Molecular Genetic Analysis of the α-latrotoxin Receptor Latrophilin in the Nematode *Caenorhabditis elegans*.

By

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Abstract

The venom of the black widow spider (BWSV) uniquely contains a family of high molecular weight proteins that cause uncontrolled vesicle release in synapses. Two membrane receptors for BWSV have been identified, one of these being latrophilin/CIRL (LPH), a member of the G-protein coupled receptor superfamily of cell-signalling receptors and the other being neurexin. In mammals, LPH and neurexin have been shown to bind BWSV, but their function is unclear.

We established *C.elegans* as a model system for studying the effects of BWSV by microinjection of venom into wild-type (N2) *C.elegans*, which showed that the venom had an acute lethal effect over a million-fold range of concentrations. BWSV treated with SDS (0.1%) or heat before injection reduced the kill rate in N2 *C.elegans* to zero, this suggests that the active component of the venom is a protein. FPLC of BWSV demonstrated that the active component of BWSV toxic to *C.elegans* resembles ε-latroinsectotoxin. Identification of a homologue of the latrophilin gene in *C.elegans*, BO457.1, induced a functional knockout of the latrophilin gene by RNA interference (RNAi). The knockout was examined for a change in phenotype, which occurred in RNAi treated worms, compared to N2, and was extensively characterised. LPH knockout *C.elegans* were completely resistant to the lethal effects of BWSV over the same concentration range as that used in the N2 worms, whereas RNAi of CYP37A1, BO286.2 and neurexin 1α homologue has no effect on BWSV toxicity.

We have shown that a *C.elegans* latrophilin homologue mediates the toxic effects of black widow spider venom in the nematode and identified a high molecular weight latrotoxin that kills *C.elegans.* Additionally, the data provide evidence for an important role of LPH in nerve cell function.

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Dedication

To my family.

…. be systematically ascetic or heroic in little unnecessary points, do everyday something for no other reason than that you would rather not do it, so that when the hour of dire need draws nigh, it may find you not unnerved and untrained to stand the test…….

William James

Abbreviations

A	Adenine
Amp	Ampicillin antibiotic
BSA	Bovine Serum Albumin
bp	Base pair
BWSV	Black Widow Spider Venom
C	Cytosine
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid (disodium salt)
EtBr	Ethidium Bromide
G	Guanine
IAA	Isoamyl alcohol
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase
kDa	KiloDalton
LB	Luria-Bertani (Medium)
LCT	Latrocrustatoxin
LIT	Latroinsectotoxin
LPH	Latrophilin
LTX	Latrotoxin
NGM	Nematode Growth Medium

PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acids
cRNA	Complementary RNA
dsRNA	Double-stranded RNA
mRNA	Messenger RNA
RNAi	RNA Interference
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodecyl Sulphate
SEC	Size Exclusion Column
T	Thymine
TAE	Tris/EDTA/Glacial acetic acid buffer
TBE	Tris/Boric acid/EDTA buffer
TBS	Tris Buffered Saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tet	Tetracyclin Antibiotics
UHP	Ultra High Purity
WWW	World Wide Web

All amino acids indicated by their standard single letter or three letter abbreviations where appropriate

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1.1 The Black Widow Spider

1.1.1 Black Widow Spider Venom

During the centuries that humans and spiders have shared the environment the human response to these ancient animals has varied between fascination and pure terror. Even though spiders are not naturally aggressive animals, some species seem to have developed venoms that not only immobilise prey, but also work in a defensive capacity. Most spiders feed on insects or arthropods (Rash & Hodgson, 2002), yet spiders such as the Black Widow Spider (*Latrodectus mactans tredisimguttatus*) have venom that can cause severe adverse effects in mammals, including man.

Clinically, the bite from a black widow spider is not usually fatal, nor a significant health problem in adult humans (Sudhof, 2001). Only in severe cases, such as when a young or old person is bitten, does latrodectism take place. Symptoms consist of muscle pain, abdominal cramps, sweating, increased blood pressure and tachycardia. Death occurs in a few, rare cases as a result of paralysis of the diaphragm.

Spider venoms, including those of the black widow, represent a source of substances, which have biological activity, that selectively target a multitude of physiological functions in both insects and mammals (Rash & Hodgson 2002). The major constituents of venoms are proteins, polyamine and polypeptide neurotoxins, nucleic acids, amino acids, enzymes and inorganic salts, though not all of these substances are necessarily biologically active.

Over the last decade the interest and volume of scientific publications studying spider toxins, especially the venom of the black widow spider, has increased immensely. This is because one of the components of black widow spider venom (BWSV), α-latrotoxin, has

shown itself to be extremely useful. α-latrotoxin is used to study synaptic transmission because it acts in a selective nature on synaptic nerve terminals to stimulate synaptic vesicle exocytosis (Grishin, 1998 & Sudhof, 2001).

The study of BWSV has not just led to the discovery of a vertebrate specific venom component which has subsequently led to the discovery of two families of synaptic receptors and their modes of action, but also the discovery of a number of different venom components which have themselves differing species specificity. BWSV is unusual compared to other spider venoms because the active components of the venom are high molecular weight proteins (Frontali *et al*, 1976).

The isolation and identification of BWSV components was carried out using fractionation on a MonoQ column (Frontali *et al*, 1976, Krasnoperov *et al*, 1992, Ashton *et al*, 2000). By further ion exchange and hydrophobic chromatographic fractionations (Krasnoperov *et al*, 1992, Ashton *et al*, 2000) each individual toxin was isolated, giving a total of seven separate venom components, one crustacean toxin, five insect toxins, and one mammalian toxin. Toxins were named α-latrocrustatoxin (α-LCT) for the crustacean toxin, α-latroinsectotoxin (α-LIT), β-latroinsectotoxin (β-LIT), γ-latroinsectotoxin (γ-LIT), δ-latroinsectotoxin (δ-LIT) and ε-latroinsectotoxin (ε-LIT) for the insect toxins and α-latrotoxin (α-LTX) for the vertebrate toxin. These toxins along with their molecular weights and species toxicity are shown in Table 1.1.

Purified toxins show a high level of specificity to particular phyla, for example, α-LCT has an LD_{50} of 100μg/kg in the crayfish *Procambarus cubensis* and *Astacus astacus*, it also has a toxic effect on crabs and shrimps (Krasnoperov *et al*, 1992). Yet there is no toxic effect of α-LCT in either mammals or insects at doses up to 5mg/kg. This is the same with insectotoxins, though they show toxicity (at different levels) in insects they also show no

cross-species toxicity, with no toxicity seen in crayfish or mammals at doses greater than 5mg/kg (Krasnoperov *et al*, 1992).

	Toxin Name						
	α-LCT	α-LIT	β-LIT	γ-LIT	δ-LIT	ε-LIT	α-LTX
Mol. Weight (kDa)	120	120	140	120	110	110	130
LD_{50} (μg/kg)	100*	15[+]	25[+]	250[+]	60[+]	1000[+]	20[=]
Yield (% total protein)	0.5	3	0.2	0.2	0.4	2	3

Table 1.1 Characteristics of Isolated Latrotoxins (Reproduced from Grishin 1998).
[+] Tested on insect larvae (*Galleria mellonella)*
* Tested on crayfish (*Procambarus cubensis*)
[=] Tested on mice

1.1.1.1 Latrocrustatoxin (α-LCT)

α-LCT is fractionated from crude BWSV by MonoQ column chromatography and is eluted in the first protein fraction; it makes up 0.5% of the crude venom and has a molecular weight of 120 kDa. The toxin causes neurosecretion at crustacean synapses. The α-LCT protein is 1385 amino acids in length and shares similar domain structure to those of α-LIT, δ-LIT and α-LTX (Grishin, 1998 & 1999). The domain structure contains an N-terminal domain of 466 aa residues, which contains two conserved hydrophobic regions, which form transmembrane segments, these areas are highly homologous in α-LIT, δ-LIT and α-LTX. Following this, LCT has a series of ankyrin-like repeats between 466 & 1183 aa, with a highly variable region at positions 921-1031 aa, a further three highly homologous regions occur in a 202 aa C-terminal end. Though this C-terminal domain is removed during toxin maturation (Grishin, 1999).

1.1.1.2 Latroinsectotoxins (LIT)

There are five latroinsectotoxins, which have been characterised in BWSV these are α-LIT, β-LIT, γ-LIT, δ-LIT and ε-LIT. These toxins are separated from the crude venom by MonoQ column chromatography; insectotoxins are eluted in both the second fraction of proteins (α, β, γ, ε-LITs) and the third fraction of proteins (δ-LIT). All insectotoxins show no toxic activity in mice or crayfish at doses of 5mg/kg body weight and above (Krasnoperov *et al*, 1992).

Data in Kiyatkin (1993), show that α-LIT is a protein 1376 amino acids in length and a p*I* of 6.38, δ-LIT cDNA encodes a protein of 1186 aa, making the amino acid sequence of δ-LIT 190 aa shorter than α-LIT (Dulubova *et al*, 1996). Alignment of the α & δ-LITs protein sequences show that they have conserved regions covering the whole protein, δ-LIT has a 38% identity with α-LIT (Dulubova *et al*, 1996) compared to the 34% identity of α-LIT with α-LTX (Grishin, 1998).

The largest similarity of the insectotoxins takes place in the N-terminal end, which is also the area in which there is most similarity between all toxin components, including LCT, LITs and LTX. Areas containing the highest levels of sequence variability take place in the same region of all toxins, these are present in residues 910-935 and 1027-1030, and these areas could be responsible for specificity of toxin binding to its presynaptic receptor. Maturation of the protein is required for functionality of the toxin. Data from Dulubova (1996) shows that with δ-LIT, C-terminal truncation is necessary for the toxin to cause neurotransmitter release.

1.1.1.3 α-Latrotoxin (α-LTX)

α-LTX is the only component of BWSV that shows toxicity in mammals (Table 1.1). α-LTX, like the other toxins in BWSV is a high molecular weight protein; it has an apparent molecular mass of 120kDa on SDS-PAGE (Frontali *et al*, 1976), though its cDNA sequence encodes a larger protein (160kDa). Expression of the full-length protein does not yield a functional toxin (Kiyatkin *et al*, 1993), although a truncated variant is active (Ichtchenko *et al*, 1998) which suggests that the protein is cleaved in the venom gland to produce the mature toxin (Kiyatkin *et al*, 1990).

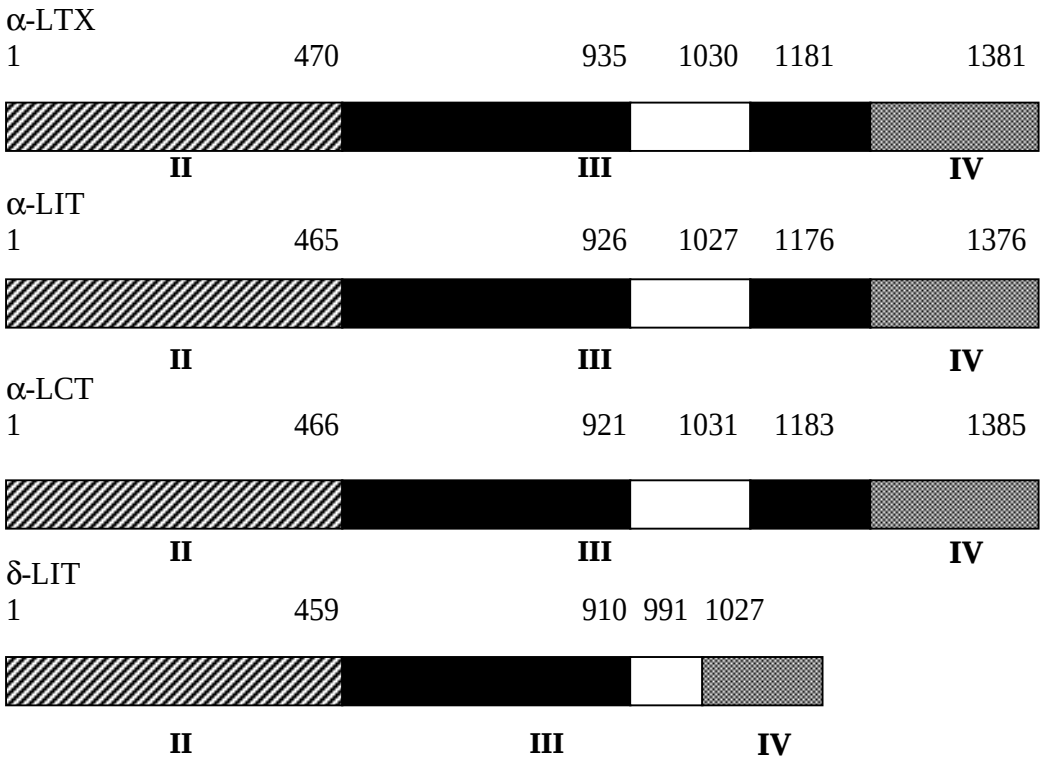


Figure 1.1 Domain structure and alignment of latrotoxins.
Three of the four domains of latrotoxin are shown for each of the aligned toxins. These toxins are α-latrotoxin, α-latrocrustatoxin and two insectotoxins α & δ. The cleaved signal peptide, Domain I is not shown. Black striped box shows Domain II, the N-terminal conserved domain, which is around 470 amino acids for each toxin. The central domain, Domain III is shown as the black box this is made up of a number of ankyrin-like repeats; it consists of between 532-717 amino acids. Within it is the white box, which represents the area of Domain III, which has the highest variation between toxin types. The final hatched box shows Domain IV the C-terminal domain, which, like Domain I is cleaved upon maturation of the toxin.

α-LTX has protein sequence homology with α-LCT, α-LIT and δ-LIT across its entire sequence length, exhibiting 37% identity with δ-LIT, and 34% identity with α-LIT (Grishin, 1998).

The toxin is composed of four domains, which are highly conserved throughout all BWSV components. Domain I is a signal peptide, which is cleaved upon maturation. Domain II is the conserved N-terminal region. Domain III is the central region made up of ankyrin-like repeats, with the fourth domain being the C-terminal end, which, like the signal peptide in Domain I is cleaved during maturation of the toxin (Kiyatkin *et al*, 1990, Dulubova *et al*, 1996, Grishin, 1998, Sudhof, 2001). A schematic representation of α-LTX is shown in Figure 1.1.

Purification of α-LTX using chromatography of BWSV shows that the main toxin is isolated in a complex with a low molecular weight protein of ~8kDa. This purified α-LTX has been shown to form a complex in solution with the low molecular weight protein (Petrenko *et al*, 1993). In the absence of divalent cations the toxin will form a dimer while the presence of cations will cause the formation of tetramers (Ashton *et al*, 2000).

Further studies into the structure/function relationship of α-LTX (Ichtchenko *et al*, 1998) using recombinant α-LTX synthesised using baculovirus expression vectors show that truncated forms of the toxin at the C-terminal end are just as potent in causing neurotransmitter release as the natural toxin (Ichtchenko *et al*, 1998), this supports the theory that the C-terminal end which is usually cleaved during maturation is not required for toxin formation of activity. Further use of toxins with the presence of increased numbers of amino acids between the conserved cysteine residues in the N-terminal region and the anykrin-like repeats region causes a loss of function of the toxin, though

this does not affect toxin binding (Ichtchenko *et al*, 1998). This indicates the importance of the distance between the cysteines and the repeat region in the function of the toxin. The 3D structure of the toxin has also been partially elucidated (Orlova *et al*, 2000) by cryo-electron microscopy. This data has shown that the latrotoxin is not a monomer, but a dimer, which can tetrimise (Orlova *et al*, 2000). The toxin has three distinct domains, head, wing and body, which are linked together. The 3D structure corresponds to the primary protein structure of α-LTX, the wing consists of residues 1-320 of the N-terminal domain, the rest of the residues are split between the body (320-982) and the head (1015-1179).

Latrotoxin monomers will assemble into stable dimers with the wings of the molecules pointing in a perpendicular direction (Ushkaryov, 2001). These assembled dimers can then spontaneously form the cyclical tetramers, in the presence of divalent cations (Ashton *et al*, 2000); this process requires significant conformational changes. The tetramers have two important features; the monomers surround a central channel that stretches the entire length of the molecule and the base of the tetramer is hydrophobic (Orlova *et al*, 2000) both of these factors are important in the action of the toxin by channel formation of the toxin and receptor based interaction.

1.1.2 Channel formation by α-LTX

α-LTX has a complex mode of action, which has still to be fully understood. Stimulation of neurotransmitter release by the toxin is thought to occur by direct formation of pores in the plasma membrane or by binding to a receptor leading to intracellular second messenger signalling causing neurotransmitter release. There is limited evidence for either of these modes of action and the possibility of collaboration between the modes of action to cause a toxin induced response.

The formation of pores or channels by α-LTX – which causes extracellular Ca^{2+} influx and cell morphology disruption (Volynski *et al*, 2000) – is one of the most important activities of α-LTX.

The discovery of the 3D structure of α-LTX (Orlova *et al*, 2000) shows that the tetramer form associated with the non-vesicular release of cations from cells (Ashton *et al*, 2000) has a distinct resemblance to a pore (Ushkaryov, 2001).

Many observations have been made regarding pore-formation and how it occurs; for example, neuroendocrine cells, which contain α-LTX receptors when exposed to toxin, will open cation channels (Hlubek *et al*, 2000), which suggest that the toxin inserts into the membrane spontaneously to form a channel *in vivo.* In *Xenopus* oocytes that do not express α-LTX receptors, the addition of toxin does not cause channel opening, only the addition of total brain mRNA will α-LTX cause Ca^{2+} channels to open, channels probably form after mRNA addition from brain as they contain α-LTX receptor proteins. Based on these different experiments it would appear that the binding/tethering of α-LTX to its receptors is necessary for the toxin to insert into membranes where it forms a non-selective cation channel (Ashton *et al*, 2000). This channel formation directly causes the release of neurotransmitters without the use of receptor based cell signalling.

Insertion of the toxin directly into the lipid bilayer of liposomes was directly observed by cryo-EM (Orlova *et al*, 2000). Based on these EM images of the toxin in the membrane four important aspects of pore formation were discovered. That the tetramer inserts into the bilayer with its base, the base fully penetrates the membrane, the upper part of the tetramer remains above the membrane with the "wings" anchoring the toxin to it and the central channel causes the opening of the membrane (Ushkaryov, 2001). Depending on conditions the channel size will be ~10-25 Angstroms in size (Ushkaryov, 2001), the size

of the pore means that other molecules can pass through the central channel including water and neurotransmitters (Davletov *et al*, 1998). Anti-α-LTX antibodies can block the pore due to the presence of the tetramer on the membrane surface (Volynski *et al*, 2000). The structure of α-LTX has a role in its interaction with α-LTX receptors in pore formation, with the N-terminal wing responsible for tethering to α-LTX receptors; this has been shown by experimentation with truncated forms of the receptor (Ichtchenko *et al*, 1998).

The model of the toxin, and its formation of channel containing tetramers, (Orlova *et al*, 2000) appear to be the best model explaining channel formation by α-LTX. It is apparent though that pore formation does not account for all the activities of the toxin, but discovery of the 3D structure has been a valuable addition to understanding α-LTX pore formation.

1.1.3 Receptor based interaction of α-LTX

α-LTX can also cause neurotransmitter release by interaction with its receptors. In order for exocytosis to occur, α-LTX has to bind to specific neuronal receptors. Receptors are localised near the active zone of the presynaptic plasma membrane. Two classes of receptor are present at these points, in equal numbers; they both bind the toxin at the same nanomolar concentrations. The main difference between these receptors, apart from their structure, is that one receptor binds α-LTX in the presence of Ca^{2+} whereas the other will bind α-LTX without the presence of Ca^{2+}.

The Ca^{2+} dependent class of receptor are neurexins, while the Ca^{2+} independent class of receptor is latrophilin or CIRL (Calcium Independent Receptor for Latrotoxin).

1.1.4 Latrophilin / CIRL

The α-LTX receptor latrophilin was isolated from bovine brain membranes by affinity chromatography (Davletov *et al*, 1996 & Krasnoperov *et al*, 1996). Further cloning studies have revealed that there are three closely related forms of latrophilin expressed in vertebrates (Sugita *et al*, 1998). All of these latrophilins are G-protein coupled receptors with large intra and extracellular domains and have the same domain structure (Sugita *et al*, 1998).

Figure 1.2 shows the structural domains of latrophilin. (Modified from Sugita *et al*, 1998). The N-terminal, a cleaved signal peptide is followed by four domains covering ~500 amino acids. The first domain is a lectin-like domain, the second a factomedin like domain, a third domain which shows homology with the G-protein coupled receptor BAI1-3 a brain-specific angiogenesis inhibitor, and the fourth domain featuring a short cysteine rich domain. This short cysteine rich sequence before the first transmembrane repeat has a high level of identity with other G-protein coupled receptors (Sugita *et al*, 1998). The cysteine rich region may represent the site of a proteolytic cleavage signal during receptor maturation; this area has been termed the GPS region (Ichtchenko *et al*, 1999).

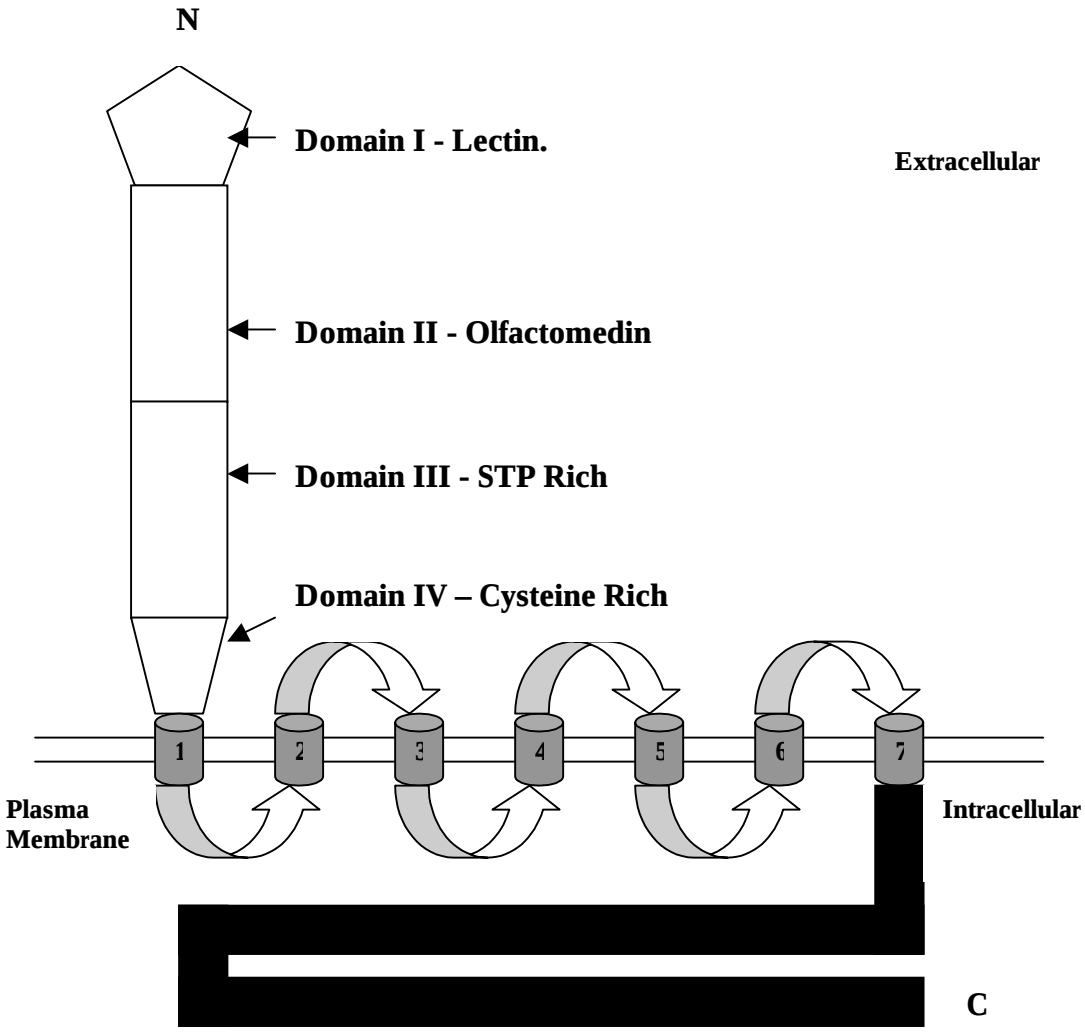


Figure 1.2 Domain structures of latrophilin/CIRL.
Four extracellular domains are present above the plasma membrane. The seven transmembrane region is similar to those in the calcitonin/secretin receptor family. Intracellular region has no domain structuring. Extracellular domains III & IV share homology with the BAI1-3 and EMR1 family of G-protein-coupled receptors respectively.

The seven transmembrane region of latrophilin is related to the calcitonin/secretin family of G-protein coupled receptors. Following the transmembrane region, latrophilin has a long intracellular C-terminal tail, which is in the region of 500 amino acids in length; this region is liable to alternative splicing (Sugita *et al*, 1998).

The three forms of latrophilin identified in vertebrates are expressed separately. Latrophilin-1 was first thought to be brain specific (Lelianova *et al*, 1997) but RNA blotting techniques show expression of the protein in all tissues at low levels (Sugita *et al*, 1998), latrophilin-2 is expressed in most tissues outside the brain, with low levels in

brain, latrophilin-3 is present only in the brain (Ichtchenko *et al*, 1999). Both latrophilin-1 & 2 bind α-LTX at nanomolar concentrations whereas the third latrophilin protein cannot bind the toxin (Ichtchenko *et al*, 1999).

The role of the latrophilins *in vivo* and the natural ligands of the receptors are unknown. By looking at their structure though, it appears that they have a physiological function possibly as cell adhesion molecules. Confirmation of latrophilin being a Ca^{2+} independent receptor was shown in Lang (1998), where synaptosomes lacking latrophilin were administered α-LTX and showed that α-LTX induced glutamate release was ablated, whereas depolarised release was normal. α-LTX induced release was prevented with and without Ca^{2+} present; this is in contrast to synaptosomes lacking neurexin, which showed glutamate release only when Ca^{2+} was present.

1.1.4.1 Latrophilin as an α-LTX receptor

The potential mechanism for latrophilin-mediated exocytosis is still unknown. After the original discovery that latrophilin is a G-protein coupled receptor it would have been expected that α-LTX triggers an intracellular G-protein linked second messenger cascade, which ultimately leads to exocytosis.

Truncation of cytoplasmic regions has no detrimental effect on latrophilin's ability to cause exocytosis when in contact with α-LTX (Hlubek *et al*, 2000). This data was generated using a cell-culture system and it is not clear whether these responses would also take place *in vivo*, meaning that C-terminal regions may still be important in starting intracellular cascades leading to neurotransmitter release. Yet, there still may be evidence for neurotransmission without intracellular signal transduction to cause exocytosis, if this is the case then what is the internal mechanism governing latrophilin-α-LTX transmitter release? It may well be that the latrophilin receptor acts as a tether for the toxin, and has

no role in the intracellular transduction of α-LTX. Published data supporting the use of the α-LTX receptors as signalling molecules mediating the neurotransmitter release caused by α-LTX is scarce, while data supporting α-LTX working as a transmembrane pore is more numerous. At this current time the argument over which mechanism governs α-LTX induced toxicity is firmly in the favour of the toxin working as a pore-forming molecule which is recruited to the plasma membrane by the latrotoxin receptor acting as a tether for the toxin. Little evidence is available regarding the activation of latrophilin in terms of signalling, though within the scope of this thesis we hope to show a role for latrophilin as a signalling molecule. This is an important area to address, as structurally latrophilin resembles a G-protein coupled receptor with characterised signalling properties

1.1.5 Neurexin

Neurexins were first isolated as possible receptors for α-latrotoxin after affinity chromatography of binding proteins on a column containing immobilised α-LTX (Ushkaryov *et al*, 1992).

