PHYSIOLOGICAL ACTIONS OF PUTATIVE NEUROPEPTIDES IN THE NEMATODES *ASCARIS SUUM* AND *CAENORHABDITIS ELEGANS*

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ABSTRACT

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Parasitic nematodes are a major cause of morbidity in humans and other animals. A better understanding of neural signalling in these animals will underpin a targeted approach to anthelmintic development. The nervous system of nematodes contains a plethora of neuropeptides, many of which have potent actions on the musculature that regulates vital behaviours, for example, locomotion, feeding and egg-laying. Here the precise physiological role of a subset of these neuropeptides has been addressed using two nematodes, a parasitic species, *Ascaris suum*, and the model genetic animal, *Caenorhabditis elegans*.

The effect of the neuropeptides on the body wall muscle of *A. suum* was determined using an in vitro preparation of the dorsal muscle strip. The direct effect of the peptide and its modulation of the response to acetylcholine (ACh) were examined. ACh, 10 μM, elicited a rapid and reversible contraction. Prior application of FLPs (FMRFa-like peptides) modified the ACh response. FLP1B (AGSDPNFLRFa) and FLP13A (APEASPFIRFa) both reduced the ACh contraction while FLP3A (SAEPFGTMRFa) and FLP21 (GLGPRPLRFa) potentiated the ACh response. FLP21 also induced a muscle contraction. None of the NLPs (neuropeptide-like proteins) tested had a direct effect on the muscle but all three reduced the ACh contraction.

To investigate the effect of neuropeptides in *C. elegans* more than 30 FLPs (encoded by 23 *flp* genes) were tested for biological activity on a semi-intact preparation of *C. elegans* pharynx. Eleven *flp* genes encoded peptides which inhibited pharyngeal activity, while eight *flp* genes encoded peptides which were excitatory. One *nlp* gene encoded a peptide which had a weak excitatory action. Three of the potent peptides (FLP13, APEASPFIRFa, inhibitory; FLP17A, KSAFVRFa, excitatory; FLP17B, KSQYIRFa, excitatory) are encoded by *flp* genes expressed in pharyngeal motorneurones and therefore have a high probability of acting through specific receptors directly on the pharyngeal muscle. The two other potent peptides FLP8 (AF1, KNEFIRFa, excitatory) and FLP11A (AMRNALVRFa, inhibitory) are only expressed in extrapharyngeal neurones and are therefore likely to act either indirectly

or as neurohormones. Intriguingly, the same neuron can express peptides which have potent but opposing biological activity in the pharynx. The physiological relevance of this counter-intuitive observation is unclear. Only five *flp* genes encode neuropeptides which had no effect on the pharynx, none of which are expressed in the pharyngeal nervous system.

Overall this suggests that the peptidergic regulation of *C. elegans* pharyngeal activity is a complex phenomenon.

In an attempt to investigate whether FLP17A and 5-HT (5-Hydroxytryptamine) (neurotransmitter in *C. elegans* pharynx) act through G-protein coupled receptors the effect of altering cAMP levels on the response to FLP17A and 5-HT was investigated. Changes in the level of cAMP were achieved by applying dibutyryl-cAMP, forskolin, ibudilast, H9-dihydrochloride and 8-Bromo-cGMP. The effects of FLP17A and 5-HT were also examined in two mutant strains of *C. elegans, egl-8* and *egl-30*, in an attempt to investigate whether they might act partly through Gαq. From this investigation it was concluded that compounds applied exogenously which potentially alter the level of cAMP in *C. elegans* pharyngeal muscle influence the excitatory responses to both FLP17A and 5-HT. Application of dibutyryl-cAMP increased the excitatory responses of both FLP17A and 5-HT. The effects of FLP17A and 5-HT were also potentiated following application of forskolin and ibudilast. H9-hydrochloride reduced the response to 5-HT and completely blocked FLP17A excitation. The responses to both FLP17A and 5-HT were greatly reduced in the two *C. elegans* mutants, egl-8 and egl-30. In conclusion the excitatory effects of FLP17A and 5-HT are mediated at least partly through G-protein coupled receptors.

These results provide further evidence that neuropeptides play important roles in the regulation of locomotion and feeding in nematodes.

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Publications

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Abbreviations

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Gluamic Acid	Glu	E
Glutamine	Glu	Q
Glycine	Gly	G
Histidine	His	H
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

5-HT	5-Hydroxytryptamine
A. suum	*Ascaris suum*
ACh	Acetylcholine
AChE	Acetylcholinesterase
AF	*Ascaris* FMRFamide-like peptide
APF	Artificial Perienteric Fluid
ARC	Accessory radular muscle
C. elegans	*Caenorhabditis elegans*
DMS	Dorsal muscle strip
EJP	Excitatory junction potential
EPG	Electropharyngeogram
FaRP	FMRFamide- related peptide
FLP	FMRFamide-like peptide
GABA	Gamma aminobutyric acid
GPCR	G-protein coupled receptor
IJP	Inhibitory junction potential
NLP	Neuropeptide-like protein peptide
NSM	Neurosecretory motorneurone
PF	*Panagrellus* FMRFamide-like peptide
SER	Serotonin receptor in *C. elegans*

CHAPTER 1

Introduction

1.1 Nematodes

The term nematode comes from the Greek words nema (thread) and eidos (form). Nematodes were first described by Aristotle (384-322 BC), and Celsus (53 BC-AD 7). Since this time 15,000 species have been identified but it is estimated that this only represents a fraction of the total (Barnes *et al.* 1993). Nematodes have been found in a diverse range of habitats, such as, the deep sea, fresh water and soil, where they have a significant effect on the nutrient cycle of the soil. As decomposers they consume fungi, bacteria and other microflora and microfauna aiding to the health of the soil. One free-living nematode *Caenorhabditis elegans,* with its fully sequenced genome, is becoming a useful biological model organism for study.

Parasitic nematodes occur in all natural communities, sometimes as benign infections, but they may also have massive economic and social impact by seriously damaging populations of domestic animals, fish and crop plants; as well as infecting man, especially throughout the developed and developing world. Parasitic worms can be found in two main phyla, the platyhelminthes and nematodes. Parasitic platyhelminthes are divided into two main classess, the flukes (trematodes), for example, *Schistosoma mansoni*, and tapeworms (cestodes), for example, *Taenia saginata.* The classification of the nematodes is complex but includes intestinal parasites, for example, *Ascaris suum* and *Ascaris lumbricoides*, and filarial parasites, for example, *Onchocersa volvulus* and *Brugia malayi.*

Intestinal nematodes currently infect over 2 billion people worldwide. It is estimated that 300 million people are ill as a result of these infections, and of those at least 50% are school aged children (World Health Report, 2000). The intestinal roundworm *A. lumbricoides* alone causes 60,000 human deaths a year (World Health Report, 1999). Nematode infection of livestock can be devastating for the local economy through lost productivity, death of livestock and increased use of foodstuffs. Plant parasitic nematodes have been estimated to cause 80 billion dollars worth of crop damage annually.

Currently, there are no vaccines against human helminth infections, and anti-helminth drugs (anthelmintics) continue to carry the burden of the efforts to control helminth disease. Most anthelmintics work by acting specifically at nematode receptors or ion channels, in particular at the neuromuscular junction, where their action results in a rapid therapeutic effect to the host.

The over-use of anthelmintics has slowly led to resistance in many strains of parasitic nematodes. The requirement, therefore, for new anthelmintics acting on novel target sites within nematodes has become of both social and economic importance.

1.2 Ascaris suum

A. suum is placed within the order Ascaridia. It mainly parasitises pigs and has a cosmopolitan distribution. Due to its size and relative abundance, *A. suum* has been used successfully to examine the physiology and pharmacology of nematodes and the mechanism of action of anthelmintic drugs (Holden-Dye *et al.,* 1988; Martin *et al.,* 1991). Female worms range from 25 to 40 cm in length and 5 mm in diameter, with males being smaller, ranging from 15 to 25 cm in length and 3 mm in diameter, (Fig. 1.1). The predilection site for *A. suum* is the small intestine of swine. Female worms are capable of producing as many as 2 million eggs daily. These thick-shelled eggs are extremely resilient and can survive for as long as five years under most farm environments.

For many years this species was considered synonymous with the human parasite *A. lumbricoides*; however, there is evidence that they are distinct species. Detailed studies revealed differences between the denticles on the ridges around the mouths of the worms, the morphology of the sex chromosomes and the protein composition of the worm's body fluid (Review by: Crompton *et al.,* 1989).

Figure 1.1 The parasitic nematode *A. suum.* (Image available on **http://www.en.wikipedia.org**)

1.2.1 *A. suum* life cycle

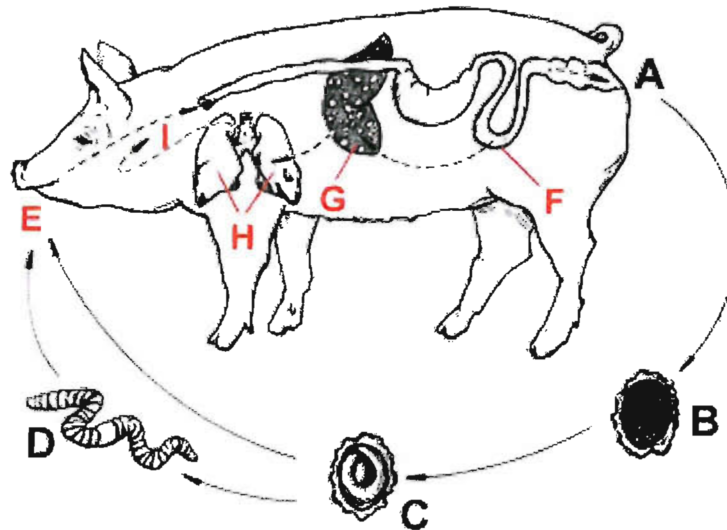


Figure 1.2 The life cycle of the parasitic nematode *A. suum*. (See text for details) (Image available on http://www.cal.vet.uppen.edu).

Eggs laid by the female worms pass via the host faeces (A) to the external environment (B). The eggs' development then depends on the oxygen tension, moisture content and temperature of their environment. Development to the infective stage occurs inside the egg via a single moult, about two weeks after expulsion from the host (C). The larvae rarely hatch and infection takes place through ingestion of the eggs with food or water (D, E). Hatching of ingested eggs is stimulated by intestinal conditions such as pH (F). Following hatching the larvae burrow into the intestinal wall and enter the hepatoportal blood stream and reach the liver within 24 hours of infection (G). In the liver the first parasitic moult from second-stage to third-stage larva takes place. Third-stage larvae continue their migration from the liver towards the heart and then the lungs via the venous system (H). Larvae break out of the alveolar capillary into the alveolous and pass through the alveolar duct to the small bronchioles and then gradually ascend the bronchial tree. Larvae then migrate from the trachea to the pharynx where they are swallowed and third-stage larvae arrive in the intestine seven to eight days after infection (F). The final two parasitic

moults from third-stage to fourth stage larvae to mature adults are completed in the small intestine (http://www.cal.vet.uppen.edu.) Fig. 1.2 summarises the life cycle of *A. suum*.

A. suum causes pathogenic effects, both in the larvae and adult stages. During the migratory period destruction of tissue and haemorrhage may occur in the liver, but the most important lesions are produced in the lungs, where the larvae cause numerous small haemorrhages into the alveoli and bronchioles. In heavy infections death from severe lung damage may occur 6 to 15 days after infection. Adult worms may be so numerous that they may become twisted into bundles in such a way that causes intestinal obstruction. Worms can also enter the stomach and be vomited, or pass up the bile duct into the liver causing blockage of the bile ducts. Human ascariasis has a number of similar effects including intestinal, biliary and respiratory obstructions; inflammation of the intestinal mucosa; protein and vitamin deficiency, which influence the nutritional status of children (Crompton *et al.,* 1989).

1.2.2 *A. suum* anatomy

A. suum are long, slender, almost featureless externally, tapered at both ends, and round in cross section. They are made up of two concentric tubes separated by a fluid filled space, the pseudocoelom. Like all pseudocoelomates the body of *A. suum* has only three main layers, the cuticle, the hypodermis and the muscle (Fig. 1.3).

The cuticle forms the outer covering of the body and consists of an outer epicuticle, a median osmophobic layer and an inner fibre layer. The basic component of the cuticle is a form of collagen associated with small amounts of lipids, which form a lattice of inelastic fibres. This collagenous lattice is important for the functioning of the nematode's hydrostatic skeleton and therefore a fundamental element underlying the workings of the locomotory system. The lattice is made up of three tough collagen fibres that spiral around the body, one in the opposite direction to the others. This arrangement and the overall elasticity of the cuticlar layers ensures that a change in length will be compensated by a change in width. This results in the body volume remaining constant, even though the turgor pressure, the length and the width change.

The hypodermis forms the middle layer of the body wall. It is beneath the cuticle and in *A. suum* is a syncytium. It projects into the body cavity along the mid-dorsal, mid-ventral and the lateral lines to form four ridges, or cords, which contain all the hypodermal nuclei (Lee and Atkinson, 1976). Two of these ridges are the lateral lines, which are thought to play a secretory role, whilst the others are the dorsal and ventral nerve cords which originate from the cranial pharyngeal nerve ring.

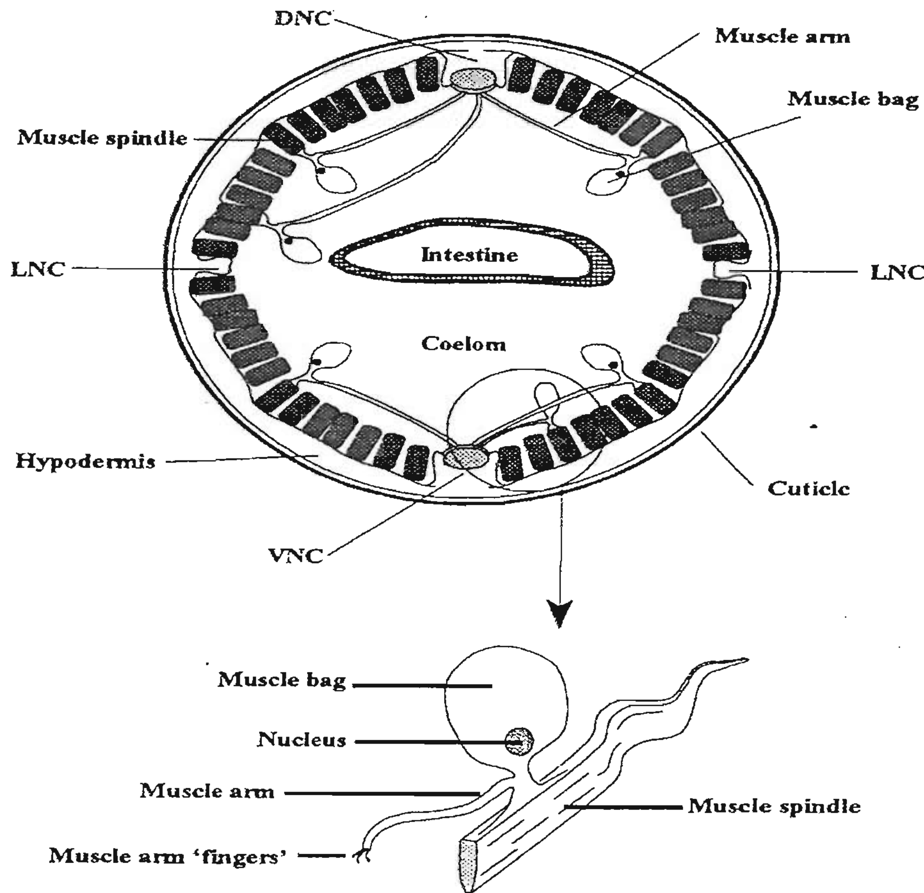


Figure 1.3 The body wall and muscle of *A. suum*. Diagrammatic representation of the body wall in *Ascaris*, showing a general structure of a somatic muscle cell. The muscle spindle, muscle bag and muscle arm are all parts of a single muscle cell. The muscle arms project to either the ventral nerve cord (VNC) or dorsal nerve cord (DNC) but do not cross the lateral line/nerve cord (LNC). The muscle arm 'fingers' interdigitate with those of neighbouring cells and form the 'syncytium' in close proximity to the nerve cord. Neuromuscular synapses are confined to this region. (Adapted from Croll and Mathews, 1977).

The somatic musculature of *A. suum* consists of longitudinal but no circular muscles. Two lateral lines divide the body musculature into a ventral and dorsal half, each innervated exclusively by one of the major nerve cords (the dorsal or ventral nerve cords). Rosenbluth (1963; 1965a,b) has described the muscle cell of *A. suum* in detail. In *A. suum* the muscle cell has three main parts (Fig. 1.3) a contractile spindle region; a ballon-shaped structure, the bag or belly which contains the nucleus; and a thin process known as the arm.

The muscle spindle contains the obliquely striated contractile apparatus of the muscle cell (Rosenbluth *et al,* 1965a) and is anchored to the hypodermis. It can range from 0.6 mm to 13 mm but its average length is 2 mm (Stretton 1976). Contraction of this muscle is associated with Ca^{++} fixation by actin and relaxation with a change in the phosphorylation state of light chain myosin (Martin and Donaheu, 1987).

The muscle bag, or belly, is a large 200 μm bag-shaped structure, which projects from the spindle and lies in the perienteric space. It contains submembrane mitochondria, the nucleus and particulate glycogen. During starvation the muscle bag shrinks as a result of glycogen depletion (Rosenbluth 1963, 1965a,b).

Each muscle cell gives rise to processes named muscle arms (Stretton 1976). Muscle arms may originate from almost any portion of the belly and synapse with nerve cells. Upon reaching the nerve cord each arm branches into thin terminal processes or 'fingers' that make contact with the nerve cord forming a muscular but not contractile structure (Rosenbluth, *et al,* 1965a,b) and they are responsible for the electrical coupling seen between adjacent cells (De Bell *et al,* 1963). Neuromuscular junctions are established between the nerve cord fibres and the syncytium. The synapses are made by short (1 – 2 μm) neuronal extensions or spines which penetrate the thin hypodermal layer and contact the syncytial membrane (Stretton, *et al.,* 1978). These synapses are associated with clear spherical 40 nm vesicles; dense core vesicles, 80 – 100 nm in diameter; giant mitochondria; and membrane thickenings on the pre-synaptic and post-synaptic membrane (Rosenbluth, *et al.,* 1965a,b). The muscle arms do not cross the lateral lines, so muscle cells of the dorsal muscle field

synapse only with motorneurones of the dorsal nerve cord and ventral muscle cells send arms only to the ventral nerve cord.

1.2.3 The nervous system of *A. suum*

The nervous system of *A. suum* is anatomically simple containing approximately 300 neurones (Stretton, *et al.,* 1978). Goldschmidt (1908) described the presence of an anterior nerve ring composed of 162 neurones, which surrounds the pharynx with a series of associated ganglia (Fig. 1.4). These ganglia, 23 in total, include the dorsal ganglion, the ventral ganglion, the two lateral ganglia, and the retrovesicular ganglion and are closely associated with the dorsal, ventral, and the two lateral nerve cords. The retrovesicular ganglion is the most comprehensively described ganglion. It contains 13 neurones and is situated about 2 cm posterior to the nerve ring on the ventral nerve cord (Goldschmidt, 1908). Four of the cells are excitatory motorneurones, two projecting to the dorsal nerve cord and two to the ventral cord (Angstadt, et al., 1989a). The remaining cells are interneurones with unbranching fibres that are confined to the ventral cord. One of these interneurones, designated 'B', extends a process posteriorly along the full length of the ventral nerve cord.

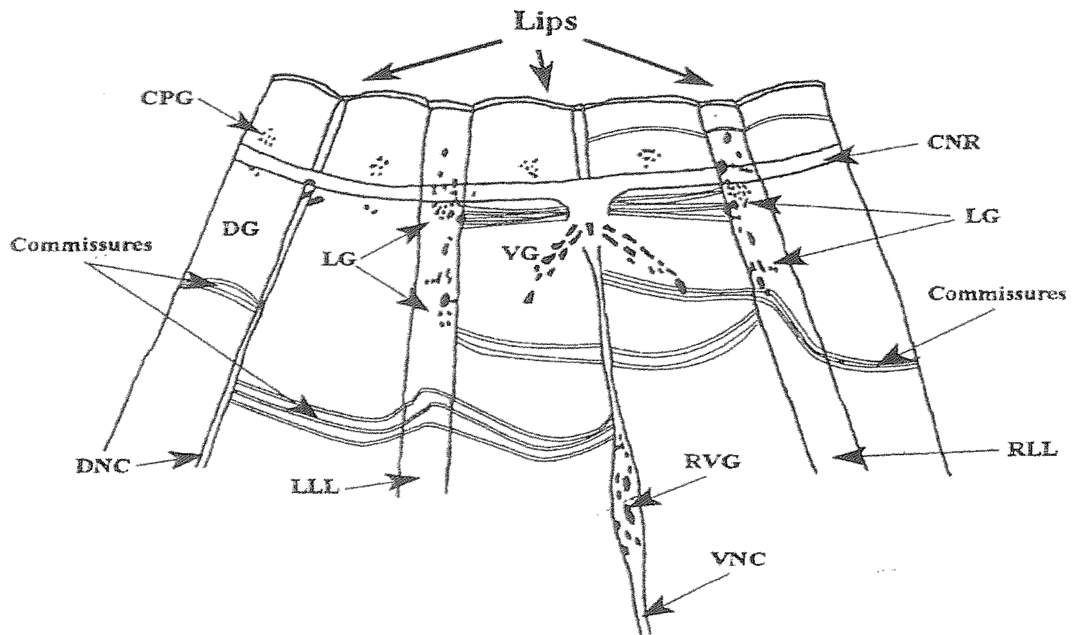


Figure 1.4. Anterior nervous system of *A. suum.* Diagram showing the anterior nervous system of *A. suum.* The circumoesophageal nerve ring (CNR) is associated with 23 ganglia and eight nerve cords. The nerve cords include the ventral nerve cord (VCN), the right lateral line (RLL), the left lateral line (LLL) and the dorsal nerve cord (DNC). The ganglia shown include six cephalic papillary ganglia (CPG), the dorsal ganglion (DG), ventral ganglion (VG), the lateral ganglia (LG) and the retrovesicular ganglion (RVG). (Figure modified from Angstadt *et al.,* 1989)

1.2.4 The Motor nervous system *of A.suum*

The somatic motor nervous system of *A. suum* contains about 90 neurones and lies in the main body portion of the worm between the head and the tail (Stretton, *et al.,* 1985). The neurones are divided into three categories (Stretton, *et al.,* 1978): motorneurones, which synapse to muscle either in the dorsal or ventral nerve cord; large interneurones that synapse onto certain motorneurones; and small interneurones that synapse onto each other or onto large interneurones.

The motor nervous system consists of five repeating units, or segments, each containing 11 motorneurones and 6 interneurones (Stretton, *et al.,* 1978). Each motorneurone consists of a straight fibre with, at most, two branch points. The 11 motorneurones are divided into seven types according to the distribution of their axons and dentrites, consisting of four dorsal and three ventral motorneurones, depending on whether they make synapses to the dorsal or ventral muscle. By recording electrical activity in muscle cells, while electrically stimulating specific motorneurones, five of the motorneurone classes could also be characterised further as either inhibitory, I, or excitatory, E, (Walrond, *et al.,* 1985). The two types of ventral motorneurones (V-1, V-2) have axonal and dendritic regions in the ventral nerve cord. The other 5 types of motorneurones have their axonal and dendritic regions in different nerve cords. One has a dorsal dendrite and a ventral axon (VI) and the other four are dorsal motorneurones, all of which have their dendrites in the ventral cord and their axons in the dorsal cord (DE1, DE2, DE3, D1), Fig. 1.5. This arrangement supports the dorsoventral partitioning of the somatic musculature, where dorsal muscle cells synapse only with motorneurone processes in the dorsal cord. The dorsal and ventral processes of VI, DE1, DE2, DE3 and D1 are connected by a lateral process called a commissure.

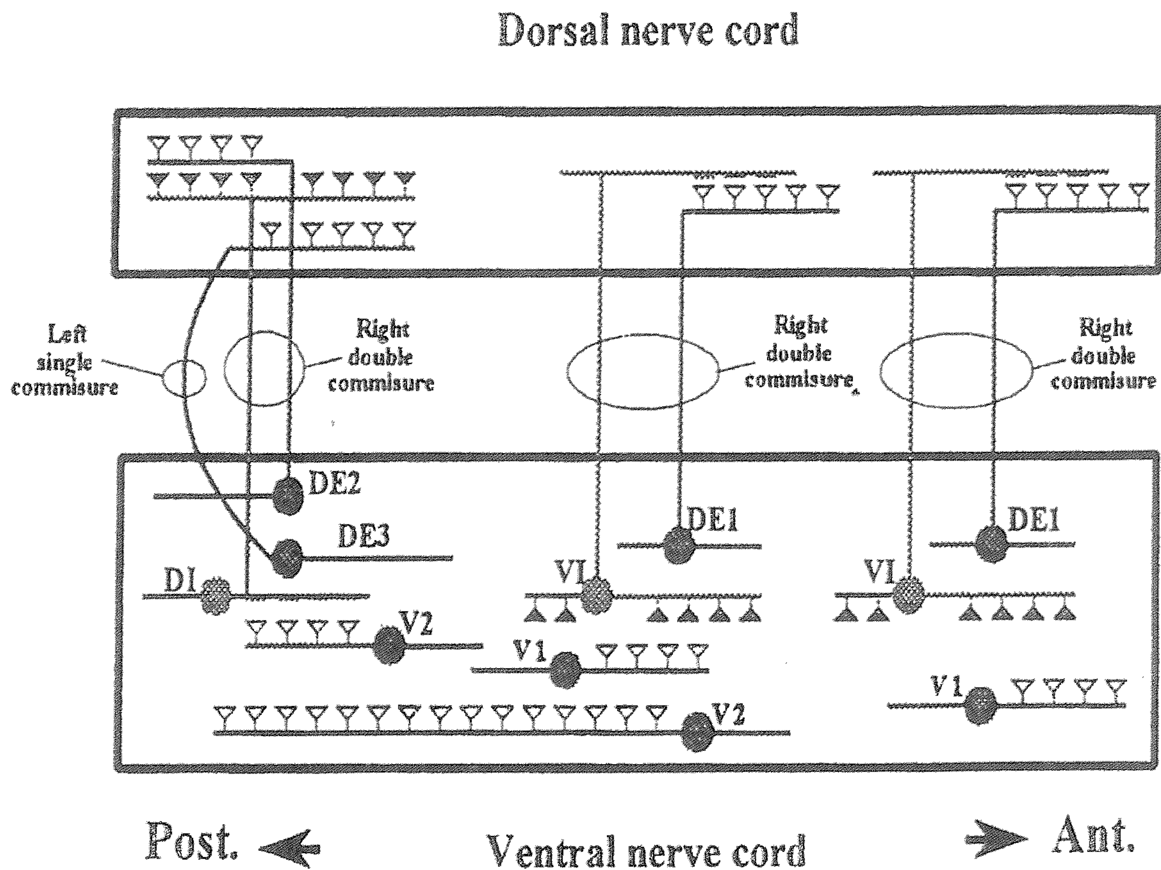


Figure 1.5. *A. suum* Motorneurones. Summary diagram of the anatomical organisation of *A. suum* motorneurones in one 'segment'. Eleven motorneurones are presented in each 'segment'. There are seven anatomical types of motorneurone but only two physiological types. (E excitatory, cholinergic; I inhibitory, GABAergic). DE1, 2 and 3, dorsal excitators; DI dorsal inhibitor; VI ventral inhibitor; V1 and 2 ventral excitators. (From Martin *et al.,* 1991)

1.2.5. Pharmacology of neurotransmission

1.2.5.1 Acetylcholine (ACh)

There is now specific evidence for ACh as a transmitter in the nervous system of *A. suum*. ACh causes a contraction when applied to body muscle strips (Baldwin and Moyle, 1949). When intracellular recordings were made from *A. suum* body wall muscle cells, ACh application caused a depolarisation in the somatic muscle cells with an increase in conductance, due mainly to sodium (Del Castillo, et al., 1963; Colquhoun *et al.,* 1991). The excitatory role of ACh is further confirmed by presence of ACh in excitatory motorneurones but not inhibitory motorneurones (Johnson and Stretton, 1985). Acetylcholinesterase (AChE), the enzyme inactivating ACh has been localised within the arms and contractile region of the muscle cells (Lee 1962). Choline acetyltransferase, the enzyme responsible for the synthesis of ACh has been localised in the commissural branches of the excitatory motorneurones as well as in the hypodermis (Johnson and Stretton, 1985).

The pharmacology of the ACh receptor has been extensively studied (Natoff 1969; Rozhkova *et al.,* 1980). The general finding, that the ACh receptor of *A. suum* somatic muscle is a nicotinic-like receptor was also supported by extensive electrophysiological studies of Colquhoun *et al.,* (1991). A range of agonists and antagonists were examined to investigate their direct effects on muscle and interaction with ACh response. In general, ganglion nicotinic agonists, such as HPPT (metahydroxyphenylpropyltrimethylammonium) and DMPP (1,1-dimethyl-4-phenylpiperazinium) were the most potent. Muscarinic agonists were less active compared to nicotinic agonists on *A. suum* body wall muscle. Pilocarpine and muscarine weakly hyperpolarized muscle cells. The antagonist profile of the *A. suum* ACh receptor was tested by employing electrophysiological techniques (Colquhoun, *et al.,* 1991). The most potent compounds were benzoquinonium and mecamylamine which are both nicotinic antagonists.

1.2.5.2 Gamma aminobutyric acid (GABA)

The distribution of GABA throughout the nervous system of *A. suum* has been estimated using immunocytochemical techniques (Johnson and Stretton, 1987; Sithigorngul *et al.,* 1989). GABA-like immunoreactivity was localised within inhibitory motorneurones of the ventral nerve cord. Commissures of inhibitory neurones that project to the dorsal nerve cord were also stained. Sithigorngul, et al., (1989) have also demonstrated the presence of GABA immunoreactive structures in the central nervous system of *A. suum.*

GABA relaxes the body wall muscle strips and hyperpolarizes the membrane potential of the muscle cells in a concentration-depended manner (Martin, 1980; Holden-Dye *et al.,* 1989). The reversal potential for GABA hyperpolarization was similar to the reversal potential for chloride ions suggesting that GABA response is a purely chloride event (Martin 1980; Holden-Dye *et al.,* 1989).

The pharmacological profile of the *A. suum* GABA receptor has been investigated further. In terms of agonist profile it resembles the mammalian $GABA_A$ receptor, with $GABA_A$ agonists, such as, dihydromuscimol and ZAPA being more potent than GABA (Holden-Dye and Walker 1988, Holden-Dye *et al.,* 1989). The *A. suum* GABA receptor was insensitive to $GABA_A$ antagonists such as bicuculline and picrotoxin. A series of azoles have been examined for antagonistic activity against the *A. suum* muscle receptor (Bascal, *et al.,* 1996). These compounds reduced the response to GABA in a non-competitive manner with IC_{50}s of less than 10 μM.

Although the *A. suum* GABA receptor has a unique pharmacological profile, the selectivity of the chloride channel has been shown to be similar to those of both vertebrates and invertebrates (Parri, *et al.,* 1991).

1.2.5.3. Glutamate

Injections of glutamate or kainate into whole *A. suum* produced paralysis in a quasi-static posture similar to the waveform seen in behaving worms (Davis and Stretton, 1996). The site of the putative glutamate receptor has been investigated and located as the DE2 motorneurones where glutamate analogues depolarise the membrane potential (Davis and Stretton, 1996; Davis, 1998a,b). The relative potency of the compounds tested was domoate > kainate > glutamate > aspartate. Quisqualate, AMPA and bromowillardine were only partial agonists while NMDA was inactive. The non-NMDA antagonists DNQX and CNQX produced partial antagonism of EPSPs and DE2 glutamate responses. Evidence for the presence of a glutamate transporter in *A. suum* hypodermis as well as DE2 motorneurones is presented by Davis (1998b).

1.2.5.4. 5-Hydroxytryptamine (5-HT, Serotonin)

In addition to the neurotransmitters, GABA and ACh there is evidence that 5-HT may play a role in regulating muscle metabolism. 5-HT can be both absorbed and synthesized by *A. suum* (Chaudhuri, *et al.,* 1988). L-Tryptophan can be absorbed through the intestine and across the cuticle and converted to 5-HT by the somatic muscle cells. In female *A. suum* 5-HT immunoreactivity is present in only one pair of neurones in the pharynx (Brownlee *et al.,* 1994a). In male *A. suum*, there is an additional set of 5 5-HT-positive neurones in the posterior 2 cm of the ventral cord (Johnson *et al.,* 1996). 5-HT injection into intact worms produces a rapid paralysis and application of 5-HT on dorsal muscle strips reduces the amplitude of ACh contractions (Reinitz and Stretton, 1996). 5-HT can abolish spontaneous slow potentials in VI motorneurones and decreases the frequency of epsps in DE2 motorneurones (Reintz and Stretton, 1996). 5-HT stimulates glycogenolysis and increases cAMP levels (Donahue, *et al.,* 1981).

Williams *et al* (1992) investigated the effect of a range of 5-HT agonists and antagonists on binding and cAMP levels in *A.suum*. A number of 5-HT-1 and –2 agonists and antagonists were found to inhibit 5-HT binding while 5-HT-3 agonists were inactive. Recently two 5-HT receptor subunits have been cloned from *A.suum*

(Huang, *et al.*, 1997). One receptor (AS-1) has 409 amino acids while the second receptor (AS-2) is identical to AS-1 apart from lacking 42 amino acids from the carboxy terminal of the third cytoplasmic loop. The AS-2 receptor has 77% homology to a *C. elegans* 5-HT receptor (Olde and McCombie, 1997) and 60-65% homology to mammalian 5-HT-2 receptors.

1.2.6 Locomotory behaviour in *A. suum*.

Locomotory behaviour in *A. suum* consists of the generation and propagation of the body waveform. This waveform is 2-dimentional in dorsoventral plane, and it consists of an alternating series of dorsal and ventral bends. At any position along the length of the worm, a bend is generated by the contraction of dorsal or ventral muscles and the relaxation of the opposing muscles. This alternating pattern of muscle contraction and relaxation is achieved by excitatory and inhibitory motoneuronal interaction. When worms are confined within a gut-like tube, forward movement is accomplished with a large amplitude wave that starts in the region of the vulva and propagates anteriorly (Johnson and Stretton, 1980). Reversal of the waves leads to a reversal in the direction of the movement.

A synthesis of the available information about the synaptic connectivity between neurones of the motor nervous system has led to the hypothesis about the circuit that controls locomotory behaviour (Stretton *et al.,* 1985). Fig. 1.6 shows the synaptic connections between the motorneurones. The dentrites of all classes of excitatory motorneurones (the dorsal excitors DE1, DE2, DE3 and the putative ventral excitors V1 and V2) receive synapses from intersegmental interneurones (Stretton *et al.,* 1978). In contrast, the inhibitory motorneurones do not receive synapses from interneuronal input, but rather receive synapses from excitatory motorneurones. Each class of inhibitory motorneurones has its dendrite opposite its output process, and receives input from the opposite excitors (DE1, DE2, DE3, onto VI and V1, V2 onto DI). These connections mediate reciprocal inhibition between the dorsal and ventral musculature. As a result activation of a DE excites a VI and produces inhibition in the ventral muscle cells when dorsal muscle cells are excited by DE. The region of ventral inhibition, causing ventral relaxation is opposite the dorsally activated muscle

that causes a dorsal contraction, so that a dorsal bend in the body is generated. Complementary connections between ventral excitors and DI motorneurones also exist and presumably generate ventral bends.

This alternating reciprocal pattern of excitation and inhibition of dorsal and ventral muscle allows the sinusoidal pattern of movement.

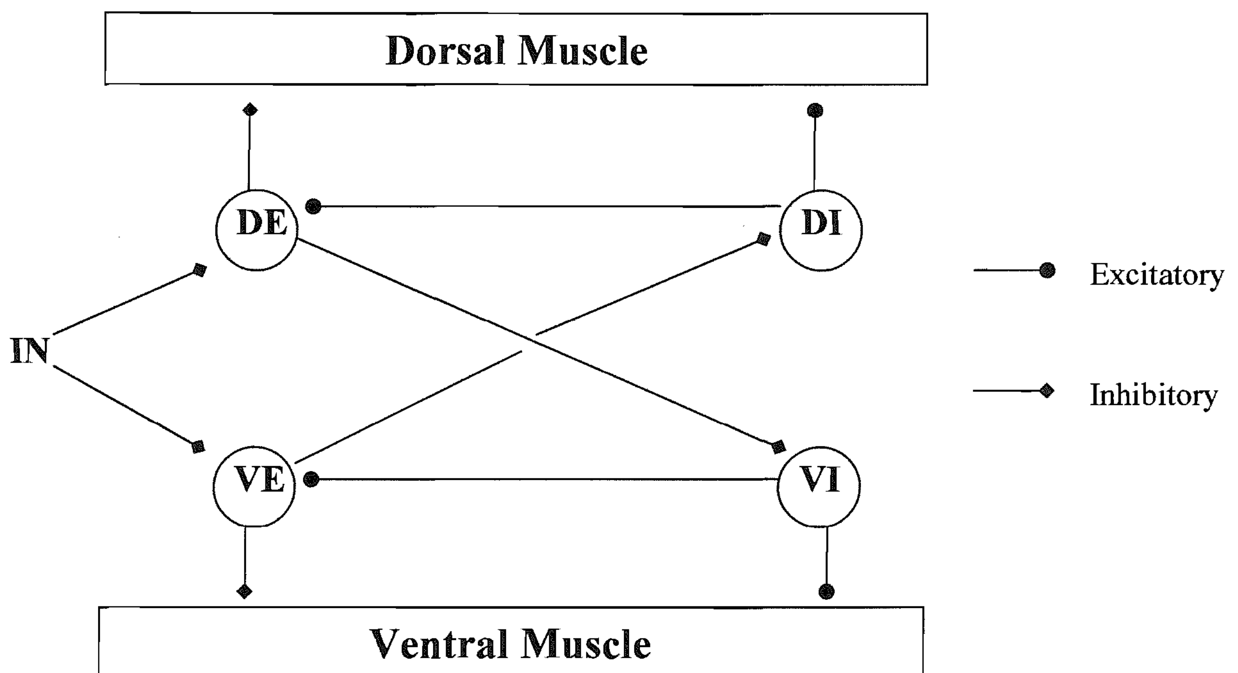


Figure 1.6. Model for motor control of *A. suum* body wall muscle. The synaptic connectivities of all dorsal excitatory motorneurones are shown as DE and the ventral excitors are shown as VE. DI and VI represent dorsal and ventral inhibitory motorneurones respectively. IN is a common interneurone that activates both DE and VE motorneurones. The generation of body waves relies upon a process of reciprocal inhibition. When the VE motorneurone stimulates the ventral muscle field it also stimulates the DI motorneurone. The dorsal field is inhibited and the body bends a bend in the opposite direction involves the dorsal excitor (DE) and the ventral inhibitor (VI) motorneurones acting in a similar manner. (Adapted from Stretton *et al.*, 1985).

1.2.7 Electrophysiological properties of *A. suum* muscle.

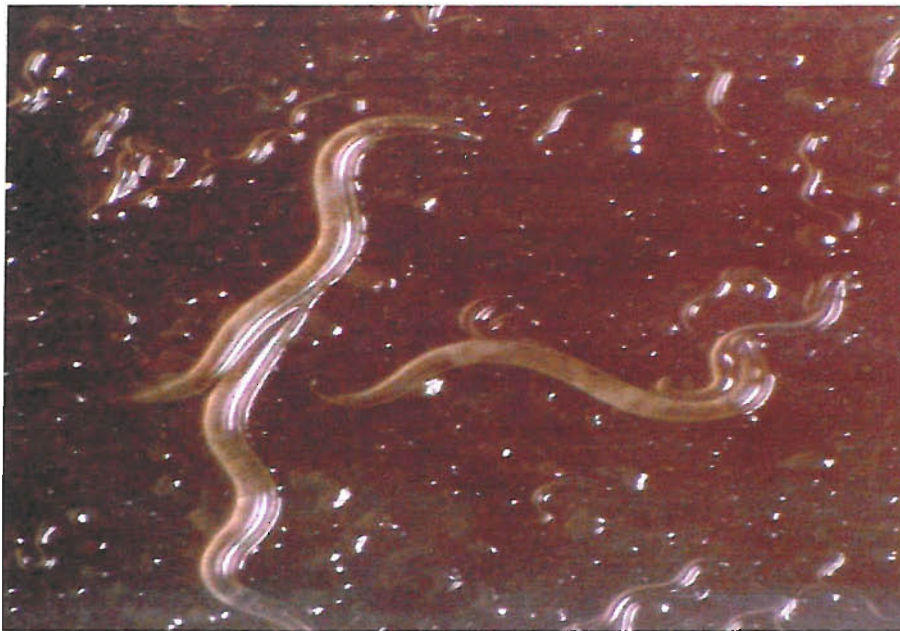
The first intracellular recordings of spontaneous depolarising potentials in the somatic muscle cells of *A. suum* were made by Jarman (1956). He described a resting membrane potential of about –30 mV on which was superimposed regular spike-like spontaneous depolarisations. The spontaneous depolarising potentials described by Jarman (1959) were shown to be myogenic in origin and to arise at the syncytium (De Bell *et al.,* 1963). Weisblat and Russel (1976) identified three types of depolarising potentials including 30 mV spike potentials with 10-50 ms duration, slow waves up to 20 mV in amplitude and 100 – 1000 ms duration and 5 mV modulatory waves lasting 3-20 s. Spike potentials are temperature dependent. Below 33 °C little activity is seen, but above 34°C activity increases with increasing temperature.

The usually low (-30 mv) membrane potential of *A. suum* appears remarkably insensitive to changes in the concentrations of ions in the bathing medium (Brading and Caldwell, 1964, 1971; Del Castillo *et al.,* 1964b). These studies failed to provide a satisfactory explanation for the behaviour of the membrane potential. Brading and Caldwell (1971) calculated, using the Goldman constant field equation, that there should be a larger additional factor contributing to membrane potential, given that permeabilities of the major ions appear low. They have suggested that this additional contribution is made by an active electogenic pump, possibly moving carboxylic acids.

1.3 *Caenorhabditis elegans*

C. elegans is a small (1 mm) free-living nematode found in the soil in many parts of the world feeding primarily on bacteria. There are two sexes, hermaphrodites and males. Hermaphrodites produce both oocytes and sperm and can reproduce by self-fertilisation, Fig. 1.7. Males produce only sperm and so to reproduce must mate with a hermaphrodite.

C. elegans is a simple organism, both anatomically and genetically. The adult hermaphrodite has only 959 somatic nuclei, and the adult male has only 1031. *C. elegans* is easily maintained in the laboratory, where it can be grown on agar plates with *Esherichia coli* as a food source.



0.1 mm

Figure 1.7 Adult hermaphrodite *C. elegans*.

1.3.1 *C. elegans* life cycle

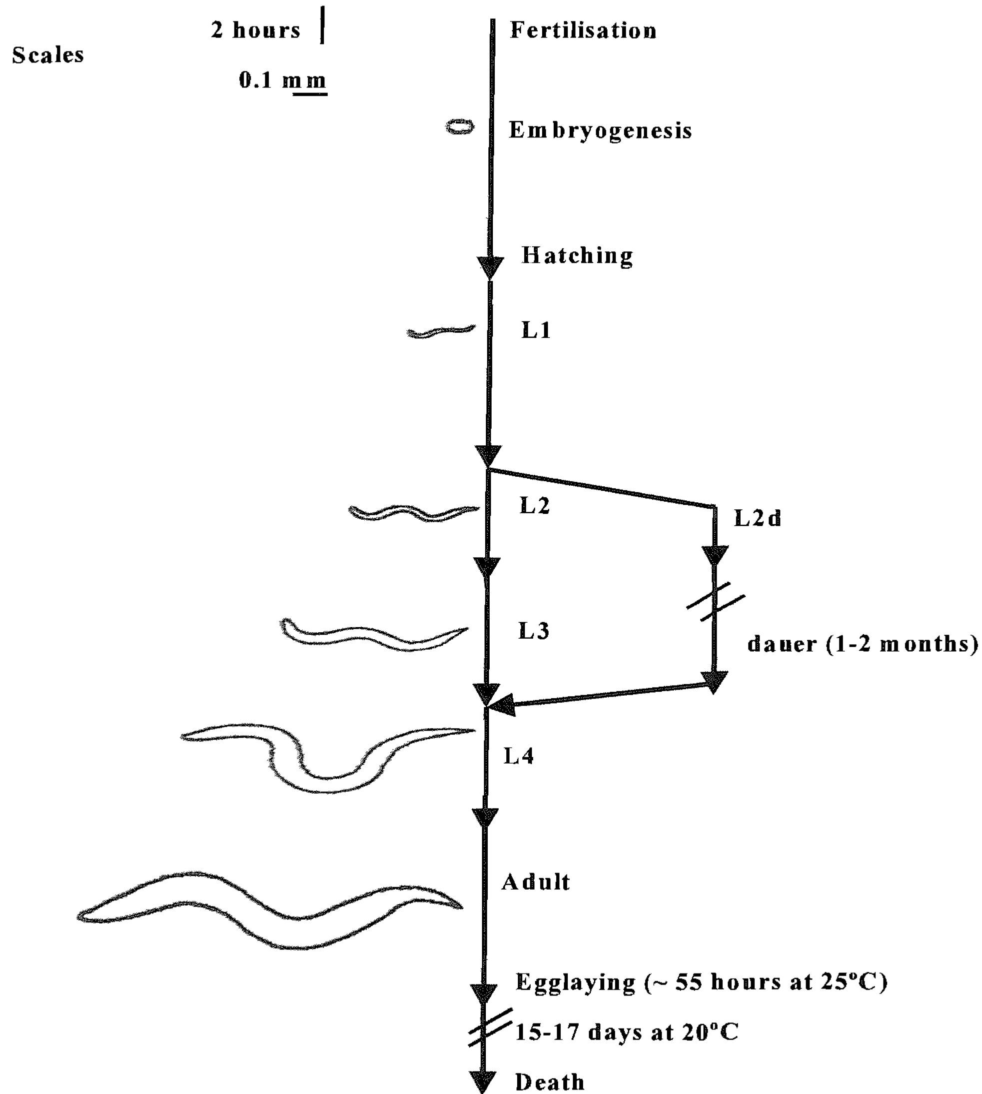


Figure 1.8 The life cycle of the free-living nematode *C.elegans*. The four larval stages (L1-4), the adult stage and the dauer stage are shown. (From Burglin *et al.,* 1998).

C. elegans have a rapid life cycle, Fig. 1.8. A single self-fertilising *C. elegans* hermaphrodite can produce around 280 hermaphrodite progeny, or up to 1000 hermaphrodites and males when mated with males. The life cycle is temperature dependent. Development from fertilisation to hatching is referred to as embryogenesis. Embryogenesis lasts 14 hours and post-embryonic development lasts 36 hours through which *C.elegans* develops through 4 larval stages (L-1 to L-4), which are punctuated by a series of moults. The mature adult arises after the fourth moult is fertile for 4 days, and then lives for an additional 10-15 days producing about 300 progeny each.

If food is limited early in development *C. elegans* will take an alternative development pathway as the L2/L3 moult to produce the dauer larva stage. This dauer L3 stage does not feed and can survive for up to 3 months, without further development. If food later becomes available then the dauer stage will develop to L4 larva stage and continue development as normal.

1.3.2 *C. elegans* as a useful biological model for study.

C. elegans is a useful biological organism for study, due to its simplicity, transparency, ease of cultivation in the laboratory, short life cycle, suitability for genetic analysis, and small genome size.

In 1998 the complete genome sequence for *C. elegans* was puplished. The *C. elegans* genome size is relatively small (9.7×10^7 base pairs of 97 Megabases), when compared to the human genome which is estimated to consist of 3 billion base pairs (3×10^9 bp or 3000 Megabases). Numerous novel molecular biological techniques exist for *C. elegans* experimentation that are not possible when examining higher eukaryotes. There are about 20,000 protein-coding genes. Identification of genes and the ability to make mutant *C. elegans* strains, which lack particular genes of interest, has also become increasingly useful in the analysis of biological pathways.

1.3.3 *C. elegans* anatomy

All nematodes share a similar anatomy. Nematodes are made up of two concentric cylinders separated by a fluid-filled cavity, the pseudocoelom, between the outer body wall and the digestive tube. The outer cylinder, coated in an extracellular collagenous cuticle, contains nematode musculature, nervous system and an excretory system. The musculature is arranged in four longitudinal strips and is attached to the cuticle via a thin strip, the hypodermis. The inner cylinder contains the pharynx and the intestine, Fig. 1.9. The majority of the animal is occupied by the reproductive system.

