and Diamond, 1982). A number of studies have investigated cAMP-dependent phosphorylation of the nAChRs, principally isolated from skeletal muscle preparations. The α , γ and δ subunits of muscle nAChRs from a number of preparations have been demonstrated to be phosphorylated *in vitro* (for example see Smith, Merlie and Lawrence Jr., 1989; Ferrer-Montiel, Montal, Díaz-Muñoz and Montal, 1991; Hoffman, Ravindran and Huganir, 1994) which provides support for the notion that the nAChR in *Periplaneta americana* could also act as a substrate of PKA. The predominant action of PKA-dependent phosphorylation on nAChR function shown to date is to increases the rate of desensitisation (see Albuquerque *et al.*, 1986; Miles *et al.*, 1987; Middleton, Rubin, and Schuetze, 1988). This does not seem to be the case in the current study, as application of the amines suppresses the control response to the same degree after ten minutes even when ACh is not regularly applied. Rather, it appears that the amines reduce the inward current mediated by cockroach nAChRs independently of the continued presence of ACh. In a sense converting them to a 'silent' state akin to that proposed to exist for hippocampal AMPA receptors (see Liao, Hessler and Malinow, 1995).

A number of putative phosphorylation sites have been identified in cloned insect receptors (see table 2b, page 24). A future direction for research might be to firstly identify whether or not the nAChR of *Periplaneta americana* is indeed phosphorylated in response to incubation with the monoamines. Initial phosphorylation assays similar to those developed by Huganir and Greengard (for examples see Miles *et al.*, 1987; also see Vijayaraghavan, *et al.*, 1990) would provide an indication as to whether the insect nicotinic AChR is a phosphoprotein. The use of acetylcholine affinity columns to purify the receptors would provide a satisfactory means of partially identifying receptor proteins. An immunoprecipitation method similar to that used by McDonald *et al.* (1998) could be used with the development of more effective antibodies for insect nAChRs in order to identify the receptor subunits involved.

The ability of the phosphatase inhibitors okadaic acid and cantharidin to prolong aminergic suppression of ACh responses is indicative of the involvement of phosphatases in reversing the phosphorylation on which modulation depends (for example see Ichinose and Byrne, 1991). Although the literature detailing the specific association of phosphatases with nAChRs is limited, one agent, okadaic acid, has previously been demonstrated to increase phosphorylation of recombinant AChR δ -subunits (Kopta and Steinbach, 1994). Okadaic acid is a potent inhibitor of all the major neuronal phosphatases, while cantharidin only inhibits PP-1 and PP-2A to any degree. Therefore, the phosphatase present in the D_f motoneurone is more than likely an invertebrate homologue of one of these two rather than a PP-2B/calcineurin phosphatase similar to that proposed to regulate PKA phosphorylation of vertebrate AMPA receptors (Banke *et al.*, 2000). Interestingly a number of vertebrate PKA systems have been identified recently that have been

found to interact closely with PP-1 phosphatases (for example see Pedarzani *et al.*, 1998). For example, it is known that PKA-mediated phosphorylation regulates the activity of PP-1 inhibitory proteins such as DARPP-32 (see the review by Herzog, Neumann, 2000). The phosphorylation of DARPP-32 reduces the activity of the phosphatases and prolongs kinase mediated phosphorylation of the PP-1-sensitive substrates. Additionally the two enzymes, PKA and PP-1, have been found to co-localise at anchoring proteins including NMDA receptor-associated proteins (Schillace and Scott, 1999; Westphal *et al.*, 1999). PKA/PP-1 signalling cascades have been proposed to mediate dopamine modulation of GABA_A currents in rat neostriatal neurones (Flores-Hernandez *et al.*, 2000) and the function of NMDA receptors in spiny neurones of the nucleus accumbens (Snyder, Fienberg, Huganir and Greengard, 1998). It would be of extreme interest to determine whether similar regulatory pathways are also present in the cockroach as this would indicate that they are a highly conserved means of controlling receptor phosphorylation states and hence of neuronal signalling.