Neurexins are a family of neuron-specific cell-surface proteins. Like latrophilin's, there are three neurexin genes present in vertebrates, called neurexin 1, 2 & 3. These genes are under the control of two distinct promoters, leading to the formation of neurexin α I, II & III and the β neurexins I, II & III (Ushkaryov *et al*, 1992 & Ushkaryov *et al*, 1994).

The structure of neurexin is of a cell-surface receptor with a large extracellular sequence, which is homologous to the laminin G-domains, O-linked glycosylation sequences, a transmembrane region and a short C-terminal sequence (Henkel *et al*, 1999).

Figure 1.3 shows the structures of neurexin α and β (modified from Sudhof, 2001).

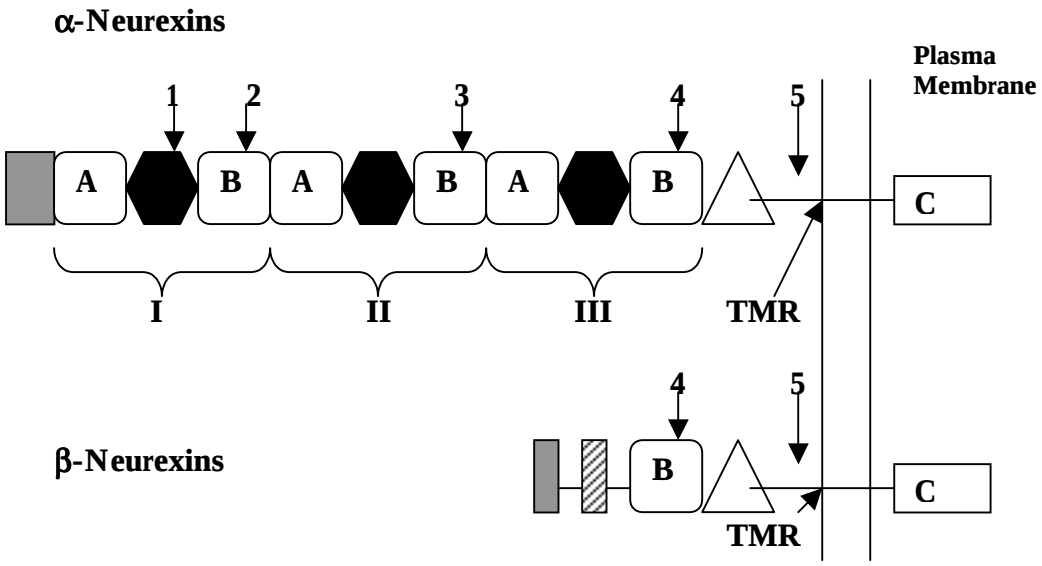


Figure 1.3 Domain structures of neurexins
Neurexins Iα-IIIα & Iβ-IIIβ share the same C-terminal domain containing the carbohydrate attachment sequence (triangles), transmembrane region (TMR) and the cytoplasmic tail (C). α-neurexins have 3 repeat sequences (I, II, III) containing epidermal growth factor (Black hexagon), flanked by LNS-A and LNS-B domains.β-neurexins contain one β-neurexin specific domain (hatched box) and one LNS-B domain. Arrowed numbers indicate alternative splice sites (1-5 for α and two for β which correspond to sites 4 & 5 for α-neurexin).

α & β neurexins only differ in the construction of their extracellular sequences, the transmembrane and intracellular C-terminus sequences remain the same. α-neurexin has an extracellular region composed of three repeats, each of these contain a central Epidermal Growth Factor (EGF) like domain sandwiched between two related LNS (LaminA, Neurexin, Sex hormone) domains A & B. These LNS domains are responsible for protein-protein interactions including the binding of α-LTX (Ushkaryov *et al*, 1992). Analysis of the structure of the sixth LNS domain (LNS-B) in α-neurexin shows that this is the same domain as the LNS domain (the only one) in β-neurexin (Sudhof, 2001).

The presence of extensive splicing sites (five in α-neurexin and two in β-neurexin) leads to many isoforms of neurexins being expressed (Ushkaryov *et al*, 1992). *In situ* hybridisations suggest that neurexins are mostly expressed in neurons, though no exact localisation of the proteins has been found. Some researchers believe that they may be

concentrated around the synapses (Sudhof, 2001). Neurexins are also conserved in vertebrates and a number of invertebrates including *C.elegans* and *Drosophila*.

1.1.5.1 Neurexin as an α-LTX receptor

Initial observations that neurexins bound to immobilised α-LTX by affinity chromatography (Ushkaryov *et al*, 1992) was supported by data showing that recombinant forms of the receptor also bind to the toxin (Davletov *et al*, 1995). Both α & β neurexins bind to α-LTX and require the presence of Ca^{2+} for this to occur. The use of neurexin 1α knockout mice (Geppert *et al*, 1998) showed that α-LTX binding to brain membranes was reduced twofold in the knockout mice when Ca^{2+} was present but was unchanged when Ca^{2+} was not present (Geppert *et al*, 1998). Further evidence for the role of neurexin in exocytosis in the presence of Ca^{2+} was shown by the depression of exocytosis in synaptosomes taken from neurexin 1α knockout mice in the presence of Ca^{2+} while release was normal in those without (Geppert *et al*, 1998). Whether this is due to its role as an α-LTX receptor or some other function of the receptor is still unclear. Neurexins are thought to be expressed near the synapses, the discovery of synaptotagmin, a synaptic vesicle membrane protein, thought to be a calcium sensor in exocytosis, which binds to the C-terminal of the receptor, suggests that neurexins produce a signal to α-LTX intracellularly. But, α-LTX will continue to cause exocytosis in mice lacking the gene for synaptotagmin making the protein non-essential for neurexin-latrotoxin transmitter release. Truncated forms of neurexin lacking C-terminal sequences also respond normally to α-LTX stimulation (Sugita *et al*, 1999). This data shows that direct binding of neurexin and α-latrotoxin results in neurotransmitter release.

1.1.6 Receptor co-operation

α-latrotoxin therefore has two receptors that it binds to with high-affinity, yet these receptors have neither sequence homology nor structural similarities. Nevertheless, PC12 cells with either of the receptors transfected into them can function on their own, cotransfection of both latrophilin and neurexin results in no enhancement of the secretion response to α-LTX (Sugita *et al,* 1998), proving that these receptors do not seen to co-operate on a functional level.

Conflicting arguments have developed though, suggesting that latrophilin and neurexin can co-operate in α-LTX induced neurotransmission in synapses. Research using synaptosomes to study the effects of Ca^{2+} dependent and independent neurotransmitter release (Geppert *et al,* 1998) show that glutamate and GABA release is the same whether Ca^{2+} is available or not, independent of toxin dose. If the receptors worked on there own then observed release with Ca^{2+} would equal the sum of Ca^{2+}-dependent and independent release, which is the case with binding, this does not the case with release, inferring that the receptors collaborate, this data doesn't mention the fact that either Ca^{2+} dependent or Ca^{2+} independent pathways could saturate the response. This data again illustrates the dichotomy of α-LTX induced neurosecretion, where arguments can be made for and against the co-operation of the two α-LTX receptors. As yet there is still no definitive evidence on interaction between the receptors.

Current thinking suggests (Sudhof, 2001) that because the receptors both bind α-LTX independently, any interaction will occur downstream of the binding, using a mechanism not yet known.

The functions and mechanisms governing the interaction between the toxin component of BWSV, α-LTX, and the receptors it binds to (latrophilin and neurexin) are still not fully resolved. The recent generation of knockout mice to the latrophilin receptor has

been a boost in the number of systems available to study the effect of latrophilin and α-LTX, but the use of mice as a model system to study protein function has significant limitations. An alternative animal model to the mouse is the nematode *Caenorhabditis elegans*.

The mouse is an animal model for biological research like *C.elegans*, but research with mice is far more expensive and time consuming, not to mention ethically fraught than that with *C.elegans*. The ease, with which *C.elegans* can be manipulated, its genetic tractability, as well as the wealth of knowledge surrounding the nematodes nervous system, makes it an ideal model for studying the mechanism of action of BWSV and associated receptors. The relatively simple nervous system and the extensive characterisation of it should make studying BWSV toxicity relatively simple.

By using a reverse genetic technique such as RNAi, a homologue of the latrophilin gene can be quickly isolated from the wealth of sequence data from the *C.elegans* genome project and the genetic function then analysed and the effects of BWSV studied in worms, which are latrophilin deficient or wild type.

1.2 *Caenorhabditis elegans*

1.2.1 *Caenorhabditis elegans* as a biological model system

In 1965 Dr Sydney Brenner selected the rhabditid nematode worm *Caenorhabditis elegans* as an experimental model to study animal development, behaviour and the nervous system in a simple metazoan (Brenner, 1974).

C.elegans is a free-living worm, which is soil dwelling and is found across the temperate regions of the world were it feeds on microorganisms. The “worm” (as it is often referred to) offers a great opportunity to study the genetics of an organism as it has a rapid life cycle, is small in size and easy to study and cultivate in the laboratory.

C.elegans is ~1mm in length with a maximum diameter in adults of ~80μm. Worms can be grown on an agar medium containing *E.coli* as a food source in Petri dishes, they have a rapid life cycle, embryo to adult development takes 3.5 days at 20°C, adults also produce a large number of offspring (~300) and are self-fertilising.

Adult hermaphrodite worms contain a total of 959 somatic cells, of which 302 are neurons. Due to the number of cells being constant throughout the wild type population, the complete cell lineage has been characterised (Wood, 1988) along with a "wiring" diagram of the nervous system and its synaptic connections (White *et al*, 1986). This is possible because of the transparent nature of the worm allowing high quality microscopic images to be taken.

1.2.1.1 Life cycle

The growth of *C.elegans* is rapid; under normal laboratory conditions the entire life cycle of the worm from egg to egg-producing adult is 3 ½ days at 20°C. *C.elegans* can grow over a range of temperatures exhibiting the normal animal behaviour of faster growth at increased temperature. The life cycle can therefore take between 3 days at 25°C to 6 days at 15°C. Population growth is greatest at 20°C with brood sizes of ~300 progeny produced over a period of four days.

C.elegans life cycle begins with embryonic development or embryogenesis, from here the first larval stage (L1) develops, anatomically these larvae appear similar to the adult worm, but smaller in size at ~250μm. Four larval stages (L1 – L4) occur during postembryonic development before a final moult leads to the adult worm.

At 20°C embryogenesis takes 14 hours, time between successive larval moults at 20°C are as follows;

L1 to L2 = 29 hours

L2 to L3 = 38 hours

L3 to L4 = 47 hours

L4 to Adult = 59 hours (all post fertilisation) (data adapted from Wood, 1988).

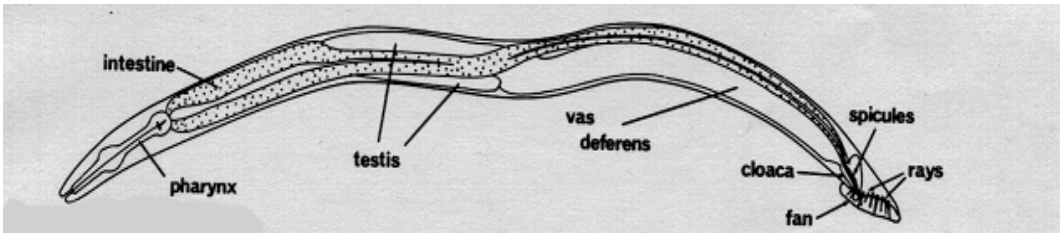
C.elegans does possess an alternative larval stage called the dauer; this is formed at the second larval moult instead of the L3 and occurs at times of high population density or when food is lacking. The dauer is a specialised larval form, which has evolved as a mechanism for surviving adverse conditions; this form of the worm is resistant to desiccation for example. The dauer is morphologically similar to the L3, yet slightly thinner, dauer will mostly remain motionless, but when touched will move faster than the L3 form, again this appears to be a survival technique. Dauer will remain viable for ~3-4 months, when conditions become more variable the dauer will moult and develop into the normal L4 larvae.

1.2.1.2 Anatomy

C.elegans is found as either male or hermaphrodite, though in normal populations only 0.1% is male. The hermaphrodite reproduces by self-fertilisation as it contains both sperm and oocytes. The male, as expected produces only sperm and therefore must mate with the hermaphrodite to reproduce. In the event of male/hermaphrodite mating the male sperm will fertilise the oocytes, this cross-fertilisation will produce a 50-50 split of males/females in the resulting brood. This is in marked contrast to hermaphrodite self-fertilisation, which generates only hermaphrodites, most populations in the laboratory are generated by self-fertilisation, hence the low level of males in a population.

Figure 1.4 shows the general anatomy of both the male (A) and hermaphrodite (B) worms (Figure from Wood, 1988).

A



B

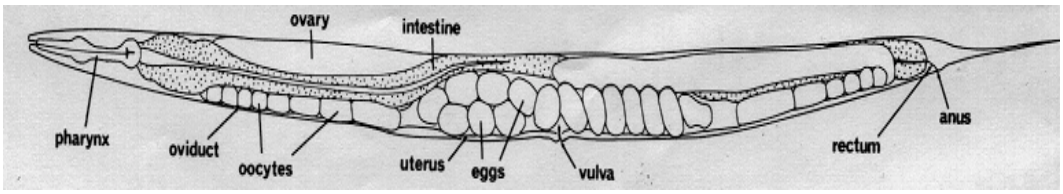


Figure 1.4 Cross sections of male and hermaphrodite *C.elegans*.
Male *C.elegans* is shown in image **A** with the hermaphrodite in image **B**. Both sexes have a similar general anatomy, and are of a similar size, though the male is a little shorter and thinner. The male also clearly shows the tail-fan array used for clasping the hermaphrodite during mating.

Both *C.elegans* sexes and most other nematodes are constructed anatomically in a similar fashion. The body is two concentric tubes separated by a fluid filled pseudocoelom, the body shape is maintained by internal hydrostatic pressure, the outer tube makes up the worms cuticle, this is mostly constructed with collagen, during each larval moult the cuticle is shed and a new one formed to allow the worm to grow. Four strips of muscle run the length of the worm attached to the sub-cuticle hyperdermis, relaxation and contraction of the two subventral and two subdorsal muscle strips causes the worms locomotion, which is observed on an agar plate as a sinusoidal wave, worms remain on the agar plate due to the surface tension of the water on the plate. Worms have a great deal of freedom in their movement, able to move forwards and backwards in response to the environment.

The mouth of the worm is at the very tip of the head, from here the gut, consisting of the pharynx and intestine run the entire length of the body; this transparent tube ends at the anus (cloaca in the male). The pharynx is responsible for the initial processing of

food in the worm; the terminal bulb of the pharynx contains a grinder that crushes the bacteria before moving it on into the intestine. The pharynx is made of 20 muscle cells, 20 nerve cells and 18 epithelial cells (Riddle *et al*, 1997). The intestine is made of 20 cells in nine rings running down the gut, contraction of these rings forces the food through the digestive tract (Riddle *et al*, 1997)

The pharynx is where most of the neuronal cell bodies are present forming a ring structure, this ring is attached to two nerve cords running the length of the worm. The nerve ring receives and integrates sensory information and connects to motor neurons in the head or along the nerve cords (Bargmann, 1998). The hermaphrodite and male reproductive systems are the areas, which have the most variation between the sexes. The hermaphrodite has two functionally independent arms; each arm contains an ovary, oviduct and a spermatheca connected to a shared uterus centred around the vulva. The uterus in the adult contains fertilised eggs and embryos, which begin development in the adult (Wood, 1988). Egg laying occurs via contractions of the vulva controlled by hermaphrodite specific neurons (Riddle *et al*, 1997).

The male has a single gonad (see Figure 1.4), which connects to the vas deferens then the cloaca at the anus. The meiotic cells which will become the sperm, are arranged down the gonad were each cell is in a later stage of spermatogenesis than the first. The mature sperm is held in the seminal vesicle prior to release. Mating is controlled by male specific neurons (Riddle *et al*, 1997).

1.2.1.3 Development

The development of *C.elegans* has also been extensively studied allowing the worm to be analysed from its initial few cells too full grown adult.

Embryogenesis occurs in two stages, the initial phase generates the 558 cells in the hermaphrodite and 560 cells in the male to form the first larval stage (Wood. 1988). The

first cell divisions produce the “founder cells”, AB, E, MS, C and D. E produces cells only found in the intestine, while D only forms body wall muscle cells (Riddle *et al*, 1997). The other founder cell groups produce cells found in all parts of the body. The second stage of embryogenesis develops the cells in the embryo into the larval shape of the worm. Differentiation of cells at this stage forms nerve and muscle cells which cause the embryo to move inside the egg, the fully formed L1 will move even while still in the egg. After hatching the worm will continue to develop, with the number of cells increasing to 959 in the hermaphrodite and 1031 in the male (Wood, 1988). During this time the reproductive organs will mature, to eventually form the active sexual organs by the time the worm reaches the adult form, sex-specific muscles are generated from a single blast cell M (Riddle *et al*, 1997). The vulva in hermaphrodites is formed from three cells in the central hypodermis; the male tail is formed from a number of hypodermal cells, and four rectal cells. The consistency of development in *C.elegans* along with the transparent nature of its body and the relatively small number of cells, which make up the body, makes the worm a perfect model for studying animal development and any mutations that may affect this development.

1.2.2 *C.elegans* nervous system

Like the whole of the organism, the nervous system of *C.elegans* has been extensively researched. In the hermaphrodite there are 302 neurons, these are connected by ~5000 synapses and ~600 gap junctions which have so far been identified (White *et al*, 1986). The structure and location of each neuron is consistent throughout hermaphrodite populations making examination of mutant populations compared to wild type relatively simple.

Another unique aspect of the worms nervous system is that only two neurons, CAN (osmoregulatory neuron) & M4 (pharyngeal motor neuron) are essential for the worms

survival (Bargmann, 1998) meaning that extensive mutations can made affecting the nervous system without the fear of inducing a non-viable animal model.

The structure of the nervous system is contained mostly in a central nerve ring located near the pharynx, ventral and dorsal nerve cords and sensory neurons located in the head. The nerve ring contains the majority of interneurons along with most of the sensory neurons. The nerve cords travel the length of the worm and contain mostly motor neurons, the head contains the majority of sensory neurons, probably because this is the part of the worm, which analyses the worm's environment.

The sensory anatomy of the *C.elegans* nervous system is comprised of neurons, which, as previously mentioned, are situated in the head region. There are some of these neurons present along the worm's body. These neurons are used for both mechanosensory and chemosensory functions, with the majority of these present in the very tip of the worm's head. The motor anatomy is responsible for body-wall locomotion, egg laying, defecation and the pumping of the pharynx (Chalfie & Sulston, 1981). Again there are a number of these neurons present in the tip of the head; these are probably responsible for touch-sensitivity (Duggan *et al*, 1998).

Serial section electron micrographs (White *et al*, 1986) have been used to identify all of the synaptic connections among neurons and muscles present in the hermaphrodite. This has led to what *C.elegans* researchers refer to as the "wiring diagram" of the worm. Though this data does not indicate if a synapse is excitatory or inhibitory it is still an invaluable tool for the scientist.

The nervous system has always been an eagerly studied aspect of *C.elegans* as the hope is that it will act as model system for the function and development of nervous systems in general. The completion of the genome sequence (*C.elegans* Consortium, *Science*: Vol **282**, 1998) has shown that many genes of vertebrate nervous systems are also present in *C.elegans* (Bargmann, 1998). The tools for analysing the nervous system *in vivo* have

mostly been comparison of fine structures of the nervous system in mutant and wild type worms by electron microscopy (EM). This is an excellent technique for looking at number and size of synaptic vesicles, but practically can be very fiddly and time consuming. Whole mount staining of the nervous system has also been used and is easier than EM, but it relies on the availability of appropriate antibodies. The most popular technique at present is the use of green-fluorescent protein (GFP) attached to neuron-specific promoters used to analyse expression patterns of neuronal proteins in *C.elegans.* This technique has the advantage of being used on live worms, allowing screening of mutants with altered neuronal structures.

Comparison of the *C.elegans* genes with molecules in the vertebrate nervous system (Bargmann, 1998) shows many similarities and a few differences between the two. Conserved systems between the two include, neurotransmitters and their receptors, synaptic release mechanisms, ligand-gated and G-protein couple receptors and second messenger pathways (Bargmann, 1998). *C.elegans* does lack rhodopsin, used for vision in vertebrates and invertebrates, some olfactory receptors in *C.elegans* are not related to vertebrate gene sequences, but do have the same properties.

1.2.3 Synaptic transmission

Communication between neurons and their target cells is achieved in *C.elegans* in the same way it is in most vertebrates, by the regulated release of neurotransmitters at synapses. Extensive study of the process of synaptic transmission has revealed that many of the molecular components of the vesicular release mechanisms as well as the respective neurotransmitters are highly conserved in metazoans, including *C.elegans* (Brownlee & Fairweather, 1999, Chacon & Sudhof, 1999). Though data has shown that proteins such as synaptobrevin are required for normal synaptic transmission in *C.elegans* (Nonet *et al,* 1997), no data as yet has been published on the effects of toxins such as

BWSV on *C.elegans* and what effect exposure to this toxin would have on the nervous system and neurotransmission in the worm, which are known to be the areas effected in mammals. Most of the classical neurotransmitters are present in *C.elegans* including acetylcholine (ACh), glutamate, γ-aminobutyric acid (GABA), serotonin and dopamine. Neurotransmitters are loaded into synaptic vesicles by vesicular transporters at the active zone of the pre-synaptic plasma membrane (Chacon & Sudhof, 1999). Specific transporters are necessary for loading different transmitters. Once the transmitter is released by vesicle fusion, it diffuses across the synaptic cleft, which separates the pre and postsynaptic membranes and binds to post synaptic receptors. Receptors on the postsynaptic membrane are specific for their transmitter; their presence on different cells determines the cellular response to the initial signal. The receptors influence electrical impulses in the post synaptic cell, from here the receptor will directly cause ion channel activation or start a second messenger system leading to ion channel activation. The transmission event is terminated by re-uptake of the transmitter from the synaptic cleft by membrane transporters.

Each of the neurotransmitters in *C.elegans* follows this process of signalling. Acetylcholine (ACh) is the primary excitatory neurotransmitter, which controls motor function in the worm. ACh is believed to be the only transmitter, which is vital for life in *C.elegans. Cha-1* mutants show no viability as they are completely deficient in ACh (Riddle *et al*, 1997). All body wall muscles, egg laying muscles and pharyngeal muscles are thought to express receptors for ACh. As an ACh signal termination mechanism *C.elegans* has the enzyme acetylcholinesterase (AChE), which hydrolyses ACh in the synaptic cleft. *C.elegans* has three classes of AChE two of which have some homology with AChE classes in vertebrates (Brownlee & Fairweather, 1999).

The GABAergic cells in *C.elegans* are responsible for inhibition of motor neurons. Therefore, GABA neurons are expressed in all body wall and enteric muscles. Unlike

ACh, GABA deficient mutants are viable, but because of their presence in body wall muscles, the mutants have a tendency to have motor defects.

Dopamine (3,4-dihydroxyphenylethylamine) is produced by hydroxylation of tyrosine by tyrosine hydroxylase too form 3,4-dihydroxy-phenylalanine (DOPA), DOPA is then decarboxylated to dopamine by aromatic amino acid decarboxylase. Dopamine inhibits locomotion and egg laying; neurons in the egg laying muscles have been shown to express these receptors.

Serotonin receptors are also expressed in the egg laying muscles, but the neurotransmitter is thought to have a stimulatory role in pharyngeal pumping as well as egg laying and is inhibitory to both locomotion and defecation. In the male worm, serotonin is an essential transmitter for initiating mating behaviour.

Glutamate acts as both excitatory and inhibitory neurotransmitter in *C.elegans.* Worms with synaptic transmission mutations were first identified by Brenner (1974). He reported that the AChE inhibitor, lannate, was toxic to *C.elegans*, and that some mutated worms showed varying resistance to the compound. Since 1974 many more mutants have been identified and characterised that are resistant to AChE inhibitors such as Aldicarb. Inhibition of AChE causes an accumulation of ACh in the synaptic cleft causing worms to become paralysed and at high concentrations to die. Therefore resistance to Aldicarb would be due to a mutation, which reduces the accumulated ACh in the synaptic space. Defects in ACh receptors should also cause resistance to AChE drugs. Infact a classical test for identifying the type of synaptic mutation in *C.elegans* is carried out by analysing a worm's response to the compounds Aldicarb and Levamisole. Worms, which are resistant to Aldicarb can be classified as presynaptic or post synaptic, presynaptic defects usually have elevated levels of ACh where levels are normal in, post synaptic mutants. Postsynaptic defects are also resistant to the AchR agonist levamisole; pre synaptic mutants have normal or elevated responses to levamisole.

As has been illustrated, *C.elegans* is a powerful genetic tool for studying gene function and loss of function. Using the genome sequence of *C.elegans* it is possible to identify homologous genes to those in mammals, such as latrophilin. One of the most recent, and most interesting methods of gene silencing in the worm is the use of RNA interference to generate gene knockouts.

1.3 RNA Interference

RNA interference (RNAi) describes the use of double stranded RNA (dsRNA) to target specific mRNAs for degradation this therefore silences their expression. RNAi is thought to be one of a broad class of RNA induced silencing phenomena, which occur in plants, animals and fungi.

The first report of RNA induced silencing showed that either sense or anti sense RNA caused a change in gene expression in *C.elegans* (Guo & Kemphues, 1995). In 1998, Fires group explained that the observed change in gene expression was due to dsRNA contamination in the sense and anti sense samples, their data (Fire *et al*, 1998 & Montgomery *et al*, 1998) showed that dsRNA caused specific gene silencing. RNAi was born. RNAi is thought to use the sequence information in the dsRNA to make a protein-RNA complex that ablates the mRNA; this makes RNAi highly specific as it only attacks genes sharing sequence with the dsRNA.

RNAi has become the major reverse genetics technique in *C.elegans* research. This is because of the completed genome sequence of the worm; there are more gene sequences than gene functions. It is therefore easier to use these sequences to uncover gene function than vice-versa, and hence reverse genetic strategies have taken on a new importance.

RNAi can be initiated in *C.elegans* by injection (Fire *et al*, 1998), soaking worms in dsRNA solution (Tabara *et al*, 1998) or feeding worms dsRNA expressing *E.coli* (Kamath *et al*, 2000). RNAi is also an important tool in the study of plants, fruit flies and hydra. RNA interference has also been shown in Zebrafish (Yin-Xiong *et al*, 2000) and mammalian cells (Elbashir *et al*, 2001).

Studies involving RNAi in *C.elegans* show why there are many methods of dsRNA induced gene silencing, this is because the mode of interference can cross cell boundaries. Therefore the site of injection or method of administration of the dsRNA is not essential for successful gene silencing. Though the other methods of RNAi (other than injection) are not as effective as injection, there are nevertheless several advantages of the feeding technique over microinjection. Feeding is a far less labour intensive method than microinjection and is better for performing RNAi on large numbers of worms. Feeding can also support a continuous population of RNAi worms as long as there is RNA producing *E.coli* present, whereas injection will only produce F1 offspring that show RNAi phenotypes, F2 progeny will not express the dsRNA-altered phenotype. The mechanism by which RNAi works has not been fully elucidated but at the moment current thinking believes that RNAi targets mRNA with the same sequence for degradation. *C.elegans* has been screened for RNAi resistant mutants (Tabara *et al*, 1999) with the goal of trying to identify the genes required to elicit RNAi. Four genes were identified called *rde-1* to *rde-4* (*rde* stands for RNAi deficient) at the moment only *rde-1* has been cloned, there are genes in both *Neurospora* and *Arabidopsis* with homology to *rde-1*, all three genes are required in there respective organisms for silencing in somatic tissues. Germline transmission of the RNAi signal requires *rde-1* and is not required for interference after (Grishok *et al*, 2000). This suggests that *rde-1* is required in the formation of the RNAi signal. The biochemistry of RNAi shows that small 21-25

nucleotides are the active dsRNA structures involved in RNAi (Elbashir *et al*, 2001), suggesting that these small fragments of RNA guide cleavage of mRNAs.