Both sexes of *C. elegans* have the same general anatomy and are of similar size, with the adult male being slightly shorter and thinner than the hermaphrodite. The mouth is at the tip of the head, while the anus of the hermaphrodite and the cloaca of the male are ventral, near to the posterior of the worm. The gut consists of the pharynx and the intestine and runs from head to tail. The body wall is separated from the gut by the pseudocoelom and presents a uniform exterior. The single-cell thick hypodermis makes up the bulk of the body wall and secretes the collagenous cuticle that covers the surface of the worm. Beneath the hypodermis, mononucleate, body-wall muscle cells are organised in four longitudinal bands, running the length of the worm in a subdorsal or subventral position. Each band is two cells wide and there are 24 muscle cells in each band, apart from the left ventral band which has 23. These muscle cells send processes to nerve cells, for neuromuscular synapse formation.

C. elegans have 959 somatic cells within the hermaphrodite, some 302 of which are neurones. Approximately 5000 chemical synapses and 600 gap junctions connect these neurones. Despite the small size and simplicity, this nervous system regulates a wide range of behaviours

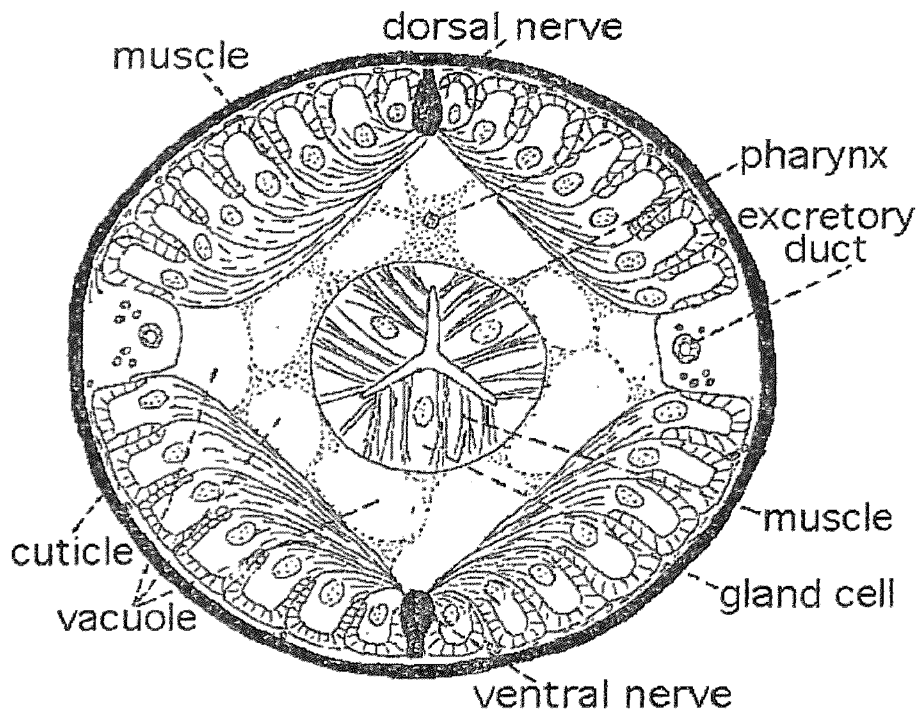


Figure 1.9 Cross section through *C. elegans* (Image available through www.biodidac.bio.uottawa.ca)

1.3.4 *C. elegans* Pharynx

In order to feed, nematodes transport food from the mouth to the intestine via a neuromuscular tube, named the pharynx. *C. elegans* pharynx is virtually a self-contained system of muscles, epithelial cells, and nerves, bounded by a basement membrane, which functions to ingest, concentrate, and process food before pumping it into the gut.

The anatomy of *C. elegans* pharynx has been studied extensively. The pharynx is composed of 34 muscle cells, 9 marginal cells, 9 epithelial cells, 5 gland cells and 20 neurones (Albertson & Thomson, 1976). The pharynx can be divided into three functional parts, which are the corpus, the isthmus and the terminal bulb, Fig. 1.10.

The pharynx consists of 8 muscle types. Five are large muscle types (pm 3, 4, 5, 6 and 7). Three are small muscle types (pm 1, 2 and 8) Pm 1 through to 4 constitutes the anterior half of the pharynx. Pm5 constitutes the middle part of the pharynx (the isthmus). Pm6 through to 8 constitutes the posterior end of the pharynx (the terminal bulb). Muscles are arranged with triradiated symmetry, forming a Y shape around the pharyngeal lumen. When these muscles contract the pharyngeal lumen opens forming a triangular shape.

In order for normal feeding to occur two motions, pumping and isthmus peristalsis, must occur. This action allows food to enter the pharyngeal lumen, to be ground up and then pass into the intestine (Doncaster, 1962; Seymour *et al.,* 1983). Pumping consists of a near-simultaneous contraction of the muscle corpus and anterior isthmus. This opens the lumen and allows liquid (containing bacteria) to flow into the corpus. The posterior isthmus however remains relaxed (closed), therefore, the bacteria remains trapped in the corpus. Relaxation of the corpus and anterior isthmus then occurs, expelling liquid back out of the mouth but retaining bacteria within the corpus (Doncaster, 1962). Isthmus peristalsis then transports bacteria from the corpus to the terminal bulb. This occurs after every fourth pump (Avery & Horvitz, 1987). The terminal bulb then acts to brake up bacteria which then pass into the intestine through the opened pharyngeo-intestinal valve (Doncaster, 1962)

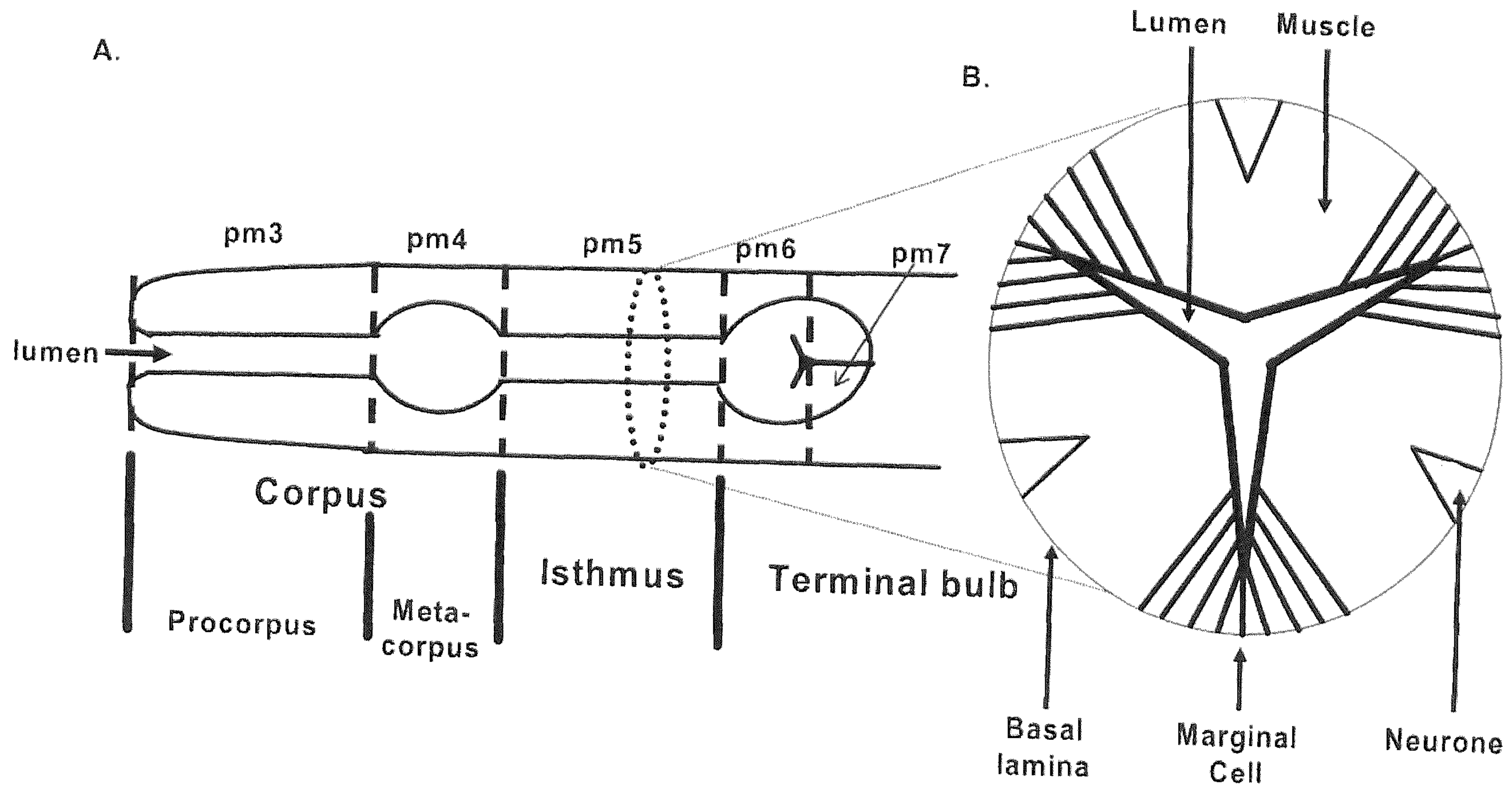


Figure 1.10. Diagram illustrating the pharyngeal anatomy of *C. elegans*. A. Lateral view. (Anterior to the left). The three functional parts of the pharynx are shown, the corpus (divided into the pro and metacarpus), the isthmus and the terminal bulb. The position of the large muscle types (pm3-7) are shown. **B.** Transverse section through the pharynx. Three muscle cells surround the pharyngeal lumen. When contraction occurs the lumen opens. Between the muscle cells and the apices of the lumen are three marginal cells. Embedded within the grooves of the basal membranes of the muscle cells are neurones (adapted from Albertson & Thomson 1976; Avery and Horvitz, 1989; Raizen & Avery, 1994)

1.3.5 The nervous system of *C. elegans*

The nervous system of *C. elegans* is simple and well described. There are 302 neurones in the adult hermaphrodite, which are interconnected by about 5000 chemical synapses and 600 gap junctions. The nervous system mediates a variety of behaviours such as several mechanosensory responses, many chemosensory responses, thermotaxis, complex responses to food, locomotion, feeding, egg-laying, defecation and male mating.

1.3.5.1 Central nervous system

The arrangement and type of neurones within both *A. suum* and *C. elegans* are largely homologous. Most of the *C. elegans* nervous system is situated in the head and is organised around the circumpharyngeal ring. The head also contains sensory receptors, together with the interneurones and some motorneurone cell bodies are situated between the corpus and the terminal bulb of the pharynx. These cells send out processes that run circumferentially around the pharynx as a fibre bundle forming the nerve ring, which forms the major neuropile in the animal. A large number of the processes in the nerve ring enter and leave on the ventral side forming the ventral nerve cord (White *et al.,* 1986).

1.3.5.2 *C. elegans* motor nervous system

The anatomy and physiology of the *A. suum* motorneurones was described by Stretton *et al.,* (1978). White et al., (1983) later showed that *C. elegans* also contains equivalent motorneurone classes. There are about 30 classes of motorneurones in *C. elegans*. These innervate body-wall, pharyngeal, egg-laying and defecation muscles. The pharyngeal and egg laying neuromuscular junctions (NMJs) are located at the terminus of motor axons or branches that extend to the muscles. Conversely, body-wall and defecation muscles have short neurone-like processes onto nearby nerve cords forming NMJ's with their motorneurones. The innervation of body-wall muscle is more complicated in nematodes than for vertebrate systems, in that, muscle cells

receive synaptic input from both excitatory and inhibitory neurones. This complexity may facilitate graded contraction of muscles using a small number of muscle cells, which results from a balance of graded excitation and inhibition by several classes of motorneurones.

Most classical neurotransmitters are present in *C. elegans,* including acetylcholine (ACh), glutamate, γ-aminobutyric acid (GABA), serotonin and dopamine. In addition, many neuropeptides are likely to have a transmitter or neuromodulator role in *C. elegans* (Nelson *et al.,* 1998a,b; Kim and Li, 2004).

1.3.5.3 Locomotion in *C. elegans*

Locomotion in *C. elegans* is achieved by wriggling the body in sinusoidal waves. This motion is produced by 95 body-wall muscle cells with longitudinal fibres that are arranged in two dorsal and two ventral quadrants (White *et al.,* 1986). Dorsal and ventral body muscles are controlled by distinct classes of motorneurones, A, B, D, AS and VC, which are defined by similarities in axonal morphologies and patterns of synaptic connectivities (White *et al.,* 1986). The alternating pattern of contraction between dorsal and ventral body wall muscle is achieved through excitatory and inhibitory motorneurone interaction, via excitatory (acetylcholine) and inhibitory (GABA) neurotransmitters.

Each motorneurone class is composed of multiple members, which are arrayed along the length of the ventral cord in repeating units (e.g. VA1 – VA6).

The A- and B-type neuromuscular junctions are arranged as complexes in which an A or B synaptic terminus acts on two postsynaptic elements, a body wall muscle and a D neurone dentrite (White *et al.,* 1986). The VD neurones receive input at the dorsal A and B type neuromuscular junctions – being activated during dorsal muscle contractions – and they form neuromuscular junctions ventrally that appear to relax the ventral muscles. The opposite set of connections exists for the DD neurones. This circuit suggests that the D neurones act as cross-inhibitors that prevent the

simultaneous contraction of the dorsal and ventral muscles (White *et al.,* 1986). Regulation of these motorneurones occurs through command interneurones which in turn receive input from sensory circuits. Therefore, these command interneurones serve to integrate and translate sensory information into the locomotory response. AVB and PVC command interneurones activate forward movement, whereas AVA, AVD and AVE activate backward movement (Fig 1.11) (Riddle *et al.,* 1997).

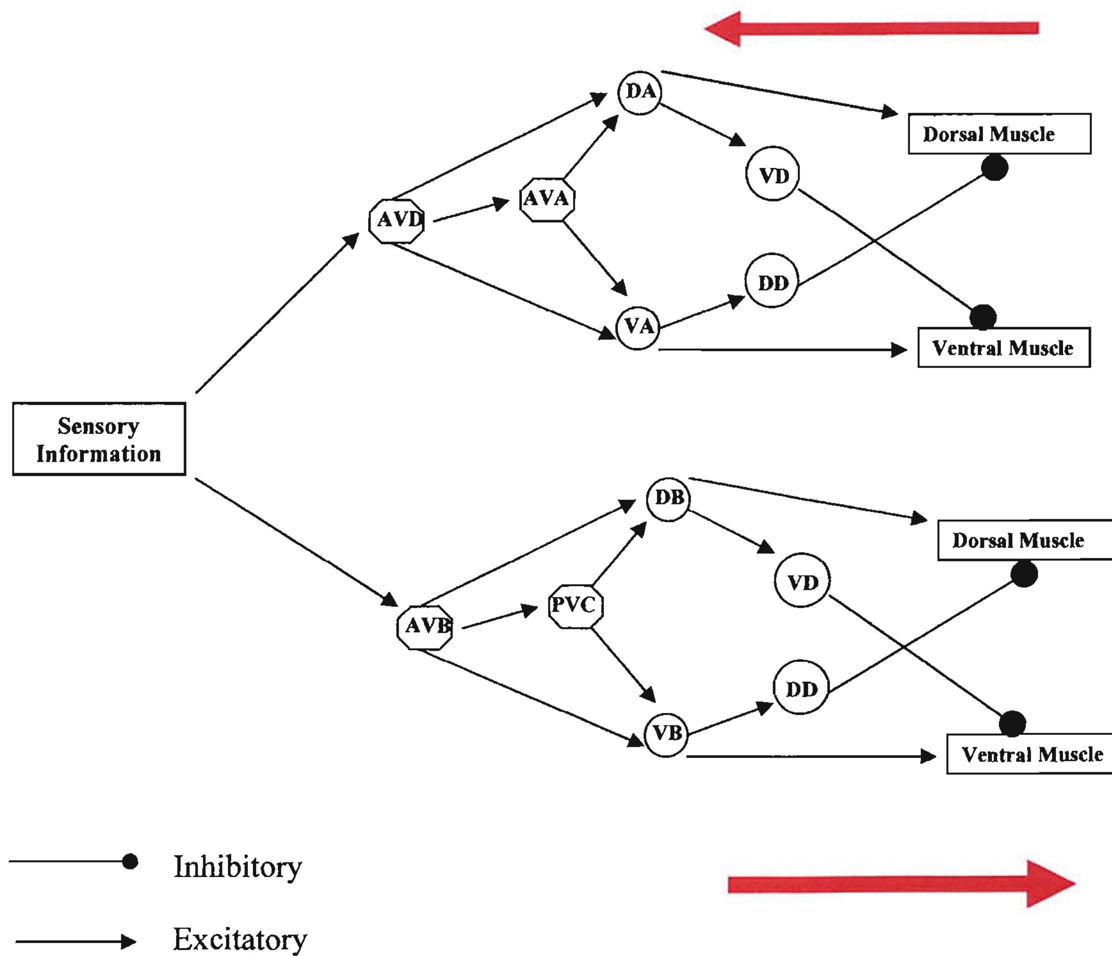


Figure 1.11 Illustration to show circuitry of *C. elegans* motor nervous system. The arrows indicate backward (upper) and forward (lower) movement, respectively. (After Riddle *et al.,* 1997)

1.4 Pharyngeal nervous system.

The pharyngeal nervous system contains 20 neurones which are made up of 14 anatomical types. They are divided into six bilaterally symmetrical pairs and eight unpaired neurones. Communication between the pharyngeal nervous system and the extrapharyngeal nervous system occurs via bilateral GAP junctions between the extrapharyngeal RIP (ring pharyngeal) interneurones and the pharyngeal I1 neurones (Avery & Horvitz, 1989). Dissection of the pharynx, and thus removing extrapharyngeal and pharyngeal nervous system interaction, has no apparent effect on pharyngeal behaviour or electrophysiology (Avery *et al.,* 1995). The nervous system controlling the pharyngeal activity is therefore a self-contained unit. A wiring diagram of the pharynx is shown in Fig 1.12.

Laser ablation of all 20 neurones does not fully inhibit pharyngeal pumping, thus the pharynx is said to be myogenic (Avery & Horvitz, 1989). However, of the 20 neurones controlling pharyngeal function, 3 neurones M4, M3 and MC are required for the normal pharyngeal activity. Nearly normal feeding occurs following laser ablation of all other pharyngeal neurones (Avery & Hoevitz, 1989; Raizen *et al.,* 1995). Only M4 appears essential for normal development as *C. elegans* has been shown to be fertile and viable following laser ablation of all the other 19 neurones (Avery & Horvitz, 1989). M4 processes synapse onto the posterior m5 muscle of the isthmus (Albertson & Thomson, 1976). Laser ablation of M4 means that the posterior region of the isthmus remains closed resulting in bacteria being trapped in the corpus (Avery & Horvitz, 1987). The closed state of the isthmus means food does not enter the intestine and the worms starve. Positive antibody staining against choline acetyltransferase and the ACh synaptic vesicle transporter in the M4 neurone suggests ACh is released from M4 (Alfonso *et al.,* 1994).

The two M3 neurones, along with the I5 neurone are important for effective transport of bacteria within the pharyngeal lumen (Avery, 1993). The M3s are located at the corpus-isthmus boundary. They have sensory endings in the posterior corpus and motor output to the corpus and anterior isthmus. These sensory endings have processes that project under the cuticle of the lumen and serve a mechanosensory

function. They sense the contraction of the pharynx and so stimulate its relaxation (Albertson & Thomson, 1976). M3s can thus control the timing of pharyngeal relaxation (Avery, 1993). M3 was shown to form fast inhibitory glutamatergic neuromuscular synapses (Dent *et al.,* 1997).

The MC neurone is the main excitatory neurone necessary for rapid pumping as laser ablation of the MC neurones in *C. elegans* grown in bacteria has been shown to result in a reduction in pharyngeal pumping (Avery & Horvitz, 1989). Stimulation of MC results in release of ACh (Raizen et al., 1995). *C. elegans* with deficient ACh synthesis have severely reduced pumping rates (Avery & Horvitz, 1990).

The activity of the pharyngeal muscle is controlled by a number of different neurotransmitters and neuromodulators. In the *C. elegans* pharynx both ACh and 5-HT increase pharyngeal pumping (Avery & Horvitz, 1990) and GABA, glutamate (Pemberton *et al.,* 2001) octopamine and dopamine (Rogers *et al.,* 2001) inhibit pharyngeal pumping. A number of neuro-active peptides, the FaRPs (FMRFamide-Related Peptides and FLPs (FMRFamide-Like Peptides), have also been shown to cause either excitation or inhibition of the pharyngeal muscle.

1.4.1 5-Hydroxytryptamine (5-HT)

5-HT is one of several biogenic amines which have been identified in *C. elegans* and has a role in the regulation of pharyngeal pumping, locomotion and egg-laying (Komuniecki *et al.,* 2004). Application of 5-HT to the pharynx increases its pumping activity up to a maximum of around 250 pumps per minute (Horvitz *et al.,* 1982; Niacaris and Avery 2003). It is likely that 5-HT acts both directly and indirectly onto *C. elegans* pharyngeal muscle (Rogers *et al.,* 2001). Pharyngeal muscle of a mutant deficient in synaptic transmission, snb-1(md247), was less responsive to 5-HT (Rogers *et al.,* 2001). However, there is also a direct 5-HT component from the release of 5-HT from pharyngeal NSM neurons where 5-HT can act as a neurohormone (Horvitz *et al.,* 1982). There are at least three 5-HT receptors in the *C. elegans* pharyngeal system. SER-1 receptors are expressed in pharyngeal muscle, SER-7b receptors are expressed in several motorneurones of the pharyngeal nervous system including MCs, M2s, M3s, M4 and M5 (Hobson *et al.,* 2006) and SER-4 is

expressed in an unidentified pharyngeal neurone (Tsalik *et al.,* 2003). From studies where these receptors have been expressed in various cell types, it has been proposed that SER-7b is associated with an increase in cAMP (Hobson *et al.*, 2003), SER-1 is 5-HT2-like in terms of structure but its pharmacology is a mix of 5-HT1 and 5-HT2 (Hamdan *et al.,* 1999) and couples to Gαq and phosphoinositide turnover while SER-4 is associated with a decrease in forskolin-stimulated cAMP levels (Olde and McCrombie 1997).

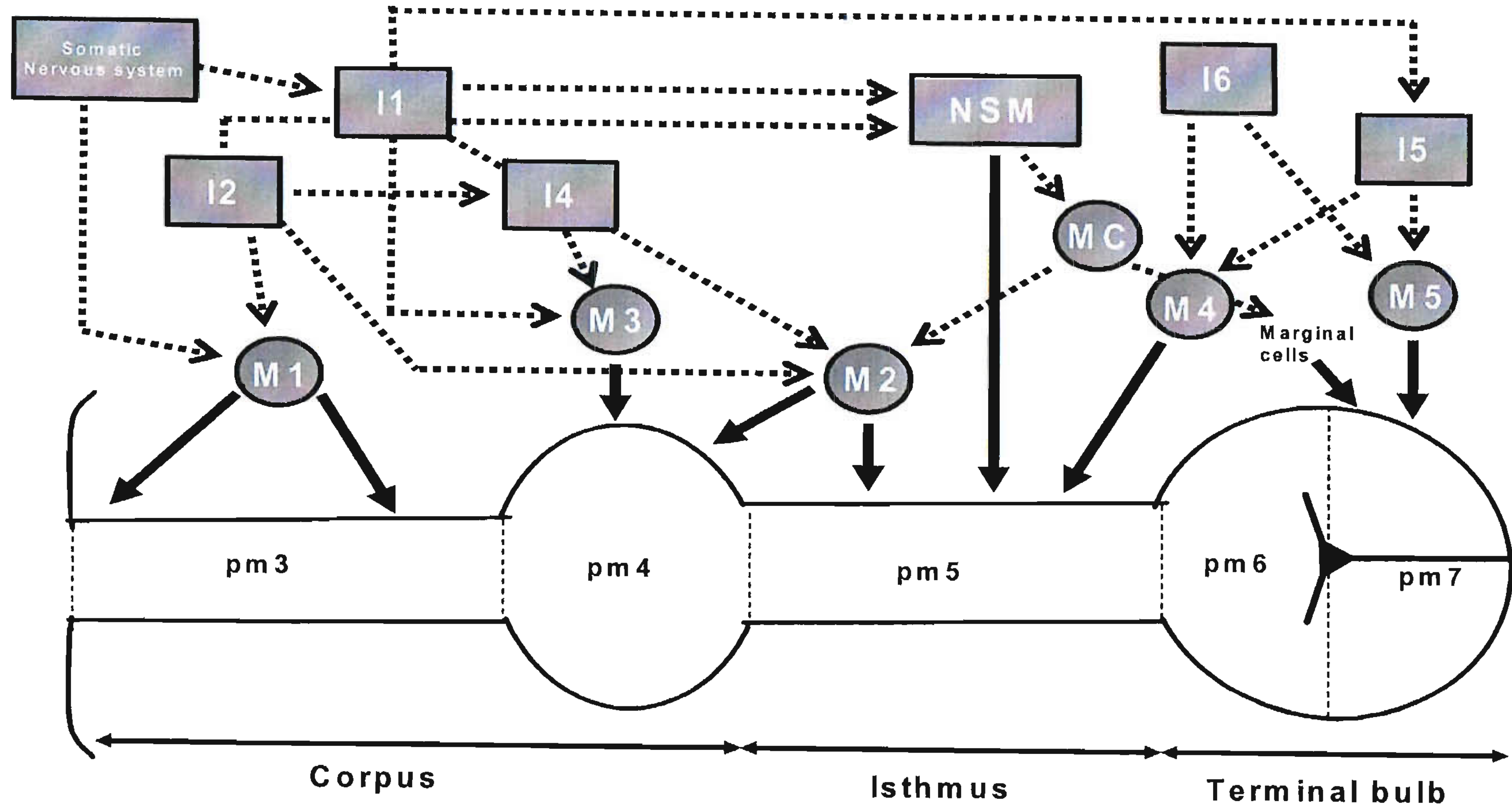


Figure 1.12 Wiring Diagram of the *C. elegans* pharynx. Neuronal connectivity of C. elegans pharynx. ○ Motorneurones , □ Interneurones, ┈┈► Chemical synapses, ──► connections between motorneurones and cells in the pharynx. (Adapted from Albertson & Thomson 1976).

1.5 Neuroactive peptides in Nematodes

Neuropeptides have been found throughout the animal kingdom, where they play key modulatory roles in a wide range of behaviours. They can be thought of as modified protein fragments, consisting of relatively short sequences of amino acid residues, from 2 up to over 50. Neuropeptides are derived from the cleavage of larger pre-pro-protein molecules, which are encoded within the genome. Following transcription and translation, the pre-pro-protein undergoes a series of post-translational modifications, which includes enzymatic cleavage into the smaller peptide molecules. Post-translational modifications, such as glycosylation, sulphation and C-terminal amidation can occur after peptides have been sequenced into storage vesicles. C-terminal amidation is thought to confer a degree of resistance to carboxypeptidases and is common among invertebrate neuropeptide families (Halton *et al.,* 1994). The modified peptides are then transferred to the presynaptic terminal by axonal transport, for storage and exocytotic release.

Differences in the general system of gene expression can lead to the diversification of peptide structures (Halton *et al.,* 1994). Such differences include: gene duplication, where peptide genes may code multiple copies of the same peptide; gene mutation, where duplicated peptides can develop divergent sequences by the action of mutational processes; differential processing, in the form of alternating splicing of the primary transcript to give different mRNA sequences from the same gene; finally differential post-translational modification, where several different peptide transmitter types may arise from a single precursor sequence.

Groups of peptides showing certain similarities are grouped into families of supposedly related peptides. One such family is the Arg-Phe-amide family of neuropeptides. RFamide neuropeptides play a key role in the physiology of the nervous and muscle systems of most, if not all, animal phyla. The first RFamide, Phe-Met-Arg-Phe-amide was, identified in the nervous system of a lamellibranch mollusc (Price and Greenberg, 1977). FMRFamides are now thought to occur in most molluscan species (Price *et al.,* 1987). FMRFamide genes have been characterised in molluscs such as *Aplysia californica* (Taussig & Scheller, 1986), *Helix aspersa* (Lutz

et al., 1990) and *Lymnaea stagnalis* (Linarce *et al.,* 1990). RFamide-like peptides are known to be distributed in the animal kingdom including vertebrates as well as invertebrates (Walker, 1992; Dockary, 2004). Interestingly, no FMRFamides have been identified in either vertebrates or arthropods. However, RFamides, for example, LPLRFamide, have been identified in the chicken brain (Dockray *et al.,* 1983). In arthropods, N-terminally extended FLRFamides occur in insects and crustacean, for example, SDRNFLRFamide, TNRNFLRFamide and PDVDHVFLRFamide (Trimmer *et al.*, 1987; Sithigorngul *et al.,* 2002; Robb *et al.,* 1989). However, in annelids, for example, in the leech, *Hirudo*, and polychaete, *Nereis*, the tetrapeptides, FMRFamide, FLRFamide, YMRFamide and YLRFamide, have been identified (Krajniak and Price 1990; Evans *et al.,* 1991). N-terminally extended FMRFamide-like peptides, for example, GNFFRFamide, and penta- and tetrapeptides with the structure F/YxRFamide, for example, RYIRFamide, GYIRFamide and YIRFamide, occur in platyhelminthes (Maule *et al.,* 1993, 1994; Johnston *et al.,* 1995, 1996; McVeigh *et al.,* 2005). The cnidarians (sea anemones/jellyfish) are the most primitive phylum to have a nervous system and while there is little evidence for the presence of classical transmitters, these animals contain RFamides, for example, pQGRFamide and pQLLGGRFamide (Grimmelikhuijzen and Graff 1986; Grimmelikhuijzen *et al.,* 1988, 1996). Finally, the echinoderms, while containing neither FRMFamides nor RFamides, do contain Famides, for example, GFNSALMFamide (Elphick *et al.,* 1991).

Unlike conventional neurotransmitters, the sequences of peptides vary between species and phyla and this makes them attractive as targets for agents that are selectively toxic to a parasite and not its host. Each putative peptide transmitter offers a multiplicity of target sites for disruption, e.g. biosynthetic pathways, pre and post synaptic receptors, inactivation mechanisms, ion channels and second messenger systems (Brownlee *et al.,* 1996).

1.5.1 FMRFamide-like peptides in nematodes

Evidence for the presence of neuropeptides in nematodes came from immunocytochemical studies in which antisera raised against a wide range of mammalian peptides showed reactivity with nematode nervous tissue, particularly tissue from *A. suum* (Sithigorngul *et al.,* 1990; Brownlee *et al.,* 1994a,b; Brownlee *et*

al., 1996). Members of the RFamide family were subsequently identified in nematodes by Stretton and his group using the pig intestinal nematode, *A. suum* (Cowden *et al.,* 1989; Cowden and Stretton, 1993). Around twenty RFamide peptides were identified in *A. suum* and divided into four groups based on their structures (Davis and Stretton, 1996). More recently this number has been revised upwards to around 30 in *A. suum* (Yew *et al.,* 2005). Neuropeptides also play a key role in the nervous system of *C. elegans* where up to 50% of the neurones demonstrate positive immunoreactivity for the FMRFamide-like peptides (Li *et al.,* 1999b). This figure is confirmed by the expression pattern of a large family of 29 genes predicted to encode FMRFamide-like peptides, the *flp* genes (for FMRFamide-like peptides) (Li, 2005; Husson et al., 2005). These are differentially expressed in just over 150 of the 302 neurones in adult hermaphrodite animals (Kim and Li, 2004). These *flp* expressing neurones are involved in a number of behaviours, including chemo-, mechano-, and thermosensation, locomotion, defecation, feeding and reproduction.

A number of the FMRFamide-like peptides are common to both *C. elegans* (designated as FLP) and *A. suum* (designated as AF), including FLP6 which is the same sequence as AF8, FLP8 which is the same as AF1, FLP16A which is the same as AF15, and FLP21 which is the same as AF9. Similarly peptides (designated as PF) first identified in the free-living nematode, *Panagrellus redivivus* (Geary *et al.,* 1992), have also been found in *C. elegans* e.g. FLPA1 (PF1), FLPA2 (PF2) and FLP6 (PF3). One *C. elegans* peptide, FLP14A, has been found in *A. suum* (Cowden and Stretton, 1993), *P. redivivus* (Marks *et al.,* 1995) and *Haemonchus contortus* (Keating *et al.,* 1995). It is therefore likely that a number of RFamide peptides are common to a range of nematode genera though it is unlikely that all RFamide peptides occur in all nematode species. For example, a recent study has shown that 23% of the genes were unique to the nematode species form which they were derived (Parkinson *et al.,* 2004).

1.5.2 Neuroactive peptides in *A. suum*

Peptide isolation in *A. suum* has revealed a family of about 30 *A. suum* FMRFamide-like neuropeptides (Yew *et al.*, 2005). Many of these peptides have been shown to affect the contractile state of *A. suum* muscle. Peptides isolated from other nematode species have also been shown to affect *A. suum* muscle. These actions are summarized in Table 1.1. In their study Yew *et al.,* (2005) used mass spectrometric analysis to identify around 40 neuropeptides but only around 30 were FMRFamide-like. In addition to the peptides listed in Table 1.1, the following have been identified in *A. suum* and given an AF number: AF18, NKFFLRKPamide, AF21, AMRNALVRFamide, AF22, NGAPQPFVRFamide, AF23, SGMRNALVRFamide, AF24, RNKFEFIRFamide, AF25, NNFLRFamide and AF26, KPNFLRFamide.

Nematode	Peptide	Sequence	Effect on *Ascaris* Muscle
A. suum	AF1	KNEFIRFa	Excitatory
	AF2	KHEYLRFa	Biphasic
	AF3	AVPGVLRFa	Excitatory
	AF4	GDVPGVLRFa	Excitatory
	AF5	SGKPTFIRFa	Biphasic
	AF6	FIRFa	Inhibitory
	AF7	AGPRFIRFa	No Effect
	AF8	KSAYMRFa	Excitatory
	AF9	GLGPRPLRFa	Excitatory
	AF10	GFGDEMSMPGVLRFa	Excitatory
	AF11	SDIGISEPNFLRFa	Inhibitory
	AF12	FGDEMSMPGVLRFa	Not Determined
	AF13	SDMPGVLRFa	Excitatory
	AF14	SMPGVLRFa	Excitatory
	AF15	AQTFVRFa	Not Determined
	AF16	ILMRFa	No Effect
	AF17	FDRDFMHFa	Excitatory
	AF19	AEGLSSPLIRFa	Inhibitory
	AF20	GMPGVLRFa	Excitatory
P. redivivus	PF1	SDPNFLRFa	Inhibitory
	PF2	SADPNFLRFa	Inhibitory
	PF3	KSAYMRFa	Excitatory
	PF4	KPNFIRFa	Inhibitory
H. contortus	AF2	KHEYLRFa	Excitatory

Table 1.1 Neuropeptides in nematodes. Primary structure, sequence comparison and action of FMRFamide-related peptides on *A. suum* muscle.

These compounds act either directly on the muscle to change its tension, or indirectly by affecting the ACh-induced contractions or GABA-induced relaxations of *A. suum* muscle. The FaRPs can greatly affect the activity of the *A. suum* muscle. Understanding where and how these peptides act on *A. suum* muscle will be important in developing novel anthelmintics to mimic the action of these peptides.

The first of the AF-like peptides to be sequenced was AF1 (KNEFIRFamide). It was extracted by Cowden et al., (1989), who also observed its bioactivity. When injected into the pseudocoelom of *A. suum*, synthetic AF1 disrupted the propagation of locomotory waves. Furthermore AF1 had effects on electrical activity observed in motorneurones of the DI and VI class. AF1 reversibly abolished slow wave potentials and reduced input resistance. By recording from muscle cells and electrically stimulating presynaptic VI and DI motorneurones, Cowden et al., (1989) demonstrated that AF1 did not block synaptic transmission, but they observed that the spontaneous IPSPs were blocked by AF1. They concluded that AF1 was indirectly modulating synaptic transmission by reducing input resistance in inhibitory motorneurones and shunting slow wave potentials. AF1 had no effect on input resistance of DE1 motorneurones and did not alter synaptic transmission between DE1 and muscle cells.

AF2 (KHEYLRFamide), a peptide showing high degree of sequence homology to AF1 was also extracted from *A. suum* by Cowden & Stretton (1993). The bioactivity of AF2 was shown to be similar to that of AF1. The bioactivity of AF2 was tested on an *A. suum* muscle strip preparation. It had multiple effects on muscle tension causing relaxation followed by an increase in muscle tone associated with superimposed rhythmical contractions and relaxations (Cowden and Stretton, 1993). Electrical activity in muscle cells was closely correlated with the effects on tension that AF2 elicited. During relaxation phases muscle cells were hyperpolarized and they depolarised in concert with rhythmical tension increase. From these observations AF2 could be acting both pre and post-synaptically (Pang *et al.,* 1995). Post-synaptically, AF2 may be increasing the probability of generating action potentials within muscle cells. Pre-synaptically, AF2 may be acting to stimulate the excitatory motorneurones causing an increase in the release of ACh. AF2 may also be acting to inhibit the inhibitory motorneurones, thus decreasing GABA release. Although a receptor for

AF2 has not been yet been identified, a G-protein involvement in AF2-triggered receptor activation has been identified, indicating AF2 may exert its action through a G-protein coupled receptor (Kubiak *et al.,* 2003c).

AF3 (AVPGVLRFamide) and AF4 (GDVPGVLRFamide) also cause a slow contraction of muscle similar to that of AF1 and AF2. Unlike AF1 and AF2, AF3 and AF4 do not stimulate any rhythmic contractions and only occasionally is the contraction preceded by an initial relaxation. AF3 and AF4 are not blocked by the nicotinic receptor antagonist mecamylamine (Trim *et al.,* 1997).

Other peptides from *Panagrellus redivivus* have also been shown to act on *A. suum* muscle. The peptides PF1 (SDPNFLRFamide) and PF2 (SADPNFLRFamide) cause a slow relaxation of *A. suum* dorsal muscle (Franks *et al.,* 1994). PF1 causes a slow chloride-independent hyperpolarisation of somatic muscle cells. PF1 may act via potassium channels as both the potassium channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4AP) inhibit the action of PF1 (Franks, 1996). PF1 also has both pre and post-synaptic actions. PF1 inhibits the excitatory junction potentials (EJPs) at the neuromuscular junction, without affecting the depolarisation of muscle cells caused by exogenous additions of ACh (Holden-Dye *et al.,* 1995). This is consistent with a pre-synaptic effect. Evidence for a post-synaptic effect of PF1 comes from the fact that PF1 inhibits the contraction elicited by exogenous applied ACh (Franks *et al.,* 1994).

PF4 (KPNFIRFamide) also causes a relaxation of muscle, however, unlike PF1 and PF2, this relaxation occurs at a relatively fast rate (Maule *et al.,* 1996). PF4 was also shown to potently affect the membrane potential of muscle cells. PF4 was three orders of magnitude more potent than GABA at producing inhibition (Holden-Dye *et al.,* 1997). The hyperpolarisation of PF4 consists of two phases, the first of which is chloride-dependent. PF4 does not act to stimulate the release of GABA since the anthelminitic ivermectin blocks the inhibitory GABA response on *A. suum* muscle but not the response to PF4 (Holden-Dye *et al.,* 1997). Therefore, there is a high possibility that a receptor for PF4 on *A. suum* muscle is allowing this fast-acting inhibitory action. The presence of an obvious endogenous PF4-like peptide within *A. suum* has yet to be identified.

1.5.3 Neuropeptides in *C. elegans*

The completion of the genomic sequence of the *C. elegans* enabled the identification of neuropeptide-like genes in *C. elegans*. Neuropeptides play a key role in the nervous system of *C. elegans* where up to 50% of the neurones demonstrate positive immunoreactivity for FMRFamide-like peptides (Li *et al.,* 1999b). This figure is confirmed by the expression pattern of a large family of 29 genes predicted to encode FMRFamide-like peptides, the *flp* genes. These are differentially expressed in just over 150 of the 302 neurones in adult hermaphrodite animals (Kim and Li, 2004). These *flp* expressing neurones are involved in a number of behaviours.

The total number of potentially RFamide peptides in *C. elegans* is surprisingly large. The *flp* genes potentially encode more than 70 different FaRPs (summarised in Table 1.2) (Li *et al.,* 1999; Husson *et al* 2005; Li, 2005). Five of the *flp* genes encode multiple copies of only one FaRP; seven genes encode one copy of one FaRP and the remaining seventeen *flp* genes encode multiple FaRPs. The first RF peptides were identified by Li and her group (Rosoff *et al.,* 1992; Rosoff *et al.,* 1993) and a further seventy peptides are predicted from post-translational processing of the *flp* gene products (Li *et al.,* 1999a; Li, 2005). The first peptide gene to be isolated from *C. elegans* was named *flp-1*. *flp-1* encodes eight FaRPs (Rosoff *et al.,* 1992; Li, 2005). Alternative splicing of the *flp-1* produces two transcripts (Rosoff *et al.,* 1993) one of which encodes seven peptides, while the other encodes only six of the eight FaRPs. To date, 35 FaRPs derived from fifteen *flp* genes have been isolated biochemically from *C. elegans*. These include FLP1 (Rosoff et al., 1993), FLP6 (Marks et al., 1998), FLP9 (Marks et al., 1999), FLP13 (Marks et al., 1997) and FLP14 (Marks et al., 1995).

Little information was known about the bioactivity of FMRFamide peptides in *C. elegans*, until advances in electrophysiological recordings from excitable cells in *C. elegans* (Goodman *et al.,* 1998; Francis *et al.,* 2003) presented the opportunity to directly determine their action. To date, the limited knowledge of the physiological role of the FLPs in *C. elegans* has been obtained largely from investigating the functional consequence of the *flp* gene knockouts. For example, a number of phenotypes have been correlated with the *flp-1* deletion including hyperactivity, loopy

movement, nose-touch insensitivity, defects in osmolarity detection, sluggishness (Nelson *et al.,* 1998a,b) and altered egg-laying behaviour (Waggoner *et al.,* 2000). The functional role of the FLP peptides can also be inferred from the expression pattern of the *flp* genes. Individual genes have discrete expression patterns in subsets of neurones that subserve specific animal behaviours (Kim and Li, 2004). These observations can be used to inform further experimental analysis of the behavioural consequences of specific *flp* gene knockouts. Rogers *et al.,* (2001) showed that a number of FMRFamide peptides affect pharyngeal pumping rates similar to the classical transmitters 5-HT and octopamine. AF1 (KNEFIRFamide), AF2 (KHEYLRFamide), AF8 (KSAYMRFamide) and GAKFIRFamide encoded by the *C. elegans* genes *flp-8, flp-14, flp-6* and *flp-5* respectively, increased pharyngeal pumping. SDPNFLRFamide, SADPNFLRFamide, SAEPFGTMRFamide, KPSVRFamide, APEASPFIRFamide, and AQTVRFamide encoded by the *C. elegans* genes *flp-1, flp-1, flp-3, flp-9, flp-13* and *flp-16* respectively, inhibited pharyngeal pumping.

The *flp* gene family represents only one neuropeptide gene family in *C. elegans*. To identify novel peptidergic neurotransmitters, the *C. elegans* genome was searched for predicted proteins with structural hallmarks of neuropeptide pre-pro-proteins (Nathoo *et al.,* 2001; Li, 2005). This search identified forty two *C. elegans* neuropeptide-like protein (*nlp*) genes encoding around 125 peptides. These *nlp* genes define a large number of sub-families of putative neuropeptides. *nlp* genes were found to be predominantly expressed in chemosensory neurones and endocrine tissues (Nathoo *et al.,* 2001). The genes also encode peptides which probably act as anti-microbials (Li, 2005). Two of the *nlp* genes *nlp-1* and *nlp-2* encode novel neuropeptides, which are weakly similar to other invertebrate neuropeptides. *nlp-1* encodes neuropeptides most similar to *A. californica* buccallin (Miller *et al.,* 1993). The nlp-2 gene encodes peptides with slight similarity to active myomodulin from *L. stagnalis* and other species (Santama *et al.,* 1994). The function of NLP peptides in the nematode *C. elegans* has not yet been investigated.

Finally there are 37 genes which encode insulin-like peptides (Li, 2005).

Gene	Peptide Sequence
flp-1	* SADPNFLRFa *SQPNFLRFa *ASGDPNFLRFa *SDPNFLRFa *AAADPNFLRFa *PNFLRFa AGSDPNFLRFa PNFMRYa
flp-2	SPREPIRFa LRGEPIRFa
flp-3	SPLGTMRFa *TPLGTMRFa *EAEEPLGTMRFa NPLGTMRFa *ASEDALFGTMRFa EDGNAPFGTMRFa *SAEPFGTMRFa *SADDSAPFGTMRFa *NPENDTPFGTMRFa
flp-4	PTFIRFa ASPSFIRFa
flp-5	*GAKFIRFa AGAKFIRFa APKPKFIRFa
flp-6	*KSAYMRFa (x6) *pQQDSEVEREMM
flp-7	SPMQRSSMVRFa (x3) TPMQRSSMVRFa (x2) SPMERSAMVRFa SPMDRSKMVRFa
flp-8	*KNEFIRFa (x3)
flp-9	*KPSFVRFa (x2)
flp-10	QPKARSGYIRFa
flp-11	AMRNALVRFa *ASGGMRNALVRFa *NGAPQPFVRFa *SPLDEEDFAPESPLQa
flp-12	RNKFEFIRFa
flp-13	*AMDSPFIRFa *AADGAPLIRFa *APEASPFIRFa *ASPSAPLIRFa *SPSAVPLIRFa ASSAPLIRFa *SAAAPLIRFa
flp-14	*KHEYLRFa (x4)
flp-15	GGPQGPLRFa RGPSGPLRFa
flp-16	*AQTFVRFa (x2) *GQTFVRFa

Gene	Peptide Sequence
flp-17	KSAFVRFa KSQYIRFa
flp-18	*GAMPGVLRFa EMPGVLRFa *SVPGVLRFa *EIPGVLRFa *SEVPGVLRFa DVPGVLRFa
flp-19	*WANQVRFa ASWASSVRFa
flp-20	AMMRFa (x2)
flp-21	GLGPRPLRFGa
flp-22	*SPSAKWMRFGa
flp-23	TKFQDFLRFa VVGQQDFLRa
flp-24	*VPSAGDMMVRFa
flp-25	DYDFVRFa ASYDYIRFa
flp-26	*FNADDLTLRFa *GGAGEPLAFSPDMLSLRFa
flp-27	GLGGRMRFa
flp-28	VLMRFa
flp-32	AMRNSLVRFa

Table 1.2. The *flp* genes in *C. elegans* and the potential FaRPs they encode; *have been biochemically isolated.

1.6 Neuropeptide Receptors

In BLAST searches of the *C. elegans* database, over 1000 G-protein coupled receptors (GPCRs) were identified. About 100 of these encode neuropeptide GPCRs (Nathoo *et al.,* 2001). Currently the majority of the predicted GPCRs in *C. elegans* are orphan receptors. To date only five GPCRs in *C. elegans* have been paired with their ligands, in contrast to the numerous vertebrate GPCRs for which the cognate ligands have been identified. Each receptor has been linked with its most potent ligand available to date but these may not prove to be their true endogenous ligands.

Mertens *et al* (2004) cloned and characterised the FaRP receptor, VRFa receptor 1 (orphan C26F1.6), where SMVRFamide proved the most active sequence, derived from peptide (TPMQRSSMVRFamide) expressed by *flp7* gene (Nelson *et al.,* 1998).

The orphan G-protein-coupled receptor C10C6.2 was cloned by Kubiak *et al* (2003b). FMRFamide-related peptides encoded by the *flp15* gene were found to be the ligands for the C10C6.2 leading to the designation of the receptor as FLP15-R. FLP15-R activation was abolished in the transfected CHO (Chinese Hamster Ovary) cells pretreated with pertussis toxin suggesting a preferential receptor coupling to Gi/Go proteins.

Two alternatively spliced *C. elegans* GPCRs, T19F4.1a and T19F4.1b were cloned and functionally characterised by Mertens *et al* (2005). The T19F4.1b receptor was found to be 30 amino acids longer than T19F4.1a and the difference in amino acid constitution was exclusively conferred to the intracellular C-terminal region, suggesting a potential difference in G-protein-coupling specificity. Both receptors were found to be activated by peptides encoded by the *flp2* gene precursors: SPREPIRFamide (FLP2A) and LRGEPIRFamide (FLP2B).

The orphan *C. elegans* GPCR Y59H11AL.1 receptor which was categorised in the tachykinin-like group of receptors was cloned by Mertens *et al* (2005). Several FLPs, the most potent of which was SPMERSAMVRFamide (*flp-7*) were able to clearly activate the cloned receptor in a dose-dependent manner. These receptors and

their putative ligands are summarised in Table 1.3. It can be seen from the table that a receptor can bind a number of peptides and a peptide can bind to more than one receptor. The system has the potential to be extremely complex. The NPR-1 receptor has a role in feeding and the change of one specific amino acid changes feeding behaviour (de Bono and Bargmann, 1998). Interestingly both FLP18 and FLP21 activate this receptor. These peptides share a PxxLRFamide C terminal sequence.