1.5 Other factors influencing nAChR function.

The protein kinase inhibitor H7 was not particularly effective at attenuating the ability of serotonin to suppress ACh responses, which is surprising because of its reported ability to inhibit cyclic nucleotide-dependent kinases (Hidaka, Inagaki, Kawamoto and Sasaki, 1984; Hidaka and Kobayashi, 1993). However, consistent with the findings presented here, it is apparent from studies on *Aplysia* that H7 is only effective at inhibiting PKC- and not PKA-mediated effects (Braha, Edmonds, Sacktor, Kandel and Klein, 1993). It would be of interest to determine whether H7 can attenuate the Ca²⁺-dependent modulation of ACh responses initiated by

muscarinic agonist in the D_f motoneurone (David and Pitman, 1996b). The reliance of muscarinic suppression on inositol-1,4,5-triphosphate and calcium (David and Pitman, 1994, 1996b) would suggest the downstream involvement of protein kinase C in regulation of ACh responses by muscarinic agonists. The convergence of the two kinases, PKA and PKC, in the same neuronal system would not be unique. A number of neurotransmitter receptors have putative phosphorylation sites for both PKA and PKC (for example see Moss, Doherty and Huganir, 1992; McDonald *et al.*, 1998). Whereas activation of PKC and PKA both exert the same action on *Aplysia* Ca²⁺ currents (for example see Braha *et al.*, 1993), rat cerebral cortex P4 purinoceptor Ca²⁺ transients (Pintor, Gualix and Miras-Portugal, 1997), and rat hippocampal dendritic action potentials (Hoffman and Johnston, 1999), there are reports of the kinases exerting opposing actions on receptors. An example of this is modulation of rat glycine receptors expressed in *Xenopus* oocytes (Vaello *et al.*, 1994). Experiments in which muscarinic agonists and monoamines were co-applied have not been performed as part of this study but might reveal whether or not the two pathways interact. It would be of interest to see if the two agents acting via different pathways act additively to completely suppress ACh responses. If they do not act in an additive manner it would suggest that there is an interaction between the two pathways. In *Aplysia* it has been demonstrated that PKC activity can down-regulate cAMP-dependent events (see Sugita, Baxter and Byrne, 1997), whereas both kinases are known to feedback onto G-protein-linked receptors to modulate their ability to transduce the initial ligand signal (for example Raymond and Olsen, 1994).

Since nAChRs in D_f can be modulated by both muscarinic agonists and amines an obvious question arises: why are two modulatory systems with the same action required? One plausible explanation is that the muscarinic pathway acts as an

feedback mechanism to control ACh excitability and prevent excessive, repeated depolarisation. If large amounts of ACh are released by afferent presynaptic terminals it would result in a delayed suppression of the cholinergic neurotransmission at the postsynaptic membrane, mediated by the muscarinic G-protein-linked receptors. The ability of serotonin to reduce the ACh response adds the extra dimension that it is independent of the level of activity in cholinergic inputs to the neurone. Additionally it should be noted that there was no indication that suppression mediated by serotonin modulated inhibitory GABAergic responses and therefore in this aspect it differs from the modulatory action exerted by the muscarinic agonists (Pitman and David, 1996b). Muscarinic agonists exerted mixed effects on GABA responses, either enhancing or suppressing the control GABA response. Muscarinic receptor activation therefore might serve to modulate the overall receptor excitability of the motoneurone, altering the responsiveness to both excitatory nicotinic ACh inputs and inhibitory GABAergic afferents, whereas the action of the monoamines appears to be specific to nicotinic receptor transmission.