Therefore, at this moment current models of dsRNA interference are as follows; dsRNA enters the organism where it interacts/activates the *rde-1* gene, small 21-25 nucleotide RNA complexes are formed which proceed to guide the cleavage of mRNA corresponding to the initially injected dsRNA.

RNAi will continue to be an important technique in *C.elegans* research as it is used in tandem with the huge amount of genome data available after the completion of the worm's genome sequencing project. As this research continues it is hoped that more data will also be generated in an attempt to further uncover the complete mechanism that governs dsRNA gene silencing, not only in *C.elegans* but also in the other organisms that this phenomenon has been seen in.

1.4 Summary

The mechanisms that govern the interactions between the toxin components of black widow spider venom (latrotoxins) and the receptors that they bind to (latrophilins and neurexins) have still not been fully elucidated. In this study we wish to examine the relationship between the BWSV and the receptor latrophilin using the nematode *C.elegans* as a model system.

The major aims of the investigation are to establish *C.elegans* as a model system for studying the effects of BWSV in both wild type worms and in worms with a homologous gene to the latrotoxin receptor latrophilin knocked out. To produce this knockout we will use the reverse genetic technique RNA interference (RNAi). By producing this knockout we hope to characterise any mutation produced both in physical phenotype

and in response to exposure to BWSV, and to establish whether these mutations are affecting the worms neuronal function.

We hope that examining the effects of BWSV and latrophilin in *C.elegans* will allow us to examine the importance of latrophilin in nerve cell function and to help provide evidence for the role of latrophilin as a receptor for latrotoxin with a role in signalling rather than just acting as a tether for the toxin to form pores in the lipid bi-layer.

We also hope to use RNAi to examine the possible effects of gene silencing on other genes that may be involved with neurotoxic compounds such as glutamate and organophosphate toxins.

Chapter 2. Materials and Methods.

2.1 Materials

All reagents were obtained at Anal R grade purity or greater. Ethanol, glycerol, β - mercaptoethanol were from BDH. Agarose, ampicillin, ammonium acetate, chloramphenicol, cholesterol, ethidium bromide, BSA, DEPC, DTT, DMSO, EDTA, glycine, hydrogen peroxide, imipramine, isoamylalcohol, levamisole, NaCl, RNaseA, SDS, TEMED, Tris-base, and tetracycline were all obtained from SIGMA. Xylene cyanol FF, Silver stain kit, Protein Minigel kit, UnoQ and UnoS columns were from BioRad. Bacto-agar, Bacto-tryptone and Bacto-yeast extract were obtained from Difco. Acetic acid, boric acid, calcium chloride, chloroform, Coomassie blue R250, gelatin, glucose, hydroxyquinoline, magnesium sulphate, phenol, potassium acetate, potassium dihydrogenphosphate, potassium hydrogenphosphate, sodium acetate, sodium hydrogenphosphate and sodium hydroxide were provided by Fisher. Invitrogen provided the Novex Pre-cast gel systems along with all Novex loading and running buffers. All restriction enzymes were obtained from either NBL or Promega. Alkaline phosphatase, proteinase K, 1KB and 1KB+ DNA ladders were from GIBCO-BRL. Filters were obtained from Whatman. Ammonium persulphate and Bromophenol Blue were obtained from ICN. Kanamycin was obtained from Melford. IPTG was supplied by NBL. Invitrogen provided TRIZOL reagent. Pharmacia supplied RNAguard, T7, T3 and SP6 polymerases, as well as Superdex 200 size exclusion column. Promega provided all commercial plasmids as well as T4 ligase and ligase buffers. Stratagene provided *E.coli* strains, XL-1, XL-2 and JM109. Greyhound Chromatography provided Aldicarb. Ultra high purity (UHP) grade water (>13 Mohms/cm^3) was produced using an ELGA-UV

water purifier. RNAi feeding vectors for subcloning, along with bacterial strain HT115 were a kind gift from J. Ahringer (MRC, Cambridge).

Black widow spider venom was obtained from Russia and Latoxan (France). Purified alpha-latrotoxin was a kind gift from Dr Y A Ushkaryov (Imperial College, London). All other compounds used were from SIGMA unless stated in the text.

2.2 General Molecular Biology Techniques

2.2.1 *E.coli* growth media

Luria-Bertani (LB) broth (10g/L Bacto-tryptone, 10g/L NaCl and 5g/L Bacto-yeast) was made using ultra high purity (UHP) water and autoclaved for one hour. LB-broth was allowed to cool to room temperature before use. LB-agar plates were made by adding 15g Bacto-agar per litre of LB-broth prior to autoclaving. LB-agar was allowed to cool to 55^{o}C before the addition of appropriate antibiotics and poured into 9cm Petri dishes. Antibiotics used in both LB-broth and LB-agars were used at the following concentrations.

Tetracycline at 20μg/ml, Ampicillin at 50μg/ml, Chloramphenicol at 25μg/ml and Kanamycin at 10μg/ml.

2.2.2 Preparation of calcium competent *E.coli*

E.coli strains (XL-1, XL-2 or JM109) were stored as –80^{o}C glycerol (10%) stocks. Individual tubes of *E.coli* were thawed and used to streak LB-agar plates containing the appropriate antibiotics (tetracycline for XL-1 and Xl-2, JM109 have no antibiotic resistance) and incubated overnight at 37^{o}C. A single *E.coli* colony was picked from an LB-plate with the appropriate antibiotic and used to inoculate a 5ml LB-broth liquid culture. The culture was grown up overnight at 37^{o}C in a shaking incubator until a thick

bacterial growth had formed. Using 2.5ml of the overnight culture, 500ml of LB broth was inoculated in a sterile conical flask and grown at 37°C in the shaking incubator until an OD_{600} of 0.6 was reached. Cultures were chilled on ice for 20 minutes, then centrifuged at 4000g at 4°C for 15 minutes. The supernatant was then discarded and the pellet resuspended in 50ml of sterile ice cold 0.1M $CaCl_2$. Cells were then centrifuged and resuspended as described. Cells were then centrifuged once more and the supernatant discarded, the final pellet was the suspended in 2ml sterile 0.1M $CaCl_2$, 10% glycerol. Cells were placed in tubes as 200µl aliquots and stored at –80°C.

2.2.3 Preparation of electro-competent *E.coli*

Cultures were treated as in section 2.1.2; apart from after the first centrifugation where the resulting pellet was resuspended in 50ml of sterile ice cold UHP water. After further centrifugation at 4000g at 4°C for 15 minutes the pellet was washed in sterile ice cold UHP water a total of five times. After the washes, cells were resuspended in 2ml sterile UHP water, 20% glycerol and stored at –80°C as 200µl aliquots.

2.2.4 Plasmid transformation into $CaCl_2$ competent *E.coli*

100µl aliquots of $CaCl_2$ competent cells were gently thawed on ice. 25-50ng of DNA was added to the cells and allowed to incubate on ice for 30 minutes. The cell suspension was then heat shocked by immersion in a 42°C water bath for 90 seconds followed by immediate transfer back on to ice for two minutes. 1ml of LB-broth was added to the cells and the resulting mixture incubated for one hour at 37°C. After incubation, 100µl of the transformed cells were plated out onto 9cm LB-agar dishes containing the appropriate antibiotic for selection and incubated at 37°C overnight.

2.2.5 Plasmid transformation into electro-competent *E.coli*

An aliquot of XL-1 Blue electro-competent cells was thawed on ice for 5 minutes along with the electroporation cuvettes and the cuvette holder. To the cells 10-50ng of DNA or 5µl of a ligation reaction was added and further incubated on ice for 10 minutes. The DNA/cell mixture was placed in a BioRad Genepulser cuvette (1mm path length) and electroporated with a voltage of 1.8kV using the electroporater. The cells were then resuspended immediately in 1ml of LB broth and then incubated at 37°C for 60 minutes. Transformed cells were then selected by growing the bacteria on LB agar plates, which contain the appropriate antibiotics.

2.2.6 Extraction and purification of nucleic acids

2.2.6.1 Phenol Chloroform extraction of nucleic acids

Tris buffered phenol (pH 8.0) is prepared by melting 500g of crystalline phenol at 68°C. To this liquid 0.1% (w/v) of hydroxyquinoline was added. Added to this was an equal volume of 0.5M Tris-HCL (pH 8.0), solution was then stirred for 15 minutes. The two phases were then allowed to separate and the aqueous phase discarded. To the remaining phenolic phase an equal volume of 0.1M Tris-HCL (pH 8.0) was added and again stirred for 15 minutes. The phases were allowed to again separate and the pH of the phenolic phases tested. The final extraction step was repeated until the pH of the phenol is in excess of 7.8. After the phenol has equilibrated, 0.1 volumes of 0.1M Tris-HCL were added. Phenol was then stored in this state at 4°C in the dark for one month.
This solution was then used to remove protein contamination from nucleic acid samples by phenol chloroform extraction. To the nucleic acid sample one volume of phenol chloroform (1:1 v/v) was added and the mixture vortexed. The two phases were separated by centrifugation for 5 minutes at 15000g. The remaining aqueous phase was removed and kept.

2.2.6.2 Ethanol precipitation of nucleic acids

Two volumes of absolute ethanol and 0.25 volumes of 3M-sodium acetate pH 5.2 were added to the DNA solution of interest. The solution was mixed and then placed on ice for 30 minutes to precipitate the nucleic acids. The solution was then centrifuged at room temperature at 14000g for 30 minutes. The pellet was then washed in 500µl of 70% ethanol and centrifuged at 14000g for 30 minutes. The pellet was then air dried to remove any remaining ethanol, then resuspended in an appropriate buffer.

2.2.6.3 Purification of plasmid DNA by alkaline lysis method

Clones of interest were used to inoculate 5ml of LB broth containing antibiotics appropriate for the plasmid and incubated overnight at 37ºC with shaking. After incubation a 1.5ml sample of the culture was aliquoted into a 1.5ml-eppendorf tube and the cells recovered by centrifugation at 13000g for 1 minute. The pellet obtained was then resuspended in 100µl of solution I (50mM glucose, 25mM Tris-HCL pH8.0, 10mM EDTA, RNAse A 100µg/ml) by vortex mixing. To this solution 200µl of solution II (200mM NaOH, 1%SDS, made up fresh) was added, the tube was then mixed 5-6 times by gentle inversion. This solution was allowed to stand at room temperature for 5 minutes before 150µl of solution III (5M Potassium acetate, 11.5% glacial acetic acid) was added. Immediately the tube was mixed by gentle inversion before placing the tube on ice for 10 minutes. The tube was then centrifuged at 15000g for 10 minutes to remove the resulting precipitate; the supernatant is kept and transferred to a clean tube. Sample was phenol chloroform extracted (section 2.2.6.1) and then ethanol precipitated (section 2.2.6.2). The resultant pellet was washed in 70% ethanol and then allowed to dry in air. The plasmid DNA isolated was then resupended in 30µl of UHP water and stored prior to further manipulation.

2.2.6.4 Purification of plasmid DNA on Qiagen Mini prep columns

For generating sequence grade DNA a Qiagen column is used, mini prep protocols were used for small-scale preparations; maxi prep columns were used for large-scale preparations.

For a small-scale preparation, 3ml of an overnight culture was pelleted by centrifugation for 1 minute at 15000g, the pellet was then resuspended in 0.3ml of buffer P1 (50mM Tris-HCL pH 8.0, 10mM EDTA, 100μg/ml RNAse A). To this 0.3ml of buffer P2 (200mM NaOH, 1% SDS) was added, the tube mixed gently and then incubated at room temperature for 5 minutes. After incubation 0.3ml of buffer P3 (3M-potassium acetate pH 5.5) was added and the tube incubated for 10 minutes on ice, before centrifugation at 15000g for 15 minutes. While centrifugation was taking place a Qiagen-tip 20 was equilibrated with 1ml of buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100). The resulting supernatant from the centrifugation step was immediately placed onto the Qiagen column and allowed to pass through. The column was then washed 4 times using 1 ml of buffer QC (1.0M NaCl, 50mM MOPS pH 7.0, 15% ethanol). The plasmid DNA that was retained on the column was eluted from the column using 0.8ml of buffer QF (1.25M NaCl, 50mM Tris-HCL pH 8.5, 15% ethanol) and the run off collected in a clean eppendorf. The collected fraction was precipitated in 0.7 volumes of isopropanol and incubated at room temperature for 20 minutes. The solution was then centrifuged for 30 minutes at 15000g; the pellet was then washed in 100ml of ice cold 70% ethanol and centrifuged again. The pellet was then air dried and resuspended in 30μl of UHP water.

2.2.6.5 Purification of plasmid DNA on Qiagen Maxi prep columns

The Qiagen tip 500 column is used for purifying large-scale sequence grade DNA. 500ml of bacterial culture containing the clone of interest were used, and a similar protocol to that used in section 2.2.6.4 performed as described in the manufacturer's handbook. The pellet isolated was resuspended in 250µl of UHP water.

2.2.6.6 Preparation of DEPC water / solutions

Diethylpyrocarbonate (DEPC) is a potent inhibitor of RNAse activity. DEPC treated water and solutions were used throughout all RNA protocols. A stock solution of DEPC was prepared as a 1:10 solution in 100% ethanol, this solution was then added to other solutions to give a final concentration of 0.1% (v/v), and left at room temperature for a minimum of 12 hours. DEPC was then deactivated by autoclaving the treated solutions for 20 minutes at 120^{o}C.

2.2.6.7 RNA extraction from *Caenorhabditis elegans*

Total RNA was prepared from frozen pellets of *C.elegans* using TRIZOL extraction. TRIZOL reagent is a solution of guanidine isothiocyanate and phenol, which simplifies the original method of Chomczynski & Sacchi.

1ml of packed frozen worms were placed in a 15ml polypropylene tube and 4ml of TRIZOL reagent plus 50µl β-mercaptoethanol added, the tube was then vortexed for 5 minutes to lyse the worms, and then incubated at room temperature for 5 minutes. The solution was split into four 2ml-microfuge tubes and incubated at room temperature for a further 5 minutes. All 4 tubes were then centrifuged for 10 minutes at 14000g at 4^{o}C to remove insoluble material; the supernatants were then transferred to clean tubes, 200µl chloroform added to each one and incubated for 5 minutes at room temperature. All 4 tubes were again centrifuged for 10 minutes at 14000g at 4^{o}C to separate the two phases.

The aqueous phase was transferred to a clean tube where 500µl isopropanol was added; the tubes were mixed by inversion and incubated at room temperature for 10 minutes to precipitate the RNA. The tubes were then centrifuged for a third time, the supernatant was removed and the pellet washed in 100µl of 75% ethanol, this wash was performed 3 times in total before the tubes were centrifuged for 5 minutes at 14000g at 4°C. Supernatants were removed and the pellet air-dried for 10 minutes. Depending on the yield the RNA pellets were resuspended in 50-300µl of DEPC water. Samples were then stored at –20°C for short term and –80°C over the longer term.

2.2.6.8 DNA extraction from *Caenorhabditis elegans*

Worms were washed from a standard culture plate in M9 buffer (6g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.25g $MgSO_4.7H_2O$ per litre) and spun down. The supernatant was removed and the worm pellet resuspended in 100µl M9 buffer in a 1.5ml microcentrifuge tube.

To the worm pellet 400µl lysis buffer (0.2M NaCl, 0.1M Tris-HCl pH8.5, 50mM EDTA, 0.5% SDS) and 10ml Proteinase K (10mg/ml). was added to the tube and the solutions mixed and incubated for 30 minutes at 65°C. After incubation a further 10µl of Proteinase K was added and the tube was again incubated for 30 minutes at 65°C. 5µl of RNaseA (10mg/ml) was added and the tube incubated at 37°C for a further 30 minutes, before adding 500µl of phenol and mixing for 30 minutes. The tubes were centrifuged for 5 minutes at 14000g and the aqueous phase removed and placed in a clean microcentrifuge tube, to this a further 500µl of phenol was added and again mixed for 30 minutes. Tubes were centrifuged for 5 minutes at 14000g and the aqueous phase again moved to a clean microcentrifuge tube. 500µl chloroform was then added and the tubes mixed for 30 minutes for a third time. After centrifugation at 14000g for 5 minutes the

aqueous phase was removed and mixed with 500µl of 100% ethanol and incubated overnight at room temperature. Precipitated DNA was pelleted by centrifugation for 10 minutes at 14000g, and then washed in 500µl of 70% ethanol. DNA was then resuspended in UHP water (100µl for a 6cm-worm plate, 200µl for a 9cm plate).

2.2.7 Polymerase chain reaction (PCR)

Short segments of DNA were amplified using PCR primers. Primers were designed so that a high sequence identity to the target DNA was achieved. Base-pair mismatches were incorporated into the primers to generate particular restriction sites, which were subsequently used for sub-cloning purposes. PCR was performed using 5µl of 10X KlenTaq PCR reaction buffer (400mM Tricine-KOH (pH9.2 at 25°C), 150mM KOAc, 35mM Mg $(OAc)_2$, 750µg/ml bovine serum albumin), 1µl 5'-Primer (~70pmol), 1µl 3'-Primer (~70pmol), 4µl dNTP mix (1.25mM), 1.5µl target DNA (~1ng), 0.5µl KlenTaq polymerase and diluted to 50µl with UHP water. The PCR reaction was overlaid with 70µl of mineral oil to prevent the reaction mix from evaporating. PCR was performed on a Perkin Elmer DNA Thermal Cycler 480 for 30 cycles at 30 seconds denaturing at 94°C, 30 seconds annealing at 60°C and 1 minute polymerisation at 72°C. The final cycle consisted of an extension cycle of 5 minutes at 60°C followed by 10 minutes at 72°C. PCR products were then analysed by agarose (TBE) gel electrophoresis along with a size ladder, and positive and negative controls.

Primer names and DNA sequences (5'-3'): -

*elegans*LPH5'- 5' GGA TGC CCT GAA TTC GGA CGT TAT AAA ACC 3'

*elegans*LPH3'- 5' TTC AGA TCG TGT CCG AAT TCT CAC ATC AAA ACA GC 3'

Conditions: -

94°C for 30 seconds
60°C for 30 seconds
72°C for 1 minute
} For 30 cycles.

60°C for 5 minutes

72°C for 10 minutes

2.2.8 Gel extraction of PCR products by Qiagen QIAquick spin column

PCR amplified products were extracted from an agarose gel by using Qiagen's QIAquick spin column. This system purifies single- or double-stranded DNA fragments from PCR reactions. Fragments ranging from 100bp to 10kb can be purified using the spin columns in a microfuge.

To one volume of PCR reaction mix, five volumes of buffer PB were added; a QIAquick spin column was then placed in its 2ml collection tube. The PCR sample was applied to the column, and spun for 60 seconds at 15000g, this bound the DNA to the column. The flow through was discarded and 750µl buffer PE added to the column, the column was the spun again for 60 seconds at 15000g. The flow through was discarded again, before the column was spun for a third time at 14000g for 1 minute. The column was moved to a clean eppendorf tube and the DNA eluted by the application of 50µl buffer EB to the center of the spin column. The column was spun for a final time at 15000g for 1 minute to collect the DNA in the eppendorf tube. The DNA was then stored at –20°C prior to further manipulations.

2.2.9 Restriction digest of plasmid DNA

A restriction digest was performed in a reaction volume of 30µl in an eppendorf tube and incubated for 2 hours at 37°C. The digest contained 5-10µl of the plasmid DNA of interest, 3µl of 10x restriction enzyme buffer and the rest of the reaction volume made up with UHP water. 5U/µg of the restriction enzyme used digests the plasmid DNA completely over 2 hours. Reactions were terminated by ethanol precipitation, and checked for digestion by agarose gel electrophoresis.

2.2.10 Agarose gel electrophoresis

DNA was separated by non-denaturing agarose gel electrophoresis. DNA electrophoresis was performed using the Pharmacia GNA-100 gel electrophoresis kit. Agarose was dissolved in an appropriate amount of 1X TBE (45mM Tris-HCL, 44mM Boric acid, 2mM EDTA pH 8.0) to give a gel in the range of 0.7-2% (w/v). 1X TAE (40mM Tris-HCL, 1mM EDTA, 5.71% glacial acetic acid) gel is used for the gel excision of DNA fragments (section 2.2.12). Agarose was dissolved in 1X TBE/TAE by microwave heating. The dissolved agarose solution was allowed to cool to 55°C before ethidium bromide (10mg/ml stock) was added to give a final concentration of 0.5µl/ml. The agarose gel solution was then poured into the gel casting mould, which contains the well forming comb and allowed to set in the mould. After the gel has become solid the gel was placed in the electrophoresis tank and immersed in the correct running buffer (1X TBE, 1X TAE). DNA samples to be run were prepared in a 10% solution of 10X loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and then loaded into the wells formed by the comb. Samples were run at 80V for 1 hour, bands were visualised by illumination under UV light.

2.2.11 Agarose gel electrophoresis (denaturing)

Denaturing agarose gel electrophoresis was carried out as with non-denaturing electrophoresis except that when running RNA the gel mixture is made with 0.1% SDS (w/v) and the 1X TBE/TAE is made up with DEPC water rather than UHP water. Gels were also run at 65V instead of 80V per hour.

2.2.12 DNA purification by GENECLEAN

Restriction digested plasmid DNA was purified using the GENECLEAN II kit. This method is only used on 1X TAE gels; the kit contains a silica matrix (glassmilk), which DNA binds to without contaminants. DNA was resolved on a 1X TAE agarose gel with the band of interest visualised under UV light. The band was then excised using a clean scalpel blade, placed in an eppendorf tube and weighed. The fragment was then treated with 3 volumes of NaI solution (6.0M). The tube was then incubated at 55^{o}C for 5 minutes or until the fragment had dissolved. To the solution 5µl of glassmilk was added and the tube mixed gently be inversion, the solution was incubated at room temperature for 10 minutes. This allows the DNA to bind to the glassmilk matrix. The DNA-glassmilk complex was pelleted by centrifugation for 15 seconds at 15000g. The supernatant was removed and the glassmilk resuspended in 500µl of ice-cold New Wash Buffer (50% 20mM Tris-HCL pH 7.2, 0.2M NaCl, 2mM EDTA and 50% ethanol) and again centrifuged. The centrifugation step was repeated a further 2 times before the pellet was resuspended in 15µl of UHP water. This solution was then incubated at 55^{o}C for 2 minutes, this elutes the DNA from the glassmilk complex. The tube was then centrifuged for 15 seconds at 15000g. The purified DNA is then transferred to a clean eppendorf.

2.2.13 Alkaline phosphate treatment of DNA

Shrimp Alkaline Phosphatase (SAP) is used to remove the 5'-terminal phosphate from nucleic acids; this dephosphorylation with SAP prevents recircularisation of linearised vectors. 2µl of 10X SAP buffer (200mM Tris-HCL pH8.0, 100mM $MgCl_2$) was added to the 15µl of the GENECLEAN sample along with 1U of SAP and the reaction volume made up to 20µl with UHP water. The reaction was then incubated for 30 minutes at 37ºC before heat inactivation at 65ºC for 15 minutes. The resulting solution can then be used for ligation reactions.

2.2.14 Ligation of DNA

Two double stranded DNA fragments can be ligated together by the formation of a phosphodiester bond between the 5'-phosphoryl group and the 3'-hydroxyl group, this occurs in the presence of the enzyme T4 ligase. The ratio of vector DNA to insert DNA is important when performing a ligation reaction. The simplest ligation reaction is when the vector and insert DNA are in a 1:1 ratio. The total reaction volume used was 10µl, 4µl of vector DNA, 4µl insert DNA, 1µl T4 DNA ligase and 1µl 10X T4 ligase buffer (250mM Tris-HCL pH 7.6, 50mM $MgCl_2$, 5mM ATP, 5mM DTT, 25% (w/v) PEG-8000). The tube was mixed and centrifuged at 14000g for 10 seconds. The solution was then incubated overnight at room temperature. This solution can be used for transformation into either calcium or electro competent cells.

2.2.15 Production of double-stranded RNA

2.2.15.1 Template generation

Template DNA used for double-stranded RNA synthesis was prepared as a stock solution of the linearising digest. This digest stock was prepared by digesting 10µl of

template DNA with 3µl of 10X restriction enzyme buffer, 4U of the restriction enzyme being used and the total reaction mixture made up to 30µl with DEPC water. This reaction was incubated for 2 hours at 37°C.

The linearised template DNA was then cleaned up by the addition of 100µg/ml of Proteinase K and 0.1% SDS (w/v), this solution was incubated for 30 minutes at 37°C. The solution was then treated with phenol chloroform extraction and centrifugation for 1 minute at 14000g. The aqueous phase was retained and 2 volumes of absolute ethanol added to it along with 0.25 volumes of 3M-sodium acetate and left on ice for 20 minutes. The DNA solution was then centrifuged at 14000g for 30 minutes at 4°C to form a pellet before resuspension in 15µl of DEPC water.

2.2.15.2 RNA transcription *in vitro*

For RNA synthesis a large-scale transcription reaction was performed. At room temperature 5µl of 10X transcription buffer (200mM Tris-HCL pH 7.9, 30mM $MgCl_2$, 10mM spermidine, 50mM NaCl), 2µl 100mM DTT, 1µl RNAguard (Pharmacia), 7.5µl NTP mix (5mM ATP, GTP, CTP, UTP), 10µl DNA template, 2µl SP6, T7 or T3 RNA polymerase. The solution is made up to 50µl with DEPC water. The reaction mix was then incubated at 37°C for T7/T3 polymerase or 42°C for SP6 for 2 hours. After incubation 5µl DNAse I is added and the reaction incubated at 37°C for 20 minutes. Once this is completed the reaction can be purified and the RNA strands annealed.

2.2.15.3 Annealing of sense and antisense cRNA strands

The cRNA strands generated by the transcription reactions are purified and then annealed to produce double stranded RNA.

After incubation with DNAse I the RNAs were phenol chloroform treated and then centrifuged for 10 minutes at 14000g. The aqueous layer was removed and to it 2 volumes of absolute ethanol were added, the solution was then centrifuged for 30 minutes at 14000g at 4°C. The resulting pellet was washed in 1 volume of 70% ethanol and centrifuged at 14000g for 10 minutes at 4°C. Pellets were air dried and resuspended in 30µl DEPC water. To anneal the RNAs the transcription products were mixed in a 1:1 ratio and then heated to 88°C for 10 minutes.

2.3 Phage Methodologies

2.3.1 Bacterial – Phage amplification

2.3.1.1 Preparation of plating bacteria

5ml LB broth supplemented with 0.2% maltose and 10mM $MgSO_4$ in a sterile flask was inoculated with a single colony of host strain C600*hfl* (Clontech) and grown overnight with shaking at 30°C. The cells were then spun down in a sterile tube for 10 minutes at 2000g in a Beckman Centrifuge. The media was then decanted off and the cell pellet gently resuspended in 10ml of 10mM $MgSO_4$ without vortexing. To this mixture 7ml top agar (48°C) was added, quickly swirled and poured on to a 150mm LB plate. The top agar was then allowed to set and the plate incubated overnight without inverting and no more than two high at 37°C.

2.3.1.2 Plating bacteriophage

150mm LB plates containing C600*hfl* host cells in top agar were divided into 12 equal sections by drawing on the bottom of the plate. To each section 5µl of an individual bacteriophage sample was gently spotted and left to dry on the plate with the lid off. After the spots were dry, plates were incubated overnight without inversion at 37°C.