Receptor	Ligand	Reference
C 10C6.2	*flp-15* peptides	Kubiak *et al.*, 2003b
C 16D6.2 F 41E7.3 Y 58G8A.4a Y 58G8A.4b C 53C7.1 C25G6.5	*flp-18* peptides	Lowery *et al.*, 2003
C 53C7.1	FLP3	Lowery *et al.*, 2003
C 26F1.6	*flp-7* *flp-11*	Mertens *et al.*, 2004
T 19F4.1a b	*flp-2* peptides	Mertens *et al.*, 2005
C 39E6.6 (NPR-1) C25G6.5	FLP-21 FLP-18	Rogers *et al.*, 2003 Kubiak *et al.*, 2003 Rogers *et al.*, 2003 Kubiak *et al.*, 2003a
Y 59H11AL.1 C26F1.6	*flp-7* peptides	Mertens *et al.*, 2005 Mertens *et al.*, 2004

Table 1.3. Summary of *C. elegans* neuropeptide receptors which interact with identified ligands. (After McVeigh *et al.*, 2006a)

Although there are a very large number of GPCRs in nematodes little is known about the identity of the second messenger systems involved in their function. For example, both AF1 (KNEFIRFamide) and AF2 (KHEYLRFamide) excite *A. suum* body wall muscle with AF2 also having an initial inhibitory component (Cowden *et al.,* 1989; Cowden and Stretton 1993; Pang *et al.,* 1992; Pang *et al.,* 1995). The injection of 10 μM of either peptide results in a large sustained increase in the level of cAMP in *A. suum* (Reinitz *et al.,* 2000) and both AF1 and AF2 increase cAMP in body wall muscle without altering the levels of either cGMP or IP3 (Thompson *et al.,* 2003). Kubiak *et al* (2003c) have also presented binding evidence for AF2 acting through a GPCR. Both AF3 (AVPGVLRFamide) and AF4 (GDVPGVLRFamide) increase the tone of *A. suum* body wall muscle and directly depolarise the muscle cells (Trim *et al.,* 1997). AF3 decreases cAMP levels in muscle cells and this may be involved in the generation of contraction (Trim *et al.,* 1998). AF9 (GLGPRPLRFamide) has also been shown to inhibit cAMP through activation of Gi/Go proteins (Kubiak *et al.,* 2003a).

1.7 Guanine nucleotide binding protein (G-protein) coupled receptors

These cell membrane-bound receptors consist of a single polypeptide chain of 400-500 residues with seven membrane spaning α-helices. The membrane spanning domains are found to be highly conserved between different receptors, and indeed different phyla. Conversely, the extracellular amino terminus, the intracellular carboxy terminus and the long third cytoplasmic loop vary greatly in length and sequence. It is widely regarded that this cytoplasmic loop is the region of the molecule, which couples to the G-protein, since site-directed mutagenesis towards this region greatly modifies coupling with this protein (Stryer, L 1995).

The C-terminal cytoplasmic tail appears to contain phosphorylation sites where specific kinases can catalyse the coupling of phosphate groups. This has the effect of reducing the interaction of the receptor with the G-protein and is probably important as a mechanism of agonist-induced receptor desensitisation. (Stryer, L 1995)

G-proteins consist of three subunits, α, β and γ. Guanine nucleotides bind to the α subunit, which has enzymatic activity, catalysing the conversion of GTP to GDP. The hydrophobic β and γ subunits remain associated as a βγ complex with the cytoplasmic surface of the membrane and can be regarded as one functional unit, a complex which has also recognised signal transduction properties. G-proteins appear to be freely diffusible in the plane of the membrane and it is a key aspect of their function that a single pool of G-protein in a cell can interact with several different receptors and effectors in an essentially promiscuous fashion (Stryer, L 1995).

In the resting state, G-proteins are thought to exist as the unattached αβγ trimer, with GDP occupying the α subunit binding site. When the appropriate receptor binds an agonist, it undergoes a conformational change greatly increasing its affinity for αβγ. Association of αβγ with the receptor stimulates GDP/GTP exchange which in turn causes dissociation of α-GTP from the βγ subunits. α-GTP is then able to diffuse in the membrane and can associate with various enzymes and ion channels, causing either inactivation or activation. This activity is terminated when GTP is hydrolysed to GDP by means of GTPase activity, found on the α-subunit. The resulting α-GDP dissociates from the effector and reassociates with βγ, completing the cycle (Fig 1.13). The Gβγ subunit has been recognised as a signal transduction molecule in its own right; Gβγ is now known to directly regulate as many different protein targets as the Gα subunit (Clapham & Neer, 1997 for review).

This process of GDP/GTP exchange is widely regarded as an amplification step, since one receptor when stimulated can activate several G-protein molecules in turn. This amplification can be enhanced, since each of these G-proteins can remain associated with their effector molecule long enough to produce many molecules of the product. This product is often a second messenger, and further amplification occurs before the final cellular response is produced.

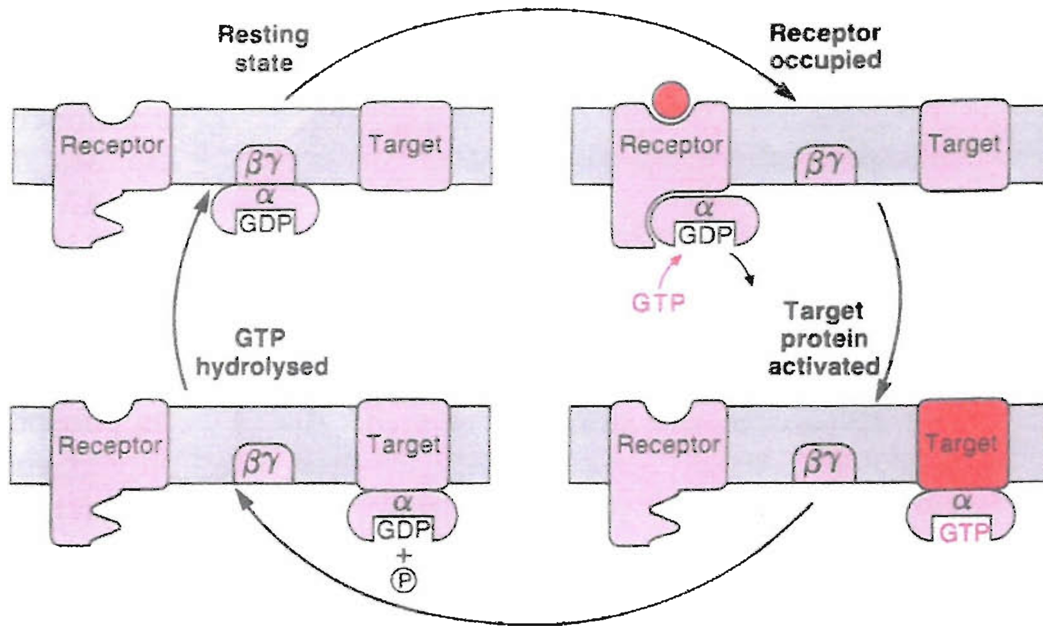


Figure 1.13. The function of the G-protein. The G-protein consists of three subunits; α,β,γ. Coupling of the α-subunit to an agonist-occupied receptor causes the bound GDP to exchange with intracellular GTP; the α-GTP complex then dissociates from the receptor. Both Gα and Gβγ are then able to interact with target protein. The α-subunit catalyses hydrolysis of the bound GTP to GDP, whereupon the α-subunit reunites with βγ. (Taken from Rang *et al.,* 1991).

G-proteins are often found to activate different functional events which is now known to result from different structures of the α-subunit of the G-protein. 23 distinct α-subunits are known and can be subdivided into 4 families based on the degree of primary sequence homology: $G\alpha_s$, $G\alpha_q$, $G\alpha_{i/o}$, $G\alpha_{12/13}$ (Guderman *et al.,* 1996). G_s and G_i are proteins which are known to stimulate and inhibit adenylate cyclase, respectively. The Gq subunits are a family which regulate phospholipase C/inositol phosphate turnover (Exton, 1996).

G proteins in *C. elegans* have been reviewed in detail by Bastiani and Mendel (2005). The *C. elegans* genome encodes 21 Gα, 2 Gβ and 2 Gγ subunits (Jansen *et al.*, 1999; Cuppen *et al.,* 2003). This extensive family of α subunits includes orthologues of the mammalian Gα family, viz, GSA-1 (Gs), GOA-1 (Gi/o), EGL-30 (Gq) and

GPA-12 (G12) (Bastiani and Mendel 2005). Gα subunits have extensive roles in *C. elegans* including locomotion, egg laying and pharyngeal pumping (Bastiana and Mandel 2005). Specific α subunits, for example, GSA-1, GOA-1 and EGL-30, are involved in the regulation of acetylcholine release by motorneurones. EGL-30 is involved in vesicle release while GOA-1 negatively regulates the EGL-30 pathway. GSA-1 also modulates the pathway (Lackner *et al.,* 1999; Miller *et al.,* 1999; Nurrish *et al.,* 1999). Raised levels of GPA-12 affect pharyngeal pumping (van der Linden *et al.*, 2003). The Gβ subunit, GPB-1, and the Gγ subunit, GPC-2, appear to function with Gα as a G protein heterotrimer in *C. elegans* (van der Voorn *et al.*, 1990; Jansen *et al.,* 2002). In contrast to the situation in the human genome where 14 and 5 genes encode γ and β subunits respectively (Milligan and Kostensis 2006) there are only two Gβ and Gγ subunits in *C. elegans* and so the functional differences must lie in the multiple Gα subunits. Multiple G protein pathways are known to function in specific cell types, for example, Go, Gq and Gs-mediated signalling occurs in motorneurones in the ventral cord (Bastiana and Mendel 2005). *gsa-1*, *goa-1* and *egl-30* are expressed in pharyngeal muscle while the first two are also expressed in pharyngeal neurones (Korswagen *et al.,* 1997; Lackner *et al.,* 1999; Bastiana *et al.,* 2003; Mendel *et al.,* 1995; Segalat *et al.*, 1995). The Gα subunit gene, *gpa-2*, is expressed in pharyngeal neurones, M1, M5 and I5 while *gpa-7*, *gpa-9* and *gpa-12* are expressed in pharyngeal muscle (Zwaal *et al.*, 1997; Lans *et al.*, 2004; Jansen *et al.*, 1999).

There are three principal targets for G-proteins, the adenylate cyclase/cAMP system; the phospholipase C/inositol phosphate system and the regulation of ion channels.

1.7.1 Adenosine 3',5'-cyclic monophosphate (cAMP)

cAMP is a labile product of the action of the membrane bound enzyme adenylate cyclase on ATP. There are at least 10 different adenylate cyclase isoforms known in mammalian systems, all of which are stimulated by $G\alpha$, and the diterpine forskolin (Sunahara *et al.,* 1996). cAMP is a second messenger in the activation of some receptor mediated events. It is readily hydrolysed to the inactive 5' AMP, by the action of cellular phosphodiesterases which are found within the cell cytoplasm.

In smooth muscle, cAMP is known to inhibit smooth muscle contraction by the activation of receptors associated with G-protein(s) coupled to adenylate cyclase, generating cAMP. This can act upon protein kinase A (PKA) and myosin light chain kinase (Fig 1.14).

As well as receptor-mediated adenylate cyclase stimulation, there are many known physiological effects on muscle mediated by an inhibition of adenylate cyclase. Such receptors are coupled to the GTP binding protein, Gi, which inhibits adenylate cyclase.

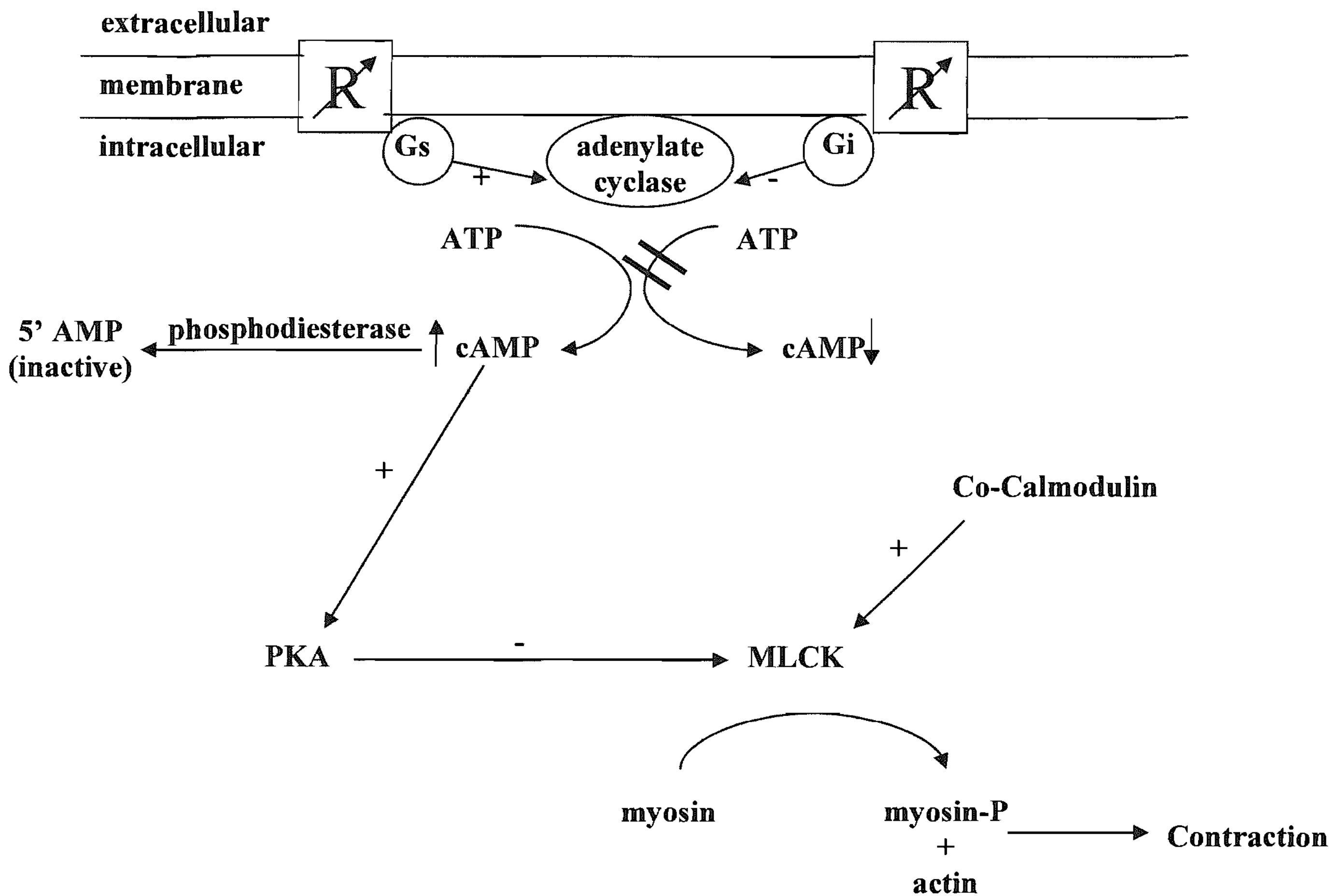


Figure 1.14. cAMP cascade. Membrane bound adenylate cyclase is responsible for the synthesis of cAMP from ATP. The enzyme can be stimulated and inhibited by GTP binding proteins, Gs and Gi respectively thus regulating levels of cAMP in the cell. cAMP stimulates protein kinase A (PKA) which inhibits myosin light chain kinase (MLCK). This results in smooth muscle relaxation (Adapted from Rang *et al.*, 1991).

1.7.2 Phospholipase C/inositol phosphate signal transduction

This is another important second messenger pathway, which results from the receptor stimulated action of the membrane bound enzyme phospholipase C (PLC) on the phospholipid phosphatidylinositol biphosphate. This process liberates diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (1,4,5 IP_3), which activates protein kinase C, and releases intracellular calcium respectively.

In *C. elegans* acetylcholine released from motorneurones is mediated through a pathway involving hydrolysis of phosphatidylinositolbisphosphate (PIP²) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Activation of EGL-30 results in raised DAG levels which leads to activation of EGL-8 (PLCβ). This promotes presynaptic localization of UNC-13, the DAG receptor protein, which increases synaptic vesicle fusion at active zones (Richmond *et al.,* 1999, 2001).

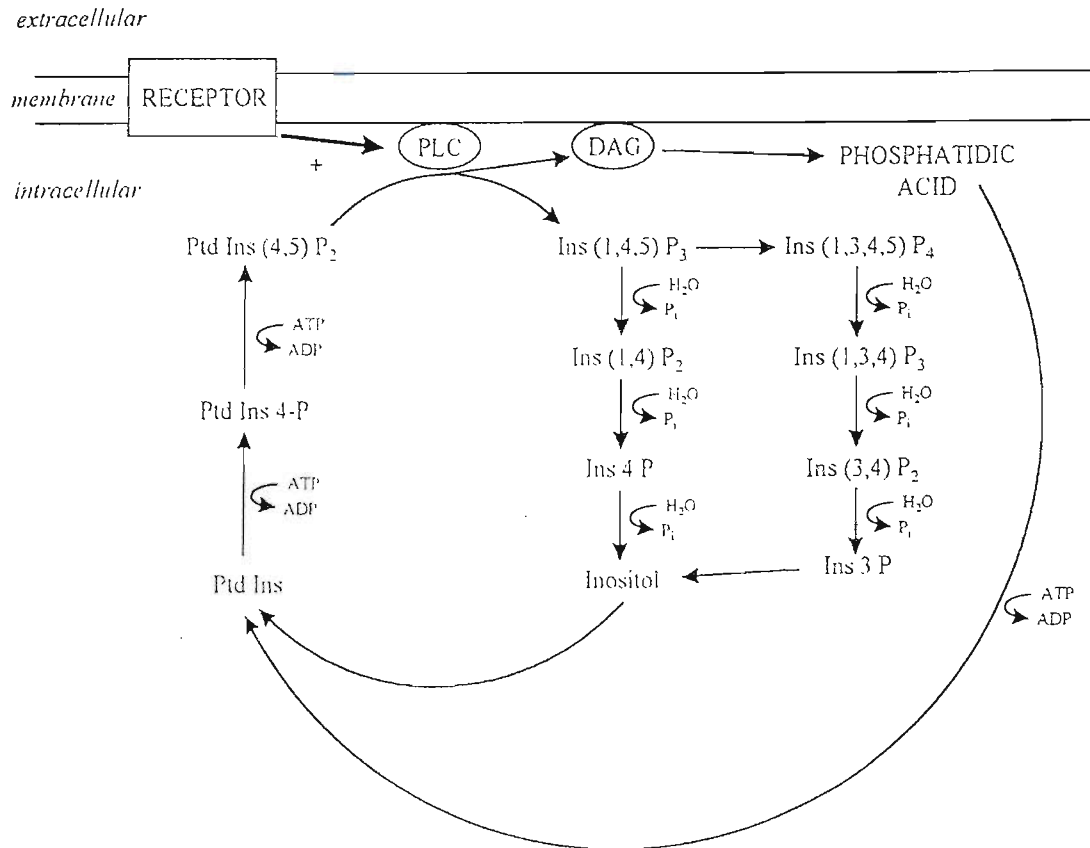


Figure 1.15. The phosphatidylinositol cycle. Activation of receptors linked to phospholipase C (PLC) via G-proteins cause the production of two second messengers, a) Ins (1,4,5)P_3 which is released into the cytosol stimulating intracellular Ca^{2+} release and b) diacylglycerol (DAG) which is membrane bound and activates PKC, thus initiating various protein phosphorylation reaction. (Adapted from Berridge, 1987).

1.8 Project Aims

From the 'Introduction" it is clear that while there is a reasonable volume of literature regarding the identification and localization of neuroactive peptides in nematodes, less is known regarding their physiology and mechanisms of action. The aim of the project was therefore to investigate the actions of endogenous neuropeptides *in vitro*, using nematode preparations. Two preparations were selected for physiological studies, the body wall muscle of the parasitic nematode, *A. suum,* and the isolated pharynx of the free-living nematode, *C. elegans*.

A. suum is a relatively large nematode, ideally suited for both physiological and pharmacological studies. It is easy to prepare sections of body wall muscle for either muscle tension or electrophysiological recordings. This preparation was used to investigate the action of neuropeptides isolated from *C. elegans*, one of which (FLP21, AF9) also occurs in *A. suum*.

Although *C. elegans* is small for physiological and pharmacological studies, its genome has been determined and many mutants are available. As it is possible to make extracellular EPG recordings from the isolated pharynx, this preparation was selected for investigating the action of FMRFamide-like peptides previously identified in *C. elegans*. Experiments were undertaken to investigate the possible second messengers involved in the excitatory effects of one FMRFamide, FLP17A (KSAFVRFamide) and the results compared with those obtained using 5-HT. Two mutants with a loss-of-function in Gαq and PLCβ were also used to study the second messengers involved in FLP17A and 5-HT excitatory effects on the pharynx.

CHAPTER 2

Materials and Methods

2.1 *A. suum* maintenance

Fresh *A. suum* were collected from the local abattoir and transported to the laboratory in a flask containing artificial perienteric fluid (APF) which had the following composition in mM: NaCl 67, CH_3COONa 67, $CaCl_2$ 3, MgCl 15.7, KCl 3, glucose 3, Trizma® base 5, pH 7.6 (with acetic acid at 37°C). In the laboratory *A. suum* were maintained in APF at 37°C for up to five days. The APF was changed twice daily.

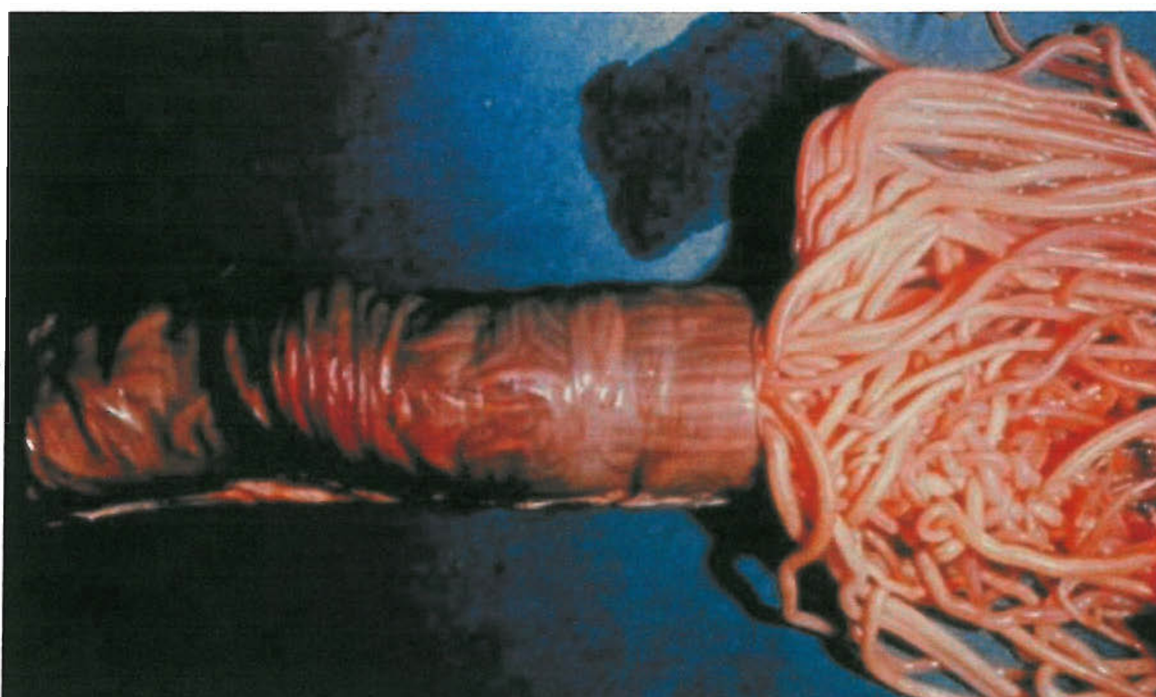


Figure 2.1. Adult *Ascaris suum*. Photograph of adult *A. suum* shown in their host environment, the small intestine of the pig. (From http://caltest.vet.upenn.edu)

2.2 In vitro muscle strip tensions.

The dorsal muscle strip (DMS) was prepared by dissecting a 1cm length of the body wall muscle immediately anterior to the genital pore of a large female *A. suum*. Dorsal and ventral muscles can be identified by the position of the three lips in the mouth of the worm. Two lips bound the ventral position of the mouth while the dorsal position of the mouth has one lip. The dorsal muscle was cut lengthwise along the

dorsal side of each lateral cord and the ventral part discarded. Any intestinal muscle remaining attached to the somatic muscle was carefully removed.

Dorsal muscle was used as it is devoid of motorneurone somata, which are only present in the ventral nerve cord. Dorsal muscle, however, does contain the dorsal inhibitory and dorsal excitatory motorneurone terminals, which have reciprocal synapses with each other.

2.3 Organ bath experiments

The *A. suum* DMS was suspended in a 15 ml organ bath. At one end a loop of cotton thread was used to secure the DMS at the base of the organ bath. The muscle strip was connected by a thread to a 2g isometric transducer, which lay above the organ bath, Fig. 2.2. The preparation was subjected to a 1g load and maintained at 37°C with a heated water jacket. Drugs were added to the organ bath using a syringe. The concentration of the drug in the syringe was adjusted such that the desired concentration in the organ bath achieved after thorough mixing. To aid mixing the organ bath was gassed with air for about 30 seconds. To wash the tissue, fresh APF was perfused through the organ bath and the overflow is removed by a vacuum pump. A hard copy of data was obtained on a flat chart recorder (BBC, Goerz, Metrawatt Austria).

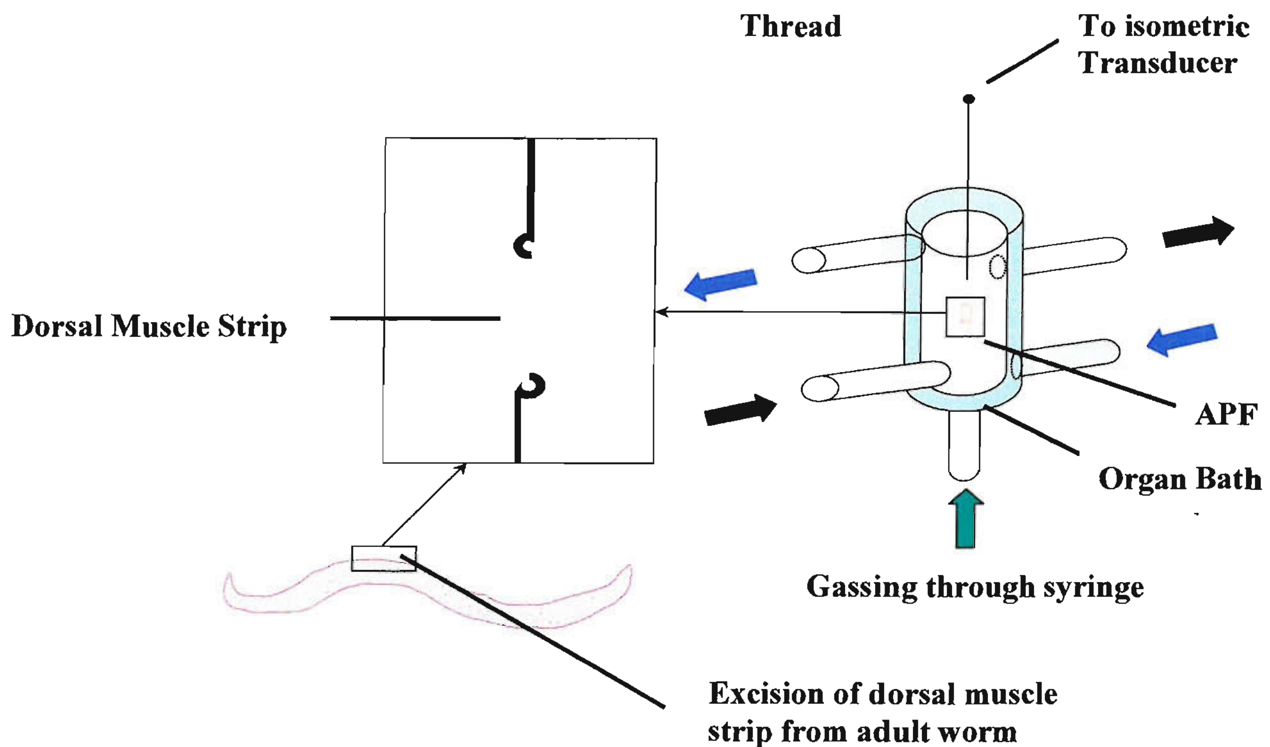


Figure 2.2. The organ bath set-up. Dorsal muscle (1cm) were removed from *A. suum* and placed in an organ bath containing APF at 37°C. The muscle was tied to 2 g isometric transducer and changes in muscle tone were measured.

2.4 Electrophysiological studies

Intracellular recordings were made from *A. suum* muscle cells using a two electrode-recording technique. The DMS was pinned cuticle side down on a Sylgard® elastomer 184 (Dow Corning Wiesbaden, Germany) lined perspex chamber and continuously perfused with APF at 32-34°C, via a fine-bore tube.

Individual muscle cell bags were impaled with 2 microelectrodes containing 10 mM KCl in 3 M potassium acetate (10-30 MΩ) connected to an Axoclamp 2A amplifier. One electrode was used to record, membrane potential and the second to pass current pulses (20 nA, 0.2 Hz, 500 msec). The chamber was grounded using a 3 M KCl agar bridge/AgCl electrode. Input resistance could be estimated from the electronic potential resulting from current injection. Bath temperature was continuously monitored with a thermometer placed adjacent to the muscle strip. Hard copy of data was recorded on a Gould model 35 2-channel chart recorder (Gould instruments, Ohio, USA) (Fig. 2.3)

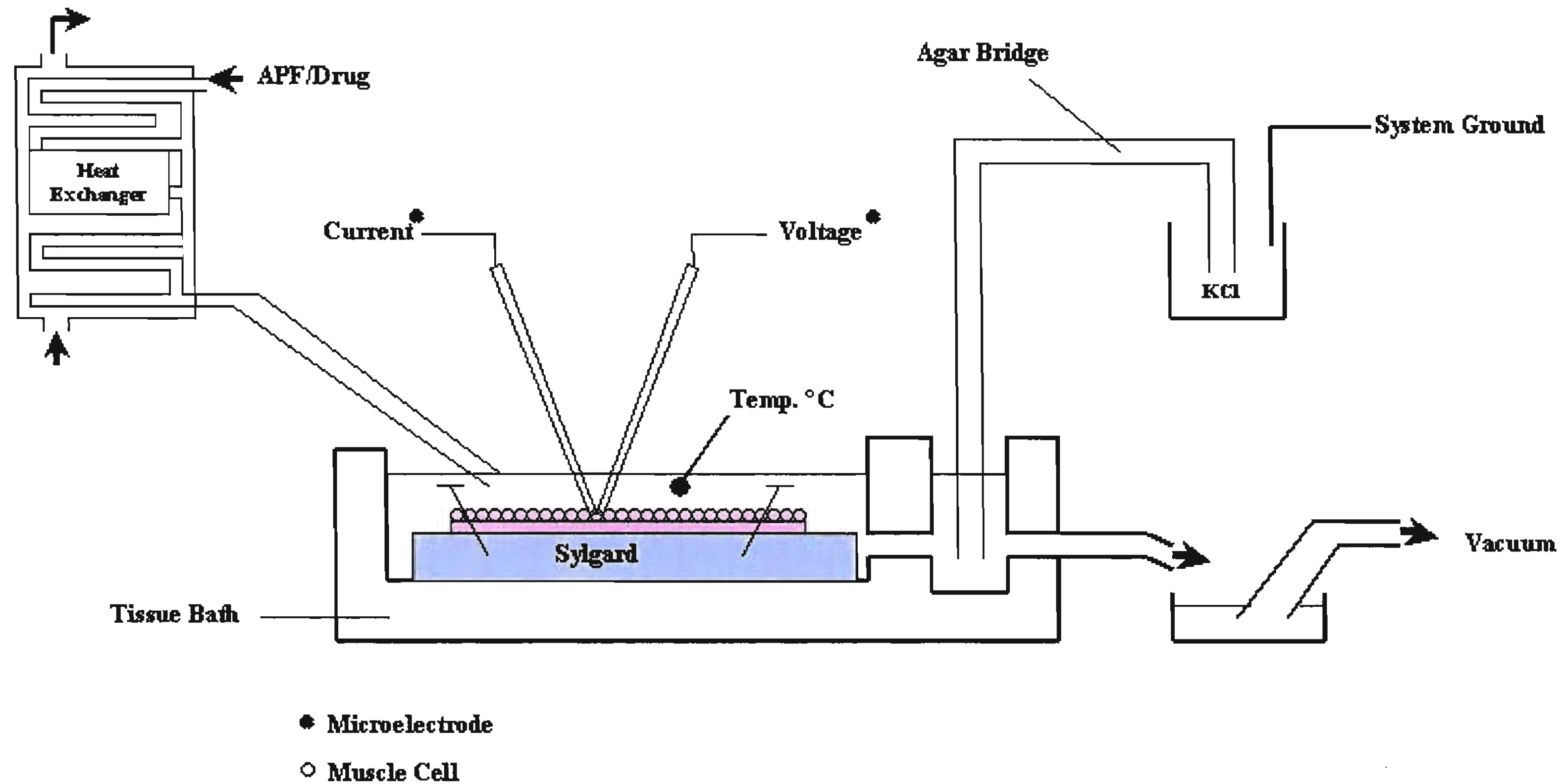


Figure 2.3. The electrophysiological set-up. Schematic representation of the two microelectrode set-up used for electrophysiological experiments on the *A. suum* dorsal muscle strip.

2.5 Drugs

ACh (Sigma Chemical Co., Poole UK) was prepared on the day of the experiment in APF. Peptides were synthesized either by Alta Bioscience (Birmingham, UK) or Southampton Polypeptides Ltd (Southampton, UK) to approximately 90% purity. 1mM stocks were stored at -20°C in distilled water.

For organ bath experiments ACh was added to the tissue for 30s and peptides were added for 5 min prior to the addition of ACh. For all electophysiological experiments the tissue was perfused with ACh for 30s and with peptides for 5 min prior to the addition of ACh.

2.6 Data Analysis

For each individual muscle strip tension experiment a consistent response to 30 μM ACh was first obtained. This value was selected since it gave a submaximal contraction of the muscle. The peak response was measured and deemed the maximal response. Subsequent responses were normalised with respect to maximal responses.

2.7 Statistical comparison

Data are presented as the mean ± S.E.M of 'n' experiments (a muscle strip from a different animal was used for each experiment). Statistical significance was determined using either a paired or unpaired two tailed Student's t-test with a significance level of $p<0.05$. (Pang *et al.,* 1994). The ACh excitation curve was fitted to the modified logistic equation using Graph pad prism (version 3.0 San Diego California).

2.8 Culturing of *C. elegans*

C. elegans were cultured under standard conditions (Brenner, 1974). Nematode growth medium (NGM) for the *C. elegans* was made, pre-autoclave, with the following composition per 500ml; NaCl 1.5g, Agar 10g, Peptone 1.25g, distilled water 487.5ml. Post-autoclave the medium was supplemented with the following 1M sterile solutions; $CaCl_2$ 0.5ml, $MgSO_4$ 0.5ml, KH_2PO_4 12.5ml, cholesterol 0.5ml of 5mg/ml stock.

Hermaphrodite animals were fed and grown on a bacterial lawn (*E. coli,* OP50 strain) and picked for experiments from 3-5 day old plates. Wild type Bristol N-2 were obtained from *Ceanorhabditis* Genetics Centre. *egl-8* (n488) were obtained from Horvitz Lab and *egl-30* (ad810) from Leon Avery Lab.

2.9 Dissection of *C. elegans* pharynxes

Individual *C. elegans* were placed in a Petri dish containing modified Dent's saline of the following composition (mM): NaCl 144; $MgCl_2$ 1; $CaCl_2$ 1; KCL 6; glucose 3 HEPES 5; pH 7.4, and transiently cooled to immobilize them. The anterior region was sectioned from the animal by cutting, using a razor blade, just posterior to the pharynx. The preparation, consisting of the pharynx, nerve ring and enteric nervous system, was transferred to the recording chamber on a microscope stage, where all experiments were carried out at room temperature.

2.10 Electopharyngeogram (EPG) recording

The method for recording EPGs was adapted from Avery *et al.,* (1995). Suction pipettes were pulled from borosilicate glass (Harvard Instruments, glass diameter 1mm). Tip diameter ranged from 20 to 40 μM depending on the size of the worm. The suction pipette was filled with Dent's saline and mounted in a holder with a tubing port through which suction can be applied. The pipette was lowered into the recording chamber and placed close to the anterior end of the preparation, Figure 2.4. Suction was applied to hold the preparation in place. The suction pipette was

connected to an Axoclamp 2B recording amplifier. The reference electrode was a silver chloride-coated silver pellet in 3M KCl connected to the recording chamber by an agar bridge. Extracellular voltage recordings were made in 'bridge' mode and the extracellular potential was set to 0 mV using the voltage off-set immediately prior to recording. Data were acquired using Axoscope (Axon Instruments) and stored for subsequent analysis.

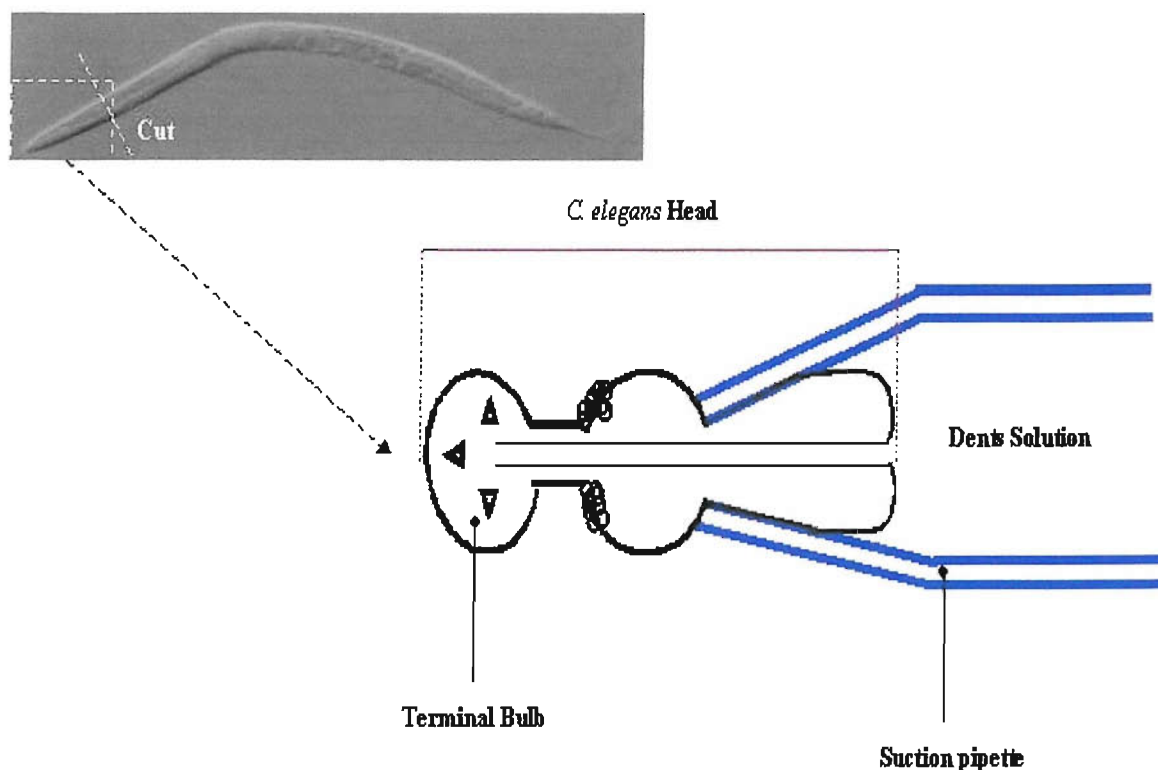


Figure 2.4 Experimental set-up for recording EPGs from *C. elegans* Pharynxes. The pharynx was exposed by cutting just above the pharyngeo-intestinal valve. A suction pipette is then placed on the nose of the worm. Suction is then applied to hold the preparation.

The volume of the recording chamber was 1ml. Initial recordings were made from pharynxes in Dent's saline for two minutes. The peptides were applied to the preparation by removing and replacing solutions with a 1ml pipette. The frequency of pharyngeal pumping was measured for 1 min before the peptide application, and for 1 min after peptide application. The effect of the peptides on the frequency of the

pharyngeal pumping is expressed as a percentage change compared to the resting rate immediately before the addition of the peptide. Following a 2 min application of the peptide the preparation was washed for 5 min and recovery was measured during the final 1 min.

The resting frequency of EPGs is relatively slow ~0.1 Hz. Therefore, in order to more accurately quantify the actions of inhibitory peptides, in some experiments the preparations were stimulated with 500 nM 5-HT throughout the experiment. In these experiments Dent's saline was replaced with Dent's containing 500nM 5-HT for 2 min. The concentration is sub-maximal (Rogers *et al.,* 2001) and stimulates a consistent background level of pharyngeal pumping against which the inhibitory actions of drugs can be measured. This was followed by application of the peptide in 500 nM 5-HT for 10 min and a subsequent wash, also in Dent's with 5-HT.

For the experiment where the levels of cAMP were altered, initial recordings were made from pharynxes in Dent's saline for 2 min. FLP17A or 5-HT was applied to the preparation by removing and replacing solutions with a 1 ml pipette. This was performed three times for each change to ensure that the majority of the saline in the chamber was exchanged. The frequency of EPGs was measured for 1 min following application of FLP17A or 5-HT. Following the 2 min application of either FLP17A or 5-HT, the preparation was washed for 5 min and recovery was measured during the final 1 min. The test compounds, dibutyryl-cAMP, forskolin, ibudilast, H9-dihydrochloride or 8-bromo-cGMP, were applied in the same way as FLP17A or 5-HT to determine their direct effect on pumping. They were then applied with FLP17A or 5-HT and left for 2 min. The preparation was then washed as above and the pumping rate measured in the absence of the compounds. Forskolin was dissolved in DMSO solvent and ibudilast in ethanol. The final DMSO and ethanol concentrations applied to the pharynx were 0.1%.

2.11 Drugs

Peptides were synthesized by either Alta Bioscience (Birmingham, UK) or Southampton Polypeptides Ltd (Southampton, UK), to approximately 90% purity. 1mM stocks were stored at -20°C in distilled water. 5-HT creatinine sulphate was

obtained from Sigma, Poole, UK. dibutyryl-cAMP sodium salt, 8-Bromo-cGMP sodium salt, forskolin, ibudilast and H9-dihydrochloride, were all obtained from Tocris (Bristol, UK).

2.12 Data Analysis

The results are expressed as the percentage change in frequency, ± S.E.M. for 'n' individual pharynxes. Each peptide was tested on a separate pharynx.

$$\text{Change \%} = \frac{[(\text{Freq. during addition Hz})-(\text{Freq. before addition Hz})]}{(\text{Freq. before addition Hz})} \times 100$$

Significance was measured using paired, two tailed Student's t-test (Rogers *et al.,* 2001). Concentration-response curves were plotted by fitting the data to the modified logistic equation (GraphPad Prism, San Diego, USA) and EC_{50} values are given with 95% confidence limits.

CHAPTER 3

Effect of FLP and NLP peptides on *Ascaris suum* somatic muscle

3.1 Introduction

Since the late 1980s, immunocytochemical studies involving the application of antisera raised to a wide range of peptides have established that nematodes possess an extensive and varied peptidergic component in their nervous system (Sithigorngul *et al.*, 1990; Brownlee *et al.*, 1994a,b; Brownlee *et al.*, 1996). Among peptide immunoreactivities demonstrated, FMRFamide staining predominates, with up to 75% of nerve cells in *A. suum* (Brownlee *et al.*, 1994a) and 50% in *C. elegans* (Li, *et al.*, 1999b) displaying FMRFamide-like immunoreactivity. Members of the RFamide family were subsequently identified in *A. suum* (Cowden *et al.*, 1989; Cowden and Stretton, 1993). As a result of earlier studies around twenty RF peptides were identified in *A. suum* and divided into four groups based on their structures (Davis and Stretton, 1996). More recently another ten RFamides have been identified using mass spectrometry (Yew *et al.*, 2005).

A gene encoding six FMRFamide-like peptides in the parasitic nematode *A. suum* has been isolated (Edison *et al.*, 1997). It contains novel sequences for AF13, AF14 and AF20 (Davis and Stretton, 1996; Edison *et al.*, 1997), in addition to the peptides AF3, AF4 and AF10 previously described (Davis and Stretton, 1995).

Neuropeptides also play a key role in the nervous system of *C. elegans* since up to 50% of the neurones demonstrate positive immunoreactivity for FMRFamide-like peptides (Li et al., 1999b). A large family of twenty-nine genes predicted to encode FMRFamide-like peptides, the *flp* genes, are expressed in just over 150 of the 302 neurones in adult hermaphrodites (Kim and Li, 2004). These *flp* expressing neurones are involved in a number of behaviours, including chemo-, mechano- and thermosensation, locomotion, defecation, feeding and reproduction.

The FLP family represents only one neuropeptide gene family in *C. elegans*. Forty-two *C. elegans* neuropeptide-like protein (*nlp*) genes were predicted from the *C. elegans* genome (Nathoo *et al.*, 2001; Li 2005). These *nlp* genes define at least 11 sub-families of putative neuropeptides with unique motifs. *Nlp* genes were found to be predominantly expressed in neurons and the endocrine tissues (Nathoo *et al.*, 2001). Many of the *nlp* genes are expressed in chemosensory neurones while other

NLPs have anti-microbial properties and their genes are expressed in the hypodermis (Li 2005). Currently there are around 125 NLPs, of which a significant number are not amidated.

A number of the FMRFamide-like peptides are common to both *C. elegans* (designated FLP) and *A. suum* (designated AF). These include FLP6, FLP8, FLP11A, FLP12, FLP14, FLP16A and FLP21 which are the same sequence as AF8, AF1, AF21, AF24, AF2, AF15 and AF9, respectively (Yew *et al.,* 2005).

The aim of this study was to investigate the physiological effects, of synthetic replicates of a number of *flp* and *nlp* peptides, predicted from the *C. elegans* genome, on *A. suum* somatic muscle. To provide an insight into the effect of a number of FLP and NLP peptides an *in vitro* preparation of the dorsal muscle strip of the intestinal parasite *A. suum* was used. The *A. suum* dorsal muscle strip preparation provides a bioassay for peptides that have a physiological role in the motor nervous system. The muscle preparation consists of the dorsal muscle and the dorsal nerve cord, which contains projections of the excitatory and inhibitory motoneurones. Changes in *A. suum* muscle state can therefore be measured using tension recording. This will allow the peptides' effects on *A. suum* muscle directly or following treatment with neurotransmittes to be investigated. As most of these peptides had no action on the basal muscle tension their action on the contraction elicited by ACh was determined. ACh has previously been shown to elicit contraction by activation of a nicotinic receptor on the somatic muscle (Colquhoun *et al.,* 1991). ACh caused a concentration – dependent excitation of *A. suum* muscle with an EC_{50} of 9 ± 2 μM which agrees with previous values.

Four *C. elegans* FMRFamide-like peptides were chosen which probably act on different receptors but only two are true FxRFamides. The other two have TMRFamide and PLRFamide as their C terminal sequences. One peptide, GLGPRPLRFamide is also found in *A. suum* and is named AF9. In addition three *C. elegans* NLPs were also tested for activity.

The use of electrophysiological techniques on *A. suum* muscle bag cells has provided an insight into the action of neurotransmitters in controlling muscle

contraction and relaxation. (Martin *et al.,* 1991; Walker *et al.,* 2000; Stretton *et al.,* 1978).

3.2 Results

3.2.1 The effect of varying ACh concentrations on *A. suum* dorsal muscle strips (DMS).

The excitatory transmitter ACh caused a concentration dependent contraction of *A. suum* muscle DMS (EC_{50} = 9.2 μM, n = 6, 95 % confidence limits 8 to 11 μM Fig. 3.1) in a manner similar to that previously reported (Baldwin & Moyle, 1949; Trim *et al.*, 1997), confirming that this technique is viable for investigating the mechanism of action of neuropeptides.