Is there a pathway that up-regulates ACh activity in the D_f motoneurone? Apart from the suggestion that a G_i protein might serve to counteract aminergic suppression, there is one other line of evidence to suggest that there is such a ‘positive’ pathway. The calmodulin (CaM) agents W-7 and KN-62 both suppressed ACh responses when applied to the preparation in the absence of amines. This would suggest that knocking out CaM-mediated events affects the ability of the ligand-gated ion channel to function. This suggests that CaM may normally functioning to maintain nAChR function. W-7 acts at a wider range of targets, inhibiting the interaction of CaM with its substrate proteins. The action of KN-62 is specific to CaM kinase II, a kinase that has been implicated in increasing presynaptic

neurotransmitter release in squid neurones by interacting with the synapsin class of vesicle protein (see for example Llinás *et al.*, 1985). This would certainly explain the increase in somatic potentials recorded after addition of KN-62 to the bath. As for postsynaptic effects, CaM has been demonstrated to not only stimulate but also maintain a basal level of phosphorylation of *Torpedo* nAChRs (Smilowitz, Hadjian, Dwyer and Feinstein, 1981). Calmodulin activity, stimulated by the *cis*-unsaturated fatty acid oleic acid, is also known to enhance ACh responses of *Torpedo* ACh receptors expressed in *Xenopus* oocytes (Nishizaki, Ikeuchi, Matsuoka and Sumikawa, 1997). CaM activity also counteracts the suppressive action of serotonin on ACh response recorded from *Helix pomatia* neurones (Dyatlov, 1991). In *Helix* LPL1 and RBc4 neurones, serotonin suppresses ACh responses by a cAMP-dependent mechanism, whereas Ca^{2+} influx enhances the responses by an action sensitive to the CaM inhibitor chloropromazine.

It is not inconceivable that local Ca^{2+} influx, even that resulting from nAChR cation channel conductance, could trigger CaM-dependent events local to the receptors and maintain their function, acting as a process of positive feedback (for references see Augustine and Neher, 1992; Ghosh and Greenberg, 1995). Increased release of ACh from presynaptic terminals however stimulates muscarinic G-protein-dependent receptors that initiate a more global increase in intracellular Ca^{2+} concentration by an IP_3 -dependent release from intracellular stores (Simpson, Challiss and Nahorski, 1995; David and Pitman 1996b). This large surge in $[\text{Ca}^{2+}]_i$ along with the formation of DAG results in PKC activation, which acts as a feedback mechanism by which nAChR function is suppressed, and the action of CaM opposed (Chakravarthy, Morley and Whitfield, 1999). Additionally there is the likelihood that Ca^{2+} -dependent events also regulate amine-induced modulation by altering both

adenylyl cyclase (Choi, Wong, Dittman, and Storm 1993; Yoshimura and Cooper, 1993; Cooper, Mons, and Karpen 1995; Xia and Storm, 1997; Baker *et al.*, 1998) and phosphodiesterase activity (Polli and Kincaid, 1994). The possibility that CaM regulates PDE activity in D_f is tentatively supported by the irreversibility of the action of serotonin applied to cells incubated with W-7. This would suggest that an enzyme responsive to CaM is involved in the mechanism underlying reversal of the suppression. This irreversibility is unlikely to be attributable to CaMK II activity, as the effect of serotonin and the CaMK II inhibitor KN-62 are both reversed after prolonged washing. A more likely candidate is a CaM-dependent PDE. The action of W-7, therefore, being to inhibit CaM-activated hydrolysis of the cyclic nucleotide second messenger and thus prolonging the modulation of the ACh response. Such PDEs have previously been shown to co-localise with areas receiving dopaminergic innervation in the mouse brain (Polli and Kincaid, 1994). The intracellular messenger pathway responsive to the amines is illustrated in figure 61 (page 290), along with the two hypothetical Ca²⁺-dependent systems outlined above.