2.3.1.3 Picking bacteriophage plaques

Plaques of interest were cored from the plate (removing the top agar only) using an inverted 1000µl Gilson tip, and transferred to a microcentrifuge tube containing 500µl SM buffer (5.8g NaCl, 2g $MgSO_4.7H_2O$, 50ml Tris-HCl (pH7.5), 5ml 2% (w/v) gelatin, in 1 litre UHP water) and 20µl of chloroform. Tube was then vortexed to release the phage particles into the SM buffer. Tube was then incubated at 4°C overnight. This phage stock is stable for up to 6 months at 4°C.

2.3.2 *In vivo* excision

2.3.2.1 Preparation of host cells

Separate overnight cultures of XL-1 Blue MRF' cells, supplemented with 0.2% (w/v) maltose and 10mM $MgSO_4$ and XLOLR cells in LB broth were grown at 30°C overnight. XL-1 and XLOLR cells were gently spun down at 1000g for 15 minutes and resuspended in 10mM $MgSO_4$ at an OD_{600} of 1.0.

2.3.2.2 Single clone excision

200µl XL-1 Blue MRF' cells at OD_{600} of 1.0, 250µl of phage stock (containing $>1x10^5$ phage particles) and 1µl ExAssist (Stratagene) helper phage ($>1x10^6$ pfu/ml) were added to a 15ml Falcon tube and incubated for 15 minutes at 37°C. 3ml of LB broth was added and the tube incubated at 37°C with shaking for 3 hours. After incubation, the Falcon tube was heated at 70°C for 20 minutes, the tube was then spun at 1000*g* for 15 minutes. Supernatant was gently decanted into a clean sterile 15ml Falcon tube, this stock contains the excised phagemid packaged as filamentous phage particles and can be stored at 4°C for up to 2 months. To plate the excised phagemid, 100µl of the phage supernatant was added to 200µl XLOLR cells in 10mM $MgSO_4$ (OD_{600}=1.0) in a 1.5ml microfuge tube

and the tubes incubated at 37ºC for 15 minutes. 200µl of this cell mixture was plated onto LB-Ampicillin plates; plates were then incubated overnight at 37ºC.

2.3.3 Extraction of bacteriophage DNA

LB-Ampicillin plates were checked for individual colony growth. Colonies were picked and grown to confluence in LB broth supplemented with ampicillin at 37ºC overnight. Bacteriophage DNA was then extracted using standard DNA protocols (see section 2.2.6.4).

2.4 General *Caenorhabditis elegans* Methodologies

2.4.1 Maintenance of *C.elegans* stocks

Worms were maintained on NGM agar (3g NaCl, 17g Agar, 2.5g Peptone, in 1 litre high purity water. Autoclave, then add 1ml 1M $CaCl_2$, 1ml 1M $MgSO_4$, 25ml Potassium phosphate pH6, and 5mg/ml cholesterol) usually in 9cm petri dishes seeded with P90C a disabled *E.coli* mutant lacking the lac operon. This deletion prevents survival of the culture outside the lab. After the hot NGM agar had cooled, the bacterial suspension was plated out using sterile techniques. Plates were then incubated overnight at 37ºC to allow the bacterial lawn to grow. Cultures require subculturing every 7-10 days.

2.4.2 Subculture of stocks

Using sterile technique, the agar layer from 4-day (or older) worm cultures was cut into 5-6 sections using a flamed scalpel blade. Agar pieces were transferred onto fresh bacterial lawns on NGM agar plates and sealed with Parafilm (to prevent drying). Worms were routinely cultured at 15ºC for 3-4 days before experimental use.

2.4.3 Harvesting stocks from plates

Worms were washed from the culture plates with approx 1-2ml K-medium (2.39g KCl, 3.099g NaCl, in 1 litre of high purity water. Autoclave and store at 15°C) or M9 buffer (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml 1M $MgSO_4$ in 1 litre of high purity water. Autoclave and store at 15°C) into a 50ml centrifuge tube on ice. The tube was then filled to around the 50ml mark with worms suspended in buffer and left to settle for 30 minutes at 4°C. The supernatant was removed with a pipette (liquid removed should be treated with bleach for decontamination) and further M9 buffer or K-medium added to the worm pellet. This is repeated several times to remove most of the bacteria in the supernatant.

2.4.4 Synchronised populations

Worms were washed from plates as in 2.3.3, and the suspension passed through a 5-micron filter (Wilson sieve) so that only L1 and L2 larvae were present in the filtrate. Worms were then centrifuged at 3000rpm for 30 minutes and plated out onto fresh agar plates for 2-3 days at 24°C or 4-5 days at 15°C. The development of such synchronised cultures is checked daily, and worms harvested as young adults once they start producing eggs and young.

2.4.5 Freezing worms as stocks

Wild type (N2) and mutant worm strains can be stored in the frozen state indefinitely (Wood, 1988). Starting with cultures of just starving L1 and L2 larvae, worms were washed off into 1ml of M9 buffer and an equal volume of freezing solution (5.85g NaCl, 6.8g KH_2PO_4, 300g Glycerol, 5.6ml 1M NaOH, in 1 litre high purity water. Autoclave then add 3ml 0.1M $MgSO_4$) added. After mixing, 0.5ml aliquots were transferred into freezing vials and placed in Styrofoam boxes at -70°C. Thawed worms can be seeded

onto fresh bacterial lawns on 9cm NGM agar plates and healthy worms picked off the next day and transferred onto fresh plates.

2.4.6 Egg isolation and cleaning

Alkaline hypochlorite dissolves all worm tissues except eggs, which are largely resistant, and will also destroy almost all bacterial and fungal contaminants. 5ml of bleach (15% Sodium hypochlorite) and 5ml of 2M NaOH were placed on a small P90C seeded NGM plate between the bacterial lawn and the edge of the plate. From a second plate 10 gravid hermaphrodites (adults containing many eggs) were placed in the bleach solution, plates were then incubated overnight at room temperature. Plates containing the bleach solution were inspected to check that the adults had dissolved leaving only eggs. The hatched eggs then move over the plate to the bleach free area and are allowed to feed and develop. Plates were then incubated overnight at 20^{o}C, before being transferred to new plates.

2.4.7 Microinjection of double-stranded RNA

RNA can be injected into any part of the worm body cavity, though it is better to inject into the cytoplasmic syncytium of the gonad. Injection needles were pulled from glass capillaries using a Sutter micropipette puller. The needles were opened just before use by physically breaking the needle. Over a flame, a yellow pipette tip was drawn out. A stretch of the drawn out part of the tip was placed on a 24x50mm coverslip, and a drop of microinjection oil placed on top. Using the 5x objective, the pipette was brought into focus and the injection needle lowered toward the pipette. The needle was rubbed up and down the pipette, to check if the needle was broken pressure was applied to the needle and continually rubbed up and down the pipette until there was a steady flow of fluid from the needle under pressure. The worms were placed on injection pads made from a

thin layer of 2%-dried agarose, which immobilised the worms by depriving them of moisture. N2 worms were injected at 40X magnification using a Nikon 32 microscope and a Leitz micromanipulator, the flow of injection fluid was controlled by a Picospritzer II (General Valve Corp). Worms were gently recovered by adding a single drop of recovery buffer (M9 plus 4% glucose) and then transferring the worms to individual NGM plates.

RNA injected worms were left for 5 hours to allow pre-fertilised eggs to be laid, before being put onto new individual NGM plates.

2.4.8 Microinjection of DNA

DNA injections were performed as with double-stranded RNA injections (section 2.4.7). DNA injected worms, which were transformed, were identified by the right rolling phenotype caused by the mutant collagen gene, *rol-6,* encoded by the pRF4 plasmid. Each selected transgenic animal was placed on a separate NGM plate for propagation. Lines were established after the selectable phenotype had been successfully passed on for three generations.

2.4.9 Microinjection of Black Widow Spider Venom

Black widow spider venom was stored as a concentrated solution at –20^{o}C in venom buffer (50mM Tris-HCL pH 8.0, 50mM NaCl). Prior to injection an aliquot of venom was thawed on ice and, if necessary, diluted in venom buffer. Adult *C.elegans* were injected as in section 2.4.7, except that venom was injected into the worm body cavity not the gonads.

After injection worms were transferred to individual P90C coated NGM plates and incubated for 30 minutes at 20^{o}C. After incubation the worms were kept at 20^{o}C but were scored every 30 minutes for reaction to the venom.

2.4.10 RNA interference by double-stranded RNA (dsRNA) feeding

2.4.10.1 Bacterial induction of feeding plasmids

Single colonies of HT115 bacteria containing cloned L4440 plasmids were picked and grown in culture in LB-broth with the addition of 50μg/ml of the antibiotic ampicillin, unless indicated. Expression of dsRNA was produced as follows.

Bacteria were grown for 8 hours at 37°C, then seeded directly onto NGM plates containing 1 mM IPTG and 50μg/ml ampicillin. Seeded plates were allowed to dry at room temperature before being incubated overnight at 25°C.

Non-induced controls were produced by seeding bacteria onto plates that lack 1 mM IPTG.

2.4.10.2 RNA interference by feeding

L4 stage hermaphrodite worms were placed onto NGM plates containing seeded bacteria expressing dsRNA for each gene being examined and were incubated for 24 hours at 25°C. Then, five worms were independently replica plated onto plates seeded with the same bacteria and allowed to lay eggs for 24 hours at 25°C before being removed. Progeny were scored for phenotype after a further 24 hours at 25°C.

2.5 Behavioral assays of *Caenorhabditis elegans*

2.5.1 Volatile repellent assay

NGM plates were prepared as in section 2.4.1 but the plates, once set, were not spread with P90C bacteria.

This assay was carried out using a modified version of the method of Bargmann *et al*, (1993). 20ml – 40ml of Octanol was transferred to a microfuge tube. A paintbrush bristle was dipped into the Octanol 10-15 times to initialise the bristle with the solvent. Five

worms of the strain to be examined were transferred to each NGM plate using a worm pick and left for two minutes to adapt to the new plate. The bristle was dipped in octanol again and under a light microscope, the tip of the bristle was brought as close as possible to the head of one worm. If contact between the worm and the bristle occurred, then a new worm should be assayed on a different plate. The time was recorded from when the bristle was brought into close proximity to the *C. elegans* to initiation of backward locomotion by *C. elegans*.

One *C. elegans* per plate was tested at a time. This was to prevent other *C. elegans* on the same test plate becoming sensitised or desensitised by exposure to octanol during testing of the first worm.

2.5.2 Osmotic repellent assay

10 NGM plates were prepared as in section 2.4.1, but the plates, once set, were not spread with P90C bacteria.

Using a pipette, a ring of 2M glucose, approximately 2cm diameter was drawn out in the centre of each plate. Two worms of the strain being studied were placed in side the ring. The time was recorded from when the worms were placed inside the ring until they entered the ring or passed through the ring boundary. This procedure was repeated for each plate.

2.5.3 Analysis of defecation cycle

This assay was carried out using a modified version of the method of Thomas *et al*, (1990). Defecation was scored by direct observation of the muscle contractions that constitute the defecation motor program (Pboc, Aboc and Exp) in single animals on P90C coated NGM plates over a span of 10 consecutive cycles at 20^{o}C. The wild-type

defecation motor program consists of sequentially occurring Pboc, Aboc, and Exp (Thomas *et al*, 1990) contractions every 45-50 seconds.

2.5.4 Exposure of *C.elegans* to levamisole

This assay was carried out using a modified version of the method of Lewis *et al*, (1980). NGM was made as in section 2.4.1; 100μM stock solution of levamisole was dissolved in UHP water and diluted in the still molten NGM to give the required range of experimental concentrations. Plates were left to solidify and, when set, coated with P90C bacteria before incubation overnight at room temperature. The strain of worm to be exposed was placed on the levamisole plates as a population of 25 and left to equilibrate for 15 minutes. This is time zero. After 15 minutes exposure to levamisole the number of *C.elegans* paralysed was recorded. Paralysis was assessed by observed lack of locomotion, a rod like appearance, and a lack of movement observed after prodding the worm with a worm pick. This procedure was repeated at 15 minute intervals for a total of 3 hours.

2.5.5 Exposure of *C.elegans* to aldicarb

This assay was carried out using a modified version of the method of Lewis *et al*, (1980). NGM was made as in section 2.4.1; 1mM stock solution of aldicarb was dissolved in 100% ethanol and diluted in the still molten NGM to give the required range of experimental concentrations. Plates were left to solidify and, when set, coated with P90C bacteria before incubation overnight at room temperature. The strain of worm to be exposed was placed on the aldicarb plates as a population of 25 and left to equilibrate for 15 minutes. This is time zero. After 15 minutes exposure to aldicarb the number of *C.elegans* paralysed were recorded. Paralysis was assessed by observed lack of locomotion, a rod like appearance, and a lack of movement observed after prodding the worm with a worm pick. This procedure was repeated at 15-minute intervals for a total of 3 hours.

2.5.6 Exposure of *C.elegans* to imipramine

This assay was carried out using a modified version of the method of Reiner *et al*, (1995). NGM was made as in section 2.4.1; 5mg/ml stock solution of imipramine was dissolved in UHP water and diluted in the still molten NGM to give the required range of experimental concentrations. Plates were left to solidify and, but were not coated with P90C bacteria. The strain of worm to be exposed was placed on the imipramine plates as a population of 25 and left to equilibrate for 15 minutes. This is time zero. Pharyngeal pumping was counted using light microscopy for each concentration for 5 minutes in total.

2.6 Microscopy of *Caenorhabditis elegans*

2.6.1 Light microscopy

For examining wild type and mutant phenotypes a stereo microscope (Leitz) was used with 10x eyepiece optics and 5x and 20x objective optics. These allow analysis of adults, embryos and all other larval stages.

All specimens were mounted on agar pads (see section 2.4.7) in UHP water. Specimens were placed in the centre of the pad and, using a Pasteur pipette the majority of the water was withdrawn. The amount of water remaining is important as the specimen should not be covered with water. A coverslip was then slowly placed on the pad, with the remaining water being used to seal the slide and coverslip together.

The slide was then mounted under the microscope and the specimens examined.

2.7 Protein Methodologies

2.7.1 Preparation of black widow spider venom from venom sacs

Concentrated black widow spider venom (a kind gift from V.Krasnoperov, Russian Academy of Sciences, Russia) was prepared by grinding venom sacs in venom storage buffer (50 mM Tris HCl, 50 mM NaCl pH8) at 4^{o}C. The venom solution was then centrifuged at 14000g for 30 minutes at 4^{o}C to remove any particulate matter. Concentrated venom solution was then tested for protein concentration by the method of Bradford (see section 2.7.2) and then stored at 4^{o}C. A second stock of black widow spider venom was obtained from Latoxan, France and stored according to manufacturers instructions.

2.7.2 Bradford (Coomassie blue) protein assay

Protein concentration is determined using the method described by Bradford, 1976. Bradford reagent is the main component of this assay, it is comprised of 100mg Serva blue G dissolved in 100ml of 85% phosphoric acid and 50ml of 95% ethanol, and made up to 1 litre. 30µl sample of protein solution to be measured was added to 50µl of 1M NaOH. To this 950µl of Bradford reagent was added and the assay solution vortexed immediately. Absorbance was read at 590nm in a cuvette. The protein concentration was determined from a standard curve of bovine serum albumin (BSA) generated between the range of 0-40 µg/ml. All assay samples and standards were carried out in triplicate and the resultant means determined. The standard curve of BSA concentrations against the absorbance at 590nm produces a linear plot upon which linear regression was carried out. Linear regression was used on the sample readings so that unknown protein sample concentrations could be calculated. Only standard curves that gave rise to R^2>0.95 were used.

2.7.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Based on a modified version of that described by Laemmli (1970). SDS-PAGE was performed using BioRad Minigel electrophoresis kit. Glass plates used for casting gels were cleaned with 100% methanol to remove all traces of grease and dirt that my interfere with the casting of the gels. Separating gels consisted of a final concentration of 7.5% acrylamide/bis-acrylamide, 375mM Tris-HCl (pH8.8), 0.1% SDS, 0.001% TEMED and 0.005% Ammonium persulphate (APS), and were poured into the casting units to within 0.5-1.0cm from the bottom edge of the well comb, then overlaid with 100% ethanol. The gel was then left to polymerize for about an hour and the overlaid ethanol removed after polymerization had taken place. The stacking gel component of the gel was made immediately prior to electrophoresis. The stacking gel consists of 4% acrylamide/bis-acrylamide, 125mM Tris-HCl (pH6.8), 0.1% SDS, 0.001% TEMED and 0.005% APS and was poured over the separating gel 0.25cm from the top of the gel kit, the well comb was then inserted before the gel set. Polymerisation of the gel takes one hour. The completed gel running apparatus was then placed into the gel tank and the upper and lower reservoirs filled with an appropriate amount of electrophoresis buffer (25mM Tris-HCl (pH8.6), 192mM glycine and 0.1% SDS (w/v)). Prior to the loading of samples, wells were flushed with electrophoresis buffer to clear the wells. Samples were diluted 1:1 using 1X Laemmli sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue and 0.125M Tris-HCl (pH6.8)). Samples and standard ladder were then boiled for 3 minutes to denature the proteins. Samples and ladder were then loaded onto the gel and the gel was then run for 1 hour at 150 Volts or until the blue running dye had reached the bottom of the gel.

Gels were stained using Coomassie Blue method. Gels were stained for 20 minutes at room temperature with gentle shaking in Coomassie Blue stain (0.25g Coomassie Blue R250, 90ml Methanol: water (1:1), and 10ml Acetic acid, filtered through Whatman No1

filter prior to use). After 20 minutes, gel was destained in Coomassie Blue destain (30% Methanol, 10% Acetic acid (v/v)), gel was successively washed in destain solution until bands were clearly visible.

2.7.4 Lithium duodecyl sulphate-polyacrylamide gel electrophoresis (LDS-PAGE)

For sharper band resolution of small to mid-size molecular weight proteins at low pH, LDS-PAGE was used.

Commercially available Pre-cast 10% Bis-Tris gels (Novex) were used. Protein samples were loaded using NuPAGE® LDS sample preparation buffer (Novex) and run in NuPAGE® MOPS running buffer (Novex) at 170V as per manufacturers instructions. Gels were then stained either by Coomassie Blue stain (section 2.7.3) or, when greater sensitivity was required, silver stain (see section 2.7.5).

2.7.5 Silver staining

The basic mechanism occurring in the silver staining of macromolecules is the reduction of ionic to metallic silver. Protein bands are imaged in the gel due to differences in oxidation/reduction potentials between sites in gels occupied by protein and adjacent sites not occupied by protein. Silver stain has an increased sensitivity over other staining methods, typically sensitivity is 50 times greater than that obtained with Coomassie Blue staining.

Gels were stained using BioRads silver stain kit. Reagents were prepared as shown in the manufacturers instructions.

Gels were initially fixed in 40% Methanol, 10% Acetic acid (v/v) for 30 minutes before being washed in 10% Ethanol, 5% Acetic acid (v/v) solution twice for 15 minutes. Oxidizer reagent was then added to the gel for 3 minutes prior to the gel being washed in

UHP water for 2 minutes, UHP water washes were continued until all the yellow colour had been removed from the gel. Silver stain reagent was then added, and the gel left to soak in the stain for 15 minutes. The gel was then washed in UHP water for 2 minutes. Developer was then added and allowed to remain on the gel until the solution turned yellow, or a brown precipitate appeared. The developer was then poured off and fresh added, the gel remained in the developer for a further 5 minutes. At this point, stop solution (5% Acetic acid (v/v)) was added, the gel was removed after 5 minutes and the examined.

2.7.6 Chromatography of black widow spider venom

Black widow spider venom (Latoxan) was resuspended in 50mM Tris, 150mM NaCl, pH8.0 and subjected to size exclusion chromatography on Superdex 200 (HR10/30 Pharmacia). 1ml fractions were assayed by diluting 1:1000 in 50mM Tris, NaCl, pH8.0 and microinjected into adult *C.elegans* (see results section). Positive fractions were analysed with a 40-300mM NaCl gradient in 50mM Tris, pH8.0 on UnoQ column (BioRad), followed by 40-400mM NaCl gradient in 50mM sodium succinate, pH6.2, on UnoS column (BioRad) or 40-400mM NaCl gradient in 20mM HEPES, pH8.0, on UnoS column. Proteins were run on a 10% Bis-Tris denaturing PAGE gel in MOPS buffer, according to the manufacturers instructions, (Novex) see section 2.7.4, followed by silver staining (section 2.7.5).

2.7.7 Peptide analysis

Proteins of interest, identified after chromatography and analysis of kill ratios from microinjection into *C.elegans*, were analysed for peptide structure.

Proteins were diafiltered into 150mM Tris, pH8.16, subjected to tryptic digestion and then MS/MS analysis.

2.8 Sequence analysis

Putative homologues of genes were identified by searching the *C.elegans* genomic DNA databases.

http://www.sanger.ac.uk/Projects/C_elegans

http://www.dna.affrc.go.jp/htdocs/swsch/index.html

All clone sequence data was analysed using GCG.

http://www.accelrys.com/products/gcg_wisconsin_package/index.html

Protein homologies and alignments were identified using Pfam 6.6.

http://www.sanger.ac.uk/Software/Pfam

All WWW based software packages were used at their respective default values.

Chapter 3. Results.

3.1 Extraction of Black widow spider venom from venom sacs

To begin analysis of black widow spider venom (BWSV), venom was extracted from the venom sacs of the spider *Latrodectus mactans tredecimguttatus*.

Venom was extracted using the method described in section 2.7.1; venom concentration was calculated using the method of Bradford (1976). Concentrated venom protein was calculated at 1.2mg/ml.

Venom was analysed for the presence of high molecular weight proteins, which characterise the components of BWSV. Venom was run as a concentrated solution and a 1:10 dilution in venom buffer (50mM Tris, 50mM NaCl, pH8) on a denaturing SDS-PAGE gel as described in section 2.7.3. SDS-PAGE gel of BWSV is shown in Figure 3.1.

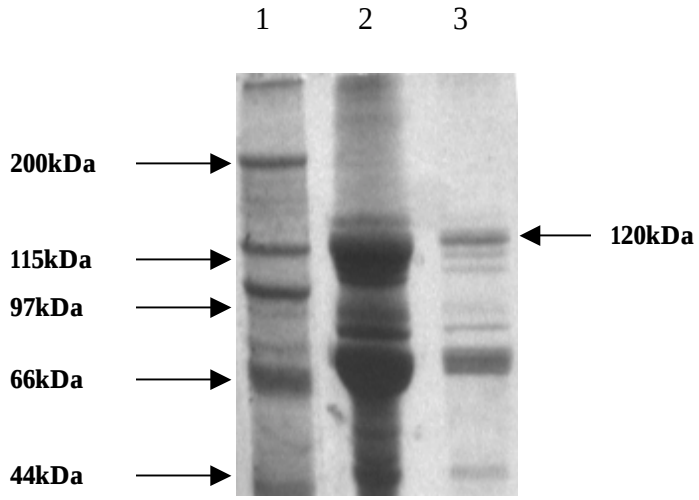


Figure 3.1 PAGE of black widow spider venom.
Black widow spider venom was run on a 7.5% acrylamide SDS-PAGE gel under denaturing conditions as described in Methods section 2.7.3. Lane **1** contains molecular weight standards. Lane **2** contains concentrated BWSV (24µg loaded). Lane **3** contains concentrated BWSV diluted 1:10 using 50mM Tris, 50mM NaCl, pH8 (2.4µg loaded). The gel was fixed and stained with Coomassie Blue (section 2.7.3). Sizes of molecular weight markers are shown by the labelled arrows on the left side of the gel, sizes of venom proteins are shown by the labelled arrows on the right side of the gel.

3.2 Effect of injected BWSV on wild-type (N2) *C.elegans*

3.2.1 Effects of BWSV on *C.elegans* over a range of concentrations

BWSV has no effects on wild-type (N2) *C.elegans* at any concentration when worms are simply steeped in venom (Bell & Mee, unpublished data). This may be due to the thickness of the cuticle surrounding the worm preventing entry of the protein molecules in venom. To bypass this problem a microinjection rig was developed to allow the injection of fluids, including venom, into *C.elegans.*

To examine the effects of BWSV on N2 *C.elegans*, a concentration range of venom was constructed and injected in adult N2 *C.elegans.* 10 worms per concentration were injected along with a negative control of venom buffer only (50mM Tris, 50mM NaCl, pH8). Worms were scored for any effects of the venom after incubation at 20°C for 2 hours.

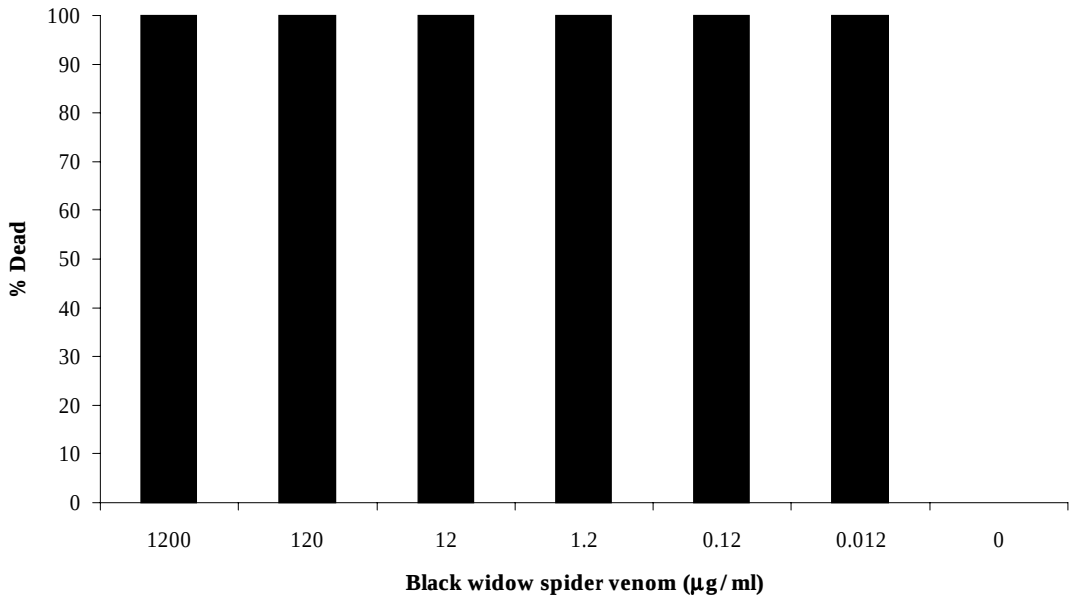


Figure 3.2 Black widow spider venom is toxic to wild-type *C.elegans.*
C.elegans (10 per point) were injected with black widow spider venom at the indicated concentration (µg/ml), or the control venom buffer (0) and allowed to recover for 2 hours at 20°C on standard NGM plates (see section 2.4.1). Animals were scored after incubation and the % of dead animals are shown. Dead worms were characterised by a paralysed, rod-like appearance and an inability to respond to heavy touch with a worm pick. Death was confirmed by re-examination at 24 hours.

BWSV was toxic over a wide range of concentrations, from 1.2mg/ml to 1.2ng/ml; at lower concentrations the toxic response from the venom was highly variable. This may be due to instability of proteins or to non-specific absorption to glass and plastic containers.

3.2.2 Effect of denatured BWSV on N2 *C.elegans*

To determine whether a protein component of the venom is responsible for the observed toxicity, venom was treated with either 0.1% SDS or heating for 10 minutes at 70°C to denature the protein components prior to injection into N2 *C.elegans*. A concentration of 1.2µg/ml was used to inject the worms: this concentration prevents instability at low concentration and is in 100-fold excess of the amount needed to kill *C.elegans* (Figure 3.2).