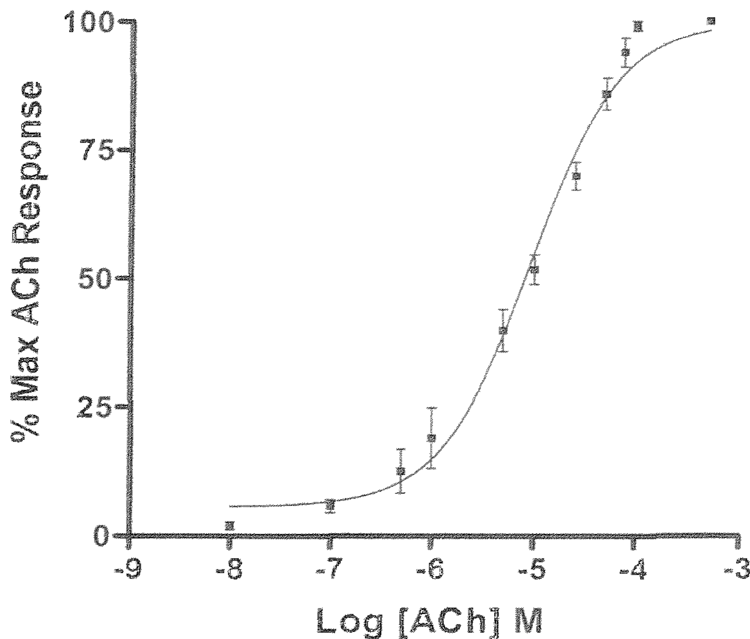
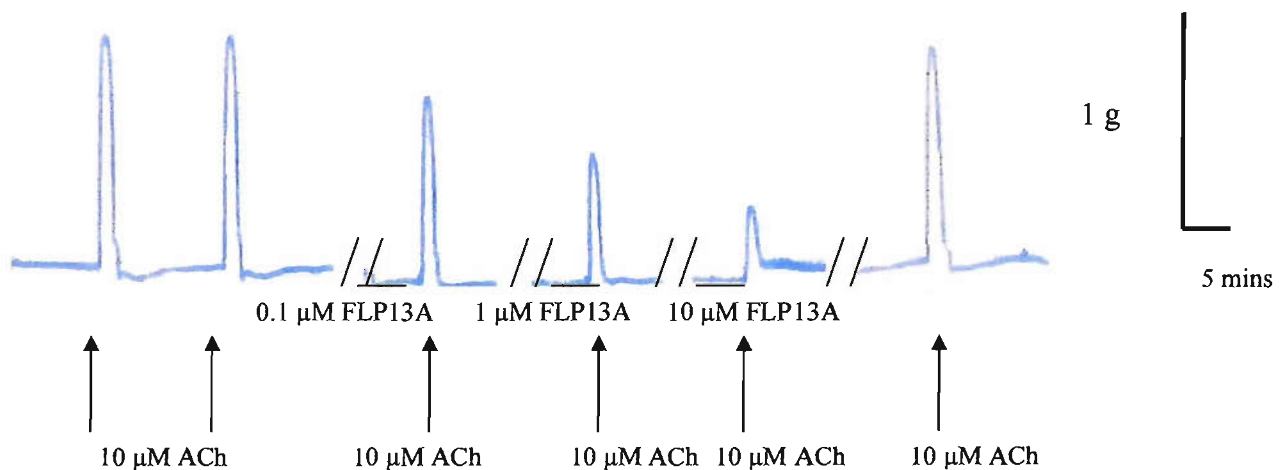


Figure 3.1 The effect of ACh on *A. suum* dorsal muscle strips (DMS). Acetylcholine causes a concentration dependent contraction of *A. suum* muscle with an EC_{50} of 9.2 μM. Each point is the mean ± S.E.M. of 6 experiments on individual DMS. Results are expressed as a percentage of the maximum response which was observed at 1 mM.

3.2.2 The effect of FLP13A (APEASPFIRFamide) on ACh induced DMS contraction.

The *C. elegans* peptide FLP13A was tested on *A. suum* dorsal muscle. FLP13A had no direct effect on DMS but concentrations of 0.1 μM, 1μM and 10 μM (n=5) applied to the muscle strip 5 min prior to ACh significantly reduced the amplitude of the contractions elicited by ACh in a concentration dependent manner (Fig. 3.2). The IC_{50} for the FLP13A effect on ACh elicited contractions was approximately 0.3 ± 0.05 μM (mean ± S.E.M.)

A



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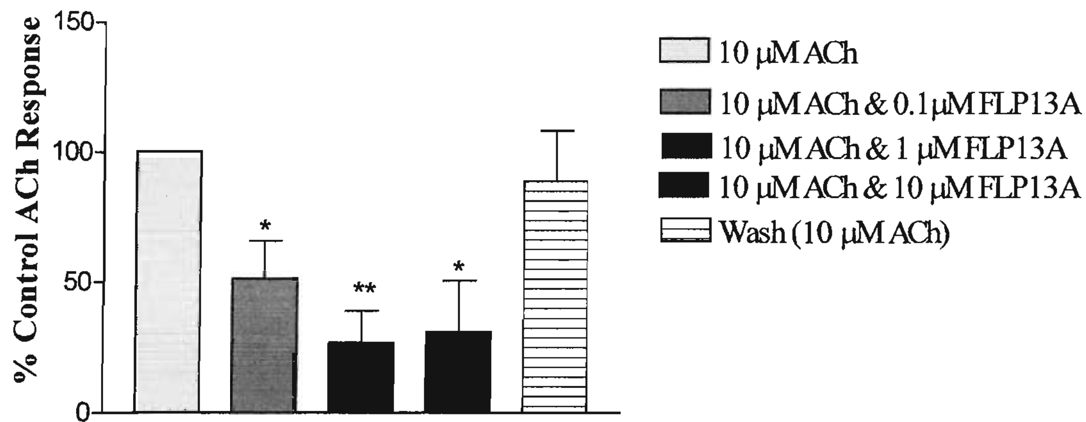
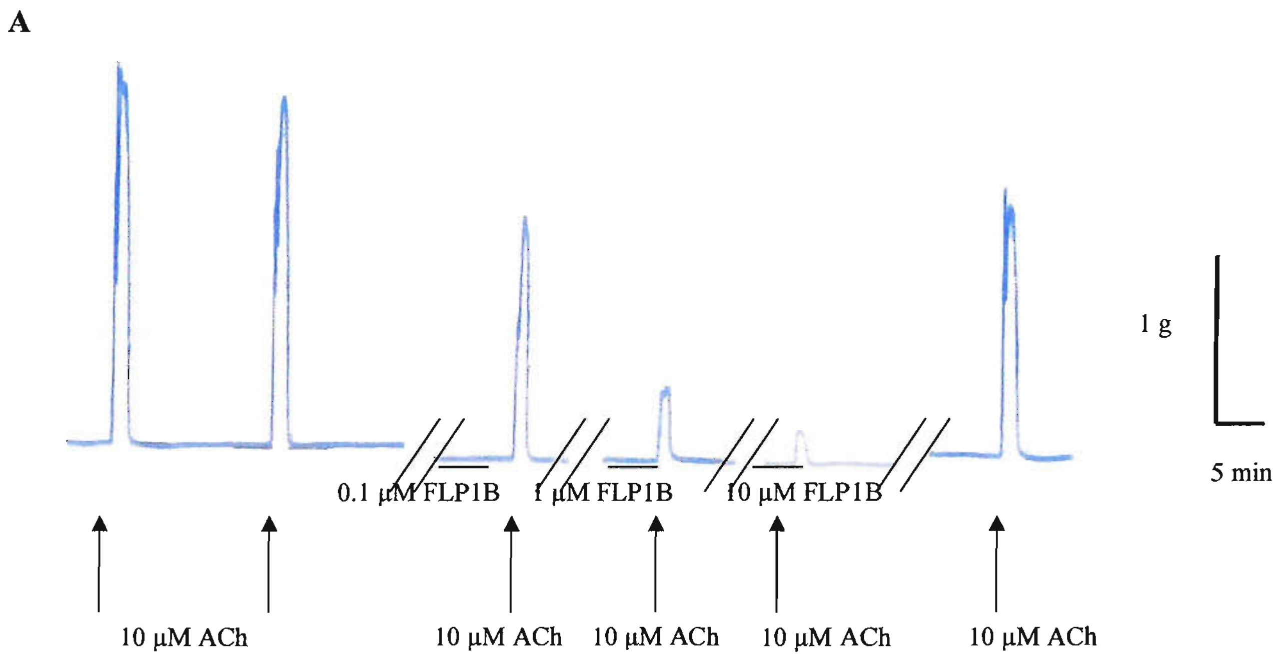


Figure 3.2 FLP13A (APEASPFIRFa) caused a concentration dependent reversible reduction of the concentration elicited by ACh. A. Example trace of the effect of FLP13A on muscle contraction elicited by ACh. Arrows indicate application of ACh and horizontal bars indicate application of FLP13A. **B.** Pooled data for different concentrations of FLP13A (n=5). The percentage inhibition of the response to ACh in the presence of FLP13A is expressed as a percentage of the amplitude of the concentration to ACh prior to the addition of the peptide. *P<0.05, **P<0.01.

3.2.3 The effect of FLP1B (AGSDPNFLRFa) on *A. suum* muscle.

FLP1B had no direct effect on DMS. Concentrations of 0.1 µM, 1 µM and 10 µM, FLP1B applied to the DMS 5 min prior to the addition of ACh, significantly reduced the amplitude of the concentration elicited by 10 µM ACh in a concentration dependent manner (Fig 3.3 n = 5) The IC_{50} for the FLP1B effect on ACh elicited contractions was 0.5 ± 0.1 µM. The effect of FLP1B was reversible upon washing indicating a non-toxic effect of the peptide on the muscle.

A



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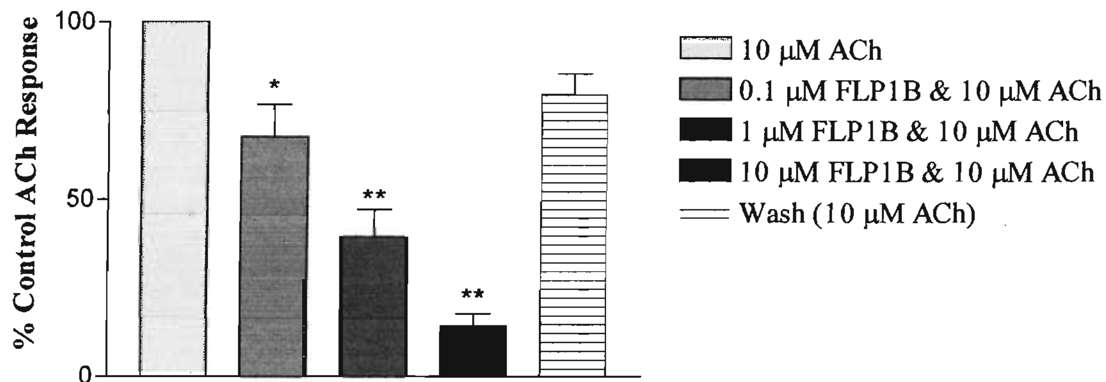
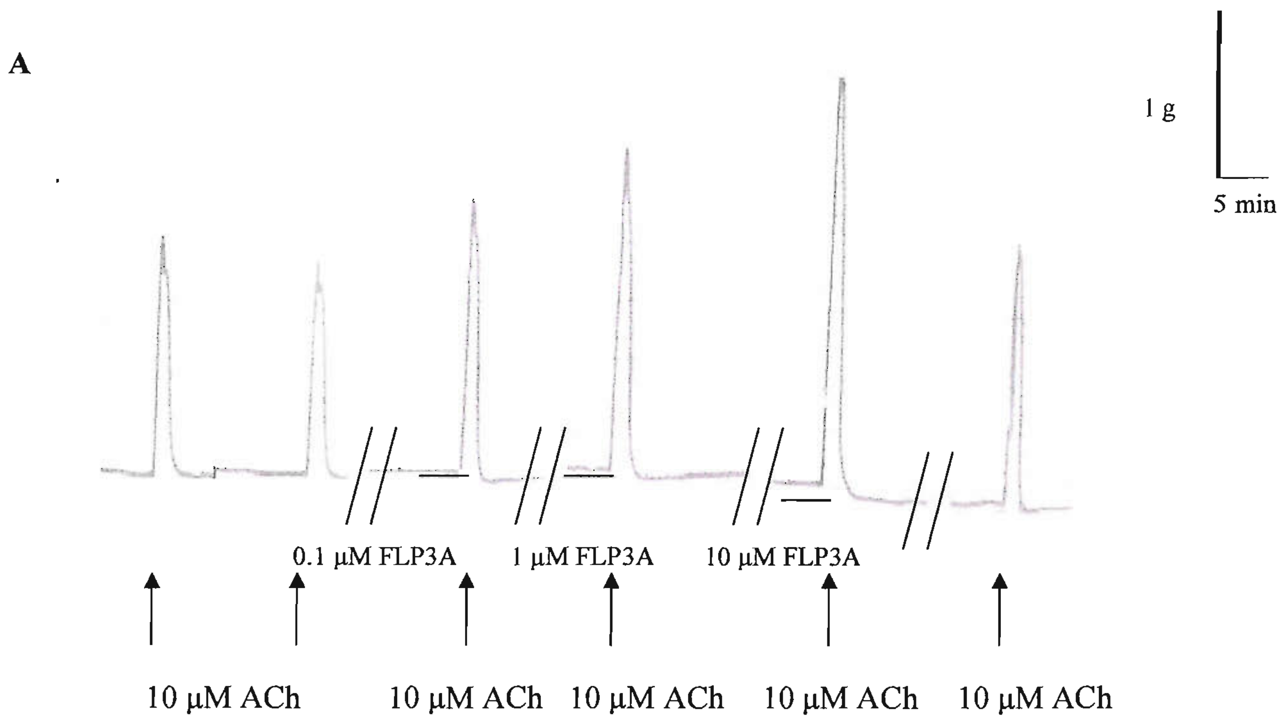


Figure 3.3. Effect of addition of FLP1B on ACh induced contractions of *A. suum* DMS. A. Representative response of a 5 minute of FLP1B application on *A. suum* DMS prior to the addition of ACh. **B.** Pooled data (n=5) for the effect of pre-incubation of the muscle with 0.1 μM, 1 μM and 10 μM FLP1B on the amplitude of the contraction to ACh. *P<0.05, **P<0.01.

3.2.4 The potentiation of ACh contractions by FLP3A (SAEPFGTMRFamide)

Muscle contractions caused by 10 μM ACh were significantly potentiated by pre-treatment of the muscle strips with various concentrations of FLP3A. The DMS were exposed to 0.1 μM, 1 μM and 10 μM FLP3A (Fig 3.4 n = 5) for 5 min prior to the addition of ACh. The EC_{50} for the FLP3A effect on ACh was 0.7 ± 0.2 μM. This effect of FLP3A was reversible upon washing. In two out of 5 preparations 10 μM FLP3A caused an average muscle contraction of 3.88g. The effect was long lasting and no full reversal of the contraction occurred following washing.

A



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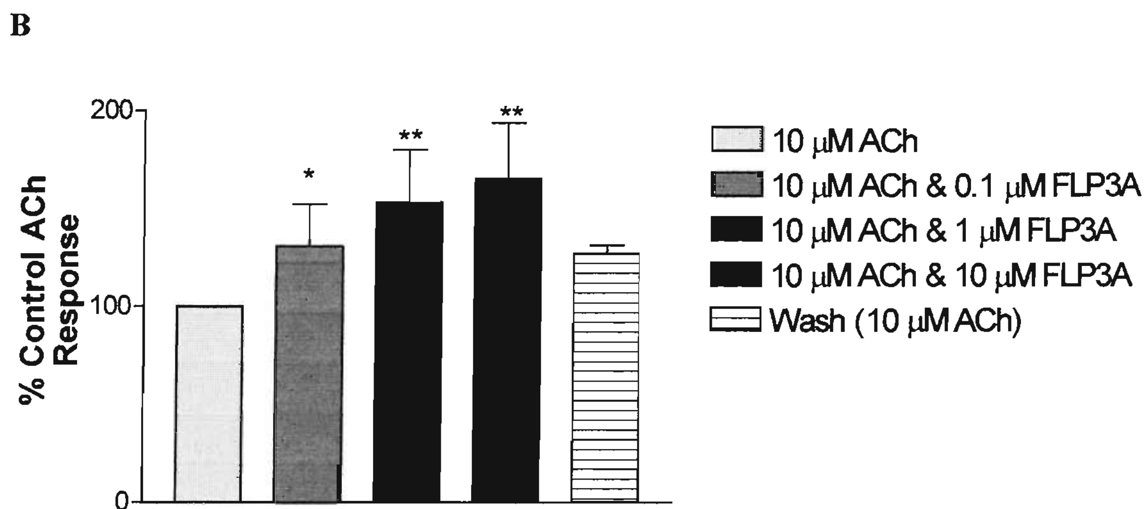


Figure 3.4 FLP3A (SAEPFGTMRFa) caused a concentration dependent reversible potentiation of the contraction elicited by ACh. A. Example trace of the effect of FLP3A on muscle contraction elicited by ACh. Arrows indicate application of ACh and horizontal bars indicate the duration of application of 0.1 μM, 1 μM and 10 μM FLP3A. **B**. Pooled data (n = 5) for different concentrations of FLP3A. *P<0.05, **P<0.01.

3.2.5 The effect of FLP21 (GLGPRPLRFa) (AF9) on *A. suum* muscle.

FLP21 had a direct effect on tension in the *A. suum* dorsal muscle strip. FLP21 elicited a potent contraction of the DMS in a concentration dependent manner (Fig 3.6 n = 2). 1 μM FLP21 elicited a mean contraction of 3.3 ± 0.8 g (Fig. 3.6 n = 2). The effect of FLP21 was long-lasting with full reversal occurring within 1 hr of washing. The effect of FLP21 on contractions elicited by ACh was also investigated. Concentrations of 0.1 μM, 1 μM and 10 μM FLP21 applied to the DMS 5 min prior to the addition of 10 μM ACh increased the amplitude of the ACh contractions in a concentration dependent manner (n = 2). The EC_{50} for the effect of FLP21 on ACh contractions was greater than 0.1 μM. This effect of FLP21 was reversible upon washing (Fig. 3.5).

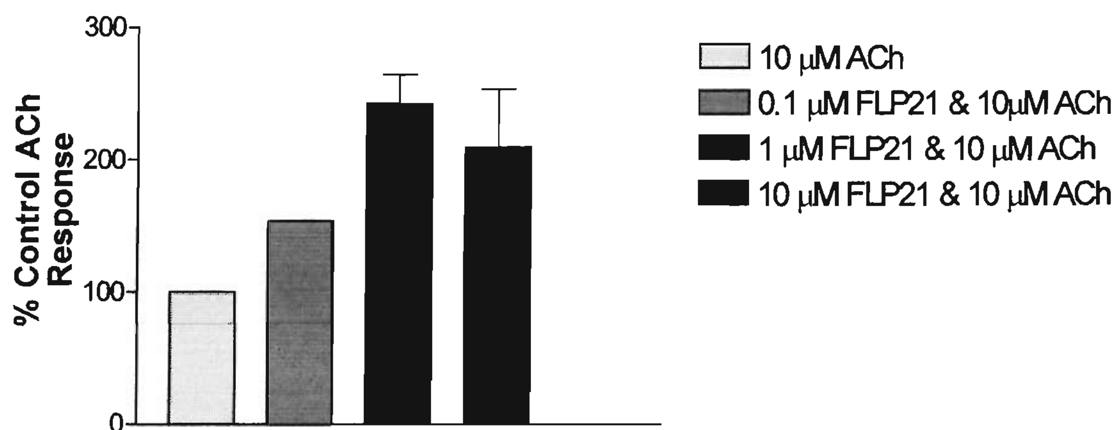


Figure 3.5 FLP21 caused a concentration dependent reversible potentiation of the contraction elicited by ACh. Pooled data for the effect of pre-incubation of the muscle with various concentrations of FLP21 on the amplitude of the contraction to ACh.

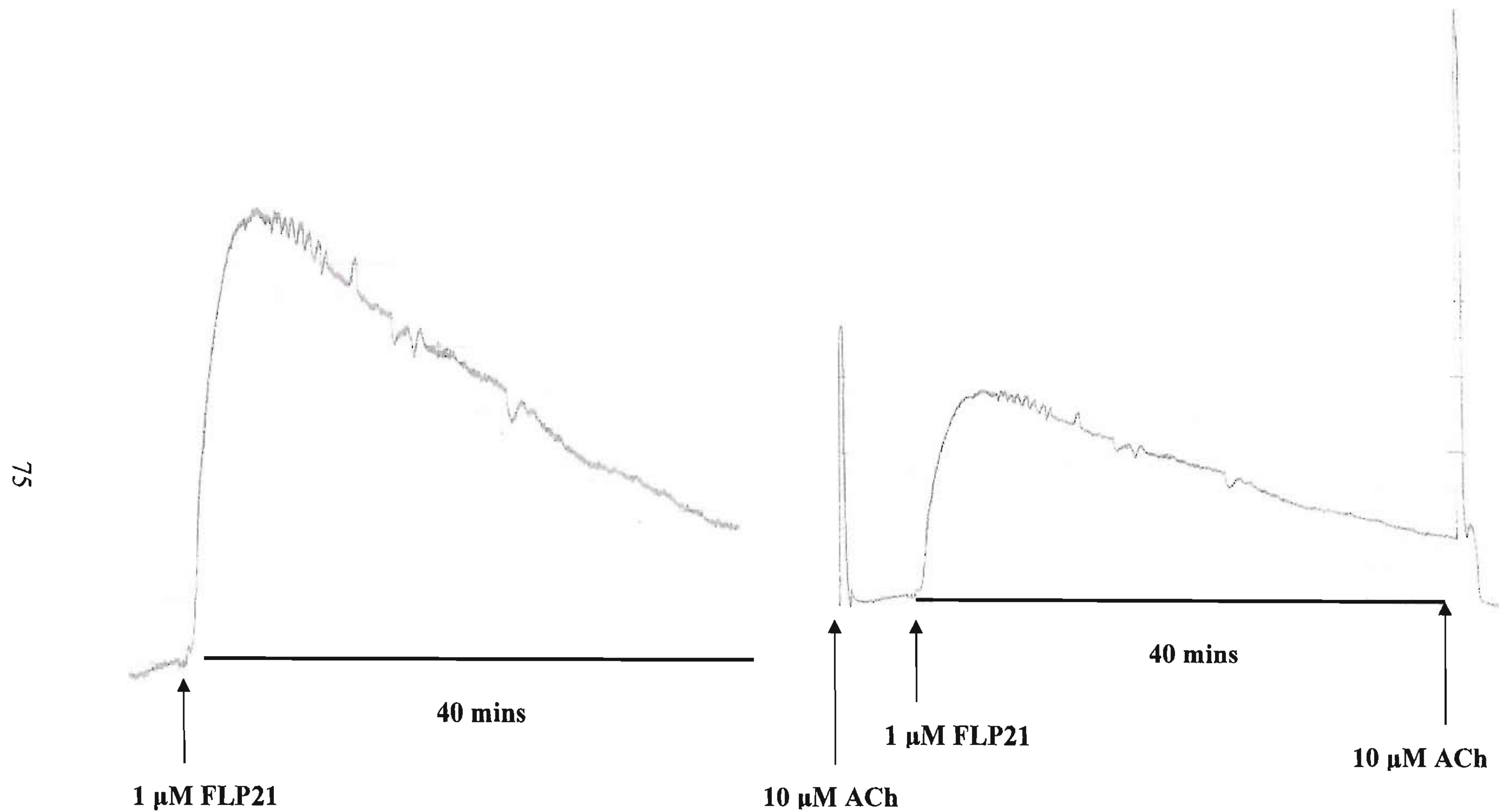


Figure 3.6 Effect of FLP21 on DMS and on amplitude of contractions elicited by ACh. A. Representative response of 1 µM FLP21 on *A. suum* DMS. **B.** Representative response of the pre-incubation of the muscle with 10 µM FLP21 on the amplitude of the contraction to ACh.

3.2.6 The effect of NLP peptides on the A. suum muscle.

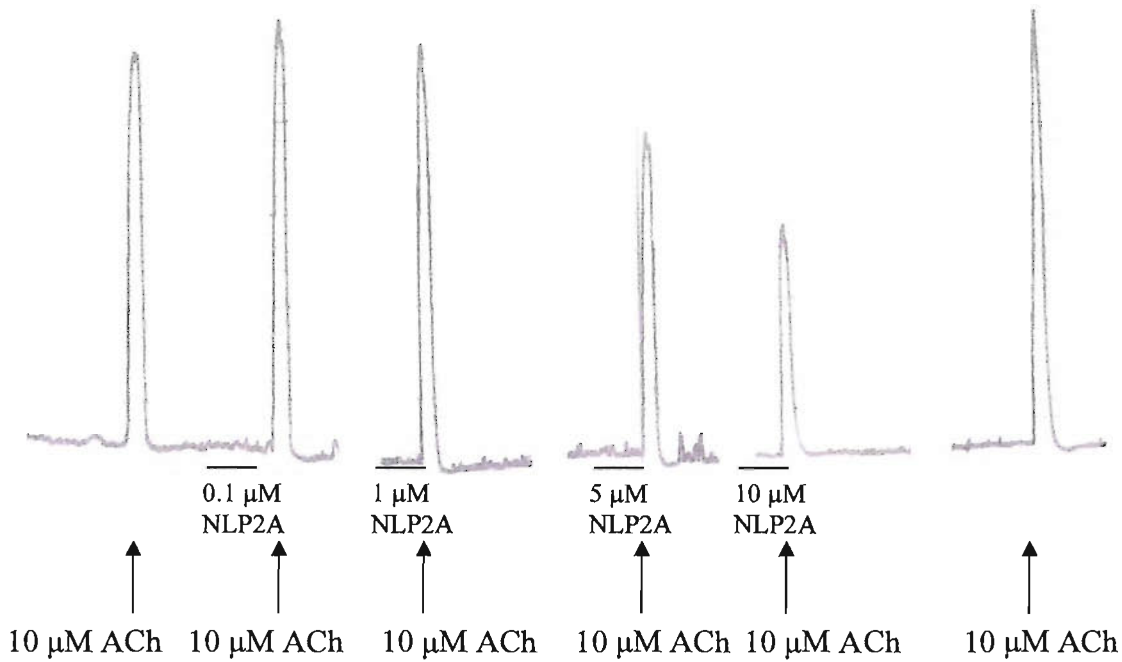
The *flp* gene family represents only one neuropeptide gene family in *C. elegans*. Forty-two, *C. elegans nlp* genes, which encode at least 11 sub-families, of putative neuropeptides have been identified from the *C. elegans* genome (Nathoo et al., 2001; Li 2005).

3.2.6.1 The effect of NLP2A on *A. suum* muscle.

The *nlp-2* gene predicted from the *C. elegans* genome encodes a number of myomodulin-like peptides. The effect of two of these peptides NLP2A (SMAMGRLGLRPa) and NLP2B (SMAYGRQGFRPa), on *A. suum* DMS was investigated.

NLP2A had no direct effect on the DMS but concentrations of 0.1 μM, 1 μM, 5 μM and 10 μM added to the DMS 5 minutes prior to the addition of ACh significantly reduced the amplitude of contractions elicited by 10 μM ACh in a dose dependent manner (Fig 3.7, n = 5). The IC_{50} for this effect of NLP2A on ACh contractions is 78 μM ± 3.8 μM. The NLP2A effect was reversible upon washing.

A



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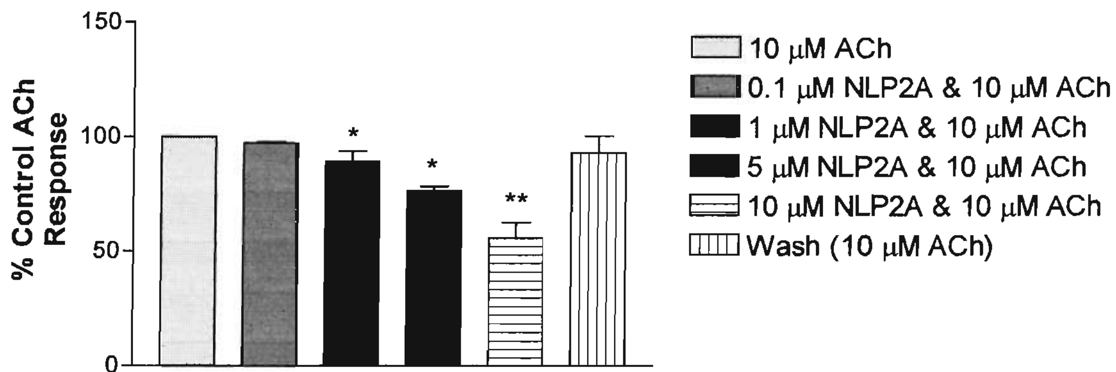


Figure 3.7 NLP2A (SMAMGRLGLRPa) caused a concentration dependent reversible inhibition of the contraction elicited by ACh. **A.** Example trace of the effect of NLP2A on muscle contraction elicited by ACh. **B.** Pooled data (n = 5) for different concentrations of NLP2A. * $P<0.05$, ** $P<0.01$.

3.2.6.2 The effect of NLP2A on *A. suum* muscle cells

The resting membrane potential of *A. suum* dorsal strip muscle cells was -24 ± 1.6 mV (n = 10) (1 experiment on 1 DMS). The mean resting input conductance was 1.11 ± 0.2 *μS*. Perfusion of ACh directly over muscle cells elicited an immediate depolarisation and an increase in input conductance. In normal APF 10 μM ACh elicited a mean depolarisation of 5.6 ± 0.3 mV (n = 13) (1 experiment on 1 DMS) and a conductance increase of 0.2 ± 0.0 2 *μS* (n = 13).

NLP2A had no direct effect on dorsal muscle cells. Application of 10 μM NLP2A on dorsal muscle cells 5 minutes prior to perfusion with 10 μM ACh significantly reduced the depolarisation caused by ACh while the input conductance was not affected (Fig. 3.8, n = 5). This effect of NLP2A was reversible upon washing.

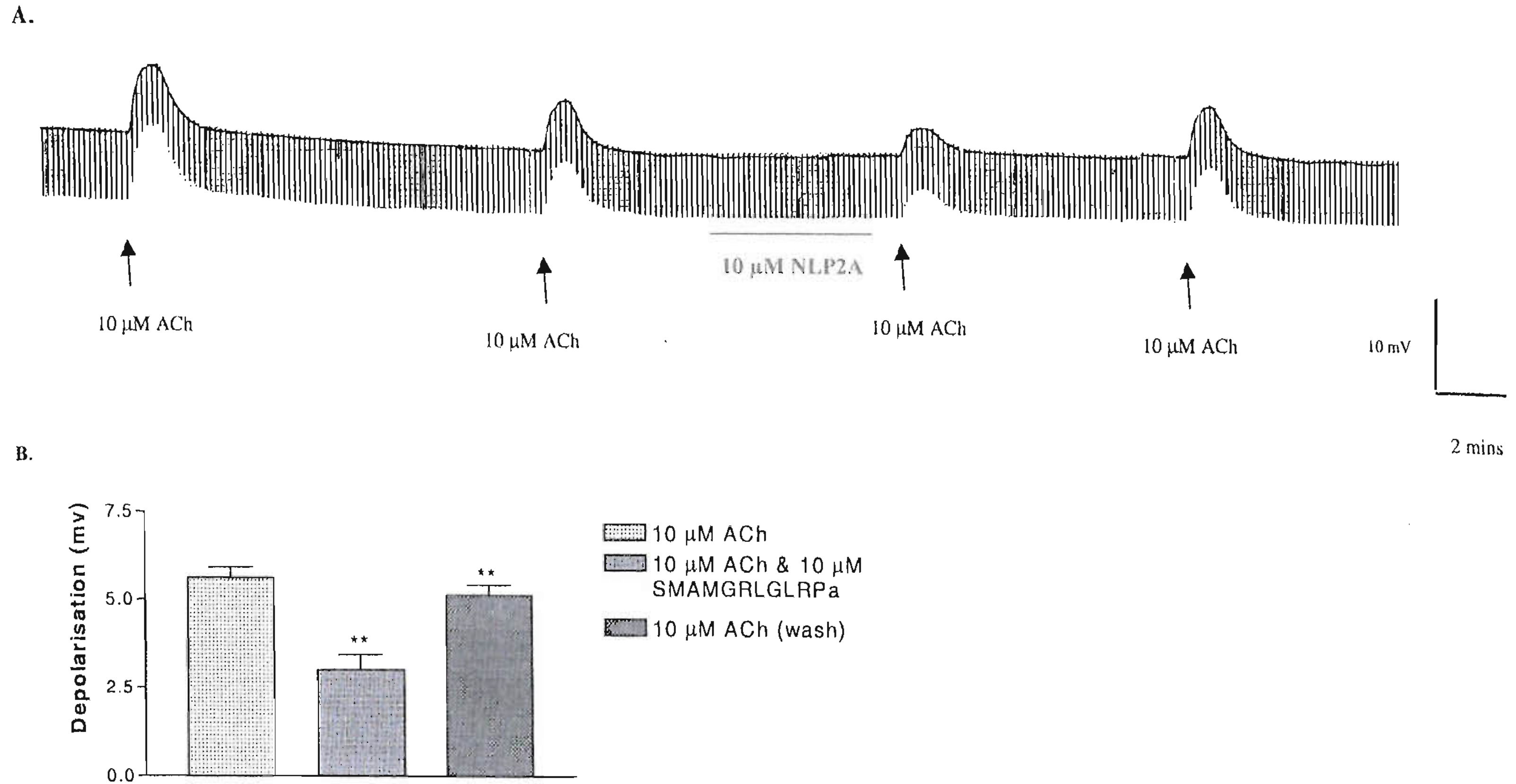


Figure 3.8 The effect of 10 µM NLP2A on depolarisation caused by 10 µM ACh on *A. suum* muscle cells. 10 µM NLP2A inhibited the depolarisation caused by 10 µM ACh. **A.** Example trace of recording from a single cell. The resting membrane potential of the cell was –28 mV and the input conductance was 1.11 *µS*. . Downward deflections in the trace were elicited by current injections (0.1Hz, 20nA, 500 ms). 10 µM ACh elicited a depolarisation of 5.6 ± 0.3 mV and an increase in input conductance of 0.2 ± 0.0 2 *µS*. In the presence of 10 µM the ACh elicited depolarisation was reduced by 3 mV while input conductance was not affected. **B.** Pooled data (n = 5) of the effect of 10 µM NLP2A on depolarisation elicited by ACh. **P<0.01

3.2.6.3 The effect of NLP2B on *A. suum* muscle.

NLP2B is encoded by the same gene as NLP2A. NLP2B had no direct effect on DMS and had a very weak inhibitory effect on the amplitude of contractions elicited by 10 μM ACh (Fig. 3.9 n = 6). The amplitude of ACh contractions in the presence of 10 μM NLP2B was reduced by 17.5 ± 4.6 % (n= 6). This effect was reversible upon washing.

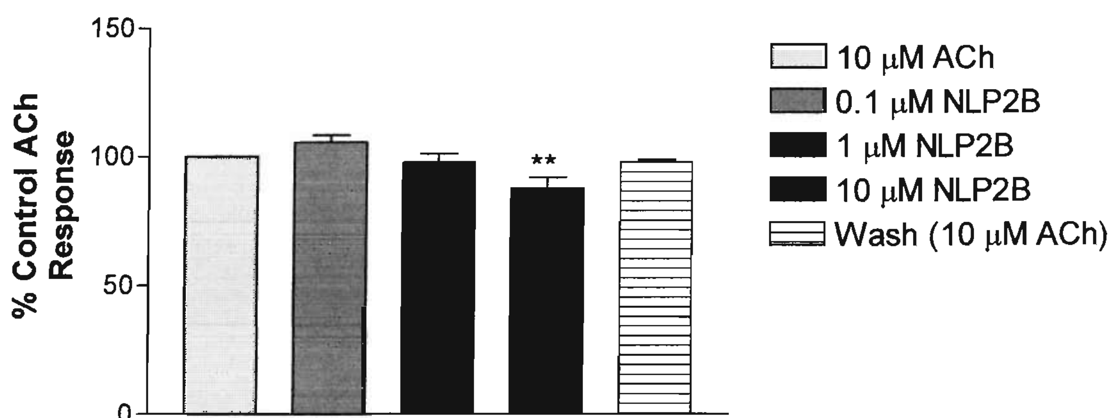


Figure 3.9 NLP2B had a weak inhibitory effect on the contraction elicited by ACh. Pooled data (n = 6) of the effect of various concentration of NLP2B on the amplitude of contraction elicited by ACh. ** P<0.05.

3.2.6.4 The effect of NLP1A (MDANAFRMSFa) on *A. suum* DMS.

NLP1A is one of the buccalin-like peptides encoded by *nlp1* gene of *C. elegans.* The effect of NLP1 on *A. suum* muscle was investigated. NLP1 had no direct effect on the DMS but a concentration of 10 μM had a weak inhibitory effect on the contraction elicited by 10 μM ACh (Fig. 3.10 n = 6). The effect of NLP1 was reversible upon washing.
The overall results are summarised in Table 3.1.

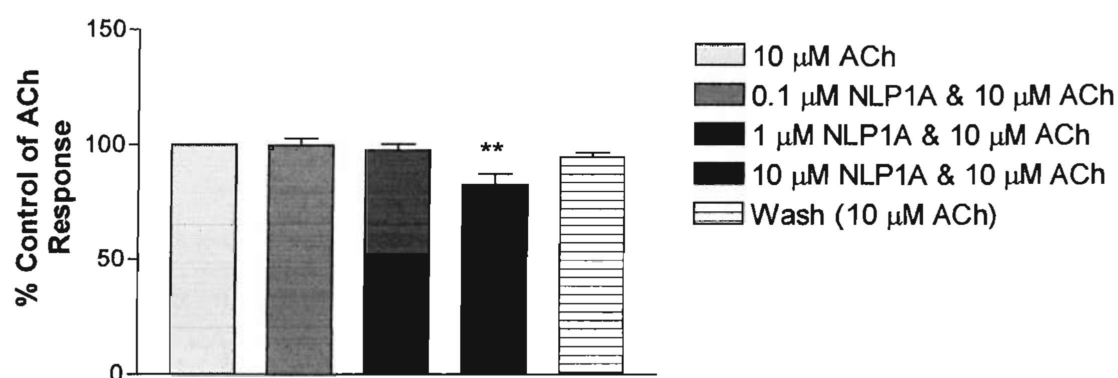


Figure 3.10 NLP1A had a weak inhibitory effect on the contraction elicited by ACh. Pooled data (n = 6) of the effect of various concentrations of NLP1 on the amplitude of contraction elicited by ACh. ** $P<0.05$.

Peptide	Direct effect	Effect on ACh contraction
FLP1B AGSDPNFLRFamide	None	Reduction IC_{50} 0.5μM
FLP3A SAEPFGTMRFamide	Direct	Potentiation EC_{50} 0.7μM
FLP13A APEASPFIRFamide	None	Reduction IC_{50} 0.3μM
FLP21 GLGPRPLRFamide (AF9)	Direct	Potentiation <1μM
NLP1A MDANAFRMSFamide	None	Reduction (weak)
NLP2A SMAMGRLGLRPamide	None	Reduction
NLP2B SMAYGRQGFRPamide	None	Reduction

Table 3.1 Summary of the actions of 4 FLPs and 3 NLPs on ACh-induced contractions of *A. suum* DMS.

3.3 Discussion

The widespread localisation of FaRP immunoreactive material in the nervous system of nematodes (Brownlee *et al.,* 1994; Li *et al.,* 1999b) suggests a role for FaRPs in the physiology of nematode locomotion, feeding and egg-laying.

Over the last few years it has become increasingly apparent that the somatic musculature of *A. suum* is subjected to modulation by a number of neuropeptides belonging to the FaRP family. For example, PF1 (SDPNFLRFa), PF2 (SADPNFLRFa) and PF4 (KPNFIRFa), isolated from *P. redivivus*, are all potent inhibitors of *A. suum* muscle (Holden-Dye *et al.,* 1995; Brownlee *et al.,* 1996; Holden-Dye *et al.,* 1996). AF1 and AF2 isolated from *A. suum* have a predominantly excitatory effect (Cowden *et al.,* 1989; Cowden and Stretton, 1993). AF3 and AF4 also isolated from *A. suum* share a common LRFamide C-terminal with AF2. AF3 and AF4 have an excitatory effect on *A. suum* muscle (Trim *et al.,* 1997). In their analysis Davis and Stretton (2001) proposed five main types of response for the 18 endogenous neuropeptides tested on *A. suum* motor nervous system.

Evidence suggests that there are a number of FaRP receptors in nematodes and so far eleven have been identified, all of which are G-protein coupled (Kubiak *et al.,* 2003a,b; Mertens *et al.,* 2004; Mertens *et al.,* 2005). Following ligand binding, the G-protein receptors either by actions on membrane ion channels or through second messenger intracellular signalling pathways, such as the elevation of intracellular calcium, changes in the intracellular concentration of cyclic AMP and/or activation of protein kinases, lead to the cellular response.

The exogenously applied FLPs and NLPs studied here may exert effects on the *A. suum* somatic muscle through G-protein receptors either presynaptically by modulating ACh or GABA release from the excitatory or inhibitory motorneurones or postsynaptically directly onto the muscle, respectively. There is also a possibility that the effect of the exogenously applied peptides is not physiologically relevant. This is particularly true for weak effects observed at high concentrations. In such cases it is

likely that the peptide may be acting via a receptor for which it is not the native ligand probably because of structural similarity to another peptide.

The biological activity of FLPs on *A. suum* somatic muscle.

The FLPs tested on the *A. suum* somatic muscle had a potent biological activity (Table 3.1) suggesting that a number of these peptides may have physiological roles in regulating the activity of the muscle. The concentration that gave a consistent response for each peptide was usually 1 μM although threshold responses were obtained at 0.1 μM. Two peptides FLP3A and FLP21 potentiated the response of the somatic muscle to 10 μM ACh while FLP21 had also a direct excitatory effect on the muscle lasting for more than 40 mins. FLP3 and FLP21 are encoded by *C. elegans flp3* and *flp21* genes respectively. FLP21 has also been isolated from *Ascaris* and is known as AF9. In previous electrophysiological studies AF9, 10 μM, produced a weak early transient hyperpolarisation, followed by a latter sustained depolarisation of about 15 mV (Davis and Stretton 2001). Injection of AF9 into the *A. suum* caused a great decrease in movement and locomotion and a decrease in head searching movements (Davis and Stretton 2001).

FLP3A and FLP21 might have presynaptic or postsynaptic actions or their effect can be partly presynaptic and partly postsynaptic as it was previously described for AF1 and AF2 peptides (Pang *et al.,* 1995). Both peptides potentiated the response of the muscle strip to ACh which is indicative of a postsynaptic effect. A presynaptic effect could have been identified by the use of ACh receptor antagonists in order to test whether they block the direct effect of the peptides on the muscle. The possibility of the involvement of second messenger systems in the response to FLP3A and FLP21 has not been investigated but it is possible that these peptides affect cAMP levels, which in turn through protein kinase A, modulates the phosphorylation state of a myosin light chain kinase-like protein that regulates the contractile elements.

FLP1B and FLP13A inhibited the response of the somatic muscle to 10 μM ACh with an IC_{50} of 0.5 μM and 0.3 μM respectively. These peptides had no direct effect on the muscle strip. FLP13A is one of the seven peptides encoded by the *flp-13* gene and has been isolated biochemically from *C. elegans* (Marks *et al.,* 1997).

FLP1B is one of the eight peptides encoded by the *C. elegans flp-1* gene. Two peptides encoded by the *flp-1* gene have also been identified in *P. redivivus* (Geary *et al.,* 1992) and were designated as PF1 and PF2. PF1 and PF2 cause a muscle relaxation in *A. suum* with a long time-course of action (Franks *et al.,* 1994). A *flp-1* deletion mutant shows hyper-locomotion among other phenotypes (Nelson *et al.,* 1998b).

There is evidence from this study that both FLP1B and FLP13A have a postsynaptic action as both peptides inhibited the contraction elicited by ACh but further studies are required to determine whether the action of these peptides is through the stimulation of GABA release. FLP1B shares common FLRFa C-terminal with PF1 and PF2 which inhibit the contraction elicited by ACh but this effect does not appear to be through GABA release (Franks 1996; Franks *et al.,* 1994).
FLP13A shares a common FIRFa C-terminal with AF1 and PF4. AF1 has a biphasic action on *A. suum* (Pang *et al.,* 1995) while PF4 causes a rapid relaxation (Maule *et al.,* 1996). This suggests that more than one receptors mediate the effects of these peptides.

The results presented in this study suggest that FaRP binding receptors are present in the neuromuscular system of *A. suum.* The IC_{50} and EC_{50} for the FLP peptides were in sub-μM concentrations suggesting that the receptors mediating the effects of these peptides have a reasonably high affinity for these ligands. FLP21 has been biochemically isolated form *A. suum* and so this peptide could have a role in the control of *A. suum* somatic muscle.

The biological activity of NLP peptides on *A. suum* somatic muscle.

The NLP peptides tested on *A. suum* muscle had a weak biological effect (Table 3.1). None of the three peptides tested had a direct effect on the muscle. NLP2B inhibited the response of the muscle to ACh with an IC_{50} of 78 μM while NLP2B and NLP1 inhibited the response to ACh with an IC_{50} of greater than 100 μM.

To date 42 *nlp* genes, encoding around 125 peptides, have been predicted from the *C. elegans* genome but none have been structurally characterised or analysed for

expression, or for their effect on neuromuscular physiology in parasitic species with the exception of one peptide NLP12, which has been shown to produce ventral coiling when injected into adult *A. suum* (Reinitz *et al.,* 2000). *nlp-12* cDNA has been characterised from *A. suum* and was found to be expressed in both head and tail tissue of adult female *A. suum* and application of NLP12 produced contraction of innervated and denarvated dorsal and ventral *Ascaris* body muscle with a threshold of 1 nM (Mc Veigh *et al.,* 2006).

The weak effect observed with the NLP peptides compared to the effect of the FLP peptides suggests that this effect is not physiologically relevant and possibly these peptides are acting through receptors for which they are not the true ligands, probably because of structural similarity to other peptides. In *C. elegans nlp-1 and nlp-2* genes are expressed in the head neurones (Nathoo *et al.,* 2001). To date it is not known whether these peptides are expressed in *A. suum.* Identification of the expression pattern of these genes will give a better insight into the physiological roles in *A. suum.* Because of their expression in sensory neurons in *C. elegans* it has been suggested that NLPs play an important role in conveying information from the environment (Li, 2005). It has also been suggested that NLPs may act as anti-microbials (Li, 2005). Such roles of NLPs in *A. suum* need to be investigated.

In conclusion the studies outlined above have only touched on the physiological role of the nematode FaRPs and NLPs. Many fundamental questions such as 'where are the neuropeptides expressed?' and 'how many receptors are there for FaRPs and NLPs' remain to be answered. Parallel comparative studies between *C. elegans* and *A. suum* with the use of deletion mutants to highlight those FaRPs and NLPs which play a key role in nematode physiology and the use of reported gene constructs which will enable the patter of gene expression to be visualised will be of some help in answering these questions.

CHAPTER 4

The role of FMRFamide-like family of neuropeptides in the pharyngeal nervous system of *Caenorhabditis elegans*

4.0 Introduction

The total number of different RFamide peptides in *C. elegans* is surprisingly large. The first were identified by Li and her group (Rosoff *et al.,* 1992; Rosoff *et al.,* 1993) and a further seventy peptides are predicted from post-translational processing of the *flp* gene products (Li *et al.,* 1999a; Li 2005). Some of these have been confirmed by biochemical isolation (Rosoff *et al.,* 1993; Marks *et al.,* 1998; Marks *et al.,* 1995; Marks *et al.,* 1997).

To date, the limited knowledge of the physiological role of the FLPs in *C. elegans* has been obtained largely from investigating the functional consequence of *flp* gene knockouts. For example, *flp-1* knockouts have defective locomotion and altered egg laying behaviour (Nelson *et al.,* 1998a; Waggoner *et al.,* 2000). The functional role of the FLP peptides can also be inferred from the expression pattern of the *flp* genes. Individual genes have discrete expression patterns in subsets of neurones that subserve specific animal behaviours (Kim and Li, 2004).

The biological activity of a small subset of the FLP neuropeptides on the pharyngeal muscle in *C. elegans* has been previously described (Rogers *et al.,* 2001).

The FLP gene family represents only one neuropeptide gene family in *C. elegans*. To identify novel peptidergic neurotransmitters, the *C. elegans* genome was searched for predicted proteins with structural hallmarks of neuropeptide pre-pro-proteins (Nathoo *et al.,* 2001). This search identified thirty-two *C. elegans* neuropeptide-like protein (*nlp*) genes. These *nlp* genes define at least 11 families of putative neuropeptides with unique motifs. *nlp* genes were found to be predominantly expressed in neurones and the endocrine tissues (Nathoo et al., 2001).

The role of the pharyngeal nervous system in regulating pharyngeal activity

There are twenty neurones in the pharyngeal nervous system and their role in regulating the pharynx has been studied by determining the consequence of laser ablation of specific neurones on the pharyngeal pumping (Avery and Horvitz, 1989).