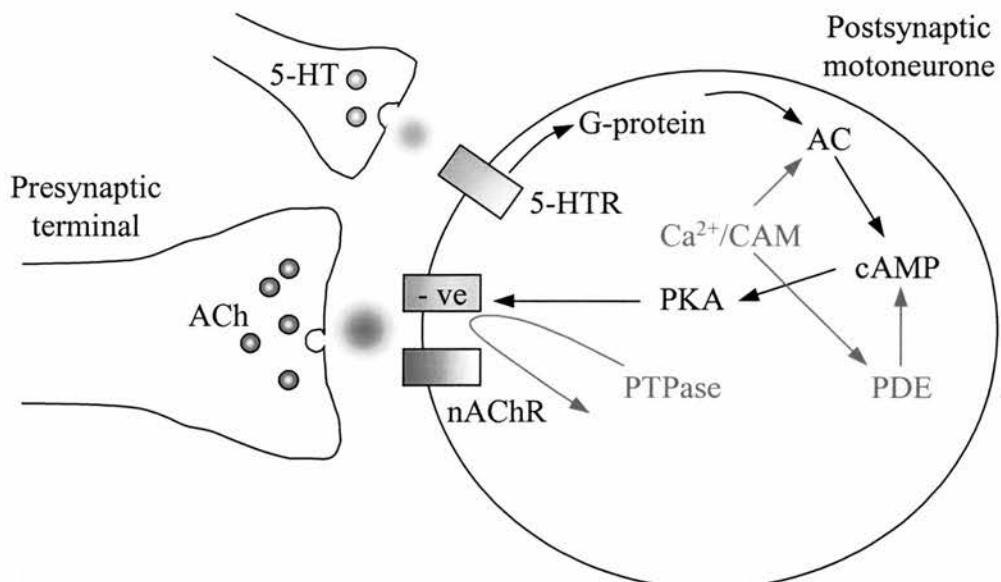
1.6 Implications for the understanding of neuronal modulation.

The findings of this research raise a number of issues about neuronal regulation in arthropod species and also have implications for our understanding of cholinergic systems and their interaction with monoamine neurotransmitters in other preparations. It represents the first time that the function of nAChRs has been shown to be modulated by a cAMP-dependent pathway positively linked to a G-protein linked aminergic receptor in an *in situ* insect preparation. However the findings and analysis should not be restricted *per se* to invertebrate application. A number of the factors implicated, for example the Ca²⁺ -dependent regulatory systems that

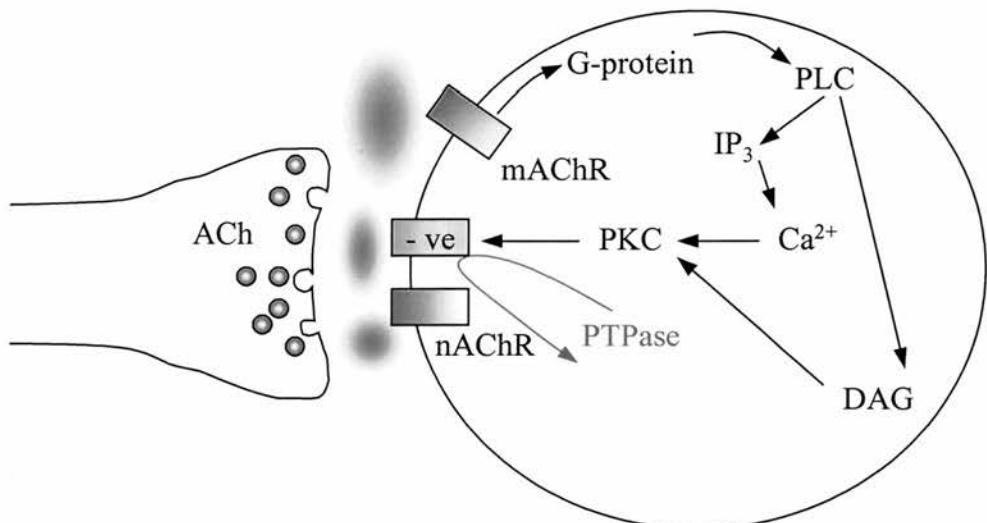
Figure 61. D_f motoneurone putative protein kinase pathways.