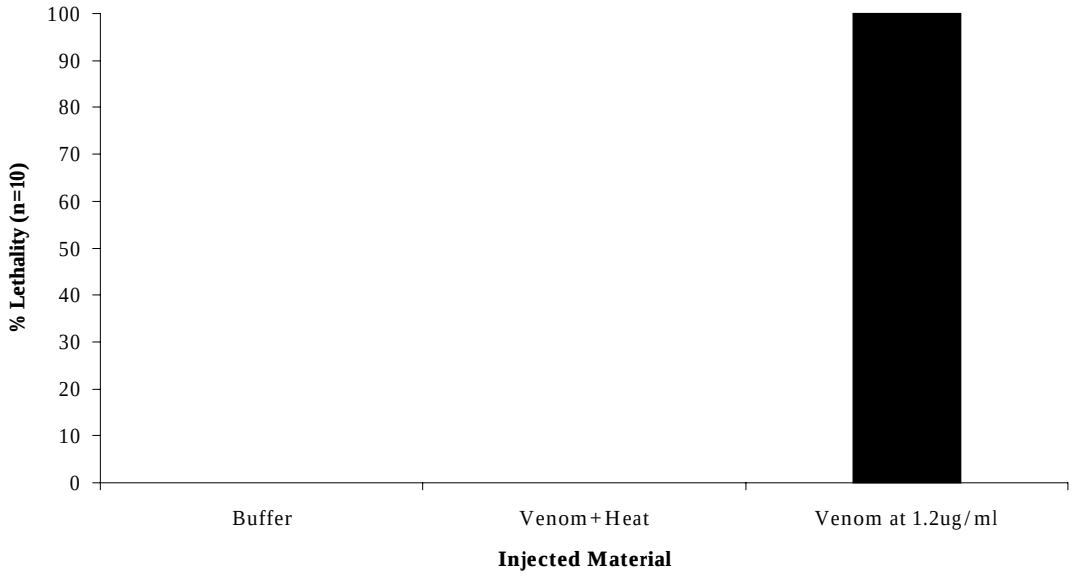


Figure 3.3 Effect of heat treatment on BWSV toxicity in *C.elegans*
BWSV at 1.2µg/ml was injected into worms (Venom), or after heating for 10 minutes at 70°C (Venom+Heat). Negative control of venom buffer only was also injected (Buffer). 10 *C.elegans* were injected per group and after incubation at 20°C for 2 hours; worms were again scored for lethality caused by venom injection.

Data shown in Figure 3.3 show that injection of BWSV at a concentration of 1.2µg/ml causes complete death of all injected animals after incubation at 20°C on standard NGM plates, this data supports the observations made in Section 3.2.1 & Figure 3.2. Injection of venom buffer (50mM Tris, 50mM NaCl, pH8) causes no death when injected into *C.elegans.* These data act as positive and negative controls for the injection of BWSV into *C.elegans* after heat treatment at 70°C for 10 minutes. The % lethality observed in these animals was 0%. This experiment demonstrates that heat- treating venom will remove the toxic effect in *C.elegans* caused by BWSV.

Figure 3.4 shows the effects of BWSV when treated with 0.1% SDS prior to injection into N2 *C.elegans*, this treatment, like pre-treatment with heat, causes the complete ablation of the toxicity of the venom.

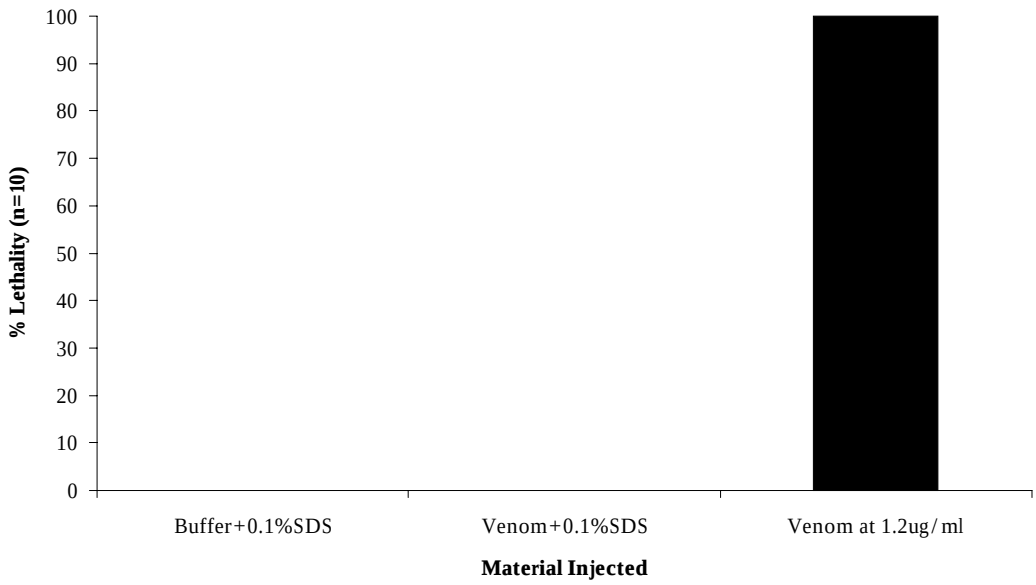


Figure 3.4 Effect of 0.1% SDS treatment on BWSV toxicity in *C.elegans*
BWSV at 1.2µg/ml was injected into worms (Venom), or after addition of 0.1% SDS (Venom+0.1% SDS). Negative control of venom buffer plus 0.1% SDS was also injected (Buffer+0.1% SDS). 10 *C.elegans* were injected per group and after incubation at 20°C for 2 hours; worms were scored for lethality caused by venom injection.

Positive and negative controls of 1.2μg/ml BWSV and venom buffer (50mM Tris, 50mM NaCl, pH8) plus 0.1% SDS respectively, were injected individually into adult *C.elegans.* Venom caused 100% lethality in the worms, while buffer plus 0.1% SDS caused 0% death. Both these data are shown in Figure 3.4. Experimental injection of 1.2μg/ml BWSV treated prior to *C.elegans* injection with 0.1% SDS, to denature the venom, showed 0% lethality in injected worms.

This data supports the argument that the active toxic component in BWSV can be denatured by thermal or chemical treatments.

Figures 3.3 and 3.4 both show that the denaturing of BWSV removes the toxicity of the venom to *C.elegans.* This therefore suggests that the component of the venom that is causing the toxicity is a protein.

3.2.3 Effects of purified α-latrotoxin on *C.elegans*

It is known that high molecular weight latrotoxins mediate the toxicity of black widow spider venom in mammals and insects.

The latrotoxin α-latrotoxin is a known toxin of mammals and is specific to vertebrates only, as it has been shown not to elicit vesicle release at invertebrate neuromuscular junctions (Frontali *et al*, 1976 & Ashton *et al*, 2000).

In order to characterise the toxic component of BWSV in *C.elegans* it is necessary to identify latrotoxins that have an effect on *C.elegans.*

Initially highly purified α-latrotoxin (kind gift of Y.Ushkaryov) at 240μg/ml was tested on adult N2 *C.elegans* using the microinjection technique that has been used previously.

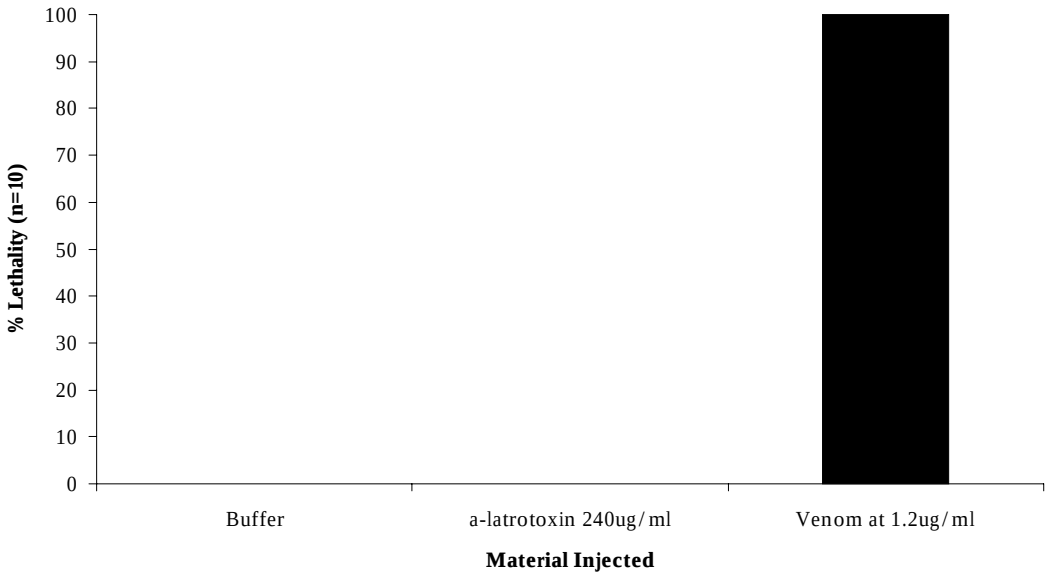


Figure 3.5 Effect of purified α-latrotoxin on *C.elegans*
C.elegans (10 per point) were injected with BWSV at 1.2µg/ml (Venom), or purified α-latrotoxin (α-latrotoxin) at 240µg/ml, or buffer control (Buffer). Animals were scored for lethality after 2 hour incubation at 20°C.

Purified α-latrotoxin showed no measurable toxicity toward *C.elegans* upon injection. Previous studies have shown that α-latrotoxin is specific to vertebrates and has no effect on insects (Krasnoperov *et al*, 1992).

The amount of α-latrotoxin in BWSV has been approximated to be 3% of total venom (Krasnoperov *et al*, 1992). Therefore, BWSV, like that used in Section 3.2.1, has an approximate α-latrotoxin content of 36µg/ml or 3% of total venom (1.2mg/ml). The experiment in Section 3.2.3 used 240µg/ml of (pure) α-latrotoxin, 6.6 times more α-latrotoxin than in total BWSV, this shows that even at very high concentrations of pure α-LTX. Pure α-LTX has no toxic effect on *C.elegans.*

Though the mechanism of latrotoxin stimulation is similar across a broad range of phyla, latrotoxins, such as α-latrotoxin only show responses in a specific animal group (Henkel *et al*, 1999). Therefore it is necessary to further characterise the components, which cause toxicity in *C.elegans*, this can be elucidated by FPLC fractionation of BWSV.

3.3 Chromatographic separation of BWSV proteins

BWSV was initially fractionated based on the method of Krasnoperov (1992). However, latrotoxin fractions were contaminated with low molecular weight proteins (data not shown), and so BWSV was purified using an initial size exclusion column (Ashton *et al*, 2000) followed by the procedure based on Krasnoperov (Krasnoperov *et al*, 1992).

3.3.1 Size exclusion standards separated using size exclusion chromatography

A Superdex 200 (HR10/30 Pharmacia) column was equilibrated into water (50ml at 0.5ml per minute), then 20mM Tris pH8, 150mM NaCl. BioRad size exclusion standards were resuspended in the same buffer: 100µl of this solution was then spun for 20 minutes at 4^{o}C and the supernatant aspirated before loading onto the column.

Two separate runs of the standards were made to check the consistency and reproducibility of separation from the column.

Chromatograms for both runs were overlaid and are shown in Figure 3.6.

670kDa, 158kDa and 44kDa standards in both runs show identical elution profiles.

The consistency of the elution profiles obtained from both runs of the standards from the size exclusion column show that the columns are working to an optimum level and there is reproducibility of separation over a number of purifications.

The column can therefore be prepared for size exclusion chromatography of black widow spider venom as the first stage of purifying and identifying the venom component toxic to *C.elegans*.

Size exclusion chromatography was utilised to remove low molecular weight protein contaminants, which are present in black widow spider venom (Frontali *et al*, 1976).

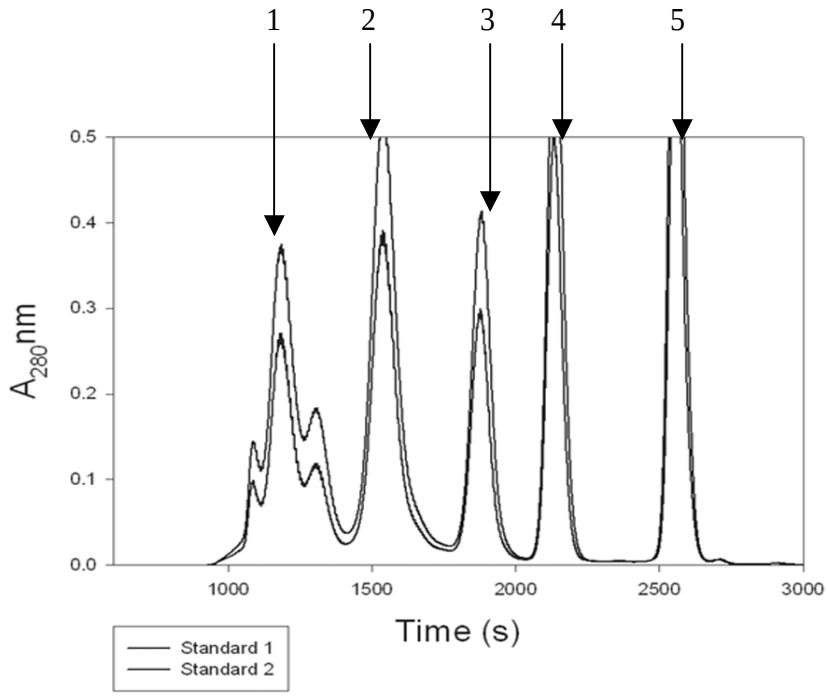


Figure 3.6 Size exclusion on a Superdex 200 column.
Size exclusion standards were dissolved in 1M Tris, 5M NaCl and loaded onto Superdex 200 column at 0.5ml/min. The absorbance at 280nm (A280nm) of the elutes is shown as the thick black line, there are 2 traces, overlaid to show the 2 runs made of identical standards. Standard sizes are as follows, **1**.thyroglobulin (bovine)670kDa, **2**.gamma globulin (bovine)158kDa, **3**.ovalbumin (chicken)44kDa, **4**.myoglobin (horse)17kDa, **5**.VitaminB12 1350Da.

3.3.2 Size exclusion separation of BWSV

13.8mg BWSV in 1ml 20mM Tris pH8, 150mM NaCl was gently mixed and then spun at 15000g for 20 minutes at 4°C. 250µl BWSV was loaded onto the column and the fractions were collected at 1-minute intervals. The chromatograph is shown in Figure 3.7. Samples were then diluted 1:1000 in venom injection buffer (50mM Tris, 50mM NaCl,pH8) and injected into adult *C.elegans* to test for toxicity. After injection worms were incubated for 2 hours at 20°C before analysis. Figure 3.7 shows % lethality for each fraction tested (n=5).

The data shows that fractions eluted from the size exclusion column that show the highest % lethality were fractions 8 through 12. These fractions correspond to proteins with a molecular weight of 170kDa to 70kDa. With peak fractions for lethality being fractions 8 & 9 with 100% lethality (n=5).

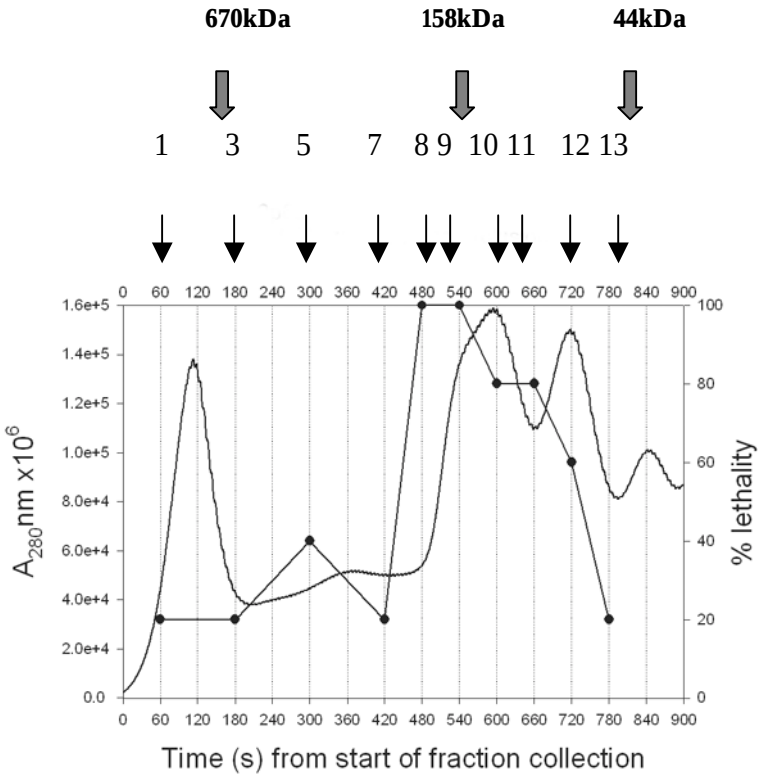


Figure 3.7 Size-exclusion chromatography of BWSV
BWSV was dissolved in 50mM Tris pH8, 150mM NaCl, and loaded onto Superdex 200 HR10/30 column at 0.5ml/minute. The absorbance at 280nm (A280nm) of the eluate is shown as a solid black line. 0.5ml fractions were collected (corresponding to numbers 1-13 at the top of the graph) and assayed by diluting 1:1000 in venom injection buffer, and microinjected into *C.elegans*, % lethality is shown (n=5) for each fraction as a thick black line with circles. The position of eluted molecular weight markers is shown by the presence of thick grey arrows at the top of the trace; these correspond with, bovine thyroglobulin (670kDa), bovine gamma globulin (158kDa) and chicken ovalbumin (44kDa).

Eluted fractions from the size exclusion column were run on a 10% Bis-Tris gel (Novex) to analyse the proteins present in each fraction. Gel image of these fractions is shown in Figure 3.8.

Fractions visualised in Figure 3.8 correspond to those tested for toxicity in *C.elegans* by microinjection shown on the chromatograph in Figure 3.7.

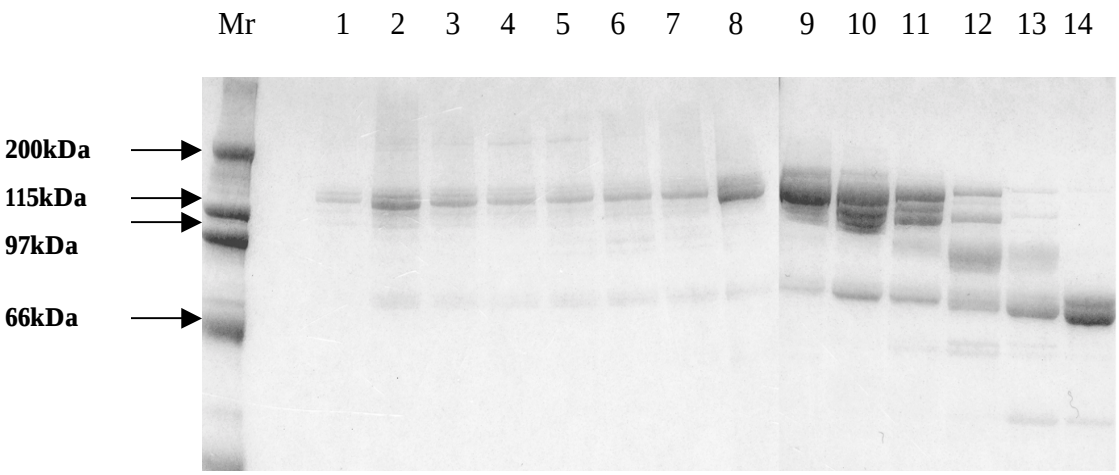


Figure 3.8 PAGE of fractions from size exclusion column.
BWSV was fractionated on Superdex 200 column (Figure 3.7) and fractions of 1ml collected and tested for toxicity in *C.elegans*. Eluted fractions were also visualised on LDS-PAGE as described in Methods section 2.7.4. 10% Bis-Tris gel (Novex) was run in NuPage Mops buffer (Novex) at 170V and stained using Coomassie Blue. Size of protein bands eluted in each fraction (1 to 14) was compared against SDS-6H (Sigma) molecular weight markers (Mr). Labelled arrows indicate size markers. Lanes 9-14 were run concurrently on a separate gel, the digital image is a composite from the two gels.

LDS-PAGE gel of the eluted fractions from the SEC shows the proteins present in the fractions, which were tested in *C.elegans* for toxicity.

Fractions 1 to 8 show proteins with molecular weights above 158kDa, fractions 9 to 14 show proteins with a molecular weight of 158kDa and below. The proteins which elute in fractions 8 and 9 appear at the same molecular weight on PAGE as those in fraction 10/11; it has previously been shown that α-LIT dimerises (e.g. Ashton *et al*, 2001). This data is consistent with dimerisation of the *C. elegans*-specific toxin.

Analysis of SEC data obtained from toxicity testing in *C.elegans* shows fractions of peak toxicity in fractions 9-12 (Figure 3.7).

Peak fractions obtained from the size exclusion column are all high molecular weight proteins.

Peak fractions, which gave the highest % lethality in *C.elegans*, were pooled as follows;

Fractions, 9, 10, 11 & 12.

3.3.3 UnoQ separation of BWSV toxic fractions obtained from SEC

Pooled samples were diluted from a concentration of 150mM NaCl to 30mM NaCl, and applied to a UnoQ1 (BioRad) column in 50mM Tris pH8, 30mM NaCl and eluted from the column using a salt gradient. The chromatograph of this purification is shown as Figure 3.9. Fractions eluted from the UnoQ1 column were collected and diluted 1:1000 in 50mM Tris, 50mM NaCl, pH8 venom buffer before toxicity testing by microinjection into adult *C.elegans*, Figure 3.9 also shows the % lethality of each eluted test fraction (n=5).

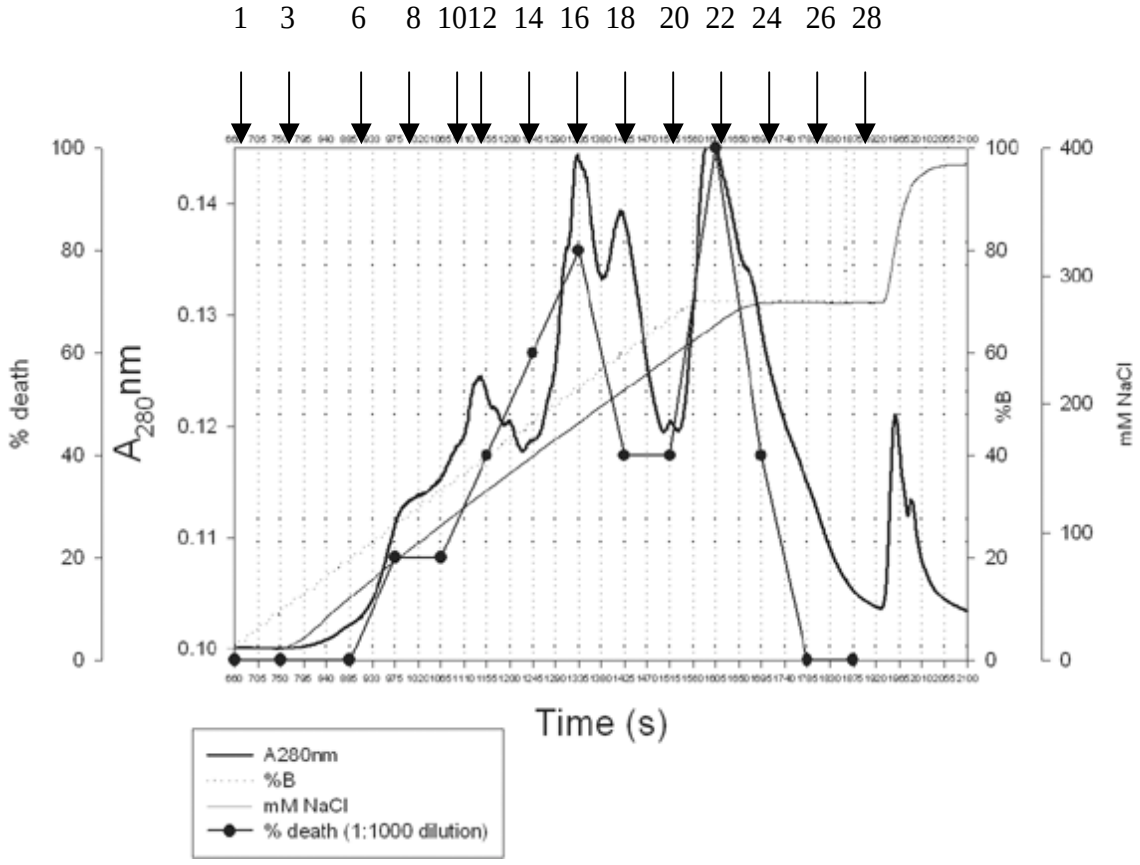


Figure 3.9 UnoQ chromatography of SEC separated BWSV fractions.
Pooled fractions (9-12) from SEC purified BWSV in 50mM Tris pH8, 30mM NaCl were loaded onto UnoQ column. The absorbance at 280nm (A280nm) of the elute is shown as a solid black line. Salt gradient elution is shown by a dotted line. 1ml fractions were collected (corresponding to numbers 1-28 at the top of the graph) and assayed by diluting 1:1000 in venom buffer, and microinjected into *C.elegans*, % lethality is shown (n=5) for each fraction as a thick black line with circles.

Eluted fractions from the UnoQ column were visualised by LDS-PAGE electrophoresis using 10% Bis-Tris gel (Novex) (Figure 3.10).

The chromatograph obtained when pooled fractions 9-12 were separated on the UnoQ column (Figure 3.9) is similar to that in Krasnoperov (1992), although this experiment used prior separation of venom on the SEC.

Therefore, fractions 1-10 correspond to the crustacea-specific toxins, 11-22 the insecto-specific toxins and 23-28 for the vertebrate-specific toxin approximately when compared to the MonoQ data in Krasnoperov (1992). Peak lethality of 80% was observed in fraction 16, while 100% lethality was seen for fraction 22.

The data also shows some activity in the crustacean-toxin fractions but this is only 20%, 0% lethality was observed in the fractions (23-28) which are believed to contain the vertebrate-specific toxin, this data supports the observations made in Section 3.2.3.

LDS-PAGE shows the fractions eluted from the UnoQ column. Only the fractions showing lethality to *C.elegans* were run, these were fractions 4-9 & 14-22. Fractions 4-9 show proteins of high molecular weight of ~115kDa, there is though, in some of the later fractions (7,8,9) low molecular weight proteins of ~66kDa which were not totally removed by the SEC purification. Fractions 14-22 contain a range of proteins, though in the 100% lethal fraction (22) there is an abundance of protein with a size in excess of 115kDa and with little low molecular weight contaminants.

As these fractions still contain some low molecular weight contamination, another round of purification was undertaken to isolate the *C.elegans* toxic component of BWSV.