Two neurones have an important role in controlling the timing of pharyngeal relaxation, M3 and I5 (Avery, 1993). In the absence of M3, relaxation of the pharynx is delayed, possibly through I5. In the absence of MC, pumping is slowed, making MC the main motorneurone responsible for rapid pumping (Raizen *et al.,* 1995). This neuron, which has been described as a neurogenic pacemaker for pharyngeal pumping (Raizen *et al.,* 1995), also has mechanoreceptor endings in the corpus muscle (Albertson and Thomson, 1976). Electron microscope analysis suggests that MC synapses onto marginal cells rather than directly onto pharyngeal muscle cells (Albertson and Thomson, 1976). Ablation of MC, M3 or I5 in larval animals causes a change in feeding behaviour and a decline in growth rate (Avery and Horvitz, 1989; Avery, 1993). The role of the motorneurones, NMSs, is less clear. They may act as neurosecretory neurones and have been shown to contain 5-HT (Horvitz *et al.,* 1982). There is also evidence that NSM directly stimulates the motorneurone MC (Niacaris and Avery, 2003). NSMs are also sensory-motor neurones (Albertson and Thomson, 1976) and may monitor pharyngeal activity and modulate neurotransmitter release of 5-HT. A 5-HT receptor (SER-1) is expressed in pharyngeal muscle and this receptor is probably the one activated when NSM is stimulated (Hamdan *et al.,* 1999). NSM also plays a role in communicating the presence of food to the rest of the animal. Currently, models for NSM function predict that it responds to the presence of food to release 5-HT, accelerate pharyngeal pumping and suppress locomotion, so maintaining the animal in an area of abundant food (Niacaris and Avery, 2003). The motorneurone M4 is essential for isthmus peristalsis, since if M4 is destroyed then peristalsis is absent and growth ceases (Avery and Horvitz, 1989). It is possible that M4 modulates excitation-contraction in the posterior isthmus (Shimozono *et al.,* 2004). A 5-HT receptor (SER-7b) is expressed on M4 and so this motorneurone can also be activated by 5-HT (Hobson *et al.,* 2003), probably released from I5 which synapses onto M4. SER-7 receptors are also expressed in MCs and M3s and so these receptors are probably involved in the regulation of pharyngeal pumping (Hobson *et al.,* 2006) A summary of the major synaptic connections of the key neurones within the pharyngeal nervous system is shown in Fig. 4.1.

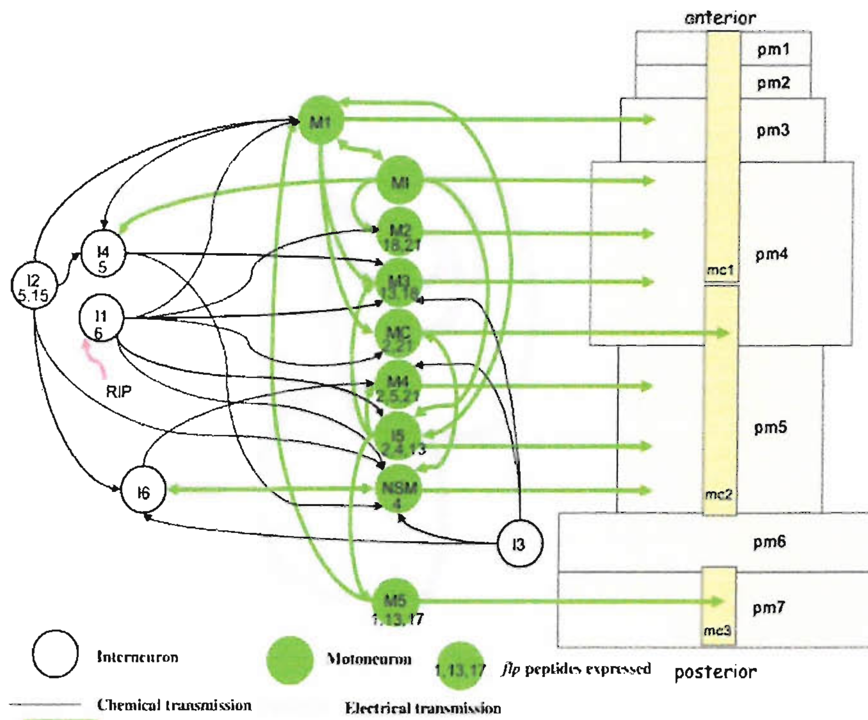


Figure 4.1. A diagram summarising the major synaptic connections in the pharyngeal nervous system of *C. elegans*. The diagram is based on Albertson and Thomson, 1976, www.wormatlas .org. and Chris Franks (personal communication). The cells of the pharynx, muscles (pm) and marginal cells (mc) are shown on the right of the diagram.

The pharmacological properties of the pharyngeal nervous system have largely been defined by the expression pattern of genes that are specific for particular neurotransmitters, by the characterisition of defects in pharyngeal pumping in animals with mutations in genes that affect the levels of specific neurotransmitters and by the phenotypic analysis of animals with mutations that affect the function of neurotransmitter receptors. There are at least three classical neurotransmitters synthesised in pharyngeal motorneurones and therefore involved in the control of pharyngeal muscle activity, acetylcholine, glutamic acid and serotonin (5-HT). Acetylcholine has been proposed as the transmitter in a number of pharyngeal motorneurones, including M1, M2, M3, M4, M5, I6 and MC (Rand *et al.,* 2000; Rainzen *et al.,* 1995). Glutamic acid is the transmitter released from M3 onto pharyngeal muscle and is important in the timing of pharyngeal muscle relaxation (Avery, 1993; Li *et al.,* 1997; Niacaris and Avery, 2003). As described above, the

NSM neurones contain 5-HT (Horvitz *et al.,* 1982) and possess varicosities, fine branches and endings on the surface of the pharynx, suggesting that 5-HT may also be released into the pseudocoelom as a neurohormone. Another amine, octopamine, detected in extracts of *C. elegans* (Horvitz *et al.,* 1982), although not identified in pharyngeal motorneurones, modifies pharyngeal muscle activity and may also modulate activity as a neurohormone (Rogers *et al.,* 2001). Recent evidence suggests that tyramine also regulates pharyngeal activity through activation of a specific tyramine receptor, SER-2 (Rex *et al.,* 2004).

The aim of this study was to determine the biological activity of at least one peptide from 23 *flp* genes in *C. elegans*. This made use of a semi-intact preparation of the dissected pharynx which retains the neural circuitry of the enteric nervous system and the anterior nervous system, including the nerve ring and the chemosensory circuits. This preparation of *C. elegans* pharyngeal muscle provides a useful and quantitave bioassay to investigating the biological activity of the FLP neuropeptides. The pharyngeal muscle itself is myogenic since pumping continues, albeit rather inefficiently, after laser killing of the entire pharyngeal nervous system (Avery and Horvitz, 1989). However, the pharynx is dissimilar to other classic myogenic muscle systems, such as vertebrate heart, in that the activity can be very variable and intermittent. It is evident that the animal regulates pharyngeal activity in response to changes in the environment, e.g. the availability of food. The mechanism for this regulation is surprisingly complex involving several classical transmitters including acetylcholine, glutamate, 5-HT, possibly octopamine and a large number of neuroactive peptides, including FLPs (Rogers *et al.,* 2001) and probably peptides encoded by neuropeptide-like protein (*nlp*) genes (Nathoo *et al.*, 2001).

4.1 Results

4.1.1 Recording electropharyngeogram (EPG) from *C. elegans* pharynxes.

A typical example of the muscular activity of wild type *C. elegans* pharyngeal pump is shown in Fig 4.2. The first upward spike (E) marks the depolarisation of the muscle membrane that initiates muscle contraction. The large downward spike (R) marks the repolarisation of the corpus muscle that precedes corpus muscle relaxation. During the period of depolarisation, inhibitory postsynaptic potentials (IPSPs) from the M3 motorneurone are evident. Wild type *C. elegans* pump duration in the presence of 500nM 5-HT lasted 152 ± 10 ms, (n = 31).

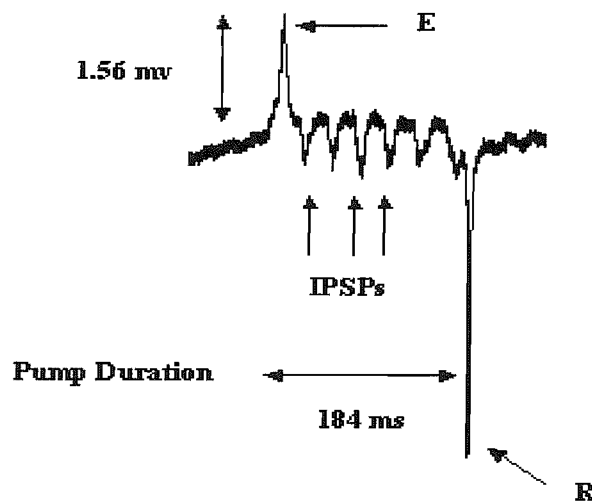


Figure 4.2. Example of an EPG trace of a single pump from wild type *C. elegans.* E and R correspond to excitation and relaxation phases of the pharyngeal muscle contraction. IPSPs are thought to arise from synaptic transmission generated from M3 inhibitory motorneurone.

4.1.2 Effect of 5-HT on wild type *C. elegans* pharyngeal pumping.

Application of 5-HT caused a concentration dependent excitation of pharyngeal pumping with an EC_{50} of 137nM (confidence limits 95%, n = 7). The onset of the response to 5-HT was rapid (within 30 s) and the effect reversed readily on washing out the drug.

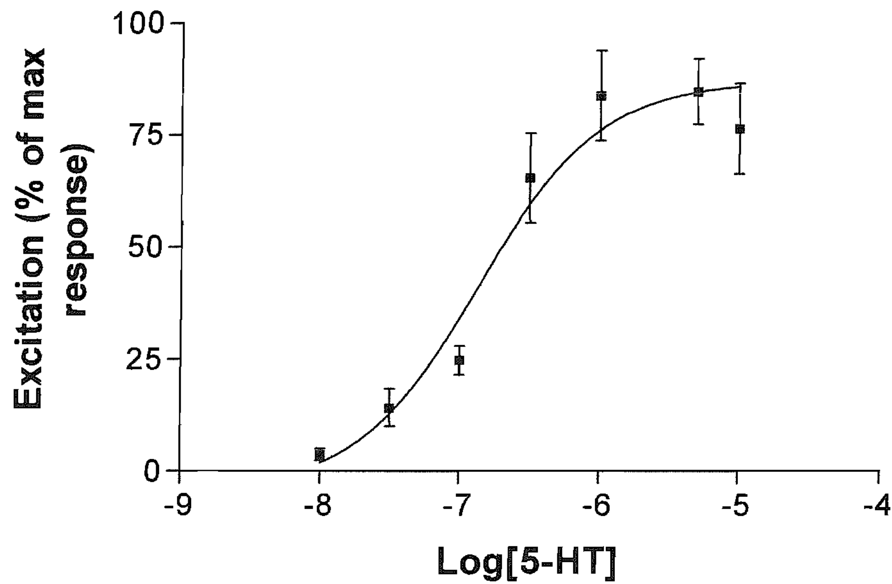


Figure 4.3 The effect of 5-HT on *C. elegans* pharyngeal pumping. Concentration-response curve for the effect of varying concentrations of 5-HT on pharyngeal pumping rates in wild type *C. elegans*. Each point is the mean ± S.E.M of 7 experiments.

4.1.3 The effect of *flp* encoded peptides on *C. elegans* pharyngeal muscle.

The resting pumping rate of the semi-intact pharyngeal preparation in wild-type animals is typically very slow, in the order of 0.5 Hz, and is probably equivalent to the rate observed in intact animals during acute starvation.

Tables 4.1, 4.2 and 4.3 summarise the results of EPG recordings of the actions of peptides expressed by the *flp genes* in wild-type *C. elegans*. At least one example of a peptide encoded by 23 *flp* genes is presented. Eight of the *flp* genes encoded peptides that were excitatory while eleven encoded peptides that were inhibitory on the pharyngeal preparation. The most potent peptides elicited responses at a threshold of 10 nM and a robust, consistent response at 100 nM. Weaker peptides did not exert consistent actions until a concentration of 1 μM was applied. Generaly, the effects were relatively fast in onset and a maximum effect was observed within 1 min of application. Following this, in the continued presence of the peptide, the response sometimes decreased, indicative of desensitisation therefore the effect of the peptide on the frequency of the EPGs was calculated from the action within the first minute of application.

The effects of excitatory peptides on the frequency of pharyngeal EPGs were determined from observations made in experiments without 5-HT, while the values for the inhibitory peptides were determined from experiments in which 500 nM 5-HT was included in the Dent's saline. However, there were four exceptions to this, FLP15A, FLP16A, FLP18A and FLP21. These peptides were all inhibitory but this was only observed when the resting frequency of pharyngeal EPGs was low, i.e. in the absence of 5-HT.

4.1.3.1 Peptides encoded by *flp* genes that had an excitatory effect on the frequency of EPGs.

Eight of the *flp* genes encoded peptides were excitatory on the pharyngeal preparation. The effects of excitatory peptides on the frequency of pharyngeal EPGs were determined from observations made in experiments without 5-HT. The effects were relatively fast on onset and maximum effect was observed within 1 min of application.

Nine of the peptides tested, encoded by 8 different *flp* genes increased pharyngeal pumping frequency in a similar manner to 5-HT. Results from EPG recording of the effects of theses peptides are summarised in Table 4.1 (see page 97).

FLP8, FLP17A and FLP17B were the most potent excitatory peptides with an effect at 100 nM (Table 4.1; Fig. 4.4; Fig. 4.6; Fig. 4.7). FLP8 increased pharyngeal pumping frequency by 267% while FLP17A and FLP17B increased pharyngeal pumping frequency by 209% and 461% respectively. FLP17A and FLP17B are encoded by the same gene *flp-17*, and exhibit very similar effects (Fig 4.4.A, 4.4.B) and are as potent as the previously described actions of FLP8 (AF1; Rogers *et al.*, 2001). FLP17A and FLP17B were tested at a range of concentrations to determine their EC_{50} values. FLP17A and FLP17B had a very similar potency. The EC_{50} for FLP17A was 189 nM (95% confidence limits 71 to 498 nM, n=5), and for FLP17B was 126 nM (95% confidence limits 35 to 450 nM, n=5 Fig 4.4.C, 4.4.D).
Of these potent peptides, only the gene encoding FLP17A and FLP17B is known to be expressed in the pharyngeal system (Kim and Li, 2004). The expression pattern for *flp-8* is extrapharyngeal.

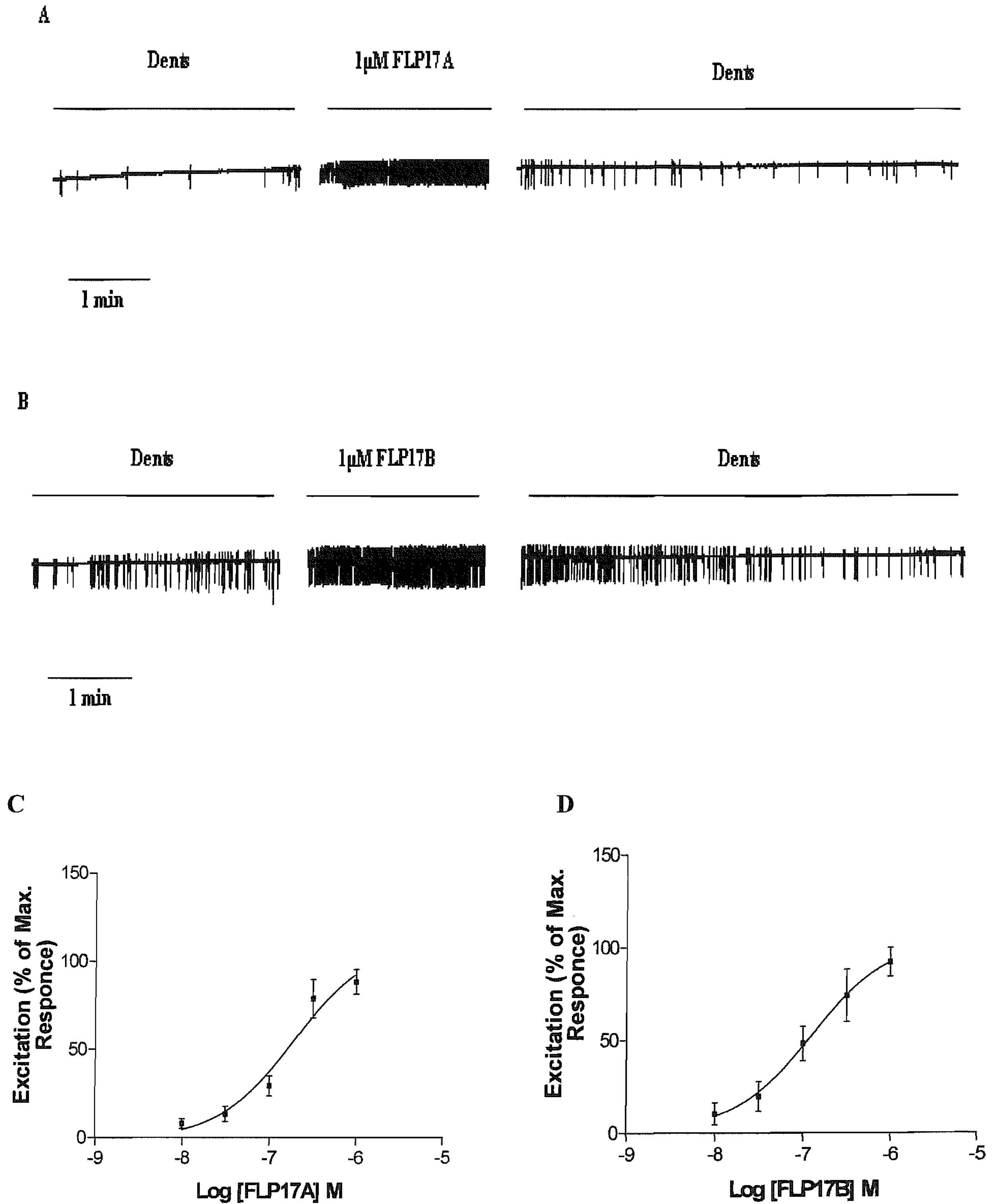


Figure 4.4 The effects of two FLP peptides encoded by the same gene, FLP17A and FLP17B, on the EPG recorded from *C. elegans* pharyngeal muscle. A and B, show an EPG recorded from an individual pharynx. For each trace, the first section shows the pharyngeal activity before the addition of the peptide. These recordings

were made in Dent's saline. Brakes in the trace indicate a short break in the recording (<1 min) during which the fluid in the recording chamber was exchanged, for Dent's saline with 1 μM FLP and subsequently with Dent's saline to test for reversal of the peptide effects. C and D. Concentration-response curves for the effects of FLP17A and FLP17B, respectively. For these experiments, 5 individual pharynxes were tested for responses to 10 nM to 1 μM for each peptide. Each point is the mean ± s.e.m of 5 determinations.

FLP14A was the most effective peptide at increasing the frequency of pharyngeal pumping at 1 μM with a change in frequency of 1482% (Table 41). The .peptides encoded by the *flp-14* gene were of particular interest as, unlike the peptides encoded by most of the other *flps*, they differ significantly from each other in their C-terminal i.e. the sequence of FLP14A is KHEYLRFamide compared to FLP14B, SLLDYRFamide and FLP14C, EIVFHQISPIFFRFamide (Nelson *et al.,* 1989b). FLP14C had no effect on pharyngeal activity at concentrations up to 1 μM whereas FLP14B elicited an inhibition (Table 4.1, 4.2 and 4.3; Fig 4.5; Figure 4.8). The expression pattern of *flp-14* is not known.

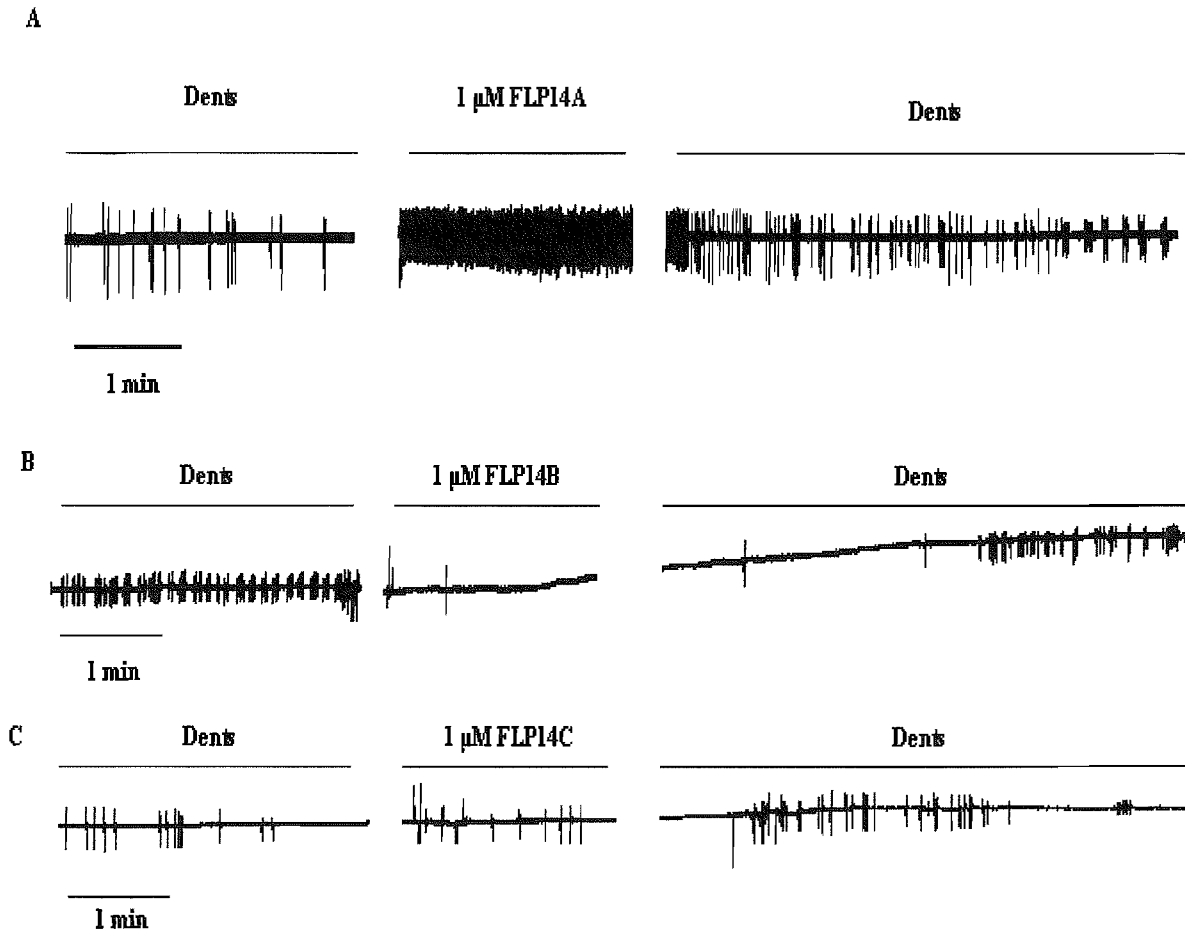


Figure 4.5. The effects of three FLP peptides encoded by the same gene, FLP14A (KHEYLRFa), FLP14B (SLLDYRFa) and FLP14C (EIVFHQISPIFFRFa), on the EPG recorded from *C. elegans* pharyngeal muscle. Each of the panels shows an EPG recorded from an different pharynx. For each panel, the first section shows the pharyngeal activity before the addition of the peptide. All recordings were made in Dent's saline. Breaks in the recording (<1 min) indicate exchange of the fluid in the recording chamber, for Dent's saline with 1 μM FLP and subsequently with Dent's saline to test for reversal of the peptide effects. The duration of the peptide application was 2 min.

FLP22, FLP5A, FLP6, FLP4A and FLP2A were also excitatory but consistent effects were only observed at 1 μM (Table 4.1; Fig. 4.6; Fig. 4.8). The effects of all these excitatory peptides were readily reversible following a wash for 5 mins.

FLP 22 is a peptide encoded by the *flp-22* gene (Li *et al.,* 1999a) and has an extrapharyngeal expression. FLP22 caused a reversible increase in pharyngeal pumping when applied at a concentration of 1 μM. The change in frequency in pharyngeal pumping was 854 ± 270%; n=4; $p<0.05$.

FLP5 is expressed in the pharynx in the I2, I4 interneurones, in the M4 motorneurone and in pharyngeal muscle (Kim and Li, 2004). FLP5 caused a reversible increase in pharyngeal pumping in a similar manner to FLP22 with a percentage increase in frequency of 813 ± 437%; n=5; $p<0.02$.

FLP6, a peptide encoded by *flp-6* gene, is also expressed in the pharynx in I1 and I4 interneurones. FLP6 weakly excited pharyngeal pumping in a reversible manner. The percentage change in pharyngeal pumping frequency in the presence of 1 μM FLP6 was 341 ± 86%; n=5; $p<0.02$.

FLP4 is expressed in I5, I6 interneurones and in the NSM motorneurone. FLP4 caused a weak increase in pharyngeal pumping frequency of 310 ± 92 %; n=5; $p<0.05$. In further experiments FLP4 caused a delayed excitation (n=4) or a weak inhibition (n=5).

FLP2, a peptide encoded by the *flp-2* gene, is expressed in I5 interneurone and in MC and M4 motorneurones. FLP2 caused the weakest excitation of pharyngeal pumping when applied at a concentration of 1 μM. The percentage change in pharyngeal pumping frequency was 269 ± 6%; n=7; $p<0.05$.

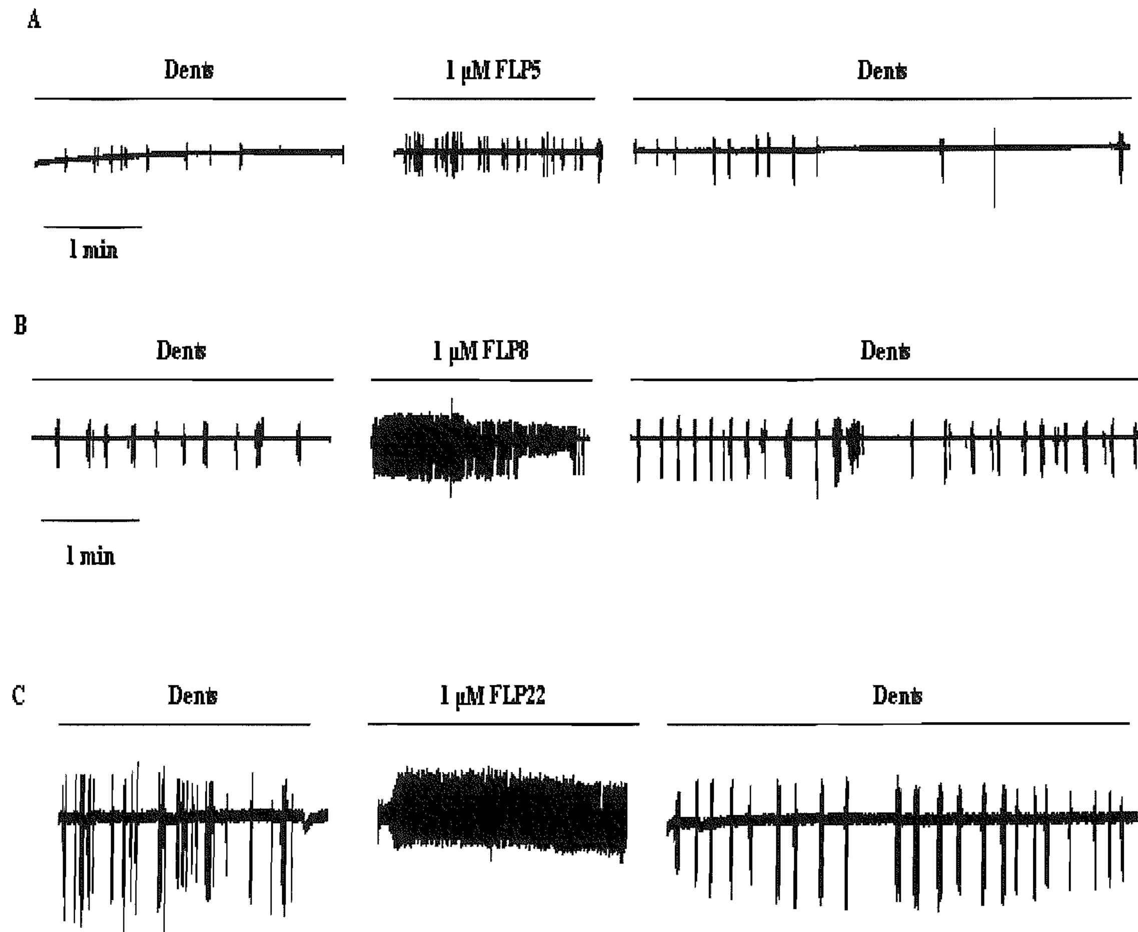


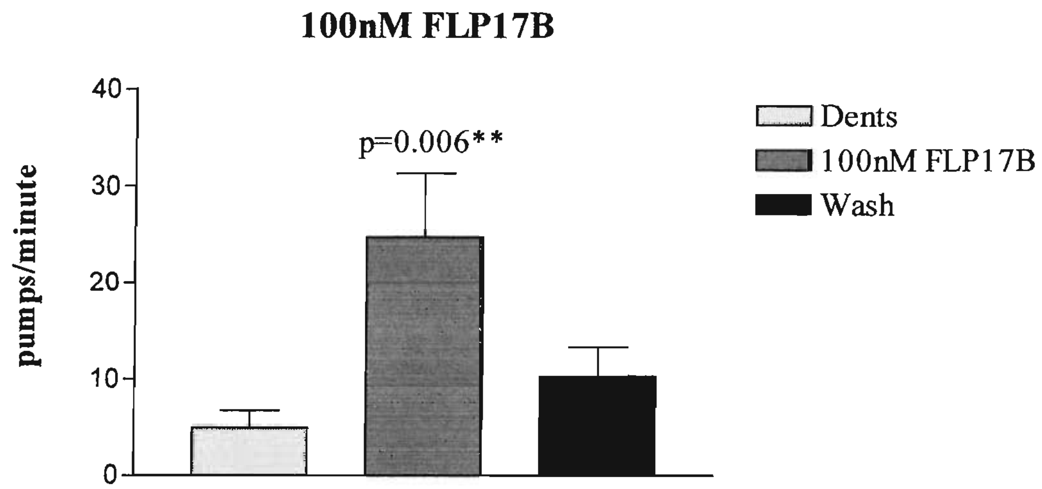
Figure 4.6. The effects of FLP peptides on the electropharyngeogram (EPG) recorded from *C. elegans* pharyngeal muscle. Each of the panels (A-C) shows an EPG recorded from an individual pharynx. For each panel, the first section shows the pharyngeal activity before the addition of the peptide. These recordings were made in Dent's saline. Each deflection is a single pharyngeal pump but because of the relative slow time-base individual pumps cannot always be resolved if the pumping activity is fast. Brakes in trace indicate a short break in the recording (<1 min) during which the fluid in the recording chamber was exchanged, for Dent's saline with 1 µM FLP and subsequently with Dent's saline to test for reversal of the peptide effects.

Gene	Expression in pharyngeal system[1]	Sequence	PEPTIDE	Action	Conc.	% Change in Frequency
flp-17	M5	KSQYIRFa	FLP17B	Potent Excitation	100 nM	+461 ± 138; n=5; p<0.02
flp-8	None	KNEFIRFa	FLP8 (AF1)	Potent Excitation	100 nM	+267 ± 80; n=4; p<0.05
flp-17	M5	KSAFVRFa	FLP17A	Potent Excitation	100 nM	+209 ± 49; n=6; p<0.05
flp-14	Not determined	KHEYLRFa	FLP14A (AF2)	Excitation	1 μM	+1482 ± 292; n=5; p<0.001
flp-22	None	SPSAKWMRFa	FLP22	Excitation	1 μM	+ 854 ± 270; n=4; p<0.05
flp-5	(I2), I4,M4, pm	GAKFIRFa	FLP5A	Excitation	1 μM	+ 813 ± 437; n=5; p<0.02
flp-6	I1, I4	KSAYMRFa	FLP6 (AF8)	Excitation	1 μM	+341 ± 86; n=5; p<0.02
flp-4	I5, I6, NSM	PTFIRFa	FLP4A	Excitation[2]	1 μM	+310 ± 92; n=5; p<0.05
flp-2	I5, MC, M4	SPREPIRFa	FLP2A	Excitation	1 μM	+269 ± 6; n=7; p<0.05

Table 4.1: Excitatory FLP peptides and their effect on *C. elegans* pharyngeal muscle. Peptides encoded by *flp-17, flp-8, flp-14, flp-22, flp-5, flp-6, flp-4 and flp-2* genes had an excitatory action on pharyngeal muscle. Peptide expression, sequence and the % change in frequency of pharyngeal pumping is presented.

[1]Kim and Li (2004). [2]The response to FLP4A was variable. In further experiments it elicited a delayed excitation (n=4) or a weak inhibition (n=5).

A.



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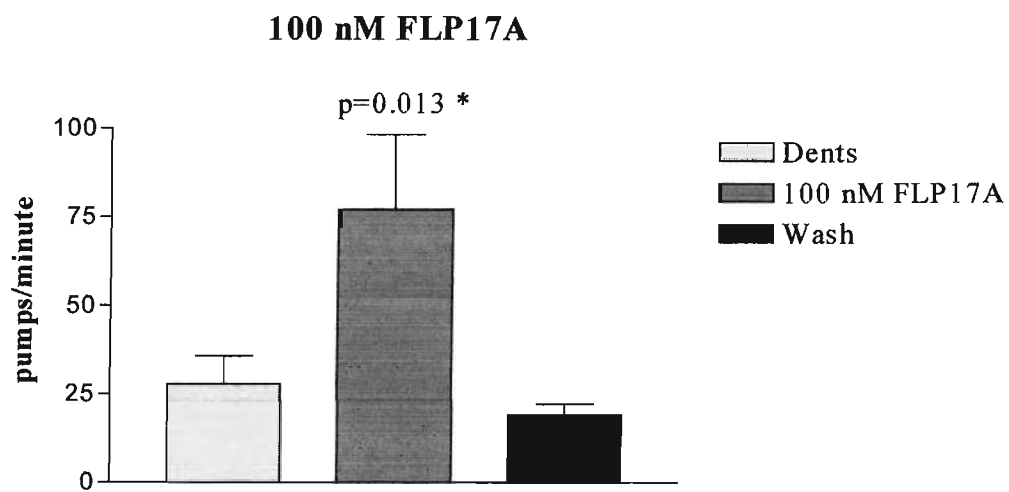

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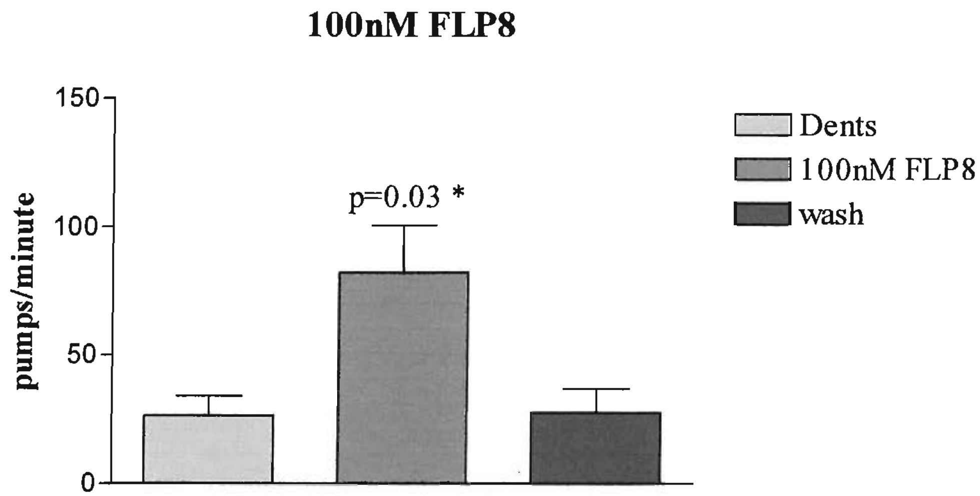
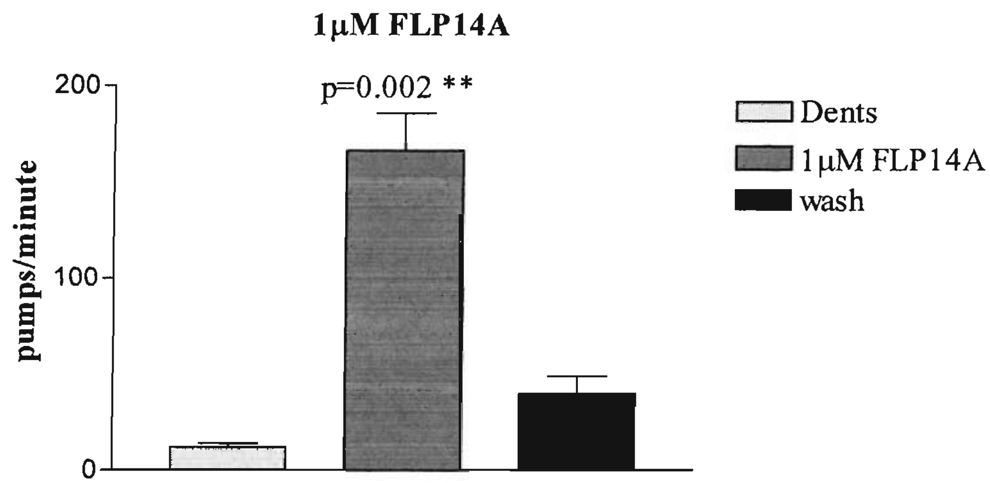


Figure 4.7. The effect of FLP peptides on the rate of pharyngeal pumping. A, B, C show pooled data of the effect of FLP17B (n = 5), FLP17A (n= 6) and FLP8 (n=4) peptides respectively on the rate of pharyngeal pumping. The control is the rate of pharyngeal pumping in Dent's saline.

A.



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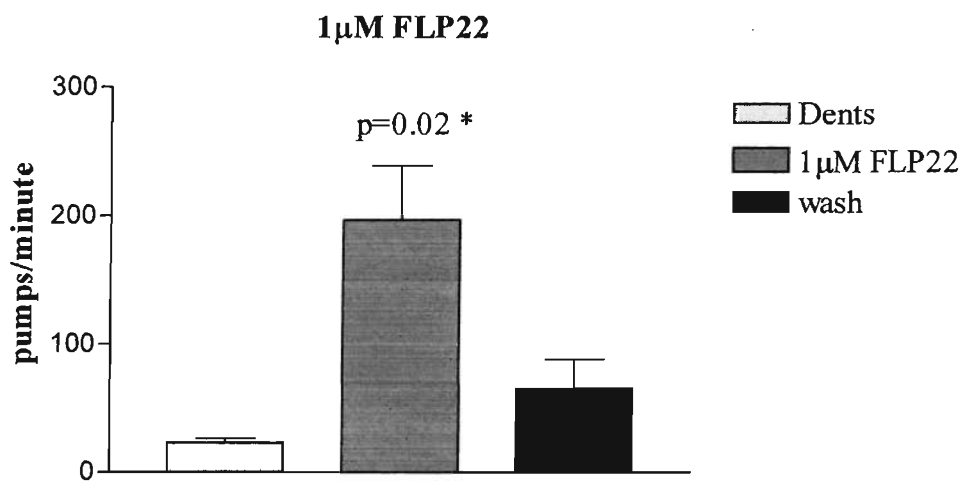

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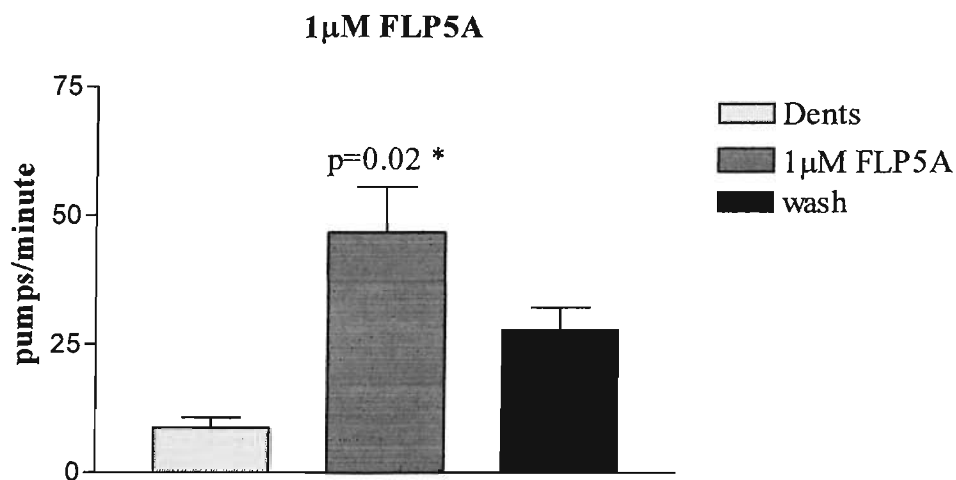

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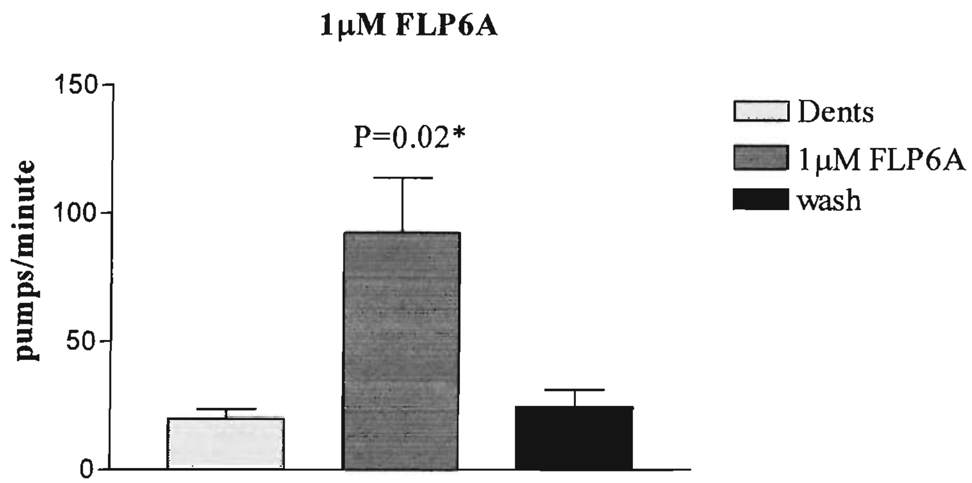

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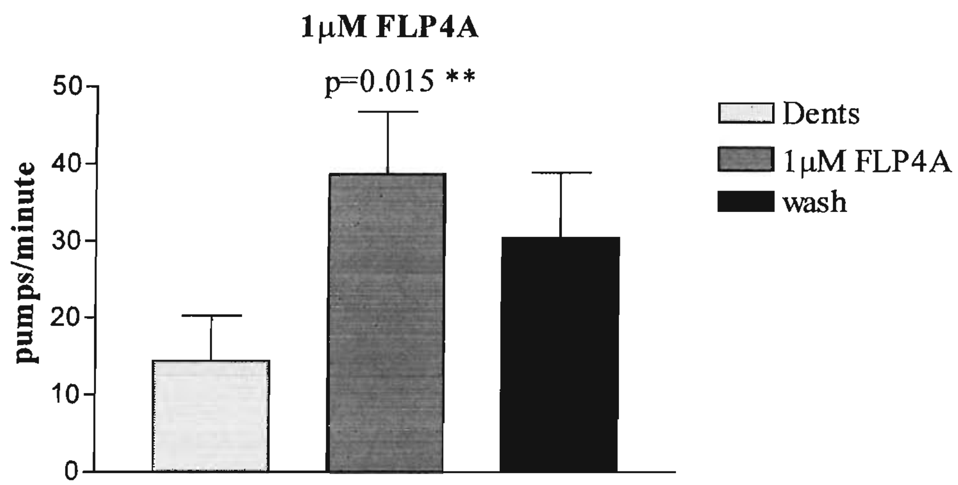


F.

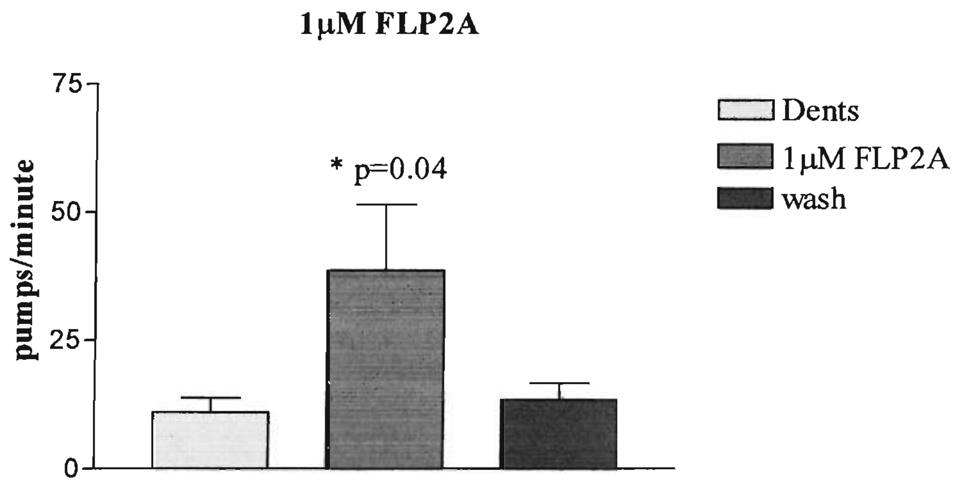


Figure 4.8 . The effect of FLP peptides on the rate of pharyngeal pumping. A, B, C, D, E and F show pooled data of the effect of FLP14A (n=5), FLP22 (n=4), FLP5A (n=5), FLP6 (n=5), FLP4A (n=5) and FLP2A (n= 7) peptides respectively on the rate of pharyngeal pumping. The control is the rate of pharyngeal pumping in Dent's saline.

4.1.3.2 Peptides encoded by *flp* genes that had an inhibitory effect on the frequency of EPGs.

Twelve FLP peptides encoded by 12 different *flp* genes had an inhibitory action on the frequency of pharyngeal pumping (Table 4.2). The effects of inhibitory peptides on the frequency of pharyngeal EPGs were determined from experiments in which 500nM 5-HT was included in Dent's saline. However, there were four exceptions to this, FLP15A, FLP16A, FLP18A and FLP21. These peptides were all inhibitory but this inhibition was only observed when the resting frequency of the pharyngeal EPG was low i.e. in the absence of 5-HT. In the presence of 5-HT the responses of these peptides were inconsistent.

Gene	Expression in pharyngeal system[1]	Sequence	PEPTIDE	Action	Conc.	% Change in frequency
flp-13	I5, M3, M5	APEASPFIRFa	FLP13A	Potent inhibition	100 nM	-74 ± 8 n=6; p<0.02
flp-11	None	AMRNALVRFa	FLP11A	Potent inhibition	100 nM	-50 ± 11; n=5, p<0.05
flp-3	None	SAEPFGTMRFa	FLP3A	Inhibition	1 μM	-99 ± 1; n=5; p<0.01
flp-1	M5	SADPNFLRFa	FLP1A2 (PF2)	Inhibition	1 μM	-93 ± 3; n=4; p<0.01
flp-18	M2, M3	EMPGVLRFa	FLP18A	Inhibition[2]	1 μM	-90 ± 4; n=4; p<0.05
flp-14	Not determined	SLLDYRFa	FLP14B	Inhibition	1 μM	-90 ± 4; n=6; p<0.01
flp-1	M5	SDPNFLRFa	FLP1A1 (PF1)	Inhibition	1 μM	-88 ± 3; n=5; p<0.001
flp-15	I2, pm	GGPQGPLRFa	FLP15A	Inhibition[2]	1 μM	-72 ± 5; n=6; p<0.01
flp-9	Not determined	KPSFVRFa	FLP9A	Inhibition	1 μM	-63 ± 7; n=8; p<0.01
flp-16	Not determined	AQTFVRFa	FLP16A (AF15)	Inhibition[2]	1 μM	-66 ± 10; n=4; p<0.05
flp-19	None	WANQVRFa	FLP19A	Inhibition	1 μM	-64 ± 11; n=4; p<0.02
flp-21	MC, M4, M2	GLGPRPLRFa	FLP21 (AF9)	Inhibition[2]	1 μM	-66 ± 10; n=4; p<0.05

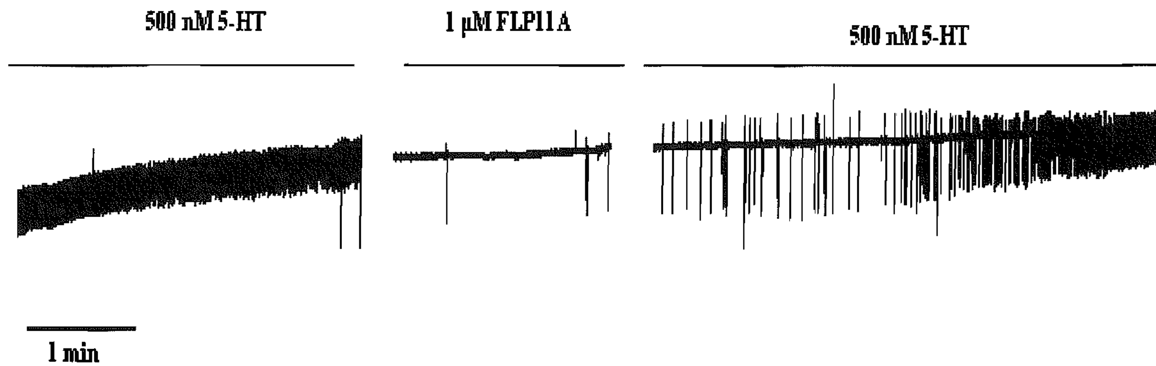
Table 4.2 Inhibitory FLP peptides and their effect on *C. elegans* pharyngeal muscle. Peptides encoded by *flp-13, flp-11, flp-3, flp-1, flp-18, flp-14, flp-15, flp-9, flp-16, flp-19* and *flp-21* genes had an inhibitory action on pharyngeal muscle. Peptide expression, sequence and the % change in frequency of pharyngeal pumping is presented.