(a) The protein kinase pathway responsive to serotonin. (b) and (c) putative kinase pathways dependent on intracellular Ca^{2+} concentrations. Pathways that function to down-regulate phosphorylation are indicated in red.

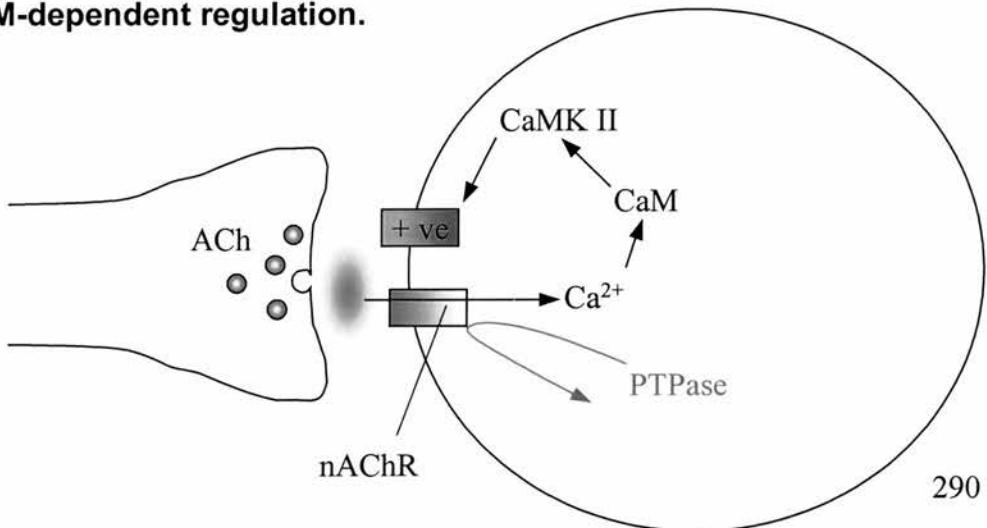
(a) Aminergic modulation.



(b) Muscarinic modulation.



(c) CaM-dependent regulation.



influence aminergic suppression, are apparent in vertebrate systems. The close resemblance of the systems revealed by the pharmacological approach used in this study to the phosphorylation-dependent pathways recently published by Greengard *et al.* (Snyder, Fienberg, Huganir and Greengard, 1998; Flores-Hernandez *et al.*, 2000) is striking, and would suggest such transduction mechanisms are common to a number of species. It also raises the possibility that nAChRs in a variety of species are likely to be susceptible to modulation mediated by intracellular messengers responsive to ligand activation of a range of metabotropic receptors. This would have considerable behavioural consequences as indicated by the recent review by Paterson and Nordberg (2000) which highlights a number of functions exerted by nAChRs in the central nervous system of vertebrates which may implicate them in a range of neuronal pathologies including epilepsy, Alzheimer's disease, Parkinson's disease, schizophrenia and anxiety and depression disorders.

One further aspect that is raised is the complexity of protein kinase signalling pathways in neurones. Interaction of such pathways has previous been studied in *Aplysia* (Braha *et al.*, 1993; Byrne and Kandel, 1996) and is implicated in the formation and maintenance of long term potentiation and depression in vertebrate systems (see for examples Bashir and Collingridge, 1992; Soderling and Derkach, 2000). The D_f motoneurone represents one of the first arthropod systems in which such complexity has been demonstrated, and further suggests conservation of multiple kinase pathways with the ability to affect the efficacy of neuronal transmission. Such regulation would enable different neurotransmitter and neuromodulator pathways to interact, to bring about behavioural adaptation.

This research was funded by MRC (UK) grant G78/5314.

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