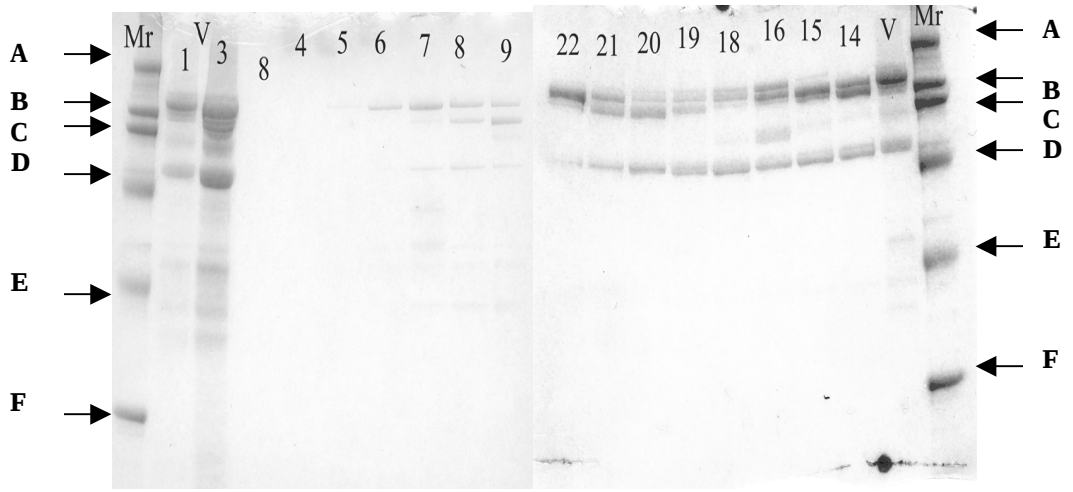


Figure 3.10 LDS-PAGE of BWSV fractions purified on UnoQ1 column.
Pooled fractions from SEC purification were fractionated on UnoQ1 column and the collected fractions analysed for toxicity in *C.elegans*. Eluted fractions were run on a 10% Bis-Tris gel to visualise proteins contained in each fraction. Gel was run at 170V and stained using Coomassie Blue. Size of eluted proteins was compared to molecular weight markers (Mr) as indicated by the labelled arrows. **A**. 200kDa, **B**. 115kDa, **C**. 97kDa, **D**. 66kDa, **E**. 44kDa, **F**. 35kDa.
Numbered lanes correspond to eluted fraction apart from V 1&3, which show unfractionated BWSV at 1µl & 3µl respectively and V, which shows 1µl-unfractionated BWSV.
This figure is a composite of two separate gels; each gel has its own Mr marker, which is used for sizing proteins on its respective gel only. Right hand markers for fractions 4-9, left hand markers for fractions 14-22.

After purification on UnoQ1 column, low salt fractions (14-16) were pooled and further purified using UnoS column, as described in Krasnoperov *et al* (1992).

3.3.4 UnoS separation of toxic fractions identified from UnoQ chromatography

Krasnoperov *et al* (1992), shows that after initial UnoQ purification, proteins eluted at the same position as the low salt fractions obtained after SEC and UnoQ separation in this thesis, contain multiple latroinsectotoxins. To continue purification of the nematode-toxic fraction, samples 14-16 from the UnoQ column were pooled and further purified on UnoS column in 50mM Na succinate pH6.2, 20mM NaCl, and run on a gradient to

0.4M NaCl. 750µl fractions were collected, diluted 1:1000 in venom buffer and microinjected into *C.elegans* (n=5) to test for toxicity.

Toxicity data obtained is shown in Figure 3.11.

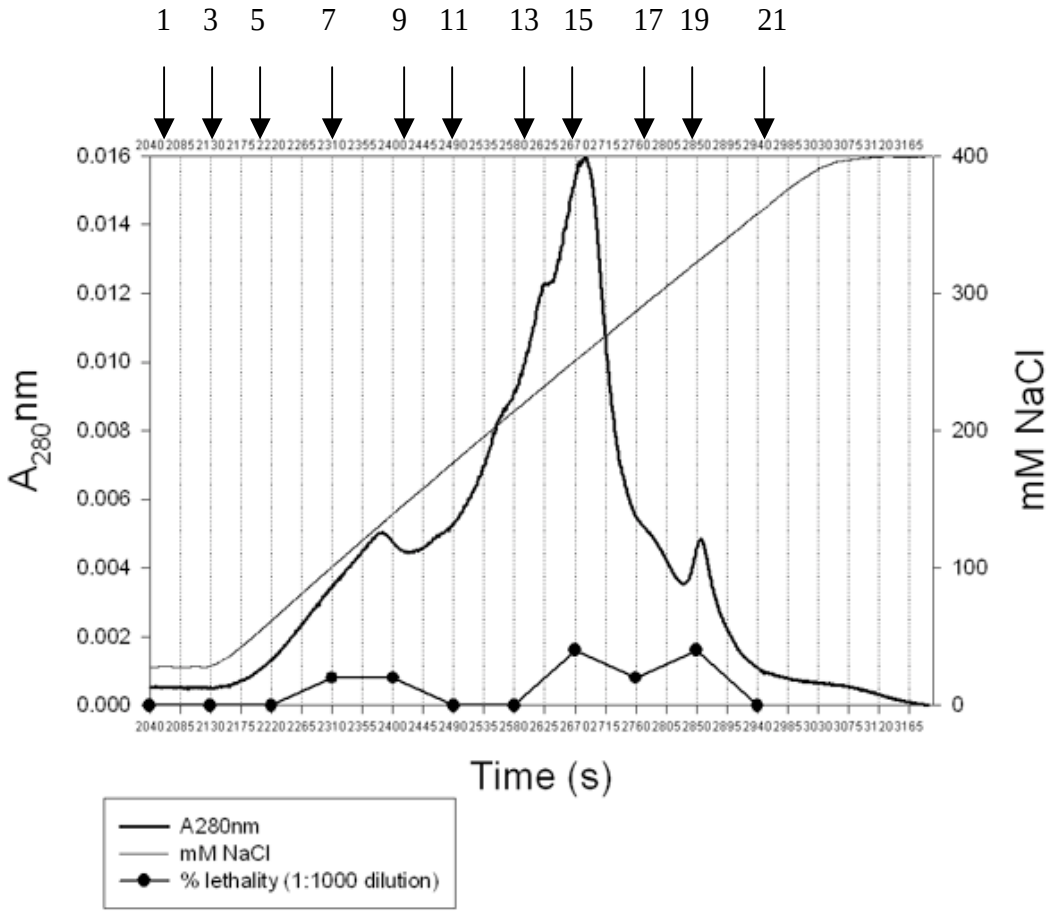


Figure 3.11 UnoS chromatography of UnoQ separated BWSV fractions.
Pooled fractions (13-22) from UnoQ1 purified BWSV in 50mM Na succinate, 20mM NaCl were loaded onto UnoS column. The absorbance at 280nm (A280nm) of the eluate is shown as a solid black line. Salt gradient (to 0.4M NaCl) elution is shown by a thin black line. 750µl fractions were collected (corresponding to numbers 1-21 on the top of the graph) and assayed for toxicity by 1:1000 dilution in venom buffer and microinjection into *C.elegans*. % Lethality is shown (n=5) for each fraction as a thick black line with circles. 1=0%, 3=0%, 5=0%, 7=20%, 9=20%, 11=0%, 13=0%, 15=40%, 17=20%, 19=40%, 21=0%.

Fractions of interest eluted from the UnoS column were visualised by LDS-PAGE electrophoresis using 10% Bis-Tris gel (Figure 3.12).

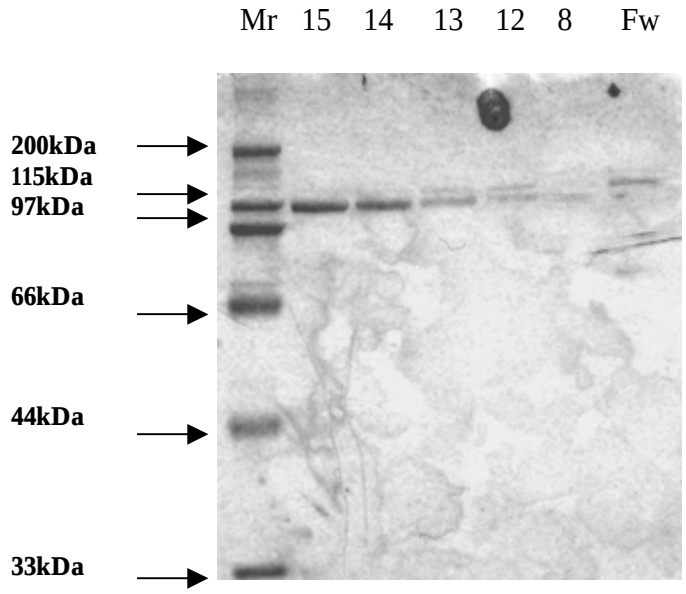


Figure 3.12 LDS-PAGE of BWSV fractions purified on UnoS column.
Pooled fractions (13-22) from UnoQ1 purification were fractionated on UnoS column and the eluted fractions collected and analysed for toxicity in *C.elegans*. Eluted fractions of interest (15, 14, 13, 12, 8 & Flow through (Fw)) were run on 10% Bis-Tris gel to visualise proteins within the fractions. Gel was run at 170V and stained using Coomassie blue. Size of eluted proteins was compared to molecular weight (Mr) markers as indicated by labelled arrows.

UnoS purification of UnoQ fractions 14-16 shows a marked decrease in the lethality of the BWSV component fractions eluted from the UnoS column. The highest % kill is present in fractions 15 & 19 (Figure 3.11) with levels of 40% lethality after a 1:1000 dilution.

Krasnoperov (1992) showed that purified latroinsectotoxins are separated under these conditions. Proteins with similar molecular mass to latroinsectotoxins are separated under UnoS conditions (Figure 3.12), and elute in the same order, and the same relative abundance, as reported by Kransoperov (1992).

Fraction 19 should not contain *C.elegans* toxic proteins; the lethality seen in this fraction may be due to a shift to the right in the elution profile of some of the later insectotoxins, and the use of different columns and venoms. LDS-PAGE of the UnoS fractions (Figure 3.12) does show that fraction 15 (40% lethality upon injection into *C.elegans*) is a high molecular weight protein <115kDa and contains no low molecular weight contaminants.

This fraction would appear to represent a homogenous BWSV component that is toxic when injected into *C.elegans.*

3.3.5 Identification of homogenous BWSV component toxic to *C.elegans*

3.3.5.1 PAGE analysis of purified *C.elegans* toxin

Purification undertaken has identified a fraction from BWSV that is potently toxic to *C.elegans* after 3 rounds of purification. Toxic fragments from each separation step were compared on an LDS-PAGE gel. Toxic fraction from UnoQ purification after separation on UnoS (fraction 15) yields a protein of 110kDa on PAGE, which has similar chromatographic properties to ε-latroinsectotoxin. Identification of *C.elegans* toxic protein is shown in Figure 3.13.

Gel was silver stained after running for extra sensitivity of protein detection.

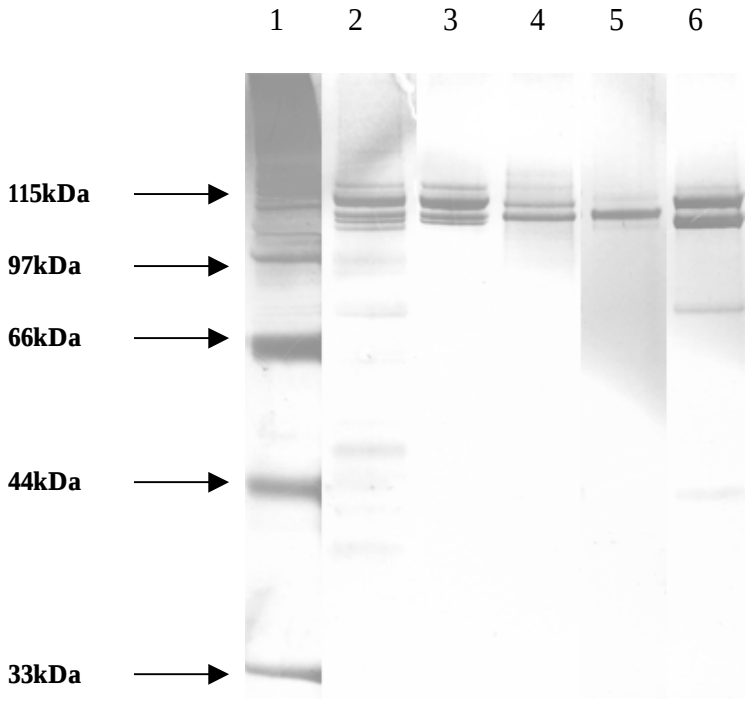


Figure 3.13 LDS-PAGE of purified BWSV showing homogenous component toxic to *C.elegans.* 10% Bis-Tris gel was run to show homogenous component toxic to *C.elegans* purified from SEC, UnoQ and UnoS columns. Molecular weight standards (**1**) are shown by labelled arrows. Neat BWSV (**2**), venom post SEC (**3**), low salt peak fractions from UnoQ (**4**), purified protein: putative epsilon latroinsectotoxin (**5**) and high salt kill peak from UnoQ (**6**) are in the remaining lanes.

3.3.5.2 Peptide sequence analysis of *C.elegans* toxic fragment

The protein obtained from BWSV purification was subjected to tryptic digestion, followed by MS/MS sequencing (John Keyte, personal communication).

This gave three peptide sequences.

EA(L/I)(L/I)GHR

(AT)FQEV(L/I)DA(L/I)(L/I)EK

(FT)TDYVNN(L/I)AEDVR

Similar results were obtained with another round of purification without the first size-exclusion column.

However, size-exclusion chromatography was included to allow the initial removal of low molecular weight components/contaminants.

3.4 Identification of latrophilin homologue in *C.elegans*

Data in section 3.3 demonstrated that particular high molecular weight proteins are toxic to *C.elegans*. Mammal-specific latrotoxins are known to bind to the mammalian receptors latrophilin and neurexin. Therefore, homologues of these receptors in *C.elegans* may be responsible for the mediation of latrotoxin-induced toxicity.

Database analysis of *C.elegans* genome (http://www.sanger.ac.uk/Projects/Celegans) was undertaken using the sequence of the latrophilin gene found in the rat.

Database searches identified two possible homologues of latrophilin in *C.elegans*, BO457.1 and BO286.2. BO457.1 was found to be more closely related to latrophilin.

Figure 3.14 shows the structure of the *C.elegans* latrophilin cDNA, which encodes a 1014 amino-acid peptide with a predicted p*I* of 7.2.

A.

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MRRNKTTYSL LQTILVACLL TVTPTFASNK PTTDESGTIS HTICDGEAAE LSCPAGKVIS        60
IVLGNYGRFS VAVCLPDNDI VPSNINCQNH KTKSILEKKC NGDSMCYFTV DKKTFTEDPC       120
PNTPKYLEVK YNCVVPATTT TTTTTTSTTT TDSSLIVDEE EEAQKDALNS DVIKPVKKKE       180
DVFCSATNRR GVNWQNTKSG TTSSAPCPEG SSGKQLWACT EEGQWLTEFP NSAGCESNWI       240
SSRNSVLSGV ISSEDVSGLP EFLRNLGSET RRPMVGGDLP KVLHLLEKTV NVIAEESWAY       300
QHLPLSNKGA VEVMNYMLRN QEIWGSWDVT KRKEFASRFI LAAEKAMVAS AKGMMTSAES       360
NVIVQPAITV EISHKIKMSS QPTDYILFPS AALWNGQNVD NVNIPRDAIL KINKDETQVF       420
FSSFDNLGAQ MTPSDVTVAI AGTDQTEVRK RRVVSRIVGA SLIENGKERR VENLTQPVRI       480
TFYHKESSVR HLSNPTCVWW NHHELKWKPS GCKLSYHNKT MTSCDCTHLT HFAVLMDVRG       540
HDLNEIDQTL LTLLTYVGCI ISIICLLLTF FAYLIFSRNG GDRVFIHENL CLSLAIAEIT       600
FLAGITRTED SLQCGIIAVA LMYMFLSALT WMLLEGYHIH RMLTEVFPSD PRRFTYLLVG       660
YIPPAIITLV AYLYNSDGFG TPDHCWLSTQ NNFIWFFAGP ACFIFCANSL VLVKTLCTVY       720
QHTSGGYLPC RHDVDSGRSI RNWVKGSLAL ASLLGVTWIF GLFWVEDSRS IVMAYVFTIS       780
NSLQGLFIFL FHVVFAEKMR KDVGHWMYRR GCGGSSNSSP NHKRHNVQRD LMSPGVNSST       840
GSDFLYNTND KYLTNSDTTN RLVYNGIMNH PNQMSVYQQH PHHQIYEQQP GTYDYATIAY       900
GDMMPGHRVA APPAYQRLAV AEGRYGSQHQ LYQGWHHRPP PEFSPPPPPL STGPPNSRHY       960
GTGSSGRRPP SSKMSDDSAY SDGSSSMLTT EVTPQGQTVL RIDLNKPSMY CQDL            1014
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B.

```
atgcgacgta acaaaacgac ttattcgttg ctccaaacga ttctggtagc ttgtctactg        60
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cacacaatat gtgatggtga agctgctgaa ctgagttgtc ctgctggaaa agtaatctcg       180
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ttggctgctg agaaagcaat ggttgcatct gcgaaaggaa tgatgacatc ggcagaatcg      1080
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aatcatcatg aactgaaatg gaaaccttct ggatgtaaac tcagttatca taataagaca      1560
atgacgtcat gtgactgtac tcatctcaca cattttgctg ttttgatgga tgtacgagga      1620
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gggactggct ccagtggacg acggccgccg agctcaaaga tgagtgatga ctctgcatat      2940
tcggatgggt catcatcgat gttgacaact gaagtgacac cacaaggaca gacggtgctt      3000
cgaattgatt tgaacaagcc gagcatgtat tgtcaagatt tatag                      3045
```

Figure 3.14 Amino acid and cDNA sequences of *C.elegans* latrophilin homologue BO457.1.
A. Amino acid sequence of BO457.1, peptide size is 1014 residues.
B. cDNA sequence of BO457.1, total cDNA size is 3045bp.
cDNA was produced and sequenced three times in total to confirm sequence data was correct. The sequence is the candidates work, not merely the result of a genefinder sequence prediction.

Although overall amino acid identity with the rat latrophilin receptor is only 29%, the region of latrophilin required for α-latrotoxin binding (residues 493-541, Krasnoperov *et*

al, 1999) has 39% aa-identity with the *C.elegans* cDNA, showing a greater degree of sequence identity in domains directly associated with toxin: receptor interaction.

Using Pfam 6.6 (http://www.sanger.ac.uk/Software/Pfam), the *C.elegans* latrophilin homologue was compared with the rat latrophilin homologue to identify further sequence identity. This search showed a number of structurally conserved domains between rat and *C.elegans* latrophilin homologues.

Figure 3.15 shows structural domains of *C.elegans* latrophilin protein aligned with the rat latrophilin protein.

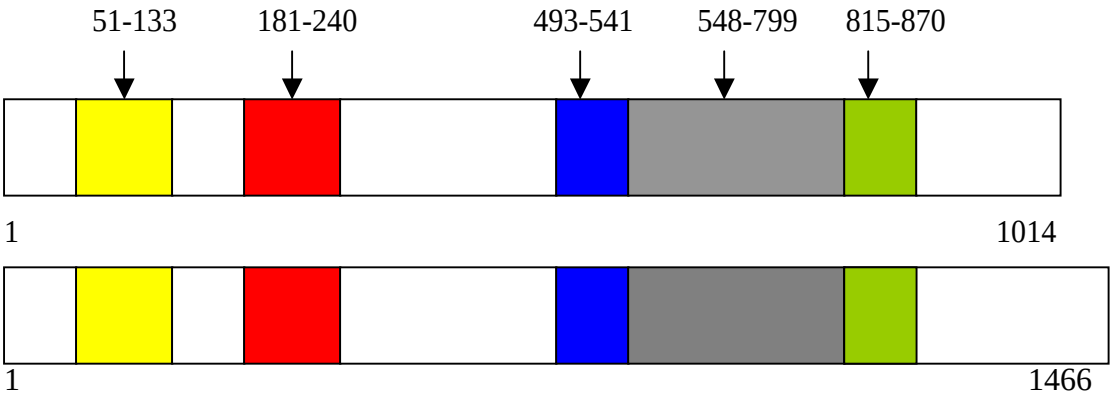


Figure 3.15 Structural domains of *C.elegans* & Rat latrophilin homologues.
C.elegans domains were identified by Pfam 6.6 search and manual alignment with the rat latrophilin sequence.
Top image shows the *C.elegans* homologue, Galactose-binding lectin domain (51-133aa) is shown by yellow box, hormone receptor motif (181-240aa) is shown by red box. G-protein coupled receptor Protease cleavage Site (493-541aa) is shown as a blue box. The 7-transmembrane secretin domain (548-799aa) is a grey box. An area of four conserved cysteines is also shown (815-870aa) as a green box. Total amino acid size is shown by labels at the base of the diagram (1-1014). Lower image shows the rat latrophilin homologue, this alignment shows the conserved protein regions with the *C.elegans* protein, the coloured boxes, which correspond to the domains shown in the above text, show conserved regions. Total amino acid size is shown by the labels at the base of the diagram (1-1466).

3.5 Extraction of RNA from *Caenorhabditis elegans*

Total *C.elegans* RNA was extracted from wild-type (N2) *C.elegans* using the TRIZOL method described in section 2.2.6.7. Further manipulations of the latrophilin gene in

C.elegans required the generation of RNA from the latrophilin cDNA. To check that these generated RNAs had not degraded on agarose gels, control total RNA from *C.elegans* was needed.

RNA quantification was determined by spectroscopy at 260nm and the integrity of the RNA was visualised by denaturing agarose gel electrophoresis (Figure 3.16). Typically, 1-2mg of RNA was isolated per 1ml of packed wild-type worms.

RNA extracted by this method gave rise to high purity intact RNA samples, as can be seen in Figure 3.16 where the 28S and 18S RNA species are clearly visible.

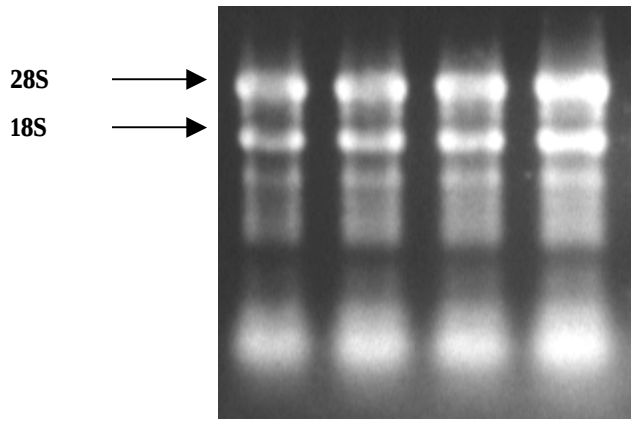


Figure 3.16 Total RNA from *C.elegans*.
Total *C.elegans* RNA was run in all 4 lanes of the gel each lane contains 2μg RNA. Gel run was 1% pre-stained, 0.5μg/ml EtBr, 0.1% SDS denaturing, 1XTBE agarose gel run at a constant voltage of 65V for 2 hours as described in Methods section 2.2.11. The gel was photographed using UV illumination.

3.6 Generation of *C.elegans* with silenced latrophilin gene by RNA Interference (RNAi)

RNA interference or RNAi is a technique, which uses double-stranded RNA (dsRNA) of a particular gene to target specific mRNAs for degradation and therefore specifically silencing that gene's function. This phenomenon has been show to occur in a number of species, but is most common in invertebrates and plants. This reverse genetic technique allows the analysis of gene function to take place via pre-identified gene sequences, rather

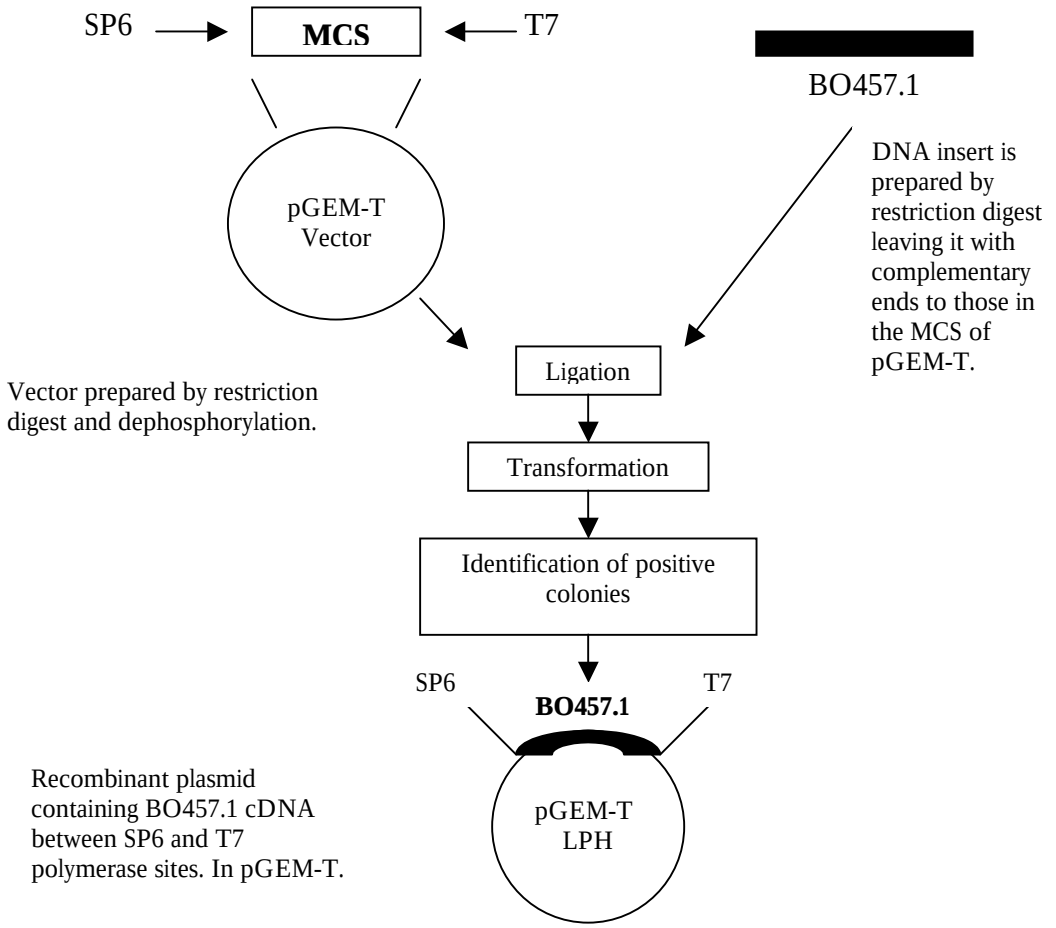
than in forward genetics where a phenotype is identified and then the sequence searched for. In this section, RNAi is used to elucidate the gene function of the *C.elegans* latrophilin gene identified in Section 3.4.

3.6.1 Sub-cloning of BO457.1 cDNA into pGEM-T vector

pGEM-T vector (Promega) was used for BO457.1 sub-cloning because it has two polymerase promoter sites (SP6 & T7), which allow the generation of cRNA fragments using *in vitro* transcription. The original BO457.1 cDNA was generated using PCR primers designed from analysis of the *C.elegans* genome sequence (Pavel Perestenko, personal communication).

The strategy for the sub-cloning of BO457.1 into the pGEM-T vector is illustrated in Figure 3.17.

Figure 3.17 Cartoon depicting the sub-cloning of BO457.1 cDNA into pGEM-T vector.



Generation of LPH: pGEM-T vector combined 3.04kb cDNA with 3.00kb pGEM-T vector giving a total plasmid size of 6.04kb. LPH:pGEM-T vector is required to be linearised to allow *in vitro* transcription to take place. To linearise for T7 transcription restriction enzymes Spe I, Not I, BstZ I, Pst I, Sal I, Nde I, Sac I, BstX I and Nsi I can be used to digest the vector at a unique site adjacent to the multiple cloning site (MCS). To linearise for SP6 transcription restriction enzymes Apa I, Aat II, Sph I, BstZ I, Nco I, and Sac II can be used.

Analysis of BO457.1 cDNA sequence showed no internal digest sites for Pst I (T7) and Nco I (SP6) enzymes.

Figure 3.18 shows pGEM-T vector and the restriction enzyme sites for linearisation.

Figure 3.19 shows LPH:pGEM-T vector restriction digested with Pst I and Nco I to check for the viability of these enzymes to linearise the vector.