[1] Kim and Li (2004). [2]These inhibitory effects were measured in the absence of 5-HT.

FLP11A and FLP13A were the most potent inhibitory peptides (Table 4.2; Fig. 4.9; Fig. 4.13). FLP13A was remarkably potent at inhibiting the pharyngeal pumping frequency at 100nM with a percentage change in frequency of 74 ± 8%; n=6, p<0.02. The *flp-13* gene is expressed in I5 interneurone and in M3 and M5 motorneurones. This result of EPG recordings confirms the earlier report of the inhibitory action of this peptide using intracellular recording techniques (Rogers *et al.*, 2001).

FLP11A, a peptide encoded by the *flp-11* gene, which is not expressed in the pharyngeal system was one of the most potent inhibitory peptides (Table 4.2; Fig. 4.9). At 100 nM FLP11A caused a percentage change in frequency of pharyngeal pumping of 50 ± 11%; n=5; p<0.05. At 1 μM FLP11A caused a complete inhibition of pharyngeal pumping. Because of its potent effect FLP11A was tested at different concentration to determine its EC_{50} value. The EC_{50} value for FLP11A was 105 nM (95% confidence limits 69 to 159 nM, n=5; Fig. 4.9).

A.



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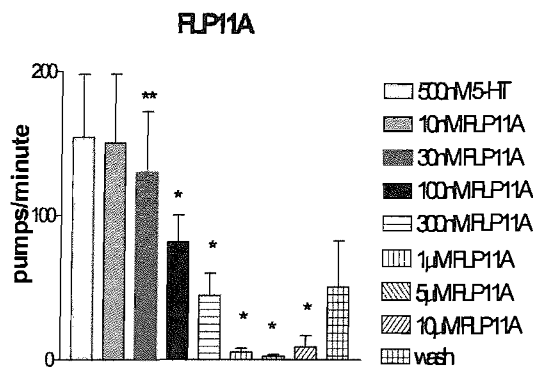

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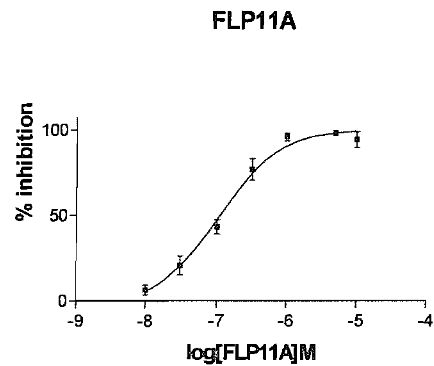
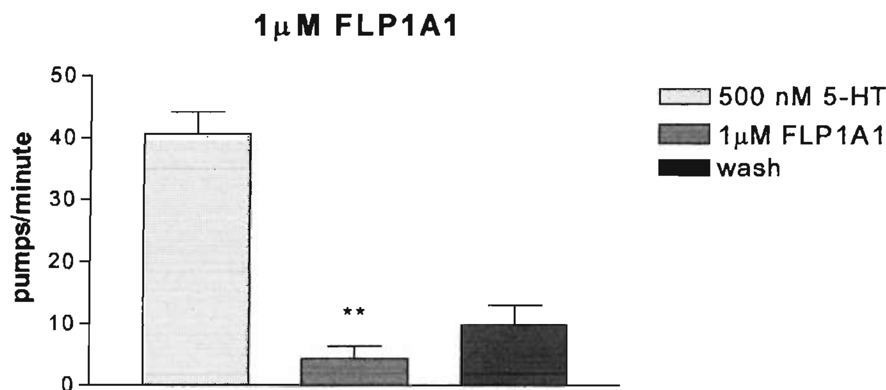


Figure 4.9 FLP11A is a potent inhibitory peptide. A shows an EPG recorded from an individual pharynx. The first section shows the pharyngeal activity before the addition of the peptide. This recording was made in 500 nM 5-HT. Brakes in trace indicate a short brake in the recording (< 1 min) during which the fluid in the recording chamber was exchanged for 500 nM 5-HT with 1 μM FLP11A and subsequently with 500 nM 5-HT to test for reversal of the peptide effects. Fig. B shows pooled data of the effect of different concentrations of FLP11A on pharyngeal pumping. Fig. C shows a concentration – response curve for the effect of FLP11A. For these experiments, 5 individual pharynxes were tested for responses to 10nM to 10 μM. Each point is the mean ± S.E.M. of 5 determinations. Recordings were made in Dent's saline with 500 nM 5-HT.

Two of the inhibitory peptides, that were very effective at 1 μM, are encoded by *flp-1* and are structurally very similar, differing only by the addition of a single alanine residue. These have been designated FLP1A1 (SDPNFLRFa) and FLP1A2 (SADPNFLRFa) (Table 4.2; Fig. 4.10). The *flp-1* gene is expressed in the M5 motorneurone. The FLP1A2 peptide was slightly more potent causing a percentage change in frequency of 93 ± 3%; n=4; p<0.01. FLP1A1 caused a percentage change in frequency of 88 ± 3%; n=5; p<0.001. However, FLP1A1 inhibition took longer to reverse.

A.



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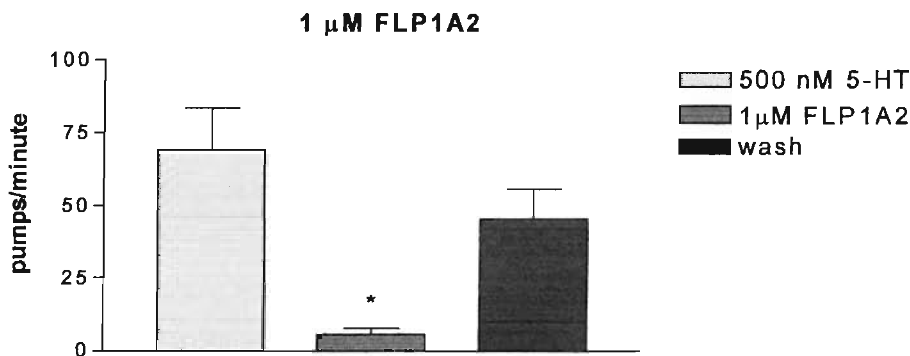


Figure 4.10. The effect of two *flp-1* encoded peptides on pharyngeal pumping. A and B represent pooled data of the effect of FLP1A1 and FLP1A2 on pharyngeal pumping respectively. Control is rate of pharyngeal pumping in 500 nM 5-HT. 1 μM peptide is added in the presence of 500 nM 5-HT and subsequently 500 nM 5-HT is added to test reversal of peptide effects. A. Each point represents the mean ± S.E.M. of 5 determinations p<0.001. B. Each point represents the mean ± S.E.M. of 4 determinations p< 0.01.

FLP3A, a peptide encoded by the *flp-3* gene, which has no pharyngeal expression was another peptide which caused a potent inhibition of pharyngeal pumping at 1 μM. At 1 μM FLP3A caused a percentage change in frequency of 99 ± 1%; n=5, p<0.01 (Fig. 4.12; Fig. 4.13). It was noted that in the presence of FLP3A the duration of individual pharyngeal pumps was significantly reduced, p< 0.001 (Fig. 4.11). For example, pump duration in the presence of 5-HT was 190.4 ± 8.48 ms, n =25 (5 measures from 5 pharynxes). Pump duration was reduced to 55.05 ± 3.09 ms, n=18 (6 measures from 3 pharynxes) in the presence of 5-HT and 1 μM FLP3A. Following wash pump duration increased to 133.1 ± 6.72 ms, n=15 (5 measures from 3 pharynxes). While no inhibitory potentials (IPs) were noted during pharyngeal pumping in 5-HT, IPs of 0.82 ± 0.05 mV, n=18 (6 measures from 3 pharynxes), appeared following addition 1 μM FLP3A. During wash there was a reduction in the magnitude of IPs to 0.38 ± 0.06 mV, n = 12 (4 measures from 3 pharynxes).

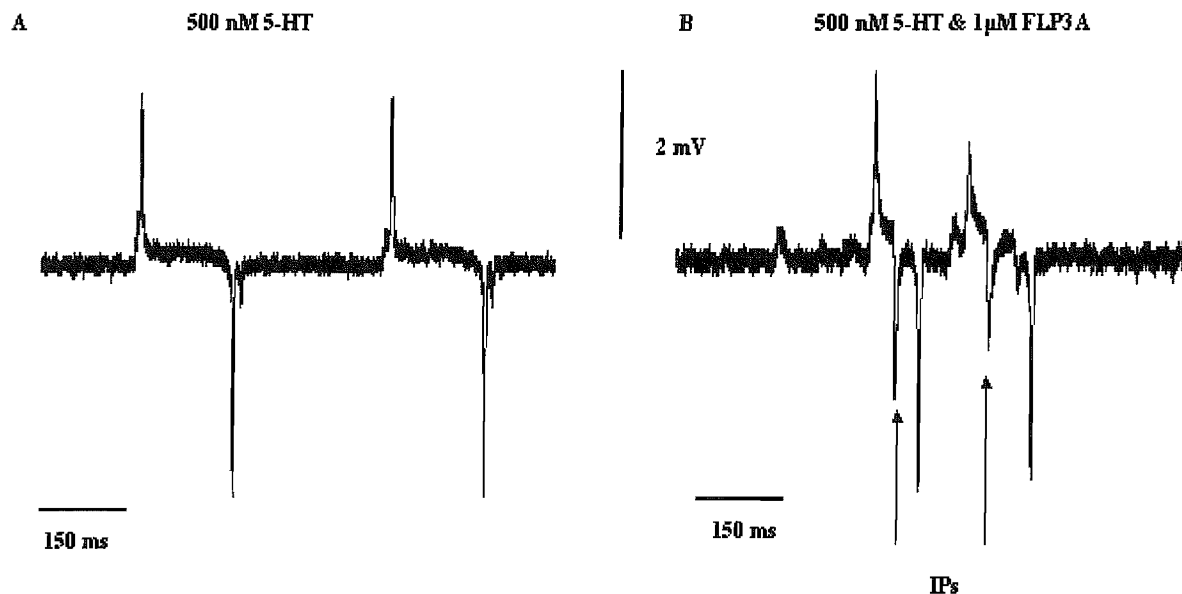


Figure 4.11 Example of EPG traces of pharyngeal pumps in 5-HT and FLP3A. A represents two pharyngeal pumps in a wild-type *C. elegans* in the presence of 500 nM 5-HT. B represents two pharyngeal pumps in the presence of 500nM 5-HT and 1 μM FLP3A.

FLP18A, a peptide expressed in M2 and M3 motorneurones, caused a potent inhibition of the frequency of pharyngeal pumping (Table 4.2; Fig. 4.12; Fig. 4.14) in the magnitude of 90 ± 4%; n=4; $p<0.05$.

FLP14B, one of the three peptides encoded by the *flp-14* gene and where its expression in the pharyngeal system has not yet been determined, caused a potent inhibition of pharyngeal pumping at a concentration of 1 μM (Table 4.2; Fig. 4.5; Fig. 4.14). The percentage change in frequency of pharyngeal pumping in the presence of FLP14B was 90 ± 4%; n=6; $p<0.01$.

FLP15A, one of the peptides encoded by the *flp-15* gene which is expressed in the I2 motorneurone and pharyngeal muscle, caused a percentage decrease in frequency of 72 ± 5%; n=6; $p<0.01$ (Fig. 4.14)

FLP9A and FLP16A are encoded by the *flp-9* and *flp-16* genes respectively. The pharyngeal expression of the *flp-9* and *flp-16* genes has not yet been determined. Both peptides were of similar potency (Fig. 4.13; Fig. 4.14, respectively) with FLP9A causing a percentage change in frequency of 63 ± 7%; n=8; $p<0.01$ and FLP16A 66 ± 10%; n=4; $p<0.05$.

FLP19A is encoded by the *flp-19* gene which is not expressed in the *C. elegans* pharynx. FLP21 is encoded by *flp-21* gene which is expressed in the MC, M4 and M2 motorneurones. Both peptides caused a reduction in pharyngeal pumping frequency (Fig. 4.13; Fig. 4.14, respectively) with FLP19A causing a reduction of 64 ± 11%; n=4; $p<0.02$ and FLP21 66 ± 10%; n=4; $p<0.05$.

In contrast to the rapid reversibility of the effects of the excitatory peptides, the recovery of pharynxes from the effects of the inhibitory peptides was more variable and depended on the peptide being tested. For example, following the inhibitory action of FLP1A2 (PF2) the pharynx recovered to 65% of the control pumping rate, whereas following FLP1A1 (PF1) the pharynx recovered less, to only 30% of the control pumping rate. The effects of FLP9, FLP16A and FLP21 showed approximately 80% recovery following a five minute wash.

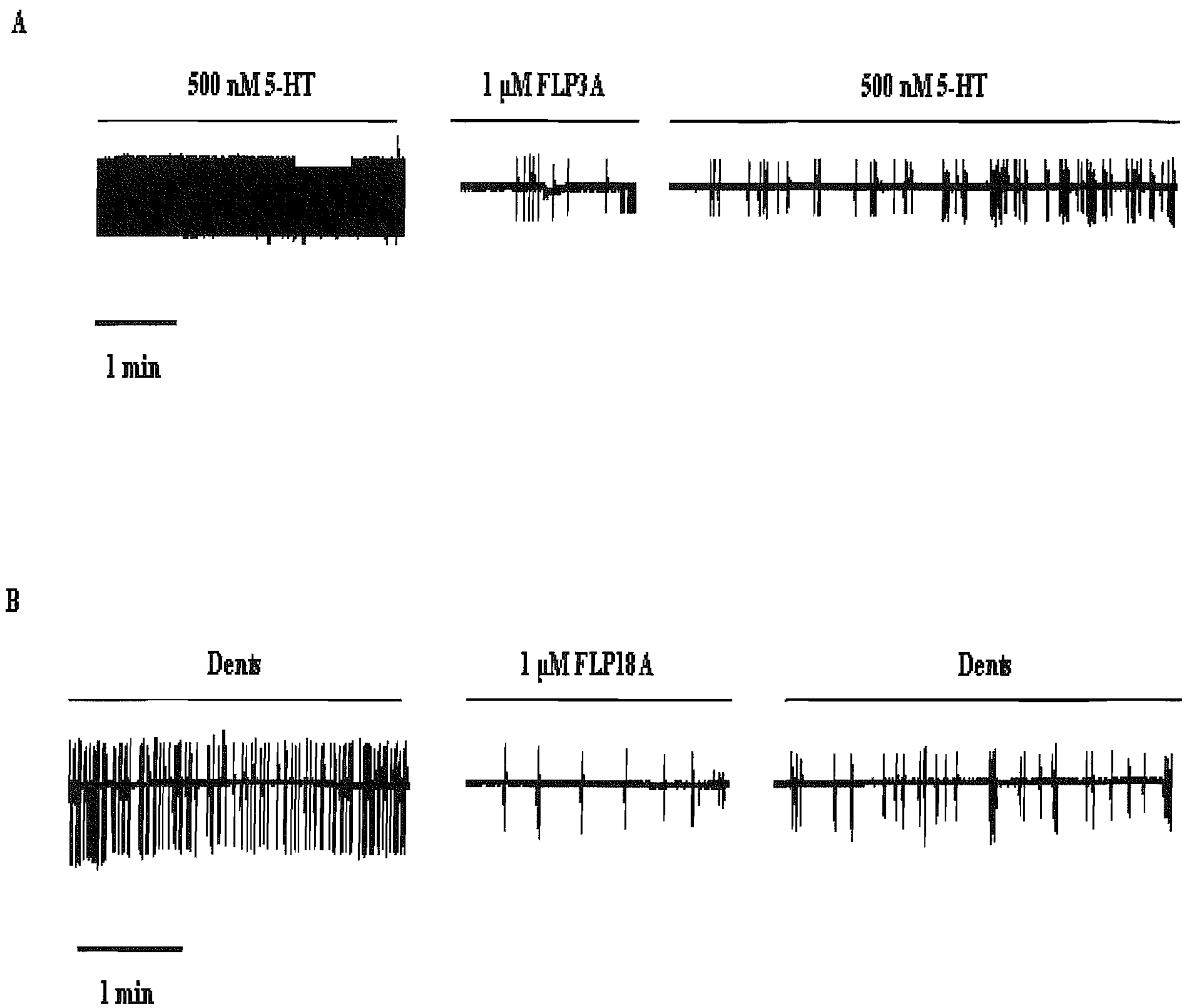
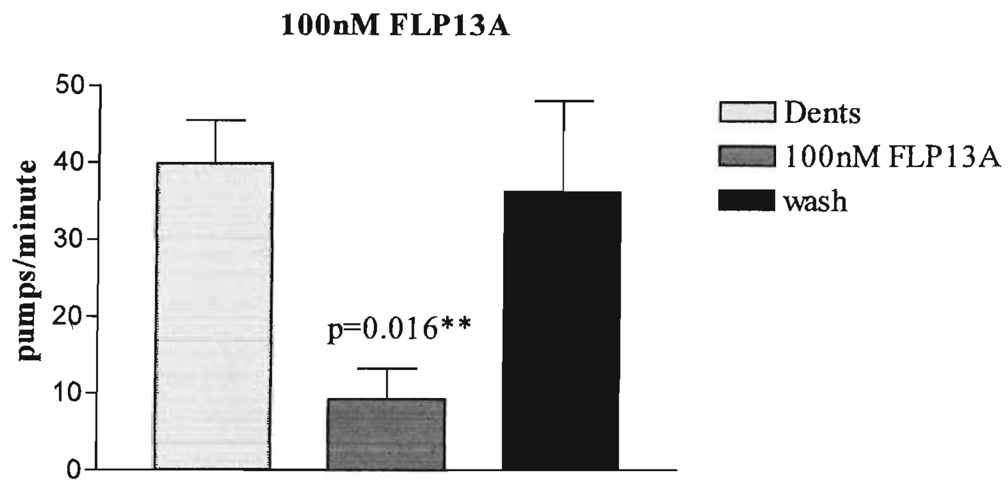
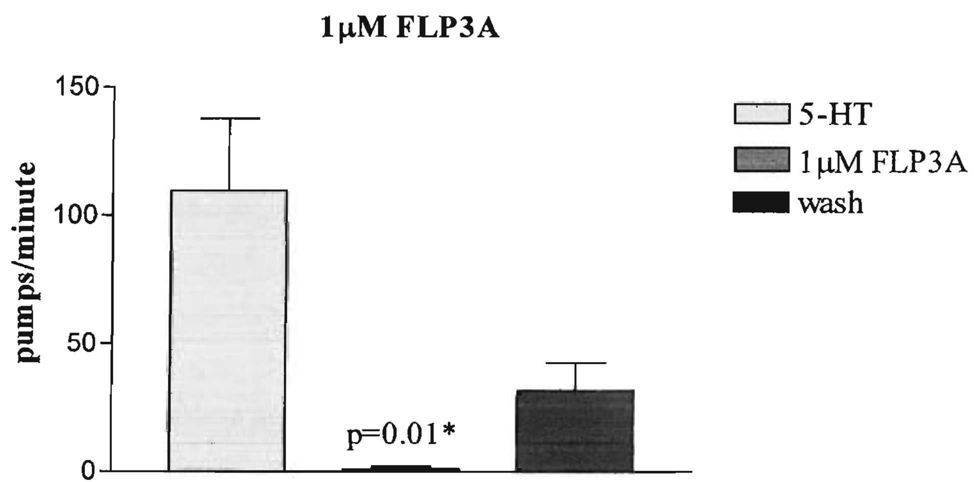


Figure 4.12 The effects of FLP peptides on the electropharyngeogram (EPG) recorded from *C. elegans* pharyngeal muscle. Each of the panels (A-B) shows an EPG recorded from an individual pharynx. For each panel, the first section shows the pharyngeal activity before the addition of the peptide. These recordings were made in Dent's saline. Each deflection is a single pharyngeal pump but because of the relative slow time-base individual pumps cannot always be resolved if the pumping activity is fast. Brakes in trace indicate a short break in the recording (<1 min) during which the fluid in the recording chamber was exchanged for Dent's saline with 1 µM FLP and subsequently with Dent's saline to test for reversal of the peptide's effects.

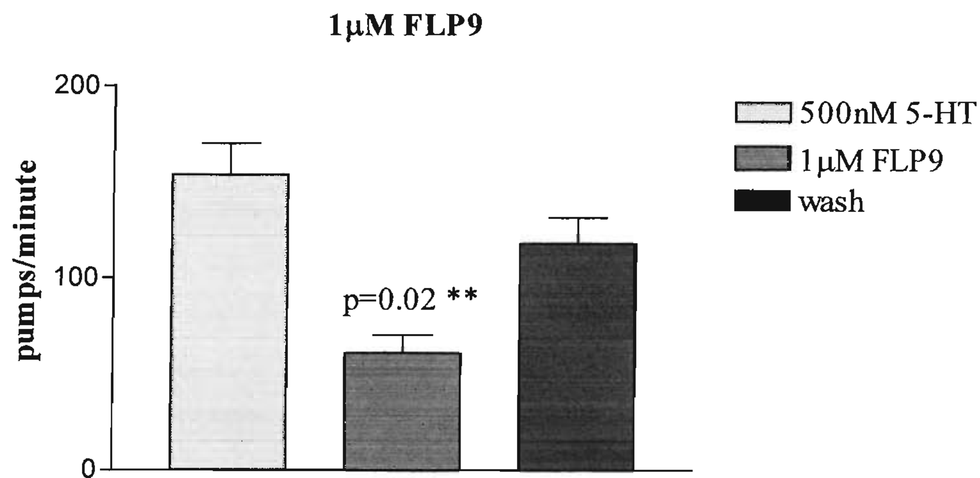
A.



B.



C.



D.

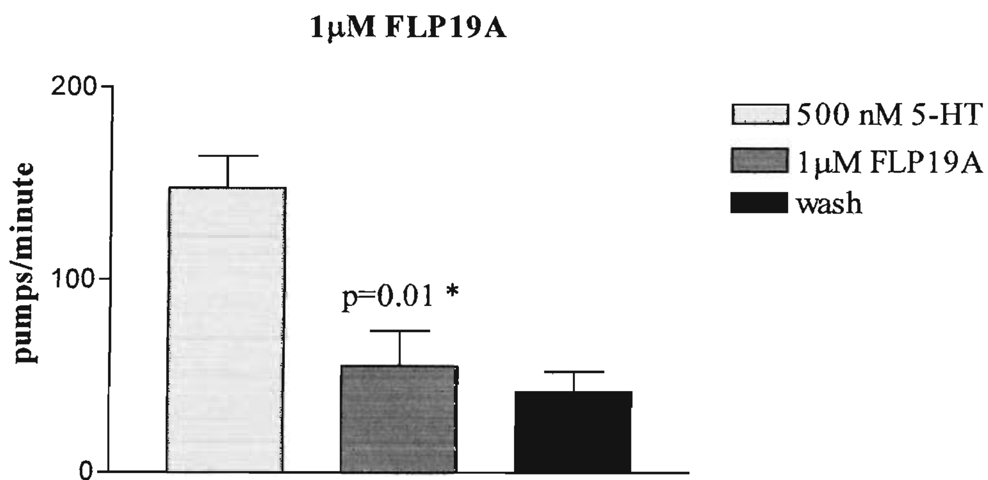
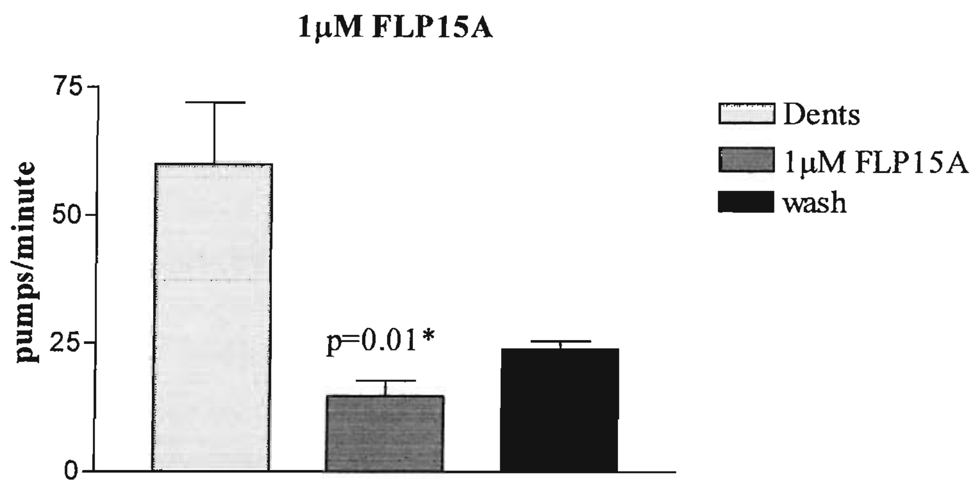
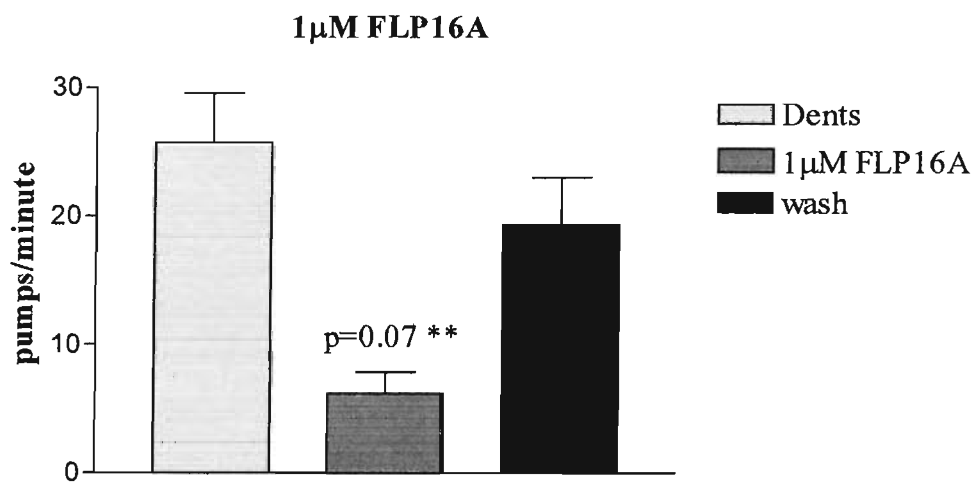


Figure 4.13. The effect of inhibitory peptides on pharyngeal pumping. A, B, C and D show pooled data of the effect of FLP13A (n=6), FLP3A (n=5), FLP9 (n=8) and FLP19A (n=4) on pharyngeal pumping, respectively. The control is the rate of pharyngeal pumping in 500 nM 5-HT.

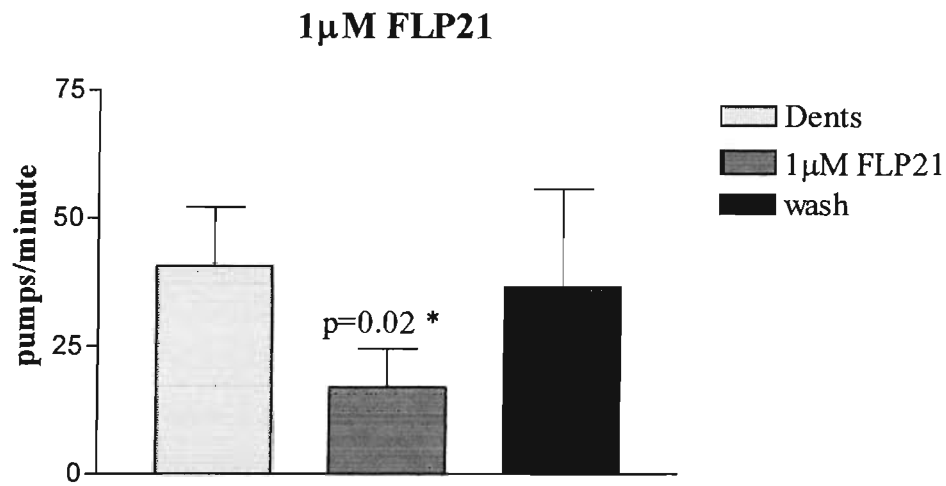
A.



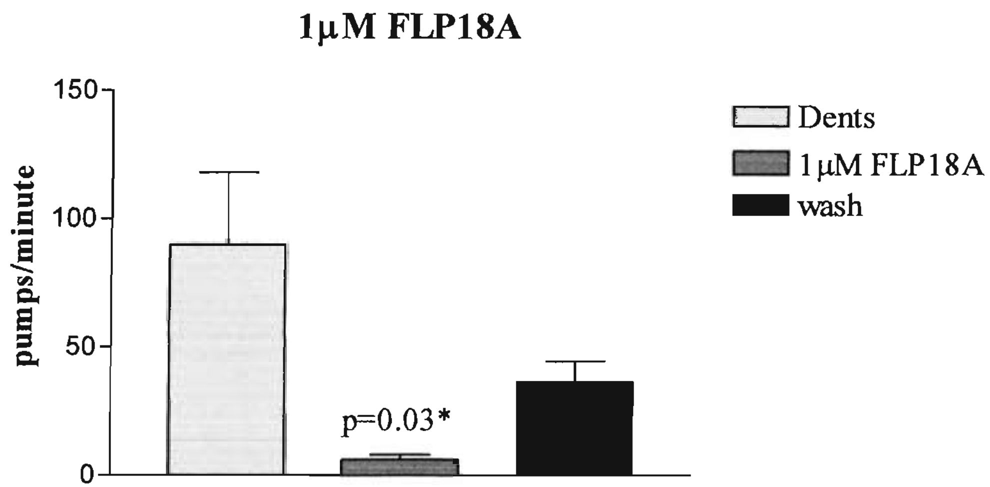
B.



C.



D.



E.

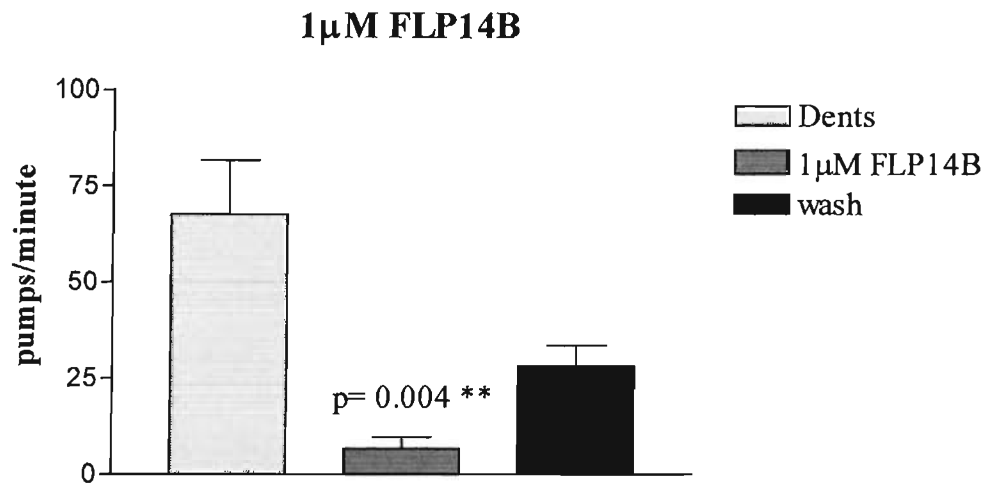


Figure 4.14. The effect of inhibitory peptides on pharyngeal pumping. A, B, C, D and E show pooled data of the effect of FLP15A (n=6), FLP16A (n=4), FLP21 (n=4), FLP18A (n=4) and FLP14B (n=6) on pharyngeal pumping respectively. The control is the rate of pharyngeal pumping in Dent's saline.

4.1.3.3 Non-Active *flp* peptides

A number of FLP peptides failed to produce a significant effect on the *C. elegans* pharynx. These peptides encoded by the *flp-7, flp-10, flp-12, flp14, flp-20* and *flp-23* genes were tested for pharyngeal activity at 1 μM (Table 4.3; Fig. 4.15). The effects of these peptides on pharyngeal pumping were determined from experiments in Dent's saline.

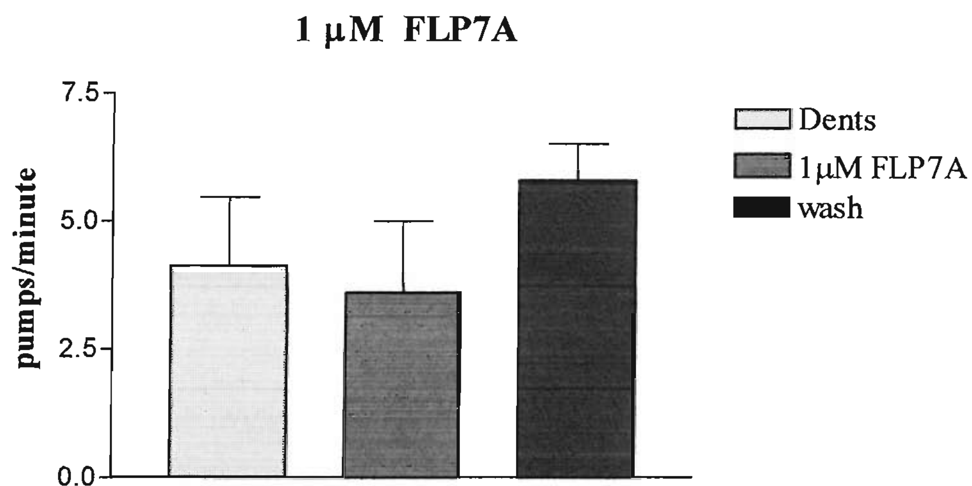
Four of the genes encoding the inactive peptides are not expressed in the pharyngeal system while for two genes their expression has not been determined.

Gene	Expression in pharyngeal system [1]	Sequence	PEPTIDE	Action	Conc.	% Change in frequency
flp-7	None	SPMQRSSMVRFa	FLP7A	No effect	1 μM	- 25 ± 12; n=5; p=0.48
flp-10	None	QPKARSGYIRFa	FLP10A	No effect	1 μM	+ 21 ± 6; n=7; p=0.76
flp-12	None	RNKFEFIRFa	FLP12A	No effect	1 μM	+20 ± 9; n=7; p=0.76
flp-14	Not determined	EIVFHQISPIFFRFa	FLP14C	No effect	1 μM	+ 25 ± 7; n=5; p=0.63
flp-20	None	AMMVRFa	FLP20	No effect	1 μM	-25 ± 11; n=6; p=0.3
flp-23	Not determined	TKFQDLRFa	FLP23	No effect	1 μM	- 22 ± 8; n=5; p=0.47

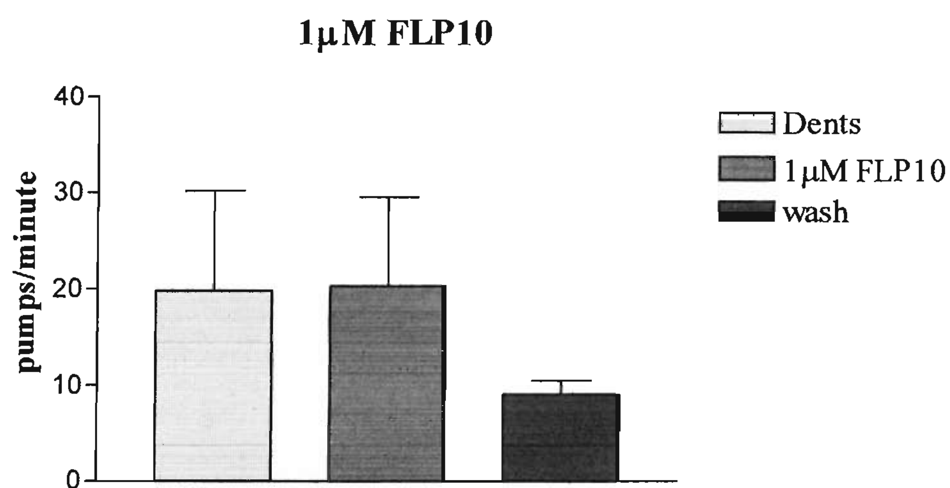
Table 4.3 FLP peptides that failed to produce an effect on *C. elegans* pharyngeal muscle. Peptides encoded by *flp-7, flp-10, flp-12, flp-14, flp-20,* and *flp-23* genes were inactive on pharyngeal muscle. Peptide expression, sequence and the % change in frequency of pharyngeal pumping is presented.

[1]Kim and Li (2004).

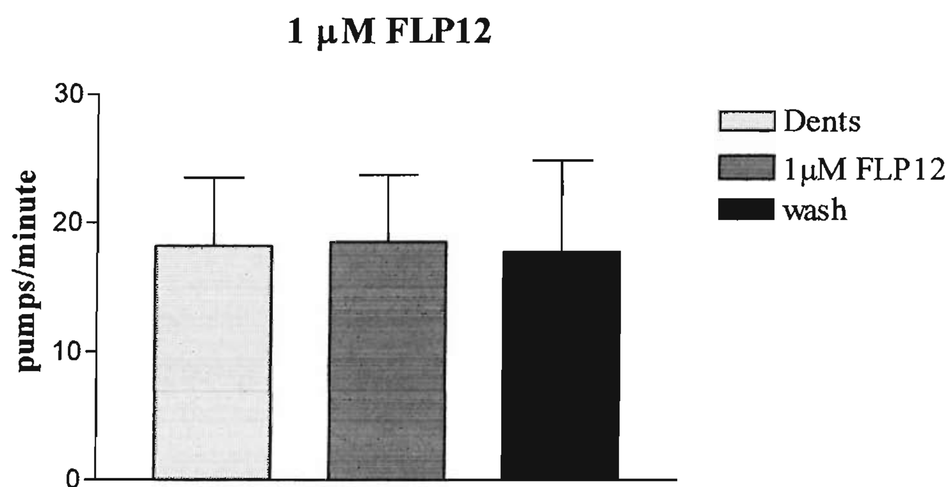
A.



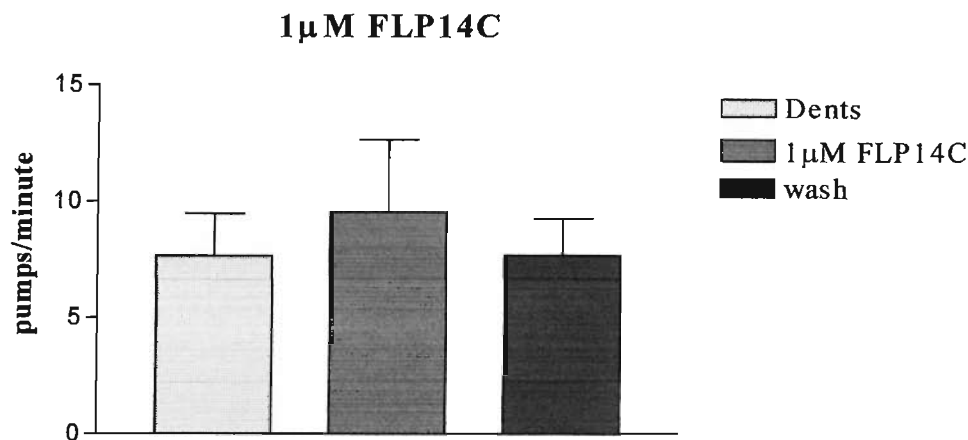
B.



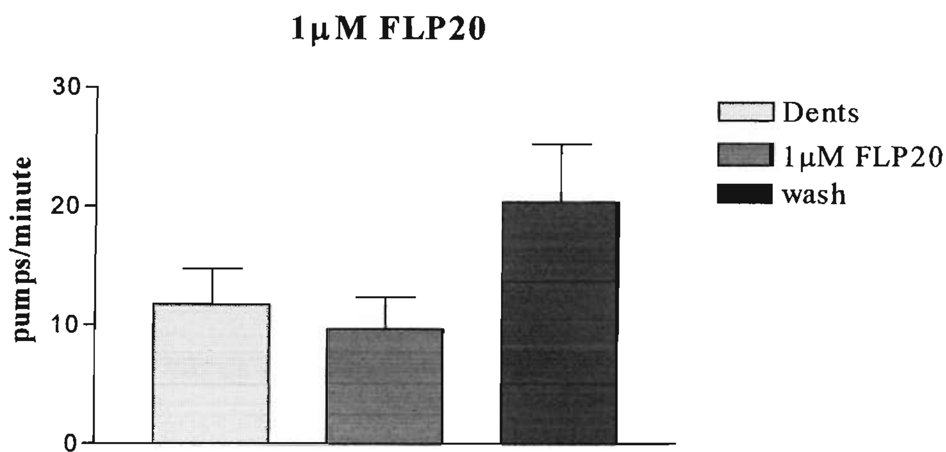
C.



D.



E.



F.

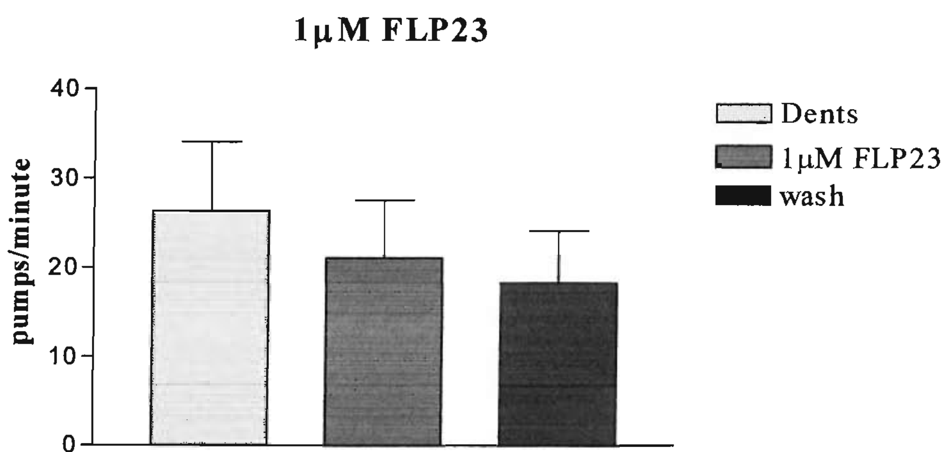


Figure 4.15. Inactive FLP peptides. A to F represent pooled data of the effect of FLP7A (n=5), FLP10A (n=7), FLP12A (n=7), FLP14C (n=5), FLP20 (n=6) and FLP23 (n=5) on the frequency of pharyngeal pumping respectively. Control is the rate of pharyngeal pumping in Dent's saline.

4.1.4 Discussion

To date, the limited knowledge of the physiological role of the FLPs in *C. elegans* has been obtained largely from investigating the functional consequence of *flp* gene knockouts.

The exogenously applied FLPs studied here may exert effects on the pharyngeal muscle in a number of different ways. Most simply, they may exert an effect via cognate receptors for the endogenous peptide which are expressed in the pharyngeal muscle. In this case it might be expected that the peptides would exert a relatively potent and reproducible effect. However, indirect effects of the exogenously applied FLPs will also be detected in this pharyngeal assay i.e. the action of the peptide may be mediated by a receptor expressed outside the pharyngeal system. Again, if this effect is mediated by a specific receptor then it would be expected that the actions of the peptide would be observed at relatively low concentrations but may be more variable. For peptides which exert a direct effect on the muscle it would be predicted that the gene encoding the native peptide would be expressed in neurones that synapse onto the muscle.
Alternatively, the relevant *flp* gene may be expressed in a neurosecretory neurone. In this case, the native peptides would be released by extrapharyngeal cells and act by diffusing through the pseudocoelom to interact with receptors either on the pharyngeal muscle or in the pharyngeal nervous system. For peptides which exert an indirect effect on the muscle, it would be expected that the relevant *flp* gene would be expressed in neurones which contribute to the neuronal circuitry regulating pharyngeal activity.
Finally, the possibility that the effect of an exogenous applied peptide is not physiologically relevant must be considered. This is particularly pertinent for weak effects observed at relatively high peptide concentrations where it is likely that the peptide may be acting via a receptor for which it is not the native ligand. If this is the case then it might be expected that the peptide in question would have strong structural similarity to a peptide that has a potent action. All these considerations have been taken into account in the discussion below, in order to interpret the observed biological activity of the FLPs with their putative physiological role in the pharynx.

To provide a foundation for this discussion, a brief overview of the neuronal regulation of pharyngeal muscle activity is provided.

The biological activity of the FLPs on the pharynx.

The potent biological activity of a large subset of the FLPs in the pharyngeal assay (Tables 4.1 and 4.2) suggests that a number of these peptides may have a physiological role in regulating the activity of the muscle and adds to the previous study reporting the bioactivity of the FLPs in the pharynx (Rogers *et al.,* 2001). The concentration required to obtain consistent responses for each peptide was normally either 100 nM or 1 μM (Tables 4.1 and 4.2) although threshold responses were often obtained at 10 nM. The results from four peptides varied depending on whether 5-HT was present or absent in Dent's saline. When FLP21, FLP18A, FLP16A and FLP15A were tested in Dent's with 5-HT they had no consistent effect whereas in Dent's without 5-HT, all three peptides inhibited pharyngeal activity, Table 4.2. This would suggest that their ability to alter pharyngeal activity is dependent on the rate at which the pharynx is pumping. However, their site of action could be within the presynaptic network rather than directly on the muscle. The other inhibitory peptides inhibited in the presence or absence of 5-HT. All the excitatory peptides were tested in Dent's in the absence of 5-HT.

Role of FLPs expressed in Pharynx.

Further insight may be gained by considering the role of the neurones in which the different *flp* genes are expressed. These expression patterns for the *flp* genes have been described by generating animals expressing transgenes consisting of the *flp* promoter sequence fused to the coding sequence for green fluorescent protein (Kim and Li, 2004; Table 4.4). The identity of the cells expressing *flps* provides an indication of the possible physiological roles of the neuropeptides, although there is the caveat that the expression patterns defined in this manner are not always unequivocal.

Using these reporter constructs it has been shown that ten *flp* genes are expressed in the pharyngeal nervous system, including six of the motorneurones

which innervate the pharyngeal muscles (Kim and Li, 2004). Interestingly, six *flp* genes are expressed in more than one of the neurones which make up the pharyngeal nervous system. One motoneurone, M5, expresses three *flp* genes. *flp* genes are also expressed in four of the pharyngeal interneurones and two *flp* genes are expressed in pharyngeal muscle.

FLP1A1, FLP1A2, FLP2A, FLP4A, FLP5A, FLP6, FLP13A, FLP15A, FLP17A, FLP17B, FLP18A and FLP 21 (Table 4.1; Table 4.2) are encoded by genes which are expressed in neurones which synapse directly onto the pharyngeal muscle (Albertson and Thomson, 1976). This is consistent, therefore, with the proposal that their actions are direct onto the muscle, and that the muscle contains receptors for these peptides. Most of these peptides are present in neurones which are key for modulation of pharyngeal activity, MC, M2, M3, M4, M5, NSM and I5, (Table 4.4, Fig. 4.2). FLP2A, which enhances pharyngeal activity, is present in three of these neurones, M4, MC and I5 (Kim and Li, 2004) and is likely to have an important role in isthmus peristalsis (M4), rapid pharyngeal pumping (MC), and motoneurone regulation. The cholinergic motoneurone M4 also expresses *flp-5* and *fp-21* and so a total of six FLPs are encoded in this cell. FLP5 peptides excite the pharynx while FLP21 (AF9) is inhibitory and so it is possible for FLPs to fine tune the action of M4 on isthmus peristalsis. The cholinergic motorneurone MC (Niacaris and Avery, 2003; Raizen *et al.,* 1995) which is responsible for rapid pharyngeal pumping (Avery and Horvitz, 1989) also contains FLP21 which could suppress the excitatory effect of MC and slow pharyngeal pumping rate. The third neuron which also contains FLP21 which could suppress the excitatory effect of MC and slow pharyngeal pumping rate. The third neuron which contains FLP2, I5, also contains 5-HT (Sawin *et al.,* 2000) and possibly glutamate (Lee *et al.,* 1999), and synapses onto several neurones in the pharyngeal nervous system, including M4, M3, M1 and possibly NSM and I4.
Pharyngeal relaxation becomes quicker when I5 is ablated (Avery, 1993) and there is evidence that I5 slows relaxation by inhibiting M3 (Avery and Thomas, 1997). This is possibly through the release of glutamate since M3 has glutamate receptor subunits (Brockie *et al.,* 2001). I5 also expresses *flp-4* and *flp-13* which excite and inhibit pharyngeal activity, respectively. However, it should be noted that the response of the pharynx to FLP4A was variable. It may be that the response of the muscle to some neuropeptides is context-dependent and although all recordings were made from

young well-fed adults there may have been differences in the feeding or nutritional status of individual animals which gave rise to this variability.

As well as making synapses onto neurones, I5 also synapses onto the muscle pm5. It is more likely therefore that the effects of FLP4A and the FLP13 peptides are predominantly direct onto the muscle. However, *in vivo*, both FLP4 and FLP13 could influence the duration of each pump by acting on M3. FLP13 peptides are also predicted to be present in M3, together with FLP18 peptides. Both groups of peptides are inhibitory, therefore could be co-released with glutamate to decrease pump duration (Dent *et al.,* 1997). I2, which expresses *flp-15*, and possibly *flp-5*, appears to play an important role in regulating pharyngeal activity since it synapses onto M1, I4, I6 and NSM, and I4 in turn synapses onto M3. FLP15A is inhibitory whilst FLP5A is excitatory thus the net effect of these peptides on the output of the pharyngeal circuit will depend on the relative amounts of the two peptides that are released at any given time.

Three *flp* genes expressed in the cholinergic motorneurone M5, *flp-1, flp-13* and *flp-17*, encode a total of fourteen FLPs. FLP1A and FLP13A inhibit pharyngeal activity while FLP17A and B are excitatory. It is interesting that FLP17A and B have a similar effect on pharyngeal activity and suggests that they activate a common receptor. If this is the case then the receptor does not distinguish between phenylalanine and tyrosine and between valine and isoleucine which may be the situation for other FLP receptors. M5 may release a cocktail of peptides that can either excite or inhibit the pharynx depending on the ratio in which they are released and their efficacy. However, from the work of Avery, (1993) M5 does not appear to play an important role in pharyngeal activity. It is not clear why it should have the potential to release so many peptides unless it does play a significant role in pharyngeal function. For example, M5 may have a role in behaviours triggered by the animal's natural environment but absent under laboratory conditions. M2, which is also cholinergic, expresses *flp-18* and *flp-21* which both inhibit pharyngeal activity. The effects of these peptides therefore oppose the excitatory effect of acetylcholine on the pharynx. *flp-6* is expressed in I1 which is the only neuron in the pharyngeal nervous system which receives an input from the somatic nervous system *via* RIP neurones. However, it is not known which transmitters and modulators are released by

RIP neurones. I1 in turn synapses onto M2, M3, MC, NSM and I5 and so FLP6 (AF8) can potentially modulate the activity of a number of key neurones in the pharyngeal nervous system and so influence pharyngeal activity, Fig. 4.2. FLP6 excites the pharynx and so it probably excites the follower neurones to I1 although this has not been proved experimentally. Two further pharyngeal interneurones, I4 and I6, express flp genes, *flp-5* and *flp-4* respectively. I4 synapses onto M1, M3 and NSM while I6, which is possibly cholinergic (Duerr *et al.,* 1997), synapses onto M4 and has tight junctions with M4 and M5. FLP5A is excitatory on the pharynx but its direct effects on these neurones is not known. However, both peptides will influence pharyngeal activity through their actions on these neurones. Two *flp* genes, *flp-5* and *flp-15,* are expressed in pharyngeal muscle. The data presented here suggest they could act as myohormones to excite and inhibit muscle activity, respectively.

Neurone	Description and putative transmitter (s)	Gene Expression[1]	Peptide Effects
MC	pacemaker neuron (ACh[2])	*flp-2; flp21*	FLP2A +; FLP21 -
M2	motoneurone (ACh[3])	*flp-18; flp21*	FLP18A -; FLP21-
M4	motoneurone (ACh)	*flp-2; flp-5; flp-21*	FLP5A +; FLP2A+; FLP21 -
M5	motoneurone (ACh[3])	*flp-1; flp-13; flp-17*	FLP17A++; FLP17B++; FLP13A- -; FLP1A1-; FLP1A2-
I6	interneurone (ACh[3])	*flp-4*	FLP4A[4]+
M3	inhibitory neurone (glutamate[4])	*flp-13; flp-8*	FLP13A - -; FLP18A-
I5	interneurone (5-HT; glutamate[4,5])	*flp-2; flp-4; flp-13*	FLP2A +; FLP13A- -; FLP4A[4]+
NSM	neurosecretory motoneurone (5-HT[6,7])	*flp-4*	FLP4A[4]+
I1	interneurone	*flp-6*	FLP6+
I2	interneurone	*(flp-5); flp-15*	FLP5A+; FLP15A-
I4	interneurone	*flp-5*	FLP5A+; FLP6+

Table 4.4 Summary of the actions of FLPs that are co-expressed in the same neurones. Pharyngeal neurones that have been reported to express flp genes are listed. Second column provides a brief descriptions of each neurone's function. The last column summarises the bioactivity of FLPs in the pharyngeal preparation of *C. elegans*. Plus signs indicate excitation; two plus signs indicate potent excitation; minus signs indicate inhibition; two minus signs indicate potent inhibition.

[1]Kim and Li, 2004; [2]Raizen *et al.,* 1995; [3]Duerr *et al.,* 1997; [4]Lee *et al.,* 1999; [5]Sawin *et al.,* 2000; [6]Horvitz *et al.,* 1982; 7Sze et al., 2000. [4]The responses to FLP4A were variable and in five preparations weak inhibition was observed.

Inactive peptides

Six of the peptides tested in the pharyngeal assay had no significant effect on the activity of the muscle. None of these are encoded by *flp* genes which are expressed in the pharyngeal nervous system. This indirectly supports the contention that the biological activity of the peptides reported here is informative about their physiological role. Two of the peptides encoded by these genes, FLP7 and FLP20, have a common C terminal of MVRFamide and so may act on a common receptor which is not expressed in the pharynx. However, two other genes, *flp-12* and *flp-23*, encode peptides with FIRFamide and FLRFamide respectively as their C terminal and might be expected to interact with the same receptors as FLP4 and FLP1 peptides, respectively. The observation that they are not active in the pharyngeal assay indicates that the relevant FLP receptors are very specific for their cognate ligand(s). FLP10 might also be expected to interact with the same receptor as FLP4, though the extended N terminal of FLP10 may prevent receptor activation. Interestingly, a shortened form of FLP10, GYIRFamide, is found in the turbellarian, *Dugesia tigrina* (Johnson *et al.,* 1996) and this peptide increases the frequency and amplitude of contractions of isolated muscle strips from the liver fluke, *Fasciola hepatica* (Graham *et al.,* 1997). It is possible that a shortened form of FLP10 might be active in *C. elegans*.

Active FLPs which are not expressed in the Pharyngeal System.

The peptides of seven genes which are not expressed in the pharyngeal system had an effect on pharyngeal activity, FLP3A, FLP8 (AF1), FLP9, FLP11A, FLP14A (AF2), FLP16A and FLP22. FLP3A is particularly interesting since it appears to act either directly or indirectly onto M3 to induce inhibitory potentials in the EPG. If these peptides have an effect *in vivo* then they must act as neurohormones by diffusing through the pseudocoelom to either act directly on pharyngeal muscle or via the pharyngeal nervous system. All these FLPs have an extended N terminal which conveys resistance to enzymatic breakdown (Price, 1986) and is consistent with a neurohormonal role. In addition, five of these FLPs, Table 4.1 and 4.2, do not have a methionine in their C terminal sequence which increases their stability since methionine can undergo oxidation.

Number of FLP Receptors in the Pharyngeal System.

From pharmacological studies using *A. suum* motor nervous system, it has been proposed there are at least five different receptors of FLPs (Davis and Stretton, 2001). Amongst the FLPs used in this *A. suum* study were four identified in *C. elegans,* FLP16A (AF15), FLP14A (AF2), FLP21 (AF9) and FLP6 (AF8). These peptides elicited different effects in *A. suum* and by inference these peptides might also act on separate receptors in *C. elegans*. From Tables 4.1 and 4.2, it can be seen that FLP16A and FLP21 inhibited while FLP14A and FLP6 excited the pharynx. If it is assumed that the same peptide ligand interacts with the same or similar receptors across the phylum, then there are at least four FLP receptors in the pharynx. Further evidence for FLP receptor subtypes is provided by studies using *A. suum* body wall and *vagina versa* muscle. In these assays there is evidence that FLP8 and FLP14A act on separate receptors (Fellowes *et al.,* 2000; Bowman *et al.,* 1996). The similarity in structure between FLP6 (PF3) and FLP17A, Table 4.1 would suggest these peptides possibly act on a common receptor. However, FLP13 peptides and FLP18 peptides, although both inhibitory, are likely to act through separate receptors as they have dissimilar structures. Furthermore, FLP13C and FLP18C have opposite effects on *A. suum* somatic muscle (Marks *et al.,* 2001) suggesting these peptides act on different receptors. From their structures and common inhibitory effects on pharyngeal activity, it is likely that all the FLP18 peptides act on a common receptor, and all FLP3 peptides act on a common receptor. Overall, these data are consistent with the presence of eight different neuropeptide receptors in the pharyngeal nervous system. The total number of putative receptors may appear excessive when compared with other families. For example, in mammals there are four defined opioid receptors, μ, δ, κ and ORL1, though there is evidence for up to four additional receptors (Waldhoer *et al.,* 2004).

As shown in Table 1.4 a number of putative FLP receptors have been identified in *C. elegans*, together with their possible ligands. The ligands for these receptors are proposed as FLP2, FLP3, FLP7, FLP11, FLP15, FLP18 and FLP21 (Kubiak *et al.,* 2003a,b; Lowery *et al.,* 2003; Mertens *et al.,* 2004, 2005; Rogers *et al.,* 2003). Out of these ligands only FLP7 is inactive on pharyngeal pumping while FLP18 and FLP21 have been discussed above. This leaves an additional three ligands,

FLP3, FLP11 and FLP15, which might act on separate receptors. From their structures it is likely that FLP11 and FLP15 act on different receptors. This suggests a total of at least eleven FLP receptors in the pharyngeal system.

CHAPTER 5

The effect of altering second messenger signaling on the response of the *Caenorhabditis elegans* pharynx to FLP17A and 5-HT

5.0 Introduction

Most, if not all, neurones contain neuropeptides in addition to classical transmitters. Neuropeptides can act as neuromodulators to modify synaptic transmission and so influence the activity of neural networks and in turn, behaviour (Nusbaum *et al.,* 2001). Most neuropeptides act through seven transmembrane domain G-protein coupled receptors (GPCRs) (Marchese *et al.,* 1999; Probst *et al.,* 1992) although there are exceptions. For example, a molluscan FMRFamide receptor is a tetramer (Lingueglia *et al.,* 1995; Cottrell 1997; Coscoy *et al.,* 1998). There is also evidence that chloride channels activated by a nematode peptide, PF4, are directly ligand-gated (Holden-Dye *et al.,* 1997; Purcell *et al.,* 2002). Physiological, pharmacological, behavioural and molecular studies suggest neuropeptides, particularly FaRPs/FLPs play fundamental roles in nematode physiology (Li, 2005). This is reinforced if their numbers are considered. For example, 38 Famides (of which 29 are RFamides) are expressed in neurones in the major ganglia, nerve ring, cords and commissures of *A. suum* (Yew *et al.,* 2005), while around 70 FaRPs have either been identified or are predicted to be encoded by 29 flp genes in *C. elegans* (Li, Kim & Nelson 1999; Husson *et al.,* 2005; Li, 2005). Many of these *C. elegans* FaRPs are expressed in identified neurones in *C. elegans* (Kim and Li 2004). Interestingly these authors have shown that more than one *flp* gene are expressed in many of these neurones, suggesting multiple roles for peptides released onto follower cells. For example, the cholinergic neurone, M5, may release up to 17 FLP peptides encoded by three genes. To complicate the situation further, some peptides excite pharyngeal pumping while others inhibit it. What are the roles for this plethora of FLPs and are they all encoded? While many FLPs have been tested for activity on *A. suum* tissues, relatively little is known regarding their cellular and molecular mechanisms of action. *C. elegans* genome data suggest there are around 60 G-protein coupled receptors (GPCRs) which may be peptide receptors (Bargmann 1998; Keating *et al.,* 2003). Gene knockout using RNAi indicates specific receptors have roles in reproduction or locomotion (Keating *et al.,* 2003a,b).

There are a considerable number of FaRP receptors in nematodes and so far eleven have been identified, all of which are G-protein coupled (Kubiak *et al.,* 2003a,b; Mertens *et al.,* 2004; Mertens *et al.,* 2005a,b; McVeigh *et al.,* 2006a). Each

receptor has been linked with its most potent ligand available to date (McVeigh *et al.*, 2006) but these may not prove to be their true endogenous ligands. For example, Mertens *et al.*, (2004) cloned and characterized the FaRP receptor, VRFa receptor 1 (orphan C26F1.6) where SMVRFamide proved the most active sequence, derived from a peptide (TPMQRSSMVRFamide) expressed by *flp-7* gene (Nelson *et al.*, 1998). Although there are a very large number of GPCRs in nematodes, as judged by evidence in the *C. elegans* genome (The *C. elegans* Sequencing Consortium 1998), little is known about the identity of the second messengers involved in their function. For example, both AF1 (KNEFIRFamide) and AF2 (KHEYLRFamide) excite *A. suum* body wall muscle with AF2 also having an initial inhibitory component (Cowden *et al.*, 1989; Cowden and Stretton 1993; Pang *et al.*, 1992; Pang *et al.*, 1995). The injection of 10 μM of either peptide results in a large sustained increase in the level of cAMP in *A. suum* (Reinitz *et al.*, 2000) and both AF1 and AF2 increase cAMP in body wall muscle without altering levels of either cGMP or IP_3 (Thompson *et al.*, 2003). Kubiak *et al.*, (2003) have also presented binding evidence for AF2 acting through a GPCR. Both AF3 (AVPGVLRFamide) and AF4 (GDVPGVLRFamide) increase the tone of *A. suum* body wall muscle and directly depolarize the muscle cells (Trim *et al.*, 1997). AF3 decreases cAMP levels in muscle cells and this may be involved in the generation of contraction (Trim *et al.*, 1998). AF9 (GLGPRPLRFamide) has also been shown to inhibit cAMP through activation of Gi/Go proteins (Kubiak *et al.*, 2003a).

5-Hydroxytryptamine (5-HT) is one of several biogenic amines which have been identified in *C. elegans* and has a role in the regulation of pharyngeal pumping, locomotion and egg-laying (Komuniecki *et al.*, 2004). Application of 5-HT to the pharynx increases its pumping activity up to a maximum of around 250 pumps per minute (Horvitz *et al.*, 1982; Niacaris and Avery 2003). It is likely that 5-HT acts both directly and indirectly on *C. elegans* pharyngeal muscle (Rogers *et al.*, 2001). These authors found that the pharyngeal muscle of a mutant deficient in synaptic transmission, snb-1 (md247), was less responsive to 5-HT. However, there is also a direct 5-HT component from the release of 5-HT from pharyngeal NSM neurones where 5-HT can act as a neurohormone (Horvitz *et al.*, 1982). There are least three 5-HT receptors in the *C. elegans* pharyngeal system. For example, SER-1 receptors are expressed in pharyngeal muscle, SER-7 receptors are expressed in a number of

pharyngeal neurones including MCs, M3s and the cholinergic motorneurones, M2, M4 and M5 and SER-4 is expressed in an unidentified pharyngeal neurone (Tsalik *et al.,* 2003; Hobson *et al.*, 2006). From studies where these receptors have been expressed in various cell types, it has been proposed that SER-7 is associated with an increase in cAMP (Hobson *et al.,* 2003), SER-1 is 5-HT2-like in terms of structure but its pharmacology is a mix of 5-HT1 and 5-HT2 (Hamdan *et al.*,1999) and couples to Gαq and phosphoinositide turnover while SER-4 is associated with a decrease in forskolin-stimulated cAMP levels (Olde and McCrombie 1997).

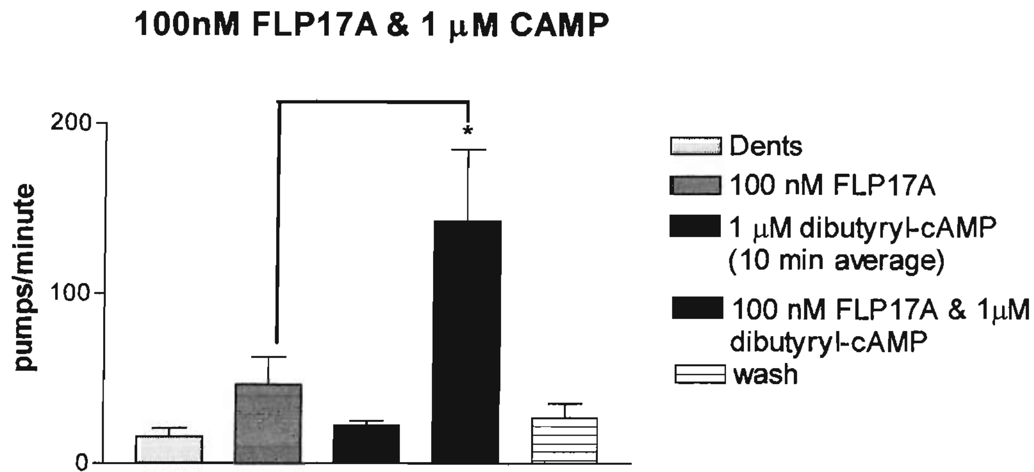
One of the aims of the work in this chapter was to investigate the effect of altering cAMP levels on the response to a FaRP, FLP17A (KSAFVRFamide). This peptide was selected because it has a potent excitatory action on *C. elegans* pharyngeal muscle. In these experiments the effects of manipulating second messenger levels on FLP17A activity were compared with those of another excitatory agent, 5-hydroxytryptamine (5-HT), (Avery and Horvitz 1990; Croll, 1975). Changes in the level of cAMP were achieved in pharyngeal muscle by applying the cell-permeable cAMP analogue, dibutyryl-cAMP (Hei *et al.,* 1991), activating adenylyl cyclase with forskolin (Seamon *et al.,* 1983) and by inhibiting the breakdown of cAMP by applying a non-selective phosphodiesterase inhibitor, ibudilast (Souness *et al.*, 1994). The effects of a protein kinase inhibitor, H-9 dihydrochloride (Hidaka *et al.*, 1984) and the membrane permeable cGMP analogue, 8-bromo-cGMP (Buchan and Martin 1991), were also examined for their effects on FLP17A excitation of pharyngeal muscle. The action of FLP17A on the pharyngeal muscle of two *C. elegans* mutants, *egl-30* (ad810) with a loss-of-function Gαq and *egl-8* (n488) with a loss-of-function PLCβ, was also tested. For comparison, the action of the above compounds was also tested on 5-HT excitation of pharyngeal muscle.

5.1 Results

5.1.1 The effect of Dibutyryl-cAMP on pharyngeal pumping.

Application of exogenous cAMP in the form of the membrane soluble dibutyryl-cAMP, 1-50μM, increased the excitatory response of 100nM FLP17A on pharyngeal muscle, Table 5.1, Figure 5.1A. In Figure 5.1A 1μM dibutyryl-cAMP increased FLP17A excitation by 323.2 ± 155.5% compared with FLP17A applied alone. This increase was significant, $P<0.05$; $n=4$. Figure 5.1B shows traces of EPGs illustrating the effect of 1μM dibutyryl-cAMP on the basic rate of pharyngeal pumping and on the response to FLP17A in one experiment. The effect of dibutyryl-cAMP was reversible following wash. Dibutyryl-cAMP added alone had a small non-significant direct effect on basal activity. Interestingly, 50μM dibutyryl-cAMP increased the FLP17A response by only 184 ± 62.7%, less than with 1μM dibutyryl-cAMP. Dibutyryl-cAMP, 500nM-10μM, also increased the excitatory response to 50nM 5-HT, Table 5.2, Figure 5.2A. The effect of 1-10μM dibutyryl-cAMP on the excitatory responses to 5-HT were significant, $P<0.05 – 0.01$. Figure 5.2C shows traces of EPGs illustrating the effect of 500nM and 1μM dibutyryl-cAMP on the response to 50nM 5-HT, which was reversible following wash.

A.



B.

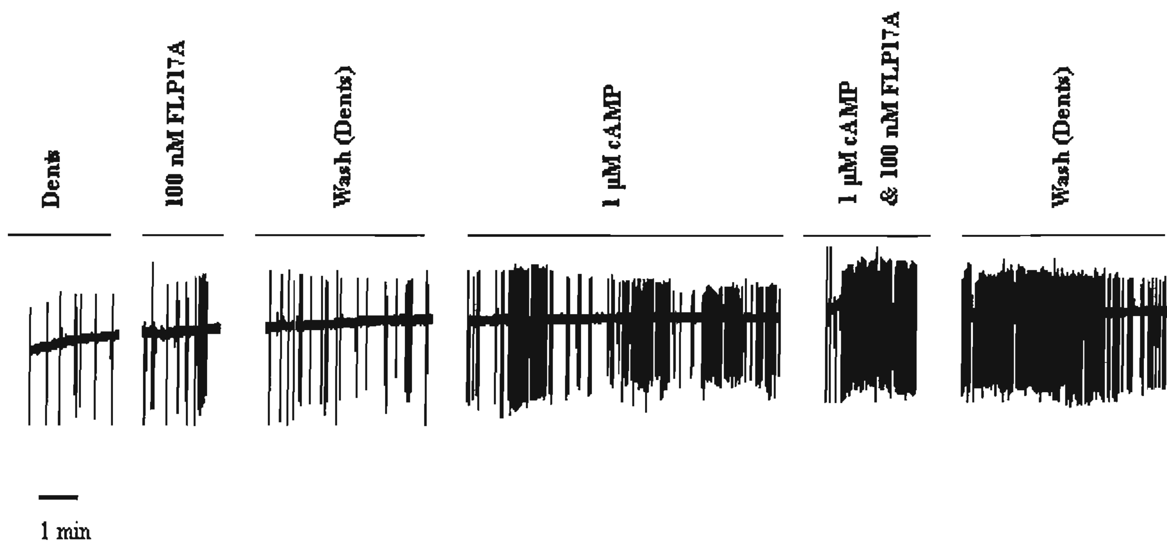
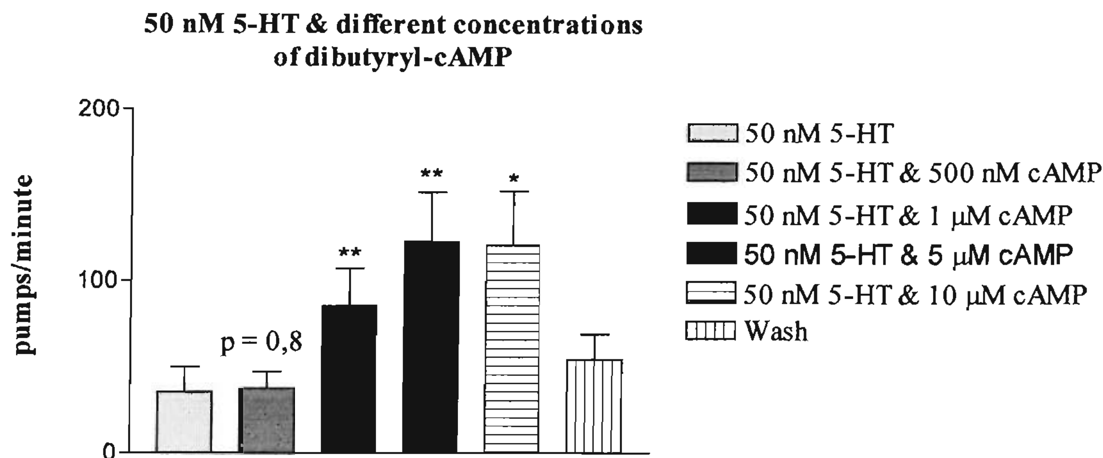


Figure 5.1. The effect of dibutyryl-cAMP on the excitatory response of FLP17A on pharyngeal pumping.

(A) Histograms to show that the response to 100nM FLP17A was enhanced in the presence of 1μM dibutyryl-cAMP; % increase was 323 ± 155.5; * $P<0.05$ n=4.

(B) Example traces to show the effect of 100 nM FLP17A, 1μM dibutyryl-cAMP and both compounds applied together on EPG activity. Following wash, the pumping rate slowly returned to control.

A.



B.

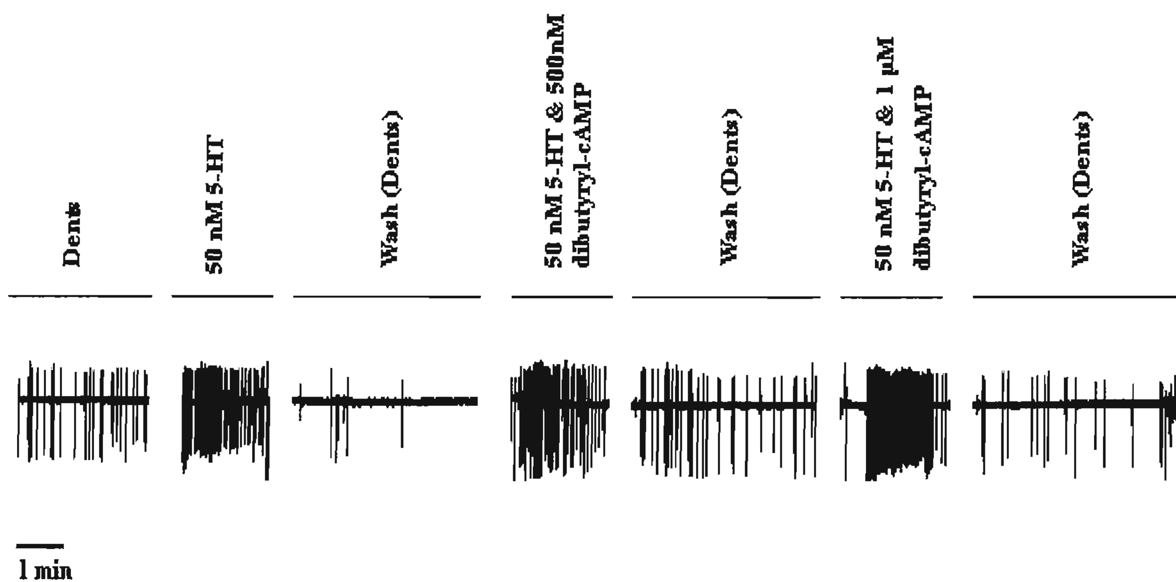


Figure 5.2 The effect of dibutyryl-cAMP on the excitatory response of 5-HT on pharyngeal pumping.

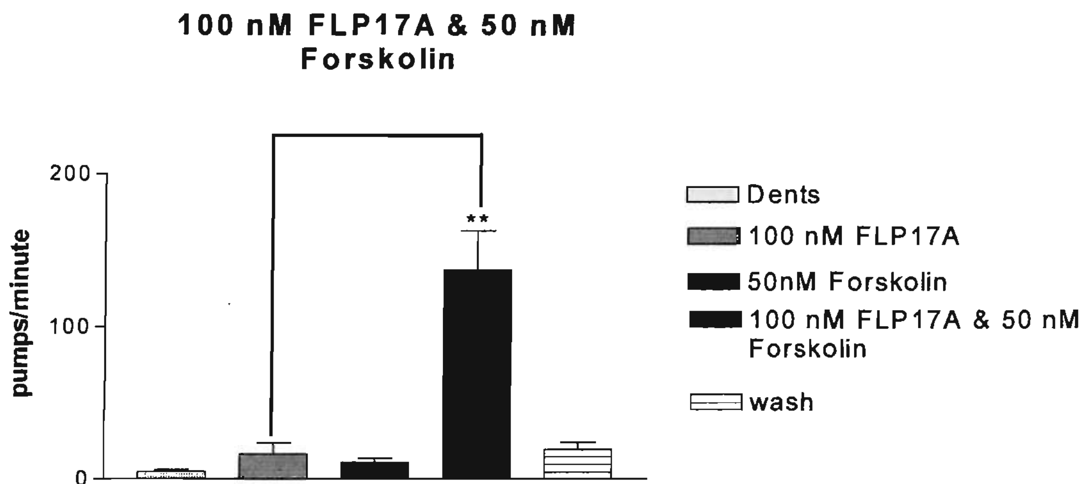
(A) Histograms to show that increasing concentrations of dibutyryl-cAMP enhance the excitatory response to 50nM 5-HT; * $P<0.05$ and * $P<0.01$; n=6

(C) Example traces to show the effect of 50nM 5-HT, 50nM 5-HT and 500nM dibutyryl-cAMP and 50nM 5-HT and 1μM dibutyryl-cAMP applied together on EPG activity

5.1.2 The effect of Forskolin on pharyngeal pumping

The activation of adenylyl cyclase by forskolin potentiated the excitatory effect of 100nM FLP17A when applied at 50nM and 1μM, Table 5.1. Forskolin was dissolved in DMSO solvent and the final concentration of DMSO applied on the pharynx was 0.1%. Figure 5.3A shows the effect of 50nM forskolin on the response to the peptide. When in the presence of 50nM forskolin, the response to the peptide increased by 1345±347%, P<0.01. These concentrations of forskolin increased the basal level of activity (Fig 5.3A, P=0.09, n=6). Figure 5.3B shows traces of EPGs illustrating the effect of 50nM forskolin on the response to FLP17A in one experiment. The enhancement of the peptide effect by forskolin was reversible following wash. Forskolin, 50nM, also enhanced the excitatory effect of 5-HT on pharyngeal muscle, Table 5.2, Figure 5.4A, with an increase in pumping frequency of 375 ± 152.7%, P<0.05. Figure 5.4B shows traces of EPGs illustrating the effect of 50nM forskolin on the response to 50nM 5-HT in one experiment. This effect was reversible following wash, Figure 5.4A.

A.



B.

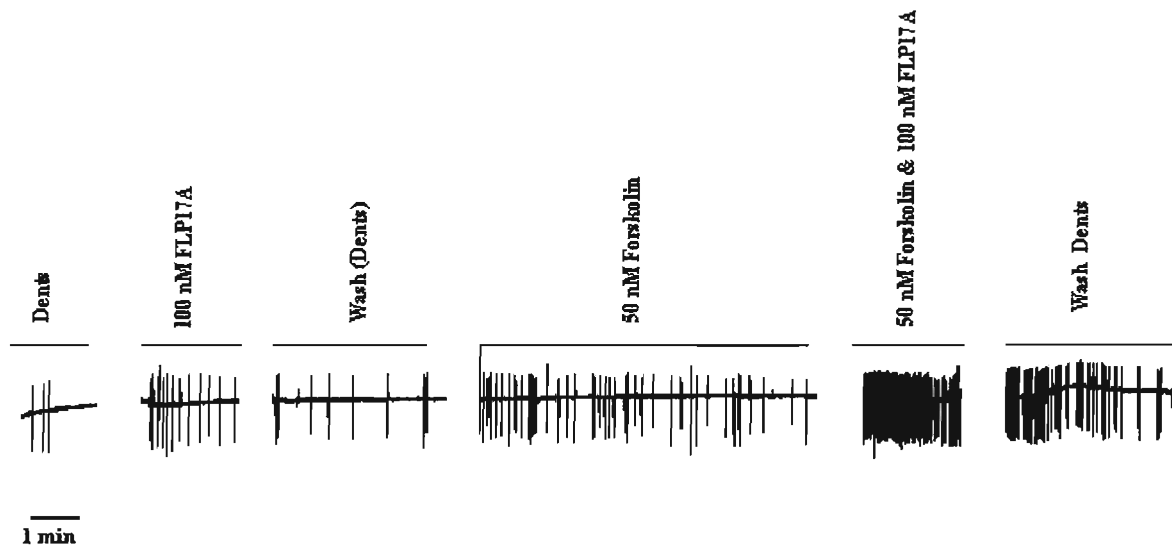
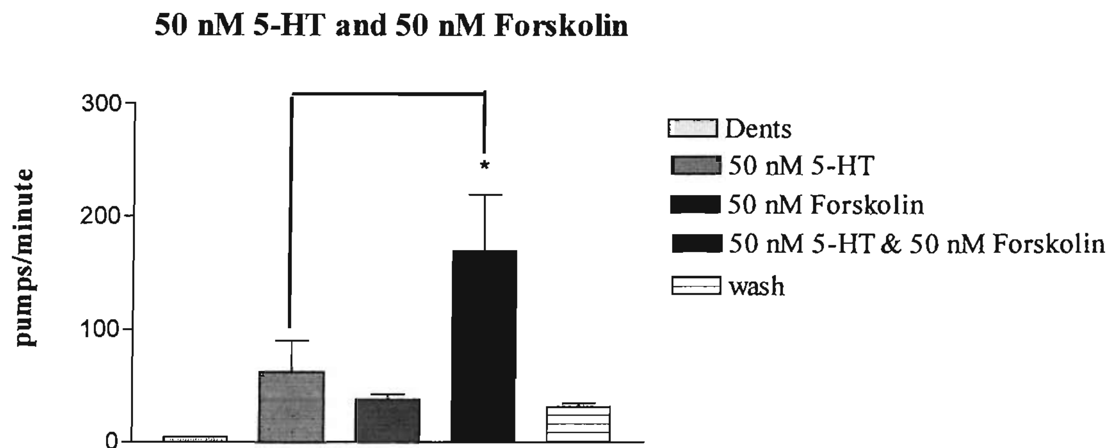


Figure 5.3. The effect of Forskolin on the excitatory response of FLP17A on pharyngeal pumping.

(A) Histograms to show the response of 100nM FLP17A was enhanced in the presence of 50 nM forskolin; % increase was 1345 ± 347; **P<0.01; n=6.

(B) Example traces to show the effect of 100 nM FLP17A, 50 nM forskolin and both compounds applied together on EPG activity. Following wash, the pumping rate slowly returned to normal.

A.



B

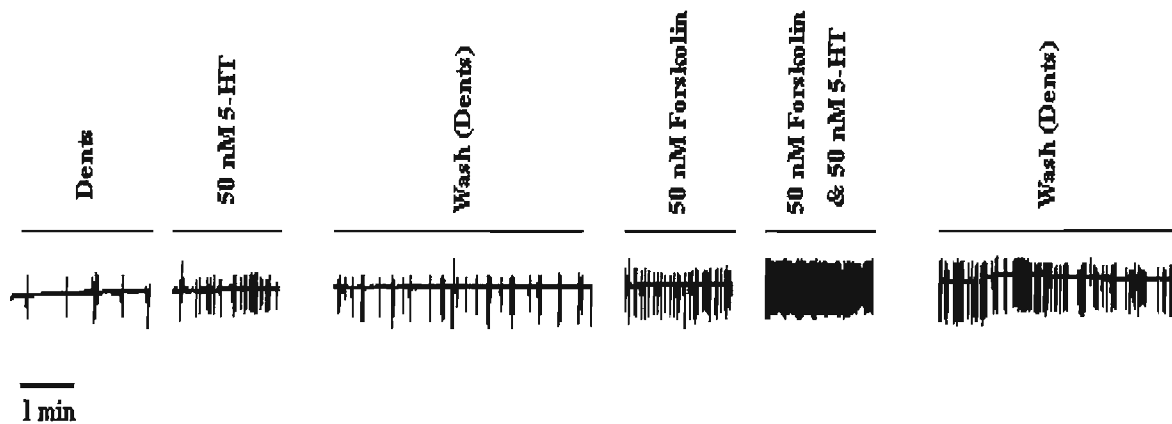


Figure 5.4. The effect of forskolin on the excitatory response of 5-HT on pharyngeal pumping.

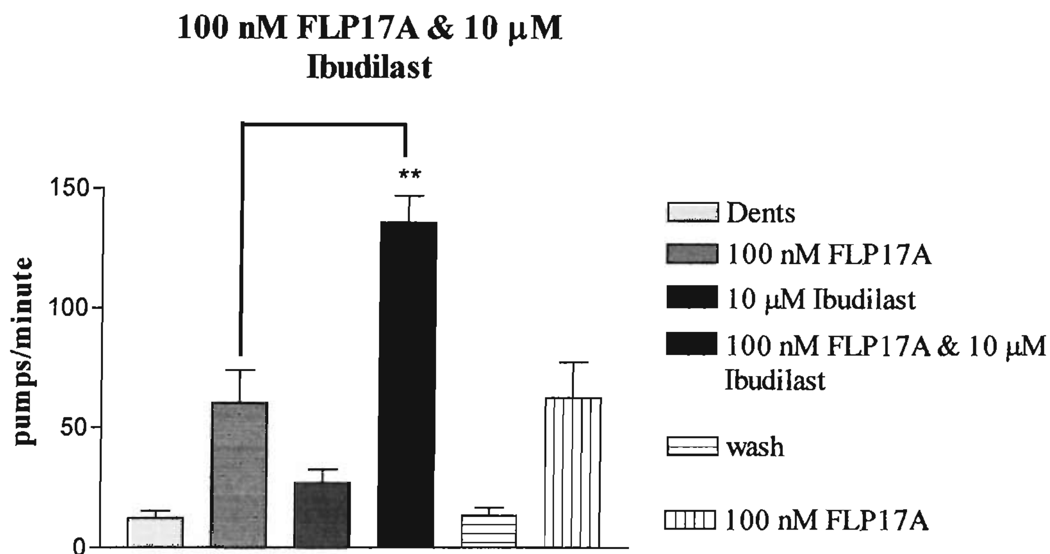
(A) Histogram to show that the response to 50nM 5-HT was enhanced in the presence of 50 nM forskolin; % increase 375.8 ± 152.7; * P<0.05; n=5

(B) Example traces to show the effect of 50 nM 5-HT, 50nM forskolin and both compounds applied together on EPG activity.

5.1.3 The effect of ibudilast on pharyngeal pumping

Application of the phosphodiesterase inhibitor, ibudilast, 10μM, enhanced the excitatory response to 100nM FLP17A on pharyngeal muscle, Table 5.1, Figure 5.5A, with an increase in frequency of 197.2 ± 85.0%. The final histogram in Figure 5.5A shows that the response to the peptide returns to pre-ibudilast levels following wash. Figure 5.5B shows traces of EPGs illustrating the effect of 10μM ibudilast on the response to 100nM FLP17A, which was reversible following wash. Ibudilast had a direct excitatory effect on basic pharyngeal activity (Fig 5.5A, P=0.16, n=5). Ibudilast, 10μM, also increased the response to 50nM 5-HT but this effect was not significant (P=0.06, n=4), Table 5.2, Figure 5.6A. Following wash, a further application of 50nM 5-HT was still slightly potentiated compared with the control 5-HT response where 5-HT was applied prior to the ibudilast. Figure 5.6B shows traces of EPGs illustrating the effect of ibudilast on the response to 5-HT. It can be seen that ibudilast clearly enhanced the response to 5-HT in this experiment and that this effect is reversible following wash. Ibudilast was dissolved in ethanol and the final concentration of ethanol applied on the pharynx was 0.1%.

A.



B

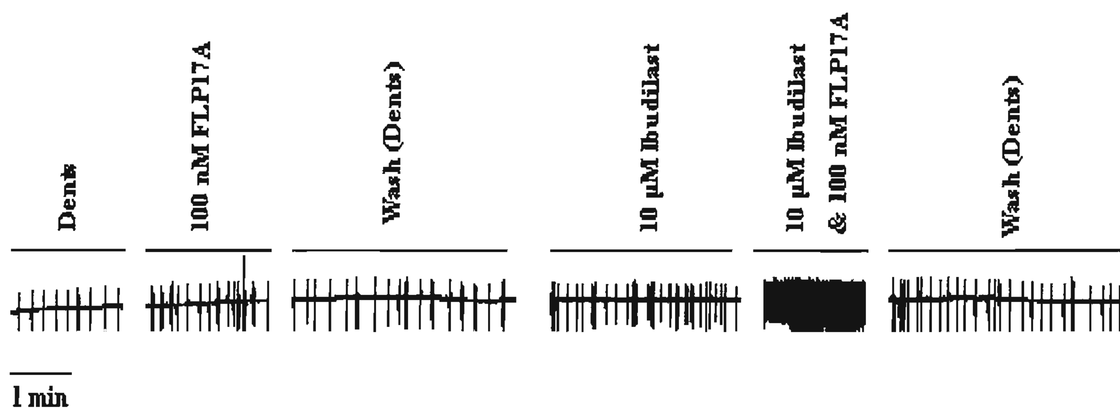
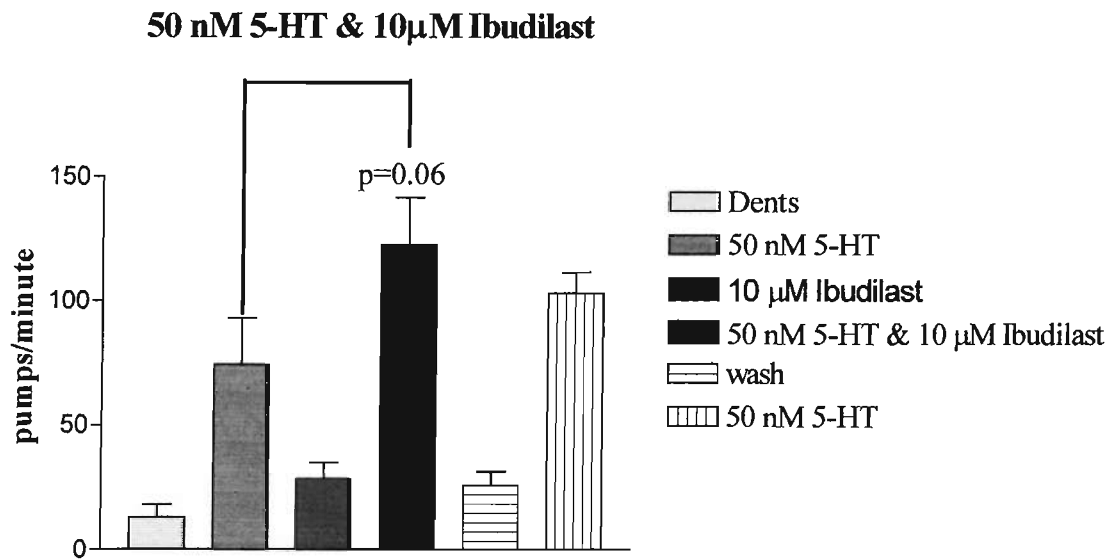


Figure 5.5 The effect of ibudilast on the excitatory response of FLP17A on pharyngeal pumping.

(A) Histograms to show that the response of 100 nM FLP17A was enhanced in the presence of 10 μM ibudilast; % increase was 197.2 ± 85; ** P<0.01; n=5

(B) Example traces to show the effect of FLP17A, ibudilast and both compounds applied together on EPG activity. Following wash, the pumping rate returned to control.

A.



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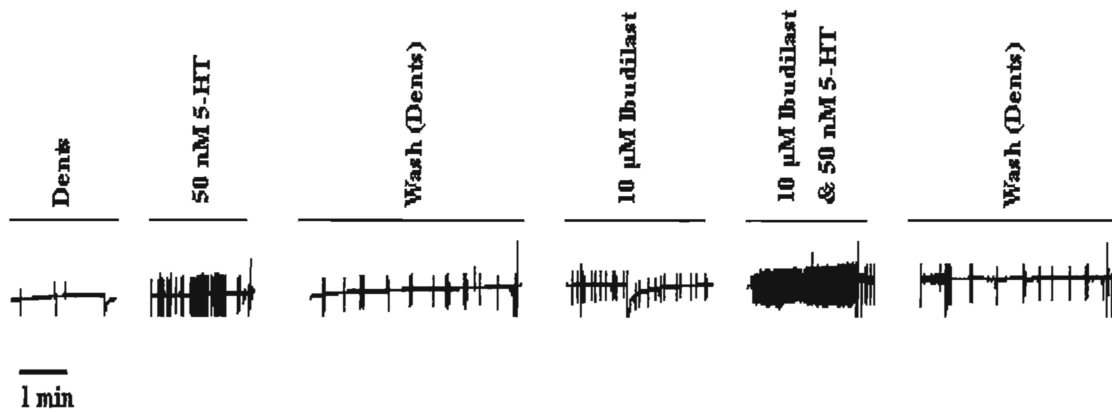


Figure 5.6 The effect of ibudilast on the excitatory response of 5-HT on pharyngeal pumping.

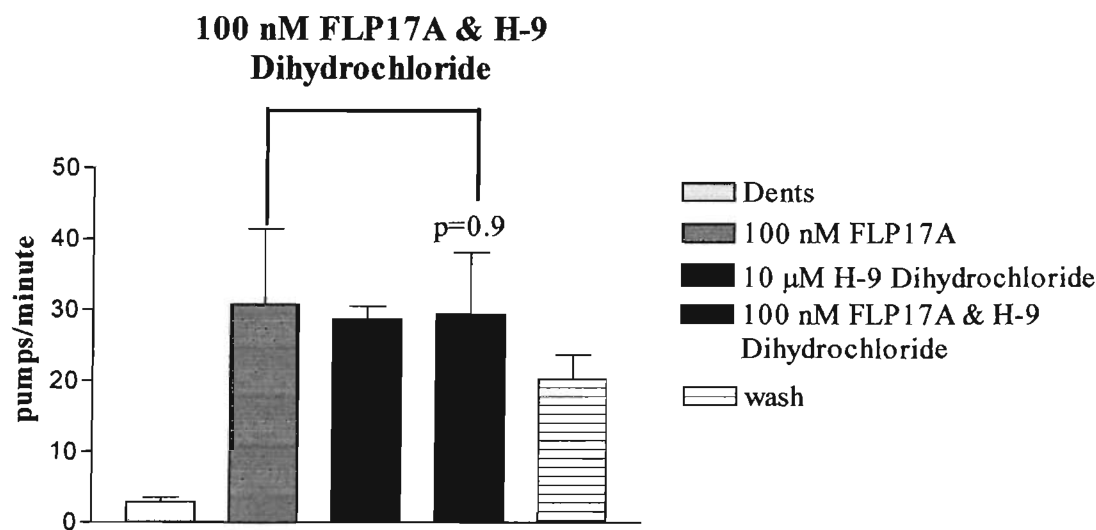
(A) Histograms to show that the response to 50 nM 5-HT was enhanced in the presence of 10 μM ibudilast; % increase 173 ± 91.4; P<0.06 (n=5).

(B) Example traces to show the effect of 5-HT; ibudilast and both compounds applied together.

5.1.4 The effect of H-9 dihydrochloride on pharyngeal pumping

When 100nM FLP17A was applied in the presence of protein kinase inhibitor, H-9 dihydrochloride, 10μM, H-9 had no apparent effect on the excitatory response to 100nM FLP17A (n=3), Table 5.1. However, in these experiments H-9 did have a direct effect on pharyngeal muscle activity often approximately equal to that of FLP17A on its own, Figure 5.7A, 5.7.B. So it is possible that all or most of the excitation seen with the mixture was due to the direct effect of H-9. In which case H-9 more or less completely blocked FLP17A excitation. The values shown in Table 5.1 for pharyngeal activity for FLP17A alone, for H-9 alone and for the mixture illustrate this point. In some experiments the response to the mixture of H-9 and FLP17A was less than for the peptide response alone. However, this compound did significantly reduce the excitatory response to 5-HT, Table 5.2, Figure 5.8A, the reduction being 32±4.96%, $P<0.01$, n=5. Figure 5.8b shows traces of EPGs illustrating the effect of H-9 on the response to 5-HT.

A.