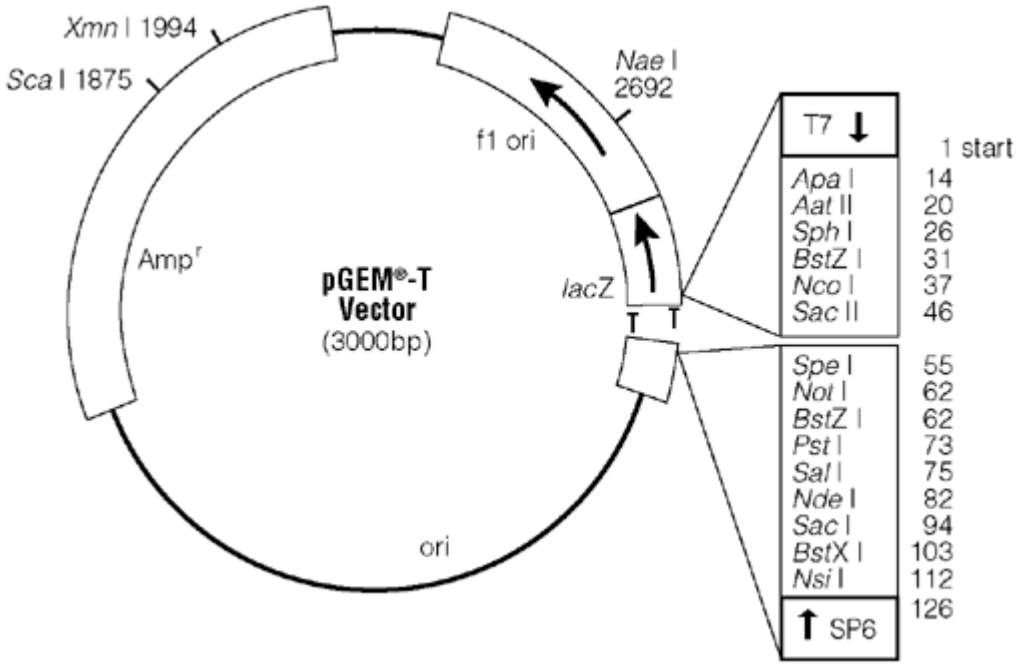


Figure 3.18 pGEM-T vector circle map.
pGEM-T vector circle map showing positions of restriction enzyme sites for use in linearising the vector (Image courtesy of Promega Inc.).

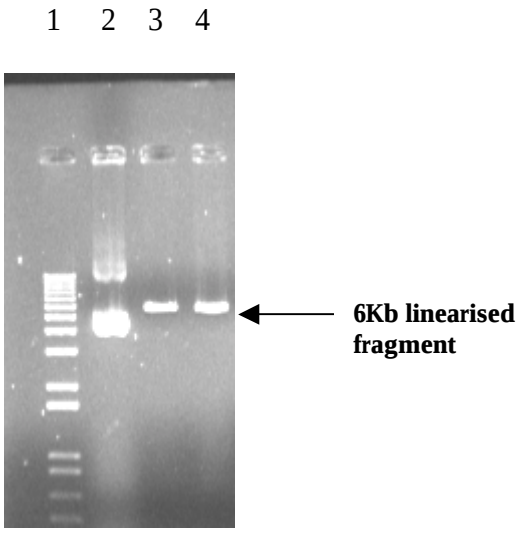


Figure 3.19 Restriction digest of LPH: pGEM-T clone.
LPH:pGEM-T clone containing BO457.1 cDNA was digested with Nco I restriction enzyme and Pst I restriction enzyme in separate reactions. Products were run on a 1% pre-stained, 0.5µg/ml EtBr, 1XTBE agarose gel at a constant voltage of 80V for 1 hour to show the presence of linearised plasmid in both digests.
Lane **1** contains 1KB+ DNA ladder, uncut LPH:pGEM-T clone in lane **2**, Pst I cut LPH:pGEM-T in lane **3** and Nco I cut LPH:pGEM-T in lane **4**. Arrows indicate position of 6Kb linearised fragment from both digests, sized by the ladder on the gel.

Both Pst I and Nco I enzymes digested LPH:pGEM-T to leave a 6Kb linearised fragment. Both enzymes were then used to prepare LPH:pGEM-T for *in vitro* transcription.

3.6.2 Generation of dsRNA complementary to BO457.1

BO457.1 cDNA was sub cloned into pGEM-T vector to give the recombinant LPH:pGEM-T vector. (Shown in section 3.6.1).

To generate sense and antisense cRNA transcripts of BO457.1, LPH:pGEM-T under went *in vitro* transcription.

Initially, LPH:pGEM-T was restriction digested with Nco I and Pst I to linearise the plasmid in both the T7 and SP6 orientation. The digested template was treated with NTP mix and the addition of SP6 RNA polymerase for the Nco I digested template and T7

RNA polymerase for the Pst I digested template. Reaction mixes were incubated at 37°C for T7 polymerase and 42°C for the SP6 polymerase, for 2 hours.

Identification of cRNA transcripts from these reactions was analysed by the presence of cRNA bands on a denaturing agarose gel.

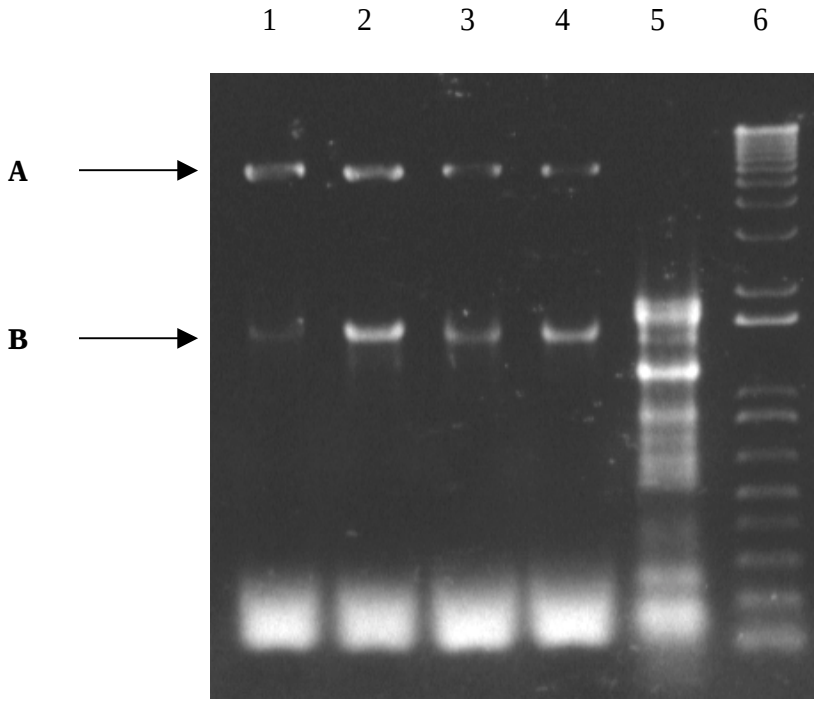


Figure 3.20 RNA transcripts from *in vitro* transcription.
LPH:pGEM-T clone digested with Nco I (lanes **1**&**2**) and Pst I (lanes **3**&**4**) were used as DNA templates for SP6 and T7 polymerase driven in vitro transcription. Products were run as duplicates on a 1% pre-stained, 0.5µg/ml EtBr, 0.1% SDS denaturing, 1XTBE agarose gel at a constant voltage of 65V for 2 hours, to show the presence of cRNA transcripts as described in Methods section 2.2.11, the gel was visualised under UV illumination and photographed.
Lane **1**&**2** contain SP6 driven *in vitro* transcription from Nco I digest. Lane **3**&**4** contain T7 driven in vitro transcription from Pst I digest. Lane 5 contains 1.5µg total *C.elegans* RNA, lane 6 contains 1KB+ size marker.
Arrows indicate presence of intact cRNA transcripts for both SP6 and T7 polymerases (**B**) and linearised plasmids (**A**).

cRNA transcripts that were generated were DNase treated to remove any remaining DNA template.

The cRNA strands were then phenol: chloroform treated and ethanol precipitated.

Annealing of both sense and antisense RNAs to form dsRNA was done by mixing in a 1:1 (v/v) ratio and heating at 88°C for 10 minutes. Once annealed, dsRNA was ready for microinjection into *C.elegans.*

3.6.3 Microinjection of LPH dsRNA into *C.elegans*

Fire *et al* (1998), stated that the microinjection of dsRNA complementary to a gene of interest into the gonad of the nematode *C.elegans* causes potent and specific gene silencing of that gene.

Since then, many changes have been made to the technique of RNAi. It is now possible to cause this gene interference by "soaking" or "feeding" worms dsRNA. Microinjection is still the most reliable method (Kamath *et al*, 2001) though more labour intensive. Adult *C.elegans* were injected with a range of substances to study the dsRNA effect.

3.6.3.1 Microinjection of dsRNA free solutions

10 adult *C.elegans* were microinjected with UHP water only using the method in section 2.4.7. Injected adults were transferred to standard NGM plates and allowed to lay eggs for 5 hours. This allows any eggs fertilised before injection to be expelled from the worm and prevent them from influencing the results.

Worms were then incubated on new NGM plates and the F1 progeny grown to the L4 larval stage, at this point, F1 progeny were examined for any phenotypic changes caused by RNAi.

Photomicrographs of UHP H_2O injected *C.elegans* F1 progeny is shown in Figure 3.21.

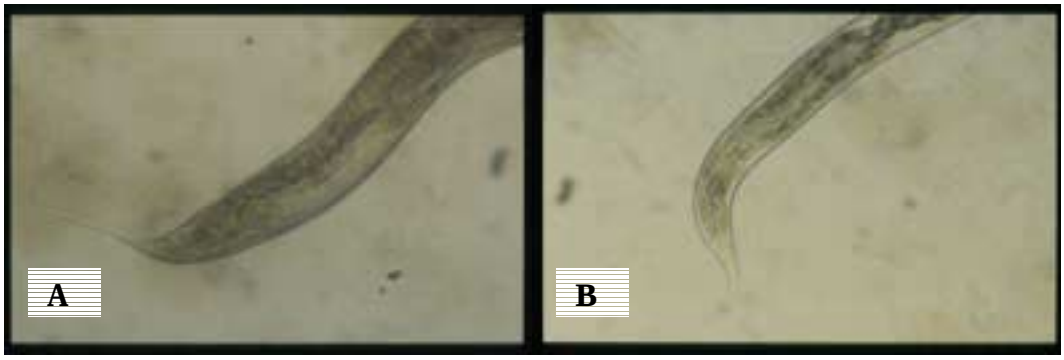


Figure 3.21 F1 progeny from *C.elegans* injected with UHP H2O
Photomicrograph taken at 40X magnification. **A**. F1 progeny of *C.elegans* injected with water, **B**. F1 progeny of *C.elegans* not injected with water.

F1 progeny are studied for phenotypic changes in *C.elegans* because RNAi is not a permanent effect. Only the injected animal and its first generation offspring are affected, second generation offspring are unaffected (Fire *et al*, 1998) by RNAi. F1 progeny are used to allow the analysis of any embryo lethal phenotypes.

Progeny from UHP H_20 injected adults showed no phenotypic changes compared to non-injected progeny, illustrating that the process of injection has no effect on phenotypic changes.

3.6.3.2 Microinjection of dsRNA complementary to the *tra-2* gene

The *tra-2* gene in *C.elegans* drives hermaphrodite development. The suppression of the *tra-2* gene causes a switch in sex from hermaphrodite to male (P.Kuwabara, personal communication). Using dsRNA complementary to *tra-2* to suppress the gene will cause an easily identifiable phenotype to develop. As male C.elegans are easily identifiable against females, this gene acts as a positive control for RNAi.

The *tra-2* clone in pBluescript was a kind gift from Dr P.Kuwabara, Sanger Centre, tra-2: pBluescript was restriction digested using BssH II to linearise the *tra-2* cDNA and T7 and T3 RNA polymerase driven transcription performed to produce sense and antisense cRNAs. From these, *tra-2* dsRNA was constructed. 10 adult *C.elegans* were microinjected with *tra-2* dsRNA using the method in section 2.4.7, dsRNA was injected at a concentration of 100μg/ml in UHP H_2O. Injected animals were transferred to standard NGM plates and allowed to lay eggs for 5 hours at 20°C to expel pre-injection fertilised eggs. Worms were transferred to new NGM plates after incubation and the F1 progeny grown to the L4 stage at 20°C. F1 progeny were analysed for change in phenotype from hermaphrodite to male.

Photomicrographs of these offspring are shown in Figure 3.22.

Figure 3.22 Photomicrographs of F1 progeny from *C.elegans* injected with *tra-2* dsRNA.
Photomicrographs taken at 40X magnification.
A. Hermaphrodite F1 Progeny **B**. F1 progeny from *tra-2* dsRNA injected *C.elegans*.
C. F1 progeny from *tra-2* dsRNA injected *C.elegans.* **D**. F1 progeny from *tra-2* dsRNA injected *C.elegans.*

Progeny from *tra-2* dsRNA injected adults show change in phenotype from hermaphrodite to male. This is shown by the presence of the male tail fan (used for mating) and the presence of the testis in the body cavity.

This data shows that the process of RNAi works with the silencing of a gene, which gave an obvious, and visual phenotype.

3.6.3.3 Microinjection of dsRNA for the latrophilin gene in *C.elegans*

Both negative (section 3.6.3.1) and positive (section 3.6.3.2) controls of RNAi have shown that the process is a viable method for inducing suppression of gene function. Therefore, 10 adult *C.elegans* were microinjected with LPH dsRNA using the method described in section 2.4.7. Double-stranded RNA was injected at a concentration of 100μg/ml in UHP water. Injected adults were transferred to standard NGM plates and

allowed to expel any eggs fertilised prior to injection with dsRNA. Worms were again transferred to new NGM plates and the F1 progeny grown to the L4 stage at 20°C. F1 progeny were analysed for any physical change in phenotype.

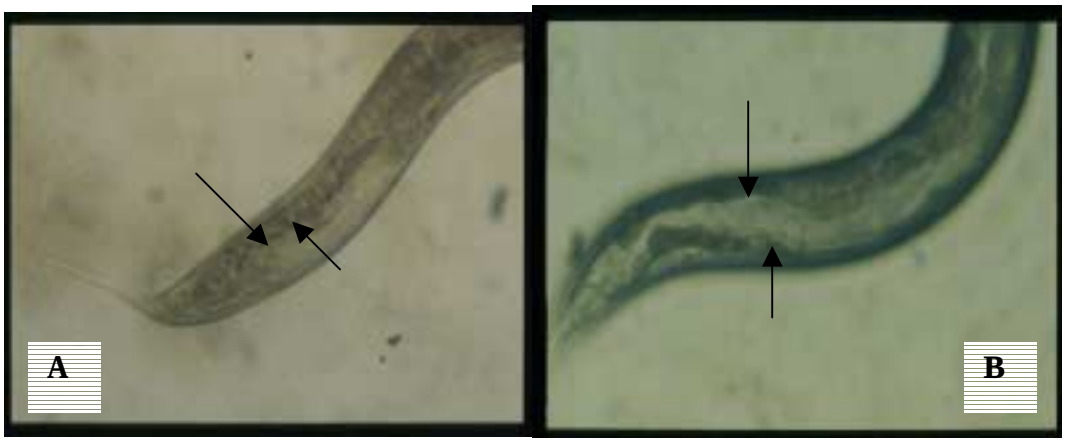


Figure 3.23 F1 progeny from *C.elegans* injected with LPH dsRNA.
Photomicrographs taken at 40X magnification.
A. Wild-type worm showing the gut (black arrows). Gut maintains the narrow intestinal passage seen in all N2 worms. **B**. Latrophilin RNAi worm showing gut distension (black arrows). Gut has expanded and some accumulation of the worm's food can be seen inside the distended organ.

Latrophilin RNAi worms, were compared to wild-type worms, and showed a distinct phenotype. Worms were constipated with an accumulation of food in the intestinal tract. This phenotype is similar to mutations seen in *aex-3* (Iwaski *et al*, 1997) and *egl-4* (Daniels *et al*, 2000) knockout *C.elegans*; both of these genes are involved in neuronal circuitry and synaptic transmission.

In addition to this visual phenotype, LPH RNAi worms were found to have an extended period of gut peristalsis. Worms were analysed using the method described in section 2.5.3, and the time taken between expulsion contractions was observed (Figure 3.24).

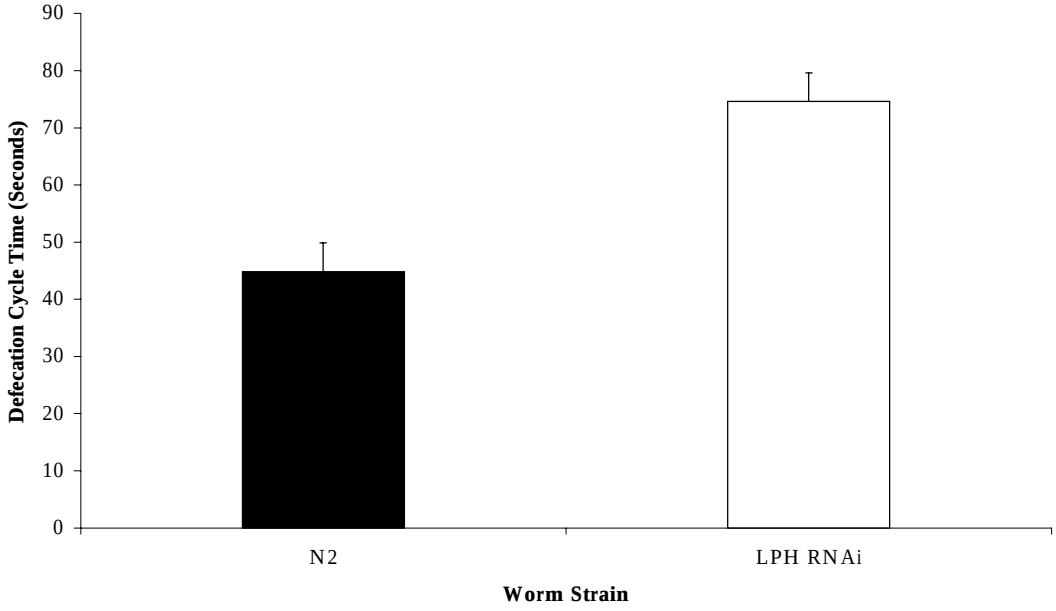


Figure 3.24 Expulsion interval in *C.elegans* defecation cycle.
Wild-type (N2) and latrophilin RNAi (LPH RNAi) worms were observed under DIC optics and the time between defecation expulsions (exp) counted for ten consecutive cycles (aboc, pboc, exp) for each of the ten animals. The results are presented as mean +/- standard deviation.

The defecation oscillator (aboc, pboc, exp) of *C.elegans* L4 larvae is characterised by the regularity of the cycle under normal temperature and nutritional status. This regularity makes it possible to distinguish between effects in aboc, pboc, exp period length or cycle variability in mutated worms.

For wild type worms the average defecation cycle is ~45-50 seconds. LPH RNAi affected worms showed a significant increase in this period, with an average cycle span of ~75 seconds.

Though there is a change in the defecation cycle, all the components of the cycle, aboc, pboc and exp were still present in the LPH RNAi *C.elegans*.

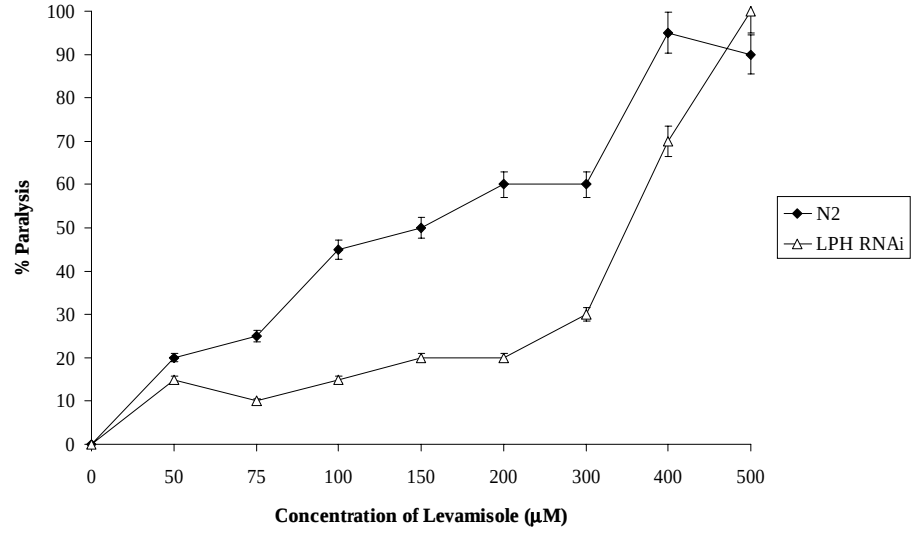
3.6.4 Sensitivity of latrophilin RNAi *C.elegans* to neuroactive agents

Worms with mutations of the gene *egl-4* have shown to have a constipated phenotype, similar to that observed in the LPH RNAi worms (Daniels *et al*, 2000). Furthermore, this

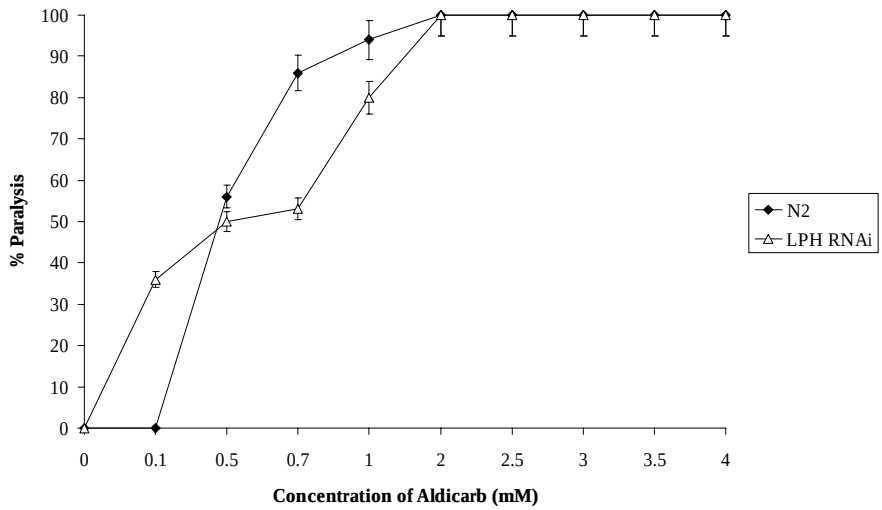
mutation has an effect on the response of the worms to neuroactive agents, such as aldicarb and levamisole.

In order to test whether there is altered neural function in LPH RNAi worms, the sensitivity of wild type and LPH RNAi worms to neuroactive agents was examined. Wild type and LPH RNAi *C.elegans* were exposed to three drugs. Levamisole, an acetylcholine receptor agonist, aldicarb, an acetyl cholinesterase inhibitor and imipramine, a seratonin re-uptake inhibitor. Both wild type and latrophilin RNAi *C.elegans* were exposed to the drugs as outlined in methods (sections 2.5.4, 2.5.5 and 2.5.6). Response of both wild type and LPH RNAi worms is shown in Figure 3.25.

A



B



C

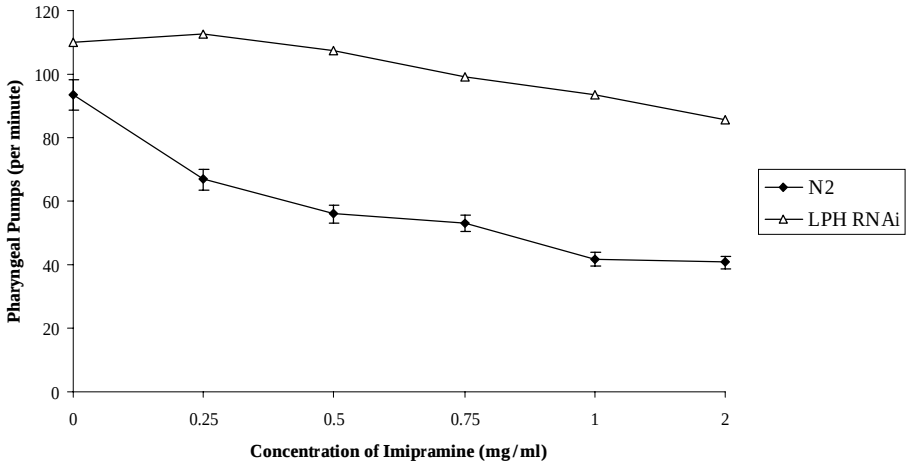


Figure 3.25 Sensitivity of latrophilin RNAi worms to neuroactive agents.
A. Wild type (N2-filled squares) or latrophilin RNAi (LPH RNAi-open triangles) *C.elegans* were exposed to levamisole and the number of worms paralysed counted. Each data point represents 25 worms per group.
B. Wild type (N2-filled squares) or latrophilin RNAi (LPH RNAi-open triangles) *C.elegans* were exposed to aldicarb and the number of worms paralysed counted. Each data point represents 25 worms per group.
C. Wild type (N2-filled squares) or latrophilin RNAi (LPH RNAi-open triangles) *C.elegans* were exposed to imipramine and the rate of pharyngeal pumping counted. Each data point represents 25 worms per group.

Latrophilin RNAi worms were less sensitive to levamisole than wild type worms, and were also less sensitive to imipramine induced decrease in pharyngeal pumping compared to wild type. However, latrophilin RNAi worms did not have significantly altered sensitivity to aldicarb. This data indicates that the silencing of the latrophilin gene in *C.elegans* has an effect on the nervous system of the worm compared to wild type worms.

3.7 Effect of LPH RNAi on BWSV toxicity in *C.elegans*

BWSV is toxic to wild type *C.elegans* over at least a million fold dilution range (Section 3.2.1). To examine what effect the inhibition of the latrophilin gene has on the toxic effects of BWSV, venom was injected into LPH RNAi worms over the same concentration range as that shown to be toxic in wild-type worms.

Adult, wild types *C.elegans* were injected with dsRNA complementary to the *C.elegans* latrophilin gene. The F1 progeny of these injected animals were grown to the adult stage at 20°C, so they were robust enough for BWSV injections.

A concentration range of BWSV (0-1200µg/ml) was set up as in section 3.2, and as in section 3.2, wild type *C.elegans* were injected with BWSV over the concentration range, along with the LPH RNAi worms.

To examine the effect of BWSV on LPH RNAi worms, 10 worms per concentration point were injected along with a negative control of venom buffer only (50mM Tris, 50mM NaCl, pH8). Worms were scored for any effects of the venom after incubation at 20°C for 2 hours.

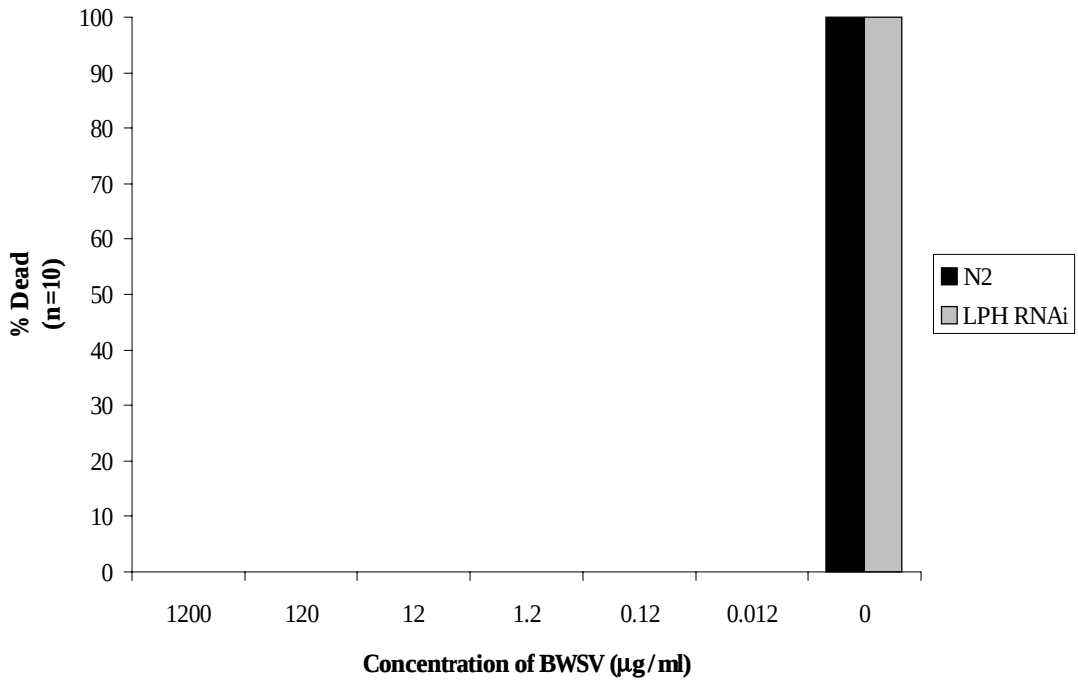


Figure 3.26 BWSV toxicity in LPH RNAi *C.elegans*.
LPH RNAi *C.elegans* (LPH RNAi) (10 per point) were injected with BWSV at the indicated concentrations (µg/ml) as were wild type (N2), or with control venom buffer (0) and allowed to recover for 2 hours at 20°C on standard NGM plates. Animals were scored after incubation and the % dead animals are shown.