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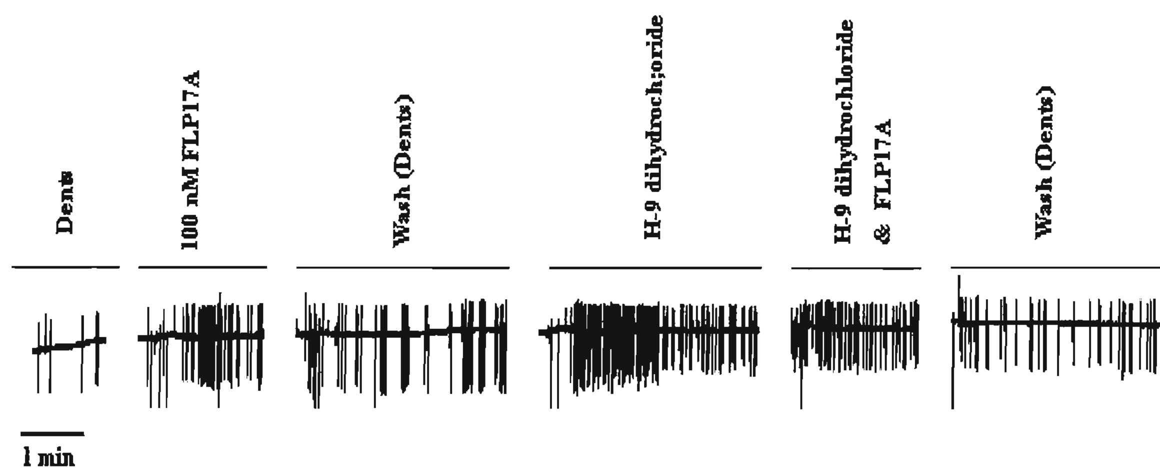
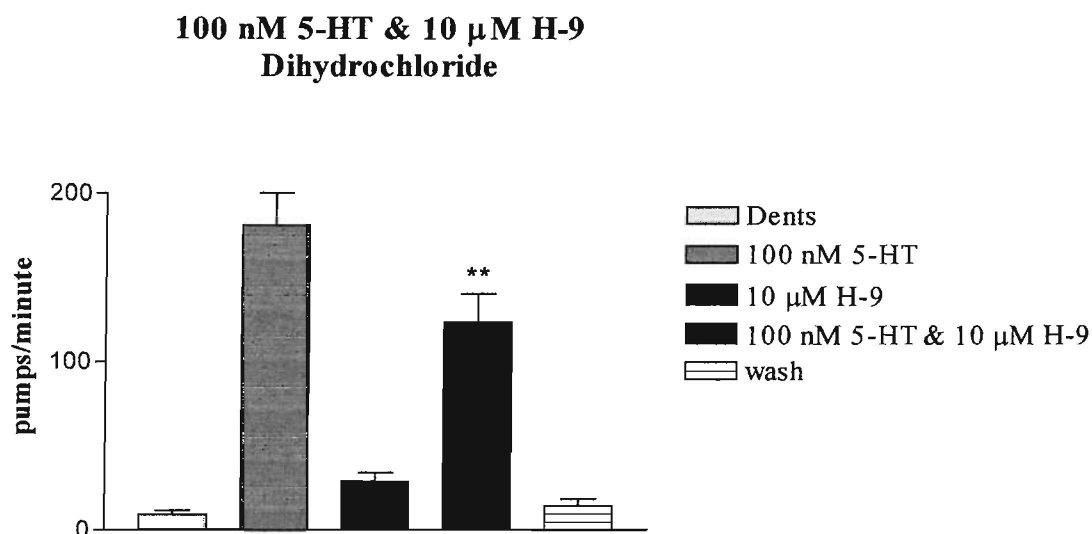


Figure 5.7 The effect of H-9 dihydrochloride on the excitatory responses of FLP17A on pharyngeal pumping.

(A) Histograms to show that the responses to 100 nM FLP17A in the presence of 10 μM H-9 (n=3);

(B) Example traces to show the effect of 100 nM FLP17A, H-9 and both compounds added together on EPG activity. Following wash, the pumping rate returned to control.

A.



B.

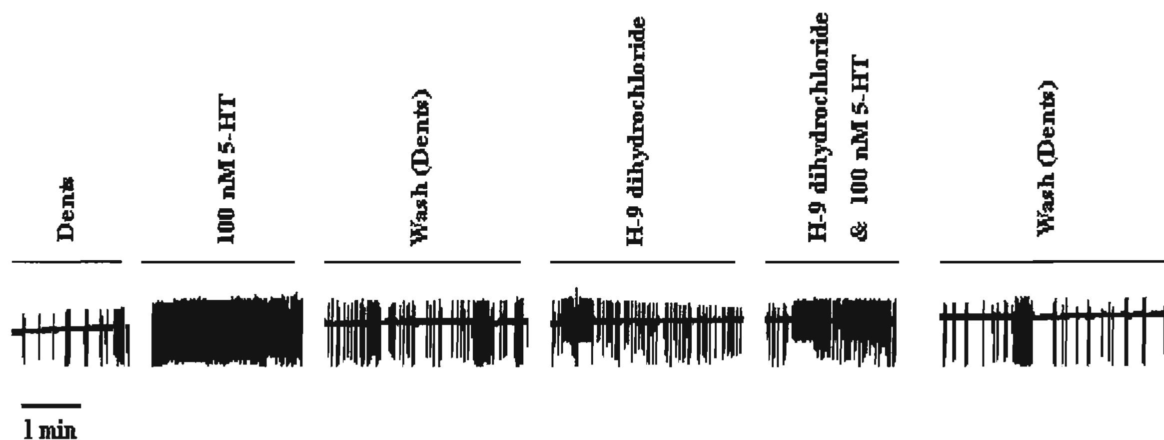


Figure 5.8 The effect of H-9 dihydrochloride on the excitatory responses of 5-HT on pharyngeal pumping.

(A) Histograms to show that the responses to 100 nM 5-HT were reduced in the presence of 10 μM H-9; % decrease 32 ± 5.0; ** P<0.01; n=5.

(B) Example traces to show the effect of 100 nM 5-HT, 10 μM H-9 and both compounds applied together on EPG activity. Following wash, the pumping rate returned to control.

5.1.5 The effect of 8-Bromo-cGMP on pharyngeal pumping.

Application of 8-Bromo-cGMP, 10μM, potentiated the excitatory response to 100nM FLP17A, although this effect was not significant (n=3), Table 5.1 Figure 5.9. This concentration of 8-Bromo-cGMP had a small direct effect on EPG activity. It was not tested for its effect on 5-HT.

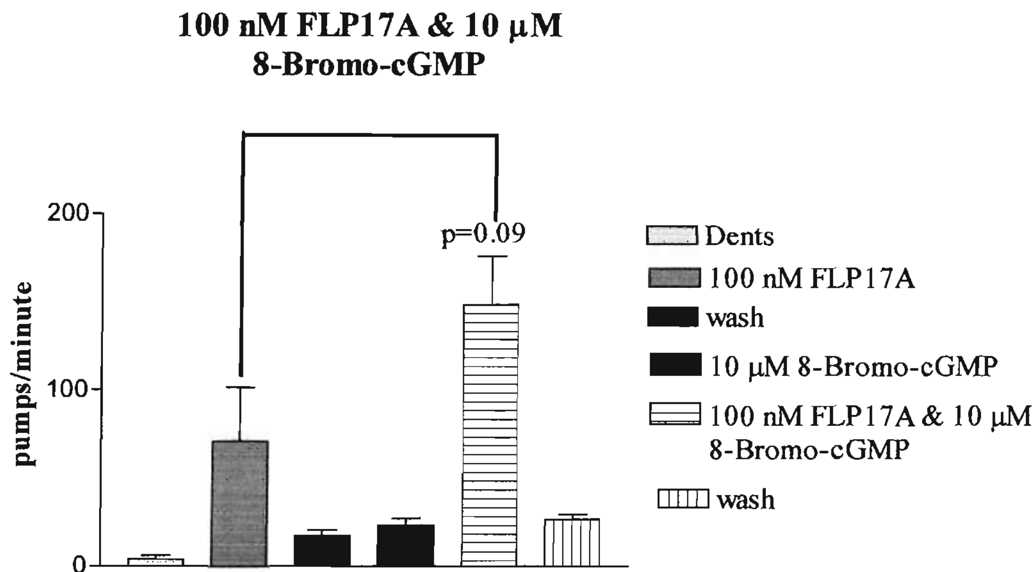


Figure 5.9. The effect of 8-Bromo-cGMP on the excitatory responses of FLP17A on pharyngeal pumping. Histograms to show that the response to 100 nM FLP17A was increases in the presence of 8-Bromo-cGMP; % increase 401 ± 390; P=0.09; n=3

	Control	100 nM FLP17A	Compound Under Test	Compound & FlP17A	Wash
cAMP 1μM	15.5 ± 4.9	46.0 ± 16.2	22.4 ± 2.4	142.0 ± 42.2 *P=0.05	26.5 ± 8.2
Forskolin 50 nM	4.8 ± 1.2	16.0 ± 7.8	10.6 ± 2.8	136.6 ±25.8 **P=0.01	19.3 ± 4.8
Forskolin 1μM	6.8 ± 1.7	11.8 ± 3.5	45.8 ± 3.9	136.6 ± 7.5 ***P=0.0001	27.4 ± 4.5
Ibudilast 10 μM	12.2 ± 2.9	60.2 ± 13.7	26.9 ± 5.4	135.6 ± 11.1 **P=0.01	13.2 ± 3.3
H-9 Dihydrochloride 10 μM	2.8 ± 0.5	30.6 ± 10.6	28.6 ± 1.8	29.3 ± 8.6 P=0.06	20.2 ± 3.3
cGMP 10 μM	3.9 ± 2.0	70.3 ±31.1	23.5 ± 3.6	148.3 ± 27.4 P=0.09	26.9 ± 2.7

Table 5.1 The effect of altering cAMP levels on the response of *C. elegans* pharynx to FLP17A. Summary of the effects of cAMP (1 μM), Forskolin (50nM and 1 μM), Ibudilast (10 μM), H-9 Dihydrochloride (10 μM) and cGMP (10 μM) on the response of the pharynx to 100 nM FLP17A. Data presented are the average of n determination and represent the number of pharyngeal pumps in 1 min.

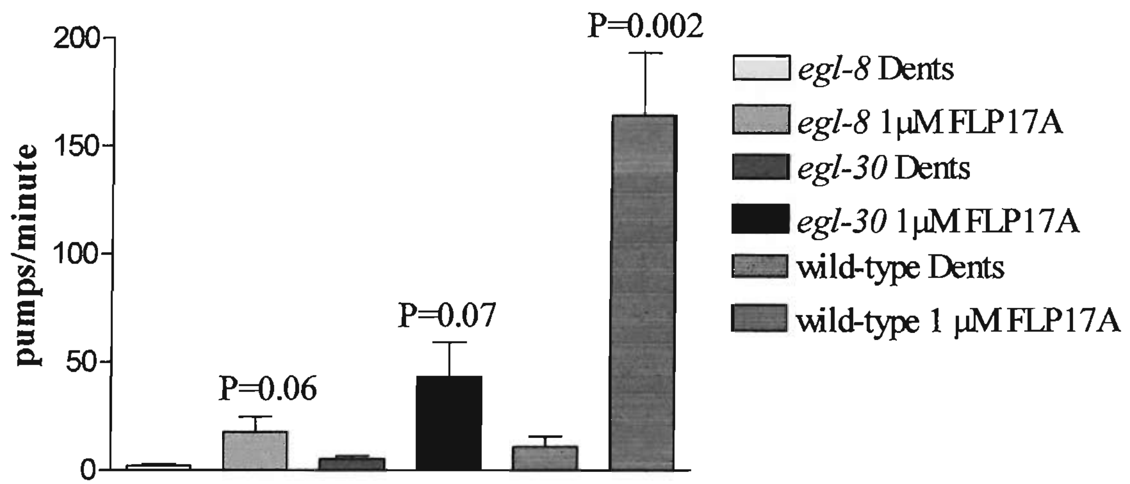
	Control	5-HT	Compound Under Test	Compound & 5-HT	Wash
cAMP 1μM	1.2 ± 0.6	35.4 ± 14.5 50 nM	---------------	122.8 ± 28.7 **P=0.06	54.3 ± 14.7
Forskolin 50 nM	4.7 ± 0.4	62.2 ± 27.5 50 nM	37.9 ± 4.5	169.0 ± 50.1 *P=0.05	31.0 ± 3.4
Ibudilast 10 μM	12.9 ± 5.2	74.0 ± 18.7 50 nM	28.4 ± 6.3	122.4 ± 18.8 P=0.06	25.8 ± 5.4
H-9 Dihydrochloride 10 μM	9.1 ± 2.4	180.4 ± 19.3 100 nM	28.8 ± 16.8	123.0 ± 16.8 **P=0.01	14.4 ± 3.9

Table 5.2 The effect of altering cAMP levels on the response of *C. elegans* pharynx to 5-HT. Summary of the effects of cAMP (1 μM), Forskolin (50nM), Ibudilast (10 μM) and H-9 Dihydrochloride (10 μM) on the response of the pharynx to 5-HT. Data presented are the average of n determination and represent the number of pharyngeal pumps in 1 min.

5.1.6 The effect of FLP17A and 5-HT on two *C. elegans* mutants, *egl-8* and *egl-30*.

The excitatory effect of FLP17A and 5-HT on pharyngeal pumping on two *C.elegans* mutants, *egl-8* (*n488*) and *egl-30* (*ad810*), was greatly reduced compared to their responses on wild type, Table 5.3, Figures 5.10A and 5.11A. In these experiments the concentration of FLP17A and 5-HT were raised to 1μM and 500nM, respectively, to obtain consistent responses in the mutants. The relative increase in FLP17A-induced excitation of pharyngeal activity which was significantly greater in the wild type *C. elegans* compared with the increase in pharyngeal activity in the two mutants, P=0.002 (n=5), compared with P=0.06 (n=5) and P=0.07 (n=5) for the action of FLP17A on *egl-8* and *egl-30*, respectively. There was a similar result with 5-HT though the differences between the action of 5-HT on wild-type and mutants was less with the levels of significance between control and 5-HT on wild-type, *egl-8* and *egl-30* being P=0.003 (n=5), P=0.01 (n=4) and P=0.002 (n=5), respectively. Figure 5.10B and 5.10C show the effect of FLP17A on EPG traces from *egl-8* (n=5) and *egl-30* *(n=5)* mutants, respectively. Figure 5.11B, 5.11C and D show the effect of 5-HT on EPGs from wild type *C. elegans* and from *egl-8* and *egl-30* mutants. In both mutants the basal pharyngeal activity is always very low.

A.



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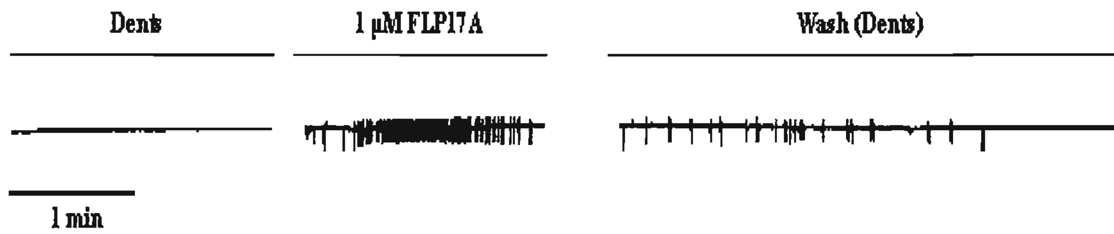
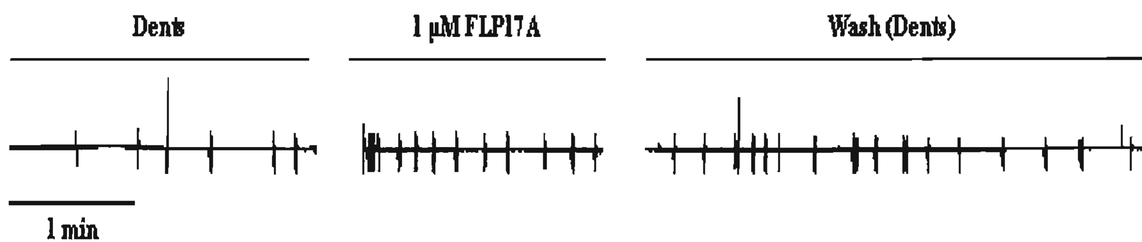


C.



D

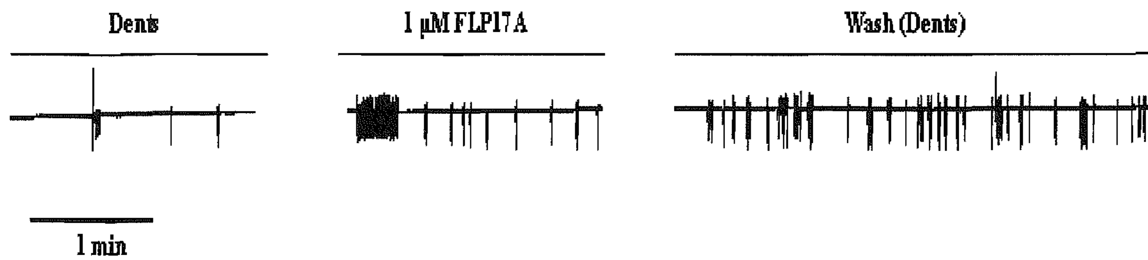
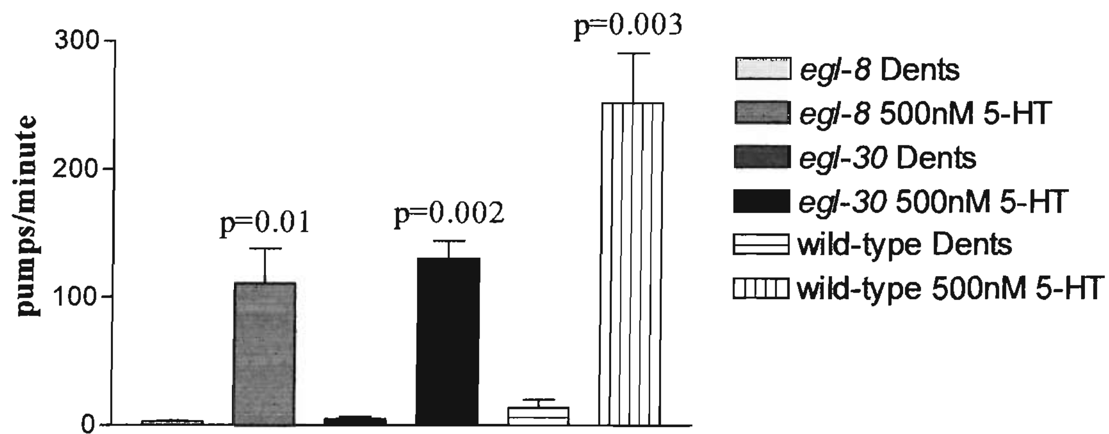


Figure 5.10 The effect of *egl-8* and *egl-30* and wild type *C. elegans* on the excitatory response of FLP17A on pharyngeal pumping.

(A) Histograms to show that the responses to 1 µM FLP17A on *egl-8* and *egl-30* were strongly reduced compared with the response on wild-type animals, n=5 in each case. (B) Example traces to shown the effect of FLP17A on wild-type animal pharyngeal pumping. (C) Example traces to show the effect of FLP17A on *egl-8* pharyngeal pumping. (D) Example traces to show the effect of FLP17A on *egl-30* pharyngeal pumping. These recordings were made in Dent's saline. Each deflection is a single pharyngeal pump. Brakes in traces indicate short brakes in the recordings (<1 min) during which the fluid in the recording chamber was exchanged for Dent's saline with 1 µM FLP17A and subsequently with Dent's saline to test the reversal of the peptide effects.

A.



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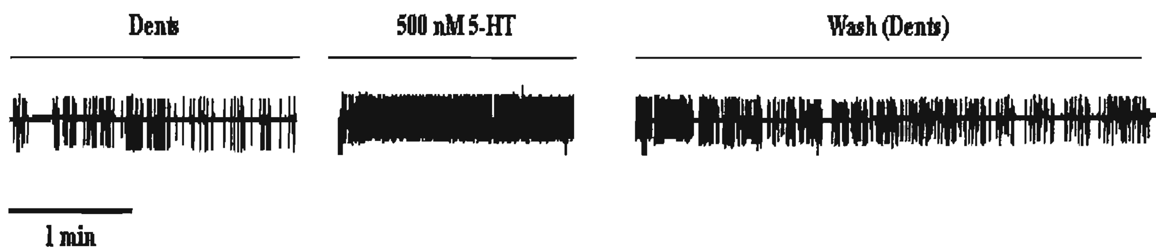

C.

D.

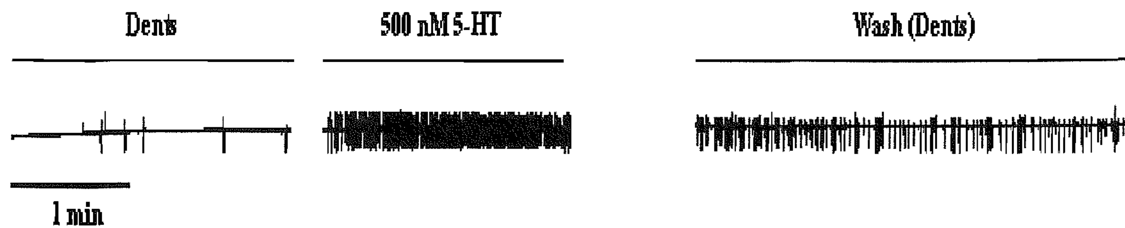

Figure 5.11. The effect of *egl-8* and *egl-30* mutants and wild-type *C. elegans* on the excitatory response of 5-HT on pharyngeal pumping.

(A) Histograms to show that the responses of 500 nM 5-HT on *egl-8* (n=5) and *egl-30* (n=4) were reduced compared with the response on wild type animals.

(B) Example traces to shown the effect of 5-HT on wild-type animal pharyngeal pumping. (C) Example traces to show the effect of 5-HT on *egl-8* mutant pharyngeal pumping. (D) Example traces to show the effect of 5-HT on *egl-30* mutant pharyngeal pumping. These recordings were made in Dent's saline. Each deflection is a single pharyngeal pump. Brakes in traces indicate short brakes in the recordings (<1 min) during which the fluid in the recording chamber was exchanged for Dent's saline with 500 nM 5-HT and subsequently with Dents's saline to test the reversal of the peptide effects.

	Control	1 µM FLP17A	Wash
egl-8	1.9 ± 0.9	17.6 ± 7.1 (n=5)	8.2 ± 3.6
egl-30	5.1 ± 1.4	43.2 ± 16.0 (n=5)	18.0 ± 3.9
WT	10.8 ± 4.6	164 ± 29.3 (n=5)	14.5 ± 6.3

	Control	500 nM 5HT	Wash
egl-8	2.7 ± 0.9	110.0 ± 27.9 (n=5)	76.8 ± 26.2
egl-30	4.9 ± 1.7	130.2 ± 13.8 (n=4)	45.0 ± 19.6
WT	13.9 ± 6.2	251.6 ± 38.9 (n=5)	37.4 ± 11.7

Table 5.3: The effect of FLP17A and 5-HT on two *C. elegans* mutant strains *egl-8* and *egl-30*. Summary of the effect of FLP17A (1 µM) and 5-HT (500 nM) on pharyngeal pumping frequency in two *C. elegans* mutant strains *egl-30* and *egl-8*. Data presented are the average of n determinations and represent the number of pharyngeal pumps in one minute.

5.2 Discussion

The present study shows that compounds applied exogenously which potentially alter the level of cAMP in *C. elegans* pharyngeal muscle influence the excitatory responses to both FLP17A and 5-HT. This increased excitation is enhanced compared with the sum of the effect of 5-HT or FLP17A and the effect of dibutyryl-cAMP, forskolin or ibudilast applied alone. This provides evidence that the excitatory effects of both 5-HT and FLP17A are altered, at least in part, through a Gs or GPCR system but where do these interactions occur within the pharynx? FLP17A will be considered first.

flp-17 is expressed in pharyngeal cholinergic motorneurone M5 which synapses onto terminal bulb muscles pm6 and pm7 and onto gland and marginal cells (Albertson and Thomson 1976). M5 receives a chemical input from I5 which contains two classical transmitters, 5-HT and probably glutamate (Sawin *et al.,* 2000; Lee et *al.* 1999) and three FLPs, FLP-2, 4 and 13 (Kim and Li 2004; Li, 2005). M5 should have receptors for these ligands and SER-7 receptors have been identified on M5 (Hobson *et al.,* 2006). Since raised levels of cAMP enhance the excitatory effect of FLP17A it would appear that at least a component of this excitation is mediated through cAMP. Previous evidence from studies mainly using *A. suum* body wall muscle suggests FLPs can activate the level of muscle cAMP. For example, both AF1 (KNEFIRFamide) and AF2 (KHEYLRFamide) can have a biphasic effect on *A. suum* body wall muscle and both peptides increase the level of cAMP in this muscle (Thompson *et al.*, 2003), with AF2 more potent than AF1. AF2 increased cAMP muscle level 85-fold above control after 60 minutes. The inhibitory peptide, PF1 (SDPNFLRFamide), had no effect on cAMP levels in body wall muscle. Interestingly, this peptide did not alter cGMP or IP3 levels in the muscle. From the studies of Thompson et al (2003) it could be concluded that peptide-induced excitation of *A. suum* body wall muscle is associated with a rise in cAMP levels. However, these authors found that using denervated preparations, AF1 and AF2 only relaxed the muscle but still activated cAMP muscle levels. These authors concluded that using *A. suum* body wall muscle strips there was no correlation between contraction induced by AF1 and AF2 and their effects to raise levels of cAMP. There is always the problem as to whether the change in cAMP level is due to receptor activation or a

secondary event following muscle activation. The potentiating effect of AF2 on the acetylcholine–induced muscle contraction of *A. suum* body wall muscle does not appear to be linked with cAMP (Keating *et al.*, 1996). Injection of FLPs into *A. suum* can alter behaviour and body length and may also alter cAMP levels. For example, both AF1 and AF2 decreased the body length and raised the level of muscle cAMP (Reinitz *et al.*, 2000). This effect appears to be selective since AF10 (GFGDEMSMPGVLRFamide) also decreased body length but had no apparent effect on cAMP levels. In contrast, these authors found that some peptides, for example, *C. elegans* FLP9 (KPSFVRFamide) increased body length but decreased cAMP levels while FLP13 (APEASPFIRFamide) increased body length without changing cAMP levels. In another study *A. suum* peptide, AF3 (AVPGVLRFamide), which contracts *A. suum* body wall muscle, reduced the basal level of cAMP in *A. suum* body wall muscle and also reduced the forskolin-induced increase in muscle cAMP (Trim *et al.*, 1998). The AF3 contraction was reduced in the presence of forskolin, indicating that the contraction was inhibited by raising cAMP. These effects induced by AF3 are probably coupled to Gi which inhibits adenylate cyclase. Thompson *et al* (2003) also observed that 5-HT had no effect on the level of cAMP in *A. suum* body wall muscle. Other studies have found that 5-HT increased cAMP levels in *A. suum* body wall muscle (Williams *et al.,* 1992).

While there have been many studies on the role of 5-HT in the regulation of *C. elegans* pharyngeal activity (Niacaris and Avery 2003), there have been few studies regarding its mechanism of action. From a parallel in the mammalian system, it may be assumed that most 5-HT receptors in *C. elegans* function through GPCRs. This is likely to be the situation with the three 5-HT receptors so far identified in the pharyngeal system (Hamdan *et al.,* 1999; Hobson *et al.,* 2003; Olde and McCrombie 1997). One of these, SER-7b, is linked with an increase in cAMP (Hobson *et al.,* 2003) and the present study would suggest that 5-HT could be acting partly through this receptor to stimulate pharyngeal activity. SER-7 receptors are located on a number of pharyngeal neurones including M2, M4, M5 and MCs but not on pharyngeal muscle cells (Hobson *et al.,* 2006) and so pharyngeal excitation would be through the release of acetylcholine to excite the pharynx (Avery and Horvitz 1990). However, it should be noted that these studies on the transduction mechanisms involved have all been conducted using cell lines and may not represent the *in vivo*

situation. Interestingly, 5-HT7 receptors from the honeybee, *Apis mellifera*, also stimulate cAMP levels when expressed in HEK293 cells (Schlenstedt *et al.*, 2006). The finding that raising the level of cAMP enhances pharyngeal muscle response to 5-HT would support an interaction between cAMP and 5-HT in this muscle. In a previous study using *A. suum* pharyngeal muscle no evidence was found for activation of cAMP by 5-HT (Trim *et al.*, 2001). These authors found evidence that 5-HT raised inositol phosphate levels in *A. suum* pharyngeal muscle, suggesting a possible link between 5-HT and SER-1 since this receptor couples to Gαq and phosphoinositide turnover in both *C. elegans* (Hamdan *et al.*, 1999) and *A. suum* (Huang *et al.*, 2002).

In an attempt to investigate whether 5-HT and FLP17A might act partly through Gαq, two mutant strains of *C. elegans*, *egl-8* and *egl-30,* were tested to determine the action of both compounds on their pharyngeal activity. *egl-30* encodes the orthologue of vertebrate Gαq (Brundage *et al*., 1996) and EGL-30 activates a number of biological processes in *C. elegans*, including pharyngeal pumping (Bastiani and Mendel 2005). EGL-30 stimulates phosphoinositide hydrolysis with the production of IP_3 and diacylglycerol (DAG) (Brundage *et al.,* 1996). *egl-8* encodes EGL-8 in *C. elegans* which activates phospholipase Cβ (PLCβ) (Lackner *et al*.,1999). In *C. elegans* the IP_3 receptor, ITR-1, is expressed in the pharynx (Baylis *et al*., 1999). At the body of neuromuscular junction both Gαq and PLCβ are required for transmitter release and so a reduction of either, as in *egl-30* or *egl-8,* respectively, will result in a reduction of transmitter released in the pharyngeal system. The response to FLP17A in both mutants was greatly reduced compared to wild type animals, Table 5.3, suggesting that part of FLP17A excitation is through activation of Gαq and hyrolysis of PIP_2.This would suggest that the main excitatory action of FLP17A is indirect through neuronal release of an excitatory transmitter, possibly acetylcholine. It would be interesting to investigate the effect of compounds, which alter phosphoinositide hydrolysis on basic pharyngeal activity and on the excitatory response to FLP17A. It would also be of interest to test the effect of FLP17A, on an *egl-30*, gain-of-function, mutant. In both mutants the basic pumping activity of the pharynx was lower compared to wild type. In the case of 5-HT, the reduction in the excitatory response of both mutants was far less, Table 5.3. This would indicate that the activation of pharyngeal muscle SER-1 receptors is only partly responsible for pharyngeal 5-HT excitation, which supports the suggestion that the excitatory action

of 5-HT in the pharynx is mediated by both pre- and postsynaptic mechanisms. This is re-inforced by the observation that in *itr-1* mutants, where overexpression of IP_3 domain acts as an IP_3 "sponge", the pharynx responds normally to 5-HT (Walker *et al.*, 2002). It would be logical to assume that part of the excitatory role of 5-HT on the pharynx is mediated through the release of 5-HT from NSMs which then activates the Gαq-coupled 5-HT muscle receptor SER-1. However, Hobson *et al* (2006) have shown using *ser-7* mutants, that 5-HT fails to activate the pharynx although SER-1 receptors are present. The role of 5-HT in stimulating pharyngeal pumping is complex. The protein kinase inhibitor, H-9 dihydrochloride, inhibits PKA, PKG and PKC (Hidaka *et al.,* 1984) and had a direct excitatory effect on pharyngeal activity. A rise in cAMP activates PKA and so inhibition of PKA by H9 will alter the response to 5-HT if this is partly mediated through cAMP and neuronal SER-7 receptors. The effect of H-9 on the response to FLP17A requires interpretation since H-9 had an excitatory effect on pharyngeal activity approximately equal to that of FLP17A, Table 5.1. When both compounds were added together there was no change in activity, suggesting that all the excitation could be due to H-9, indicating that H-9 had blocked FLP17A excitation. This would link FLP17A excitation with cAMP since the latter activates PKA, which is blocked by H-9 (Hidaka 1984). Application of cGMP increased basic pharyngeal activity and increased the effect of FLP-17A but this increase was not significant. Further experiments are required to check for any contribution to FLP17A excitation by cGMP.

Although these studies are only preliminary they indicate that through the use of a combination of selective agonists, antagonists and inhibitors together with mutants for specific genes including *acy-1* and *goa-1*, it will be possible to dissect out the signaling pathways employed by the peptides and classical transmitters located in the pharyngeal system of *C. elegans*. This will provide information regarding the possible pathways employed by peptides and classical transmitters in the pharynxes of parasitic nematodes and in turn provide possible sites for the development of novel anthelmintics in parasitic nematodes.

CHAPTER 6

Discussion

6.0 Discussion

Anthelmintics work by targeting certain channels and receptors together with biochemical processes which are specific to the parasitic nematode. Receptors which are targeted by existing anthelmintics include the excitatory ACh receptor and the inhibitory GABA receptor, both of which are found on nematode body wall muscle and are required for nematode movement.

The over usage of anthelmintics has led to a problem of resistance, where existing drugs lose their efficacy against the parasite. Anthelmintic resistance has been seen worldwide in the sheep, pig, cattle, and horse industries. Resistance results from genetic mutations within the parasitic nematode's DNA. This mutation may, for example, occur in a gene encoding for the receptor that binds the anthelmintic, conferring reduced efficacy for that anthelmintic. As this nematode is not affected by the anthelmintic it survives and is thus able to pass on this mutation and subsequent resistance. This resistance problem has encouraged the search for novel anthelmintics, which target sites specific to the nematode, and that differ from the existing commercially available drugs.

Neuropeptides play a key role in the physiology of the nervous and muscle systems of nematodes (Brownlee *et al.,* 1996; Brownlee *et al.,* 2000; Maule *et al.,* 1996; Walker *et al.,* 2004). Genes encoding FLPs have been isolated from *A. suum* (Edison *et al.,* 1997) and *C. elegans* (Li *et al.,* 1999; Li 2005; Husson *et al.,* 2005). Interestingly a recent paper has estimated that more FLPs have been identified in nematodes (85) than any other phylum (Liu *et al.,* 2006). However, the FLPs represent only one family of peptides in *C. elegans.* A family of 42 neuropeptide-like protein (*nlp*) genes has also been identified (Nathoo *et al.,* 2001; Li 2005). Neuropeptides may provide a novel target site for the development of new anthelmintics.

Peptides encoded by the *C. elegans, flp-1, flp-3, flp-13, flp-21, nlp-1* and *nlp-2* genes were tested for activity on *A. suum* body wall muscle using the dorsal muscle strip (DMS). The DMS contains muscle cells, hypodermal tissue and a section of the dorsal nerve cord and has previously been used to assess the activity of neuropeptides (Franks *et*

al., 1994; Maule *et al.,* 1994). It should be noted that peptides tested using this preparation may act presynaptically on motorneurones as well as postsynaptically on the muscle. Peptides encoded by *flp-1, flp-3 and flp-13* genes were previously shown to be active on the *C. elegans* pharynx (Roger *et al.,* 2001) and were selected to be tested for activity on *A. suum* muscle in order to compare their effects between the two nematodes. The peptide encoded by the *flp-21* gene has been isolated from *A. suum* and is designated as AF9 (Cowden and Stretton 1995). The three peptides encoded by 2 *nlp* genes were selected to be tested for activity to provide a comparison between the two families of neuropeptides identified in *C. elegans.* The peptides were tested for activity at a range of 0.1 to 10.0μM and the qualitative results from the two species are summarized in Table 6.1

The FLPs tested on the *A. suum* somatic muscle had a potent biological effect suggesting that a number of these peptides may have physiological roles in regulating the activity of the muscle.

The FLPs FLP1B and FLP13 had no direct effect on the *A. suum* muscle but affected the ability of the muscle strip to contract in response to ACh by reversibly reducing the amplitude of contractions elicited by repeated application of ACh (10 μM) in a concentration-dependent manner with an IC_{50} of 0.5 μM and 0.3 μM respectively. FLP3A and FLP21 caused a concentration-dependent reversible potentiation of the contraction elicited by ACh with an EC_{50} of 0.6 μM and > 0.1 μM respectively. FLP21 (AF9) is endogenous to *A. suum* and also had a direct effect on the muscle causing a long-lasting contraction.

In the *A. suum* neuromuscular system there are two main classical transmitters, ACh which is excitatory (Johnson and Stretton 1985) and GABA which is inhibitory (del Castillo *et al.,* 1963; Sithigorngul *et al.,* 1989). One possible mechanism of action for the FLPs could be through the excitation or inhibition of the GABAergic signaling pathway or through enhancing or inhibiting the release of ACh.

The results presented here suggest that the FLPs tested have a postsynaptic action since they affect the response of the muscle to ACh. A presynaptic effect or a partly presynaptic and partly postsynaptic effect has previously been described for other peptides including AF2 (Pang *et al.*, 1995) and further studies are required to determine whether the FLPs described here act in a similar manner.

Evidence suggests that there are a number of FaRP receptors in nematodes and so far eleven have been identified in *C. elegans*, all of which are G-protein coupled (Kubiak *et al.*, 2003 a,b; Mertens *et al.*, 2004; Mertens *et al.*, 2005). The results described in this thesis suggest that FaRP-binding receptors are present in the neuromuscular system of *A. suum*. The IC_{50} and EC_{50} for the FLP peptides were in sub-μM concentrations suggesting that the receptors mediating the effects of these peptides have a reasonably high affinity for these ligands.

Peptide	**Action on**	
	***A. suum* body wall muscle**	***C. elegans* pharynx**
FLP1B AGSDPNFLRFa	no direct effect; reduced ACh contraction;	reduced pharyngeal pumping
FLP3A SAEPFGTMRFa	induced muscle contraction in some preparations; potentiated ACh contraction;	reduced pharyngeal pumping
FLP13A APEASPFIRFa	no direct effect; reduced ACh contraction	reduced pharyngeal pumping
FLP21 (AF9) GLGPRPLRFa	induced muscle contraction; potentiated ACh contraction	reduced pharyngeal pumping
NLP1A MDANAFRMSFa	no direct effect; slight reduction of ACh contraction	
NLP2A SMAMGRLGLRPa	no direct effect; reduced ACh contraction	
NLP2B SMAYGRQGFRPa	no direct effect; slight reduction of ACh contraction;	

Table 6.1 Comparison of the actions of a series of FLPs and NLPs on *A. suum* body wall muscle and *C. elegans* pharynx.

As already mentioned, the *C. elegans* nervous system has a remarkable abundance of genes encoding NLPs and FLPs. In the present study at least one peptide encoded by *flp-1* through *flp-23* genes was tested for activity in *C. elegans* using the semi-intact preparation of the dissected pharynx. This preparation retains the circuitry of the enteric nervous system and the anterior nervous system including the nerve ring and chemosensory circuits.

The genes for many of the peptides selected for testing have either pharyngeal expression or are expressed in extrapharyngeal neurones thus providing a comparison between the location for the encoding of the peptide and its action on the pharynx. Eight of the *flp* genes encode peptides which are excitatory while eleven genes encoded peptides which were inhibitory. The most potent peptides elicited responses with a threshold of 10 nM and induced a robust, consistent response at 100 nM. Less potent peptides did not exert a consistent response at concentrations below 1 μM.

The potent biological activity of a large subset of the FLPs in the pharynx suggests that a number of these peptides may have a physiological role in regulating the activity of the muscle. FLP13A, FLP17A and FLP17B were three of the peptides that had a potent effect on pharyngeal muscle. These peptides are encoded by genes expressed in pharyngeal motorneurones, suggesting that they may act through specific receptors located on the pharyngeal muscle.

Five of the peptides tested in the pharyngeal assay had no significant effect on the muscle. None of these are encoded by *flp* genes expressed in the pharyngeal nervous system which indirectly supports the contention that the biological activity of the peptides described in this study is informative regarding their physiological role. Seven peptides, FLP3A, FLP8, FLP9, FLP11A, FLP14A, FLP16A and FLP22 were active on pharyngeal muscle with FLP8 being a potent excitor and FLP11A, a potent inhibitor of pharyngeal activity. All of these peptides are encoded by genes that are expressed in extrapharyngeal neurones, suggesting that these peptides act either indirectly or as neurohormones or their expression patterns have not been determined.

Out of the 27 FLPs tested on the *C. elegans* pharynx 21 were active. No obvious relationship was observed between the C terminal sequences and whether the responses were excitatory or inhibitory. For example, FLP17B and FLP13A end in IRFamide but the former was excitatory and the latter inhibitory. Both are encoded by genes which are expressed in motorneurones (Li 2005) and therefore are presumed to be released directly onto the pharyngeal muscle which will have receptors for both peptides. If the sequences of the two peptides are compared then the major differences are that FLP17B has a K and Q in positions occupied by D and P in the case of FLP13A. These differences may indicate that the two peptides act on different receptors and structure-activity studies would help to resolve this. Interestingly, most of the excitatory responses were mediated by heptapeptides, while most of the inhibitory responses were caused by peptides with longer N terminal sequences. From the results it can be observed that peptides with biological activity which are expressed in extrapharyngeal neurones have extended N terminals which convey resistance to enzymatic breakdown (Price 1986) which is consistent with a neurohormonal role.

The regulation of pharyngeal pumping is vital to the development, growth and reproductive ability of *C. elegans*. It is clear from this study that FLPs play an important role in the modulation of this pharyngeal activity.

The semi-intact pharyngeal preparation used in this study provides preliminary data for the biological activity of peptides on the pharynx. It cannot be assumed that a peptide always has the same action at all its effector sites, but it is reasonably certain that the FLPs encoded in neurones which synapse directly on the pharyngeal muscle have an effect as described in Chapter 4. The direct effect of neuropeptides on neurones may be different from that observed on pharyngeal pumping. Thus a neuropeptide may excite a neurone which in turn releases a classical transmitter or neuropeptide which inhibits pharyngeal activity. This can only be resolved when recordings are made from neurones within the pharyngeal network as performed by Marder and her colleagues using the crustacean stomatogastric ganglion (Weimann *et al.,* 1997).

The biological significance of neuropeptides can also be addressed from investigating the functional consequence of *flp* gene knockouts as described by Nelson *et al.* (1998) and Waggoner *et al.* (2000). The use of *C. elegans* mutant strains deficient in synaptic signaling will provide a further insight into the mechanism of action of neuropeptides. Of particular interest will be the interplay between classical transmitters and neuropeptides, and the role these neuropeptides may have in altering pharyngeal activity in response to the environment and nutritional status of the animal.

The 20 neurones which make up the pharyngeal nervous system all potentially present sites for the expression of genes encoding neuropeptide receptors. Many of these neurones are connected via either chemical or electrical synapses, Figure 4.1 (Albertson and Thomson 1976) and so the final response of the neuropeptide on pharyngeal muscle will be determined by the threshold for conduction across each synapse in the circuit. Clearly, the greater the number of synapses involved in the circuit, the greater the possibility for variation in the responses from one preparation to another. The work of Kim and Li (2004) which was discussed earlier demonstrated that 19 of the 20 pharyngeal neurones have genes which are expressed in these neurones and encode for neuropeptides. It is also likely that most, if not all, of these neurones possess neuropeptide receptors. Only MI has not been shown to possess *flp* or *nlp* genes which are capable of being expressed. Interestingly many of the neurones have more than one *flp* or *nlp* gene which can be expressed and so possess the potential to release many neuropeptides. However, by analogy with other systems, it is likely that not all these neuropeptides are released at the same time or are necessarily active (Weiss *et al.,* 1992; Vilim *et al.,* 2000).

Assuming that at least one neuropeptide is released at a synapse, then there must be a receptor on the postsynaptic membrane for it to interact with. This means there could be up to 20 neuropeptide receptors in the pharyngeal system and a single neurone is likely to have several different neuropeptide receptors. There is also the question of

selectivity of neuropeptides for their own receptors and so neuropeptides may act on more than one receptor.

Another complication in the analysis of individual neuropeptide function is that some neurones, for example, I5, which contains FLP2s, FLP4s and FLP13s, synapse onto a number of key neurones in the regulation of pharyngeal pumping. If all these peptides are released simultaneously, the effect on pharyngeal pumping could be very complex. There must be a system whereby the neuropeptides are differentially released. In the case of I5 which also contains 5-HT and glutamate, the actions of both classical transmitters have also to be taken into account. In this case, one classical transmitter excites pumping while the other inhibits but their actions on the I5 follower neurones are unknown.

It is likely that all pharyngeal neurones contain one or more classical transmitter although the transmitters have not been identified in all the neurones (Franks *et al.,* 2006). Eight neurones contain ACh, two contain glutamate and three contain both glutamate and 5-HT (Dent *et al.,* 1997; Li *et al.,* 1997; Lee *et al.,* 1999; Rand *et al.,* 2000; Sawin *et al.,* 2000; Sze *et al.,* 2000; Niacaris *et al.,* 2003). In seven neurones the classical transmitter has not yet been identified. ACh is a major transmitter onto pharyngeal muscle as it is present in five out of seven motorneurones and the MCs are also cholinergic. Thus changes in muscle cholinoceptors will potentially have a significant effect on pumping. There are at least 27 nicotinic ACh receptor subunits in *C. elegans* (Jones and Sattelle 2004) and EAT-2 has been identified in the pharynx (McKay *et al.,* 2004). 5-HT can either act directly on pharyngeal muscle since SER-1 receptors occur on the muscle (Tsalik *et al.,* 2003) or indirectly via I6, M3, M1 or M4 (Albertson and Thomson 1976). Glutamate can likewise act either directly on the muscle or indirectly via the same neurones as in the case with 5-HT. Octopamine has been shown to inhibit pharyngeal activity in *C. elegans* (Rogers *et al.,* 2001) but octopamine has not been identified in any pharyngeal neurone although tyramine-β-hydroxylase has been identified in the lateral ganglion RIC neurones (Alkema *et al.,* 2005). Since tyramine receptors occur in the pharyngeal system it is also likely that this amine has a physiological role in the pharynx (Rex and Komuniecki 2002; Komuniecki *et al.,* 2004).

RIM neurones have also been identified which contain tyrosine decarboxylase but not tyramine-β-hydroxylase, suggesting they synthesise and possibly release tyramine (Alkema *et al.,* 2005).

The only synaptic input from the extra-pharyngeal system is via the ring/pharyngeal interneurone, RIP, which makes a gap junction with I1s, allowing for sensory modulation of pharyngeal pumping (Avery and Thomas 1997). The classical transmitter synthesized in I1s is unknown but both *nlp-3* and *nlp-6* are expressed in these neurones. I1s make chemical synapses onto M2s, M3s, MCs, NSMs and I5 and so their activation can potentially modulate the activity of nine neurones out of 20 in the pharyngeal nervous system.

One approach to analyse the action of neuropeptides in the pharyngeal system would be to test their effects on pumping in animals where one or more neurones have been removed by laser ablation (Avery and Horvitz 1989). Alternatively mutants where specific neuropeptide or classical transmitter receptors are absent can be studied. Such a combined approach should provide more selective information regarding the roles of neuropeptides in the regulation of pharyngeal pumping.

In the third part of this study the effect of altering cAMP levels in the pharynx on the responses to FLP17A and 5-HT were investigated. It was shown that compounds applied exogenously which potentially alter the level of cAMP in the pharyngeal muscle, influenced the strength of the excitatory responses to both 5-HT and FLP17A, suggesting that the excitatory effects of both 5-HT and FLP17A are altered through G_s or at least a GPCR system. Previous evidence from studies mainly using *A. suum* body wall muscle suggests FLPs can increase the level of muscle cAMP (Thompson *et al.,* 2003). The role of 5-HT in the regulation of pharyngeal pumping in *C. elegans* has been studied in depth but there have been relatively few studies on the second messengers involved. Using COS-7 cells Hobson *et al.* (2003) found that *C. elegans* SER7 receptor activation induced a rise in cAMP. The present study strongly supports the proposal that the 5-HT response is associated with a rise in cAMP through activation of SER7 receptors. SER7 receptors

are not located on pharyngeal muscle which suggests that the 5-HT-induced increase in 5-HT in cAMP occurs in neurones including M2, M4, M5 and MCs, all of which synthesise ACh. A recent study using CHO cells to express *C. elegans* muscarinic GAR-3b receptors has shown that carbachol activation of these receptors stimulates cAMP production (Park *et al.,* 2006). Activation of GAR-3b receptors excites pharyngeal muscle (Franks personal communication) which agrees with the present study where cAMP potentiates the action of compounds which excite pharyngeal muscle.

The use of two mutants, *egl-8,* with a loss-of-function PLCβ and *egl-30,* with a loss-of-function Gαq, clearly showed a large reduction in the response to FLP17A while the response to 5-HT was also reduced but to a lesser extent. FLP17A is expressed in M5 (Kim and Li 2004) and so will be released onto the pharyngeal muscle when M5 is activated. This means there should be FLP17A receptors on the muscle which will be activated by direct application of FLP17A, as in the present study. Thus the excitatory response of FLP17A must involve both PLCβ and Gαq. These results re-inforce the need to determine the distribution of FLP receptors in the pharyngeal system. While there are SER1 5-HT receptors on pharyngeal muscle it is also likely that 5-HT also acts presynaptically on SER7 receptors to release ACh onto the pharyngeal muscle.

In conclusion it is clear from this study that FLPs play an important role in the modulation of pharyngeal activity in *C. elegans* and on *A. suum* somatic muscle. The role of NLPs on *A. suum* muscle is less clear since these peptides had very weak actions on the muscle. However, from their expression pattern in the *C. elegans* pharyngeal system it is likely they play a physiological role. The use of electrophysiological studies in *A. suum* and mutant strains in *C. elegans* which are deficient in synaptic transmission, will give a better insight into the mechanism of action of these neuropeptides in nematodes and provide more information regarding the importance of neuropeptides in modulating locomotion and feeding. Knockout or over-expression experiments where the expression of specific genes of interest is altered will also provide information regarding the significance of neuropeptides in regulating biological activities in nematodes. The experiments reported in this study on the mechanism of action of FLP17A and 5-HT are

only preliminary but indicate that through a combination of selective agonists, antagonists and inhibitors and the use of mutants, experiments can be devised which will assist to determine the second messenger pathways responsible for neuropeptide and classical transmitter function. These pathways may provide another approach in the development of novel targets for new anthelmintics to combat parasitic nematodes.

CHAPTER 7

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