Latrophilin RNAi worms were completely resistant to the effects of BWSV, which were shown in wild type *C.elegans* over a 10^6 fold range of venom concentrations. This demonstrates that the latrophilin gene in *C.elegans* is required for BWSV toxicity.

3.8 Effect of RNAi on BWSV toxicity in *C.elegans*

3.8.1 RNAi of CYP37A1 gene in *C.elegans*

To investigate whether the process of microinjection is related to the effects of BWSV seen in LPH RNAi *C.elegans*, an unrelated *C.elegans* gene, a cytochrome P450 known as CYP37A1, was silenced in *C.elegans* using RNAi and the gene silenced worms F1 progeny observed for any reaction to BWSV upon injection.

An EST of putative CYP37A1, yk147c9 (CE18566) was obtained from Professor Y. Kohara (National Institute of Genetics, JAPAN) as a phage clone. The Clone was subjected to phage excision (see methods) to yield clones in pBluescript SK- (Stratagene). A 0.78Kb cDNA insert of CYP37A1 was linearised from pBluescript by digest with restriction enzyme Pvu II, which excises the whole 0.78Kb cDNA fragment ready for *in vitro* transcription. *In vitro* transcription was performed using T7 and T3 polymerases to generate sense and antisense cRNA transcripts complementary to CYP37A1 in *C.elegans*.

1 2 3 4 5 6

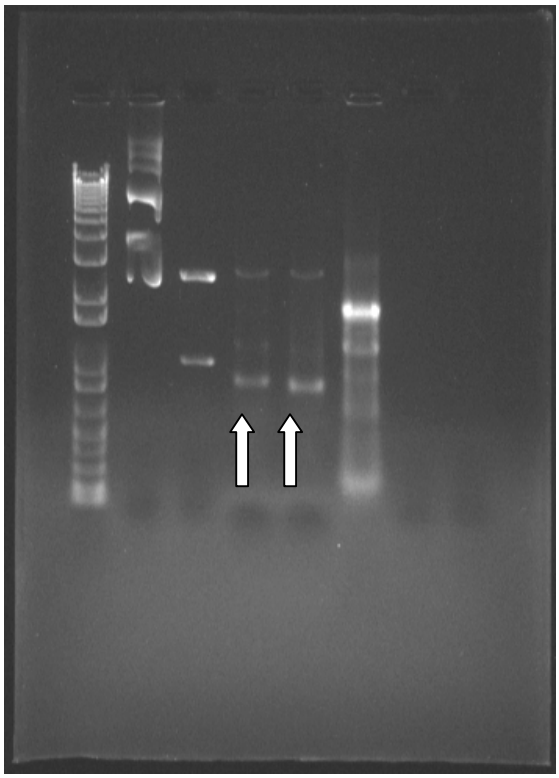


Figure 3.27 cRNA transcripts of CYP37A1 gene.
pBluescript vector containing 0.78Kb cDNA of *C.elegans* CYP37A1 (**2**) was digested by Pvu II restriction digest to remove the insert (**3**). Insert DNA was used as a DNA template for T7 (**4**) and T3 (**5**) transcription. Arrows indicate transcription products. Lane 1 contains 1KB+ DNA ladder to size fragments. Lane **6** contains total *C.elegans* RNA as a control of stability of RNA within the gel. Gel run was 1% pre-stained, 0.5μg/ml EtBr, 0.1% SDS denaturing, 1XTBE agarose gel run at a constant voltage of 65V for 2 hours.

cRNA transcripts were Dnase treated to remove the remaining DNA template and annealed at 88^{o}C for 10 minutes as described in Methods (Section 2.2.15).

10 N2 *C.elegans* were microinjected with 100μg/ml concentration of CYP37A1 dsRNA, worms were treated as in methods and the F1 progeny injected with BWSV at 1.2μg/ml concentration. After 2 hours at 20^{o}C injected worms were analysed for any effects caused by the injected venom.

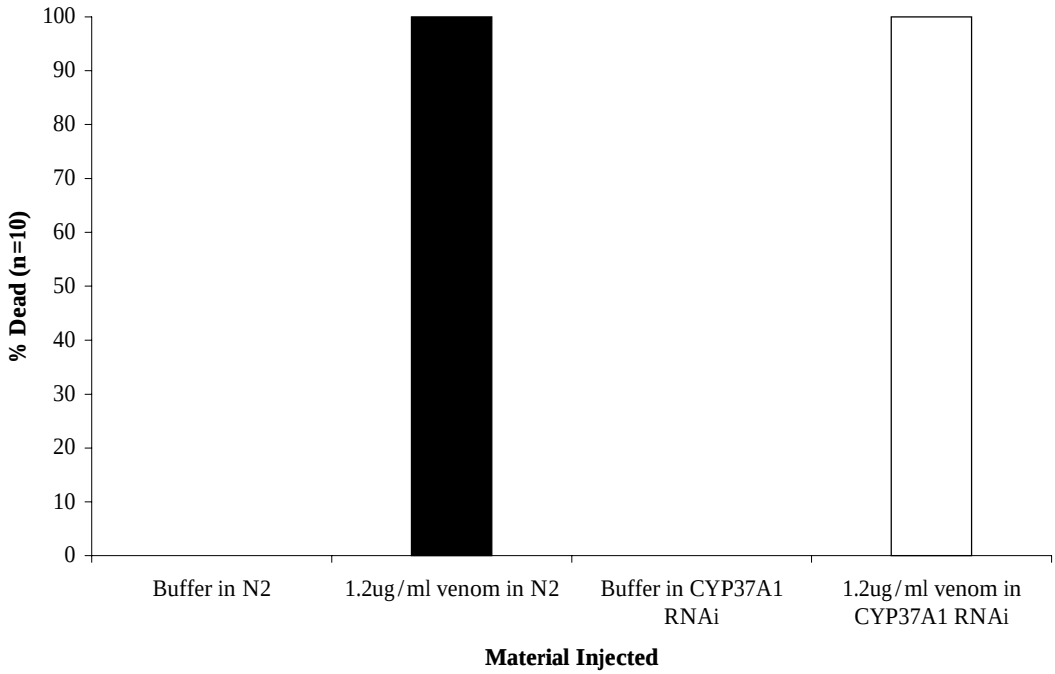


Figure 3.28 Effect of BWSV on CYP37A1 RNAi *C.elegans*.
CYP37A1 RNAi *C.elegans* (CYP37A1 RNAi) (10 per point) were injected with BWSV at a concentration of 1.2µg/ml as were wild type *C.elegans* (N2). Wild type *C.elegans* (N2+Buffer) and CYP37A1 RNAi (CYP37A1+Buffer) (10 per point) were also injected with venom buffer only as controls for the injection process. % Death of animals is shown after incubation at 20°C for 2 hours.

These results show that *C.elegans,* which undergo RNAi for CYP37A1, have the same sensitivity to BWSV as N2 worms.

Therefore, the silencing of a gene in *C.elegans* using RNAi does not affect the sensitivity of *C.elegans* to BWSV. This is important to eliminate any possible effects on *C.elegans,* which may be due to the process of RNA interference. The data in Figure 3.28 clearly shows that any effects, which occur in *C.elegans,* which have undergone gene silencing using dsRNA after injection of BWSV, are due to the venom and not the process of dsRNA interference.

This data also shows that animal death due to BWSV in CYP37A1 RNAi *C.elegans* occurs at the same concentration of venom (1.2µg/ml) as of that shown for the N2 worms.

3.8.2 RNAi of Neurexin 1α gene in *C.elegans*

Neurexin 1α is also known to bind the mammalian α-latrotoxin. Structurally, neurexins resemble cell-surface receptors with extended extra cellular sequences, a single transmembrane region and a short intracellular sequence.

To investigate whether a neurexin homologue in *C.elegans* has a role in BWSV toxicity, an EST of neurexin 1α, yk23f8 (CE25791) was obtained from Professor Y. Kohara (National Institute of Genetics, JAPAN) as a phage clone.

The Clone was subjected to phage excision (see methods, section 2.3) to yield clones in pBluescript SK- (Stratagene). A 1.01Kb cDNA insert of neurexin 1α was linearised by independent restriction digest with Sac I (for T7 transcription) and Kpn I (for T3 transcription) restriction enzymes. These enzymes linearise the plasmid for sense and antisense *in vitro* transcription.

In vitro transcription was carried out as described in methods (section 2.2.15) using T7 and T3 RNA polymerases to generate sense and antisense cRNA transcripts complementary to the neurexin 1α gene in *C.elegans*.

cRNA transcripts were DNAse treated to remove the remaining DNA template and annealed at 88ºC for 10 minutes. This annealing causes the sense and antisense cRNA transcripts to bind and form the double-stranded RNA complex, which is used to silence the neurexin 1α gene in *C.elegans*. 10 N2 *C.elegans* were microinjected with 100μg/ml neurexin 1α dsRNA. Worms were treated as in methods and the F1 progeny injected with BWSV at 1.2μg/ml. After 2 hours at 20ºC, injected worms were observed for any reaction to the injected BWSV.

1 2 3 4 5 6 7

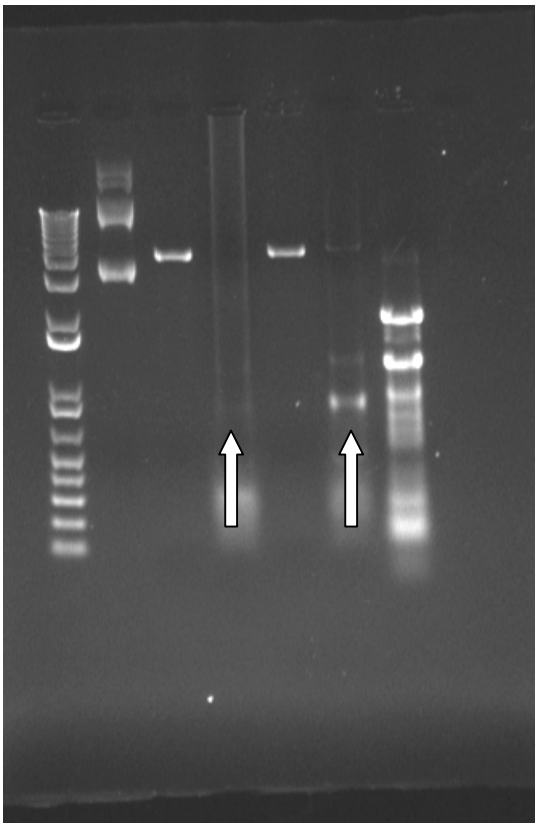


Figure 3.29 cRNA transcripts of neurexin 1α gene.
pBluescript SK- vector containing 1.01Kb cDNA of *C.elegans* neurexin 1α (**2**) was digested by Sac I digest (**3**) to linearise the plasmid for T7 polymerase transcription (**4**). Kpn I digest (**5**) was used to linearise the plasmid for T3 polymerase transcription (**6**). Arrows indicate transcription products. Lane 1 contains 1KB+ DNA ladder to size fragments. Lane **7** contains total *C.elegans* RNA as a control of the stability of RNA in the gel. Gel was run as a 1% pre-stained, 0.5μg/ml EtBr, 0.1% SDS denaturing, 1XTBE agarose gel at a constant voltage of 65V for 2 hours.

These results show that the gene for neurexin 1α in *C.elegans* has no mediatory effect on the toxicity of BWSV. Worms with the silenced gene showed the same sensitivity to injected venom as the wild-type worms. Negative controls of venom buffer only show no toxic effect in either neurexin 1α RNAi or N2 worms.

The data shown in Figure 3.30 suggest that neurexin 1α, has no effect on BWSV toxicity in *C.elegans*, in marked contrast to its effects in mammals (Sudhof, 2001).

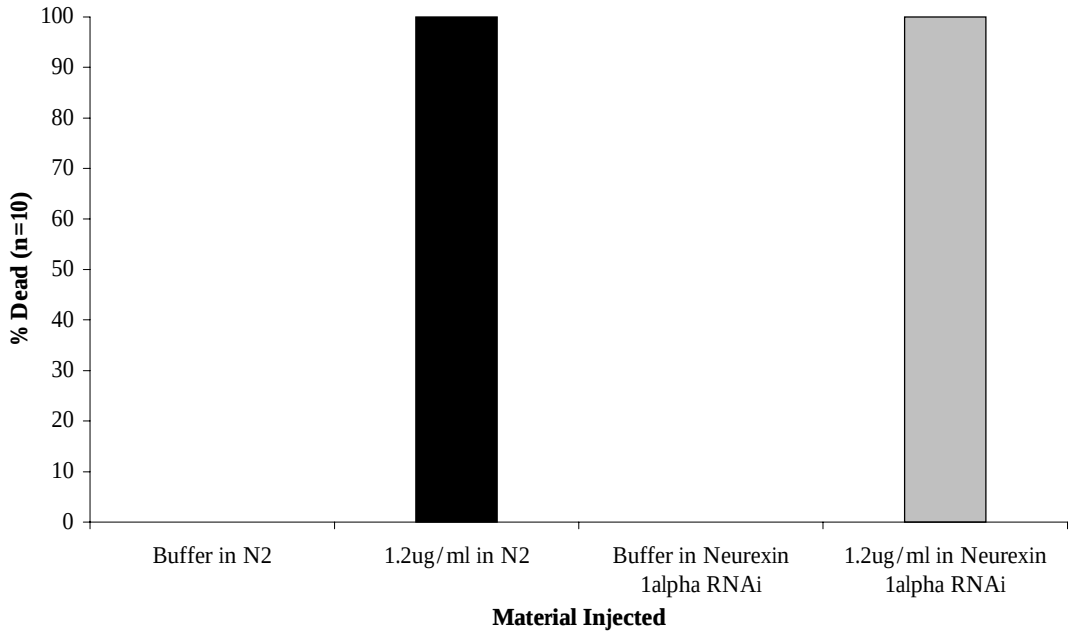


Figure 3.30 Effect of BWSV on neurexin 1α RNAi *C.elegans*.
Neurexin 1α RNAi *C.elegans* (neurexin 1 alpha) (10 per point) were injected with BWSV at a concentration of 1.2µg/ml as were wild type *C.elegans* (N2). Wild type *C.elegans* (N2+Buffer) and neurexin 1α RNAi (neurexin 1alpha+Buffer) (10 per point) were also injected with venom buffer only, as a control. % Death of animals is shown after incubation at 20°C for 2 hours.

3.8.3 Effect of BWSV on BO286.2 knockout *C.elegans*

It is possible that the latrophilin RNAi induced resistance to BWSV is the result of dsRNA interference with a gene closely related with BO457.1, such as other members of the secretin family of serpentine receptors in *C.elegans* (BO286.2, F155B9.7). To test this theory, a deletion mutant lacking the most closely related gene BO286.2 (provided by *C.elegans* Knockout Consortium) was injected with black widow spider venom.

BWSV was used at 1.2µg/ml concentration and injected into 10 wild type (N2) *C.elegans* and 10 BO286.2 gene knockout *C.elegans.* A further 10 worms from each population were also injected with venom buffer only as a control.

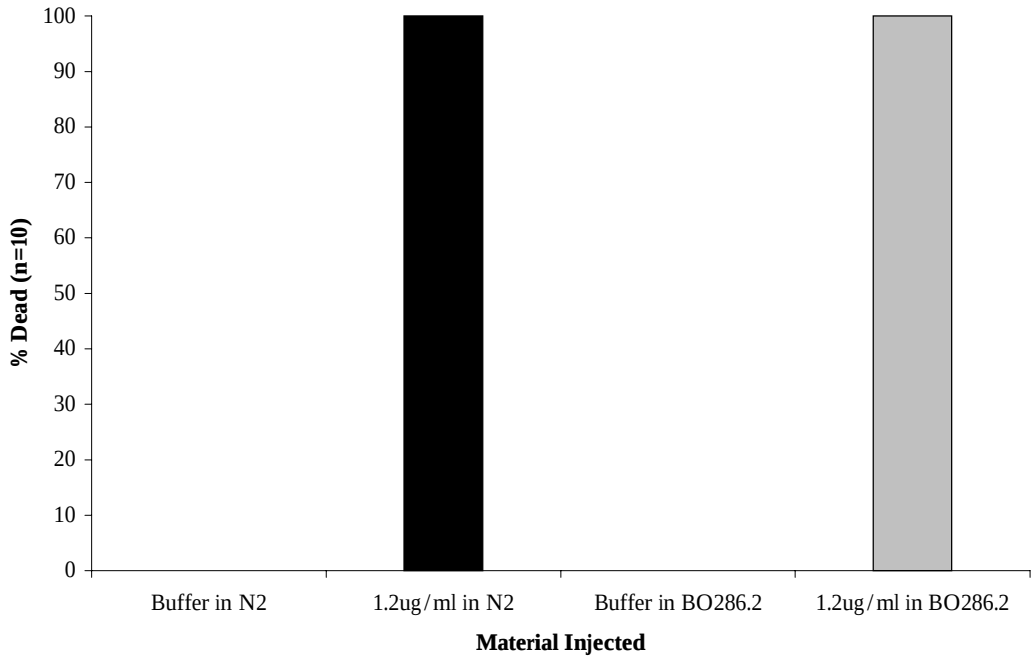


Figure 3.31 Effect of BWSV on BO286.2 knockout *C.elegans*.
BO286.2 knockout *C.elegans* (BO286.2+Venom) (10 per point) was injected with BWSV at a concentration of 1.2µg/ml as were wild type worms (N2+Venom). Wild type *C.elegans* (N2+Buffer) and BO286.2 (BO286.2+Buffer) (10 per point) were also injected with venom buffer only. % Death of animals is shown after incubation for 2 hours at 20°C.

These results show that *C.elegans* with a knockout of the gene BO286.2 have the same sensitivity to the toxic effects of BWSV as those seen in wild-type worms (Figure 3.31). BO286.2 is a closely related gene for latrophilin but has a lower sequence homology with the rat latrophilin gene than that seen for BO457.1.

This data shows that BO286.2 has no effect on *C.elegans* sensitivity to BWSV injection, as when injected with venom they show the same response as those seen in the wild-type worms.

3.9 RNAi in *C.elegans* by dsRNA feeding

The data in section 3.7 & 3.8 shows that *C.elegans* are resistant to the toxic effects of BWSV after RNAi introduced by microinjection. It was therefore decided to evaluate RNAi by feeding.

A fragment of the *C.elegans* latrophilin cDNA was isolated by PCR, from base 495-1631, having 39% identity with the latrotoxin-binding domain in the latrophilin receptor in the rat (Krasnoperov *et al*, 1999).

This 1.1Kb fragment lacks the 7-transmembrane domain, thereby obviating the possibility of non-specific cross-hybridisation with other 7-transmembrane receptors in *C.elegans*, and was used as a template for RNAi.

Studies involving RNAi have shown that the mode of interference can cross-cell boundaries (Fire *et al*, 1998). In RNAi by microinjection this crossing of cell boundaries explains why the dsRNA affects all the tissues in the worms body rather than those, which come into direct contact with the injection material. This migration of dsRNA through the organism allows other methods of dsRNA gene silencing to be used. Therefore, it is possible to induce RNAi by feeding *C.elegans* bacteria, which express the dsRNA being studied. The consumed bacteria will express dsRNA in the gut, which will migrate to all tissues throughout the worm's body (Fire *et al*, 1998).

3.9.1 Generation of pL4440: LPH

Oligonucleotides GGATGCCCT**GAATTC**GGACGTTATAAAACC and TTCAGATCGTGTCC**GAATTC**TCACATCAAAACAGC were used to PCR amplify bases 495-1631 of the latrophilin cDNA from *C.elegans*.

Agarose gel electrophoresis was used to identify and purify the PCR product; this gel is shown in Figure 3.32.

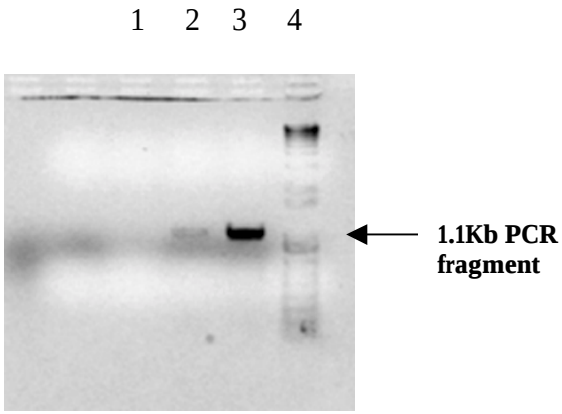


Figure 3.32 PCR of latrophilin fragment.
PCR was carried out as in methods and products run on a 1% pre-stained 0.5µg/ml EtBr 1XTBE agarose gel run for 1 hour at 80V. Lane **1** negative control, lane **2** PCR products at a 1:10 dilution in load buffer, lane **3** PCR product, lane **4**-1KB+ DNA ladder. PCR product of correct size is shown by labelled arrow.

The PCR product was extracted from the gel (section 2.2.8) and cloned into pGEM-T. pGEM-T containing the PCR fragment was EcoR I digested into the RNAi feeding vector pL4440 (gift from Julie Ahringer, CRC Cambridge). This gave the feeding vector pL4440: LPH.

pL4440: LPH plasmid was transformed into *E.coli* strain HT115 (Timmons *et al*, 2001) ready for use in RNAi feeding.

3.9.2 RNAi feeding using pL4440: LPH

RNAi feeding of *C.elegans* using pL4440: LPH plasmid was performed as described in methods along with the use of *dhc-1* as a control (Fraser *et al*, 2000).

Dhc-1 codes for the dynein heavy chain gene in *C.elegans*, which is involved in microtubule organisation in the maturing embryo. Silencing of the *dhc-1* gene will induce an embryo lethal phenotype. This is an excellent control gene to follow an RNAi feeding protocol with.

After feeding, F1 worms were analysed for phenotypes with *dhc-1* as a positive control, HT115 with no feeding vector as a negative control and pL4440: LPH as the experimental gene.

TEST GENE	n	% PHENOTYPE
Dhc-1	54	100
pL4440: LPH	62	100
No gene	56	0

Table 3.1 Responses of *C.elegans* to RNAi by feeding.

100% phenotype penetrance was observed for both *dhc-1* gene and pL4440: LPH. *Dhc-1* phenotype was the embryo lethal phenotype, with 100% embryos laid not developing. pL4440: LPH was identified as it exhibited the same constipated, elongated intestine seen in the original RNAi injections with the LPH gene, 100% of F1 progeny exhibited this phenotype.

3.9.3 Effect of BWSV on pL4440: LPH RNAi *C.elegans*

To examine whether the 1.1Kb fragment of *C.elegans* latrophilin cDNA has a mediatory role in BWSV toxicity in *C.elegans*, pL4440: LPH RNAi *C.elegans* were injected with BWSV at a concentration range of 0-1200μg/ml.

Adult *C.elegans* exhibiting the LPH RNAi phenotype were removed from the RNAi feeding plate and injected with BWSV at one of the selected concentrations. 10 worms were injected per concentration. This was repeated using wild type *C.elegans* as controls. Worms were scored for effect of the venom after incubation at 20^{o}C for 2 hours.

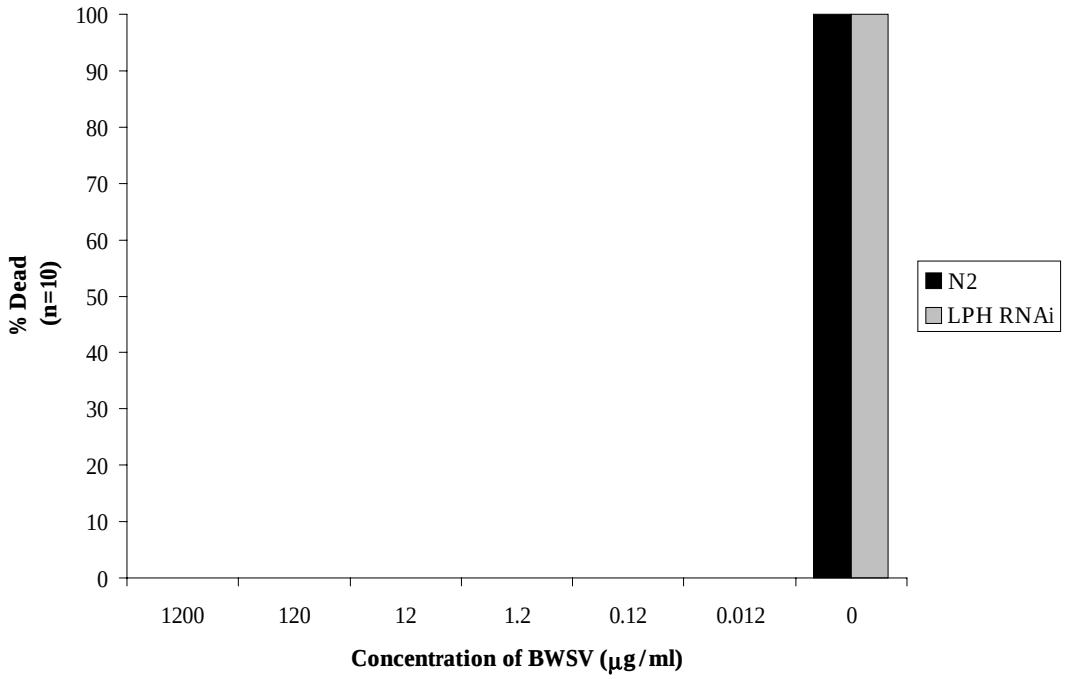


Figure 3.33 Effect of BWSV on pL4440: LPH RNAi *C.elegans*.
pL4440: LPH *C.elegans* (pL4440: LPH) (10 per point) were injected with black widow spider venom at the concentrations indicated (μg/ml) as were wild type (N2) *C.elegans*, or venom buffer only (0) and allowed to recover for 2 hours at 20°C on standard NGM plates.
Animals were scored after incubation and the % of dead animals shown.

pL4440: LPH fed *C.elegans* show identical responses to BWSV as those shown in LPH RNAi worms induced by microinjection using dsRNA from the complete LPH gene. This proves that the latrophilin gene in *C.elegans* is responsible for BWSV toxicity and the 1.1Kb fragment associated with the latrotoxin-binding site is directly responsible for the mediation of BWSV toxicity in the nematode *Caenorhabditis elegans*.

This data also shows that the section of the *C.elegans* latrophilin gene that with the highest homology with the rat latrophilin gene (39%) is integral to the response of *C.elegans* to injected BWSV. This is, as previously stated in this thesis (section 3.4), the domain responsible for latrotoxin binding.

3.10 Construction of an LPH: GFP construct

Gene fusion vectors are useful tools, allowing the expression of reintroduced genes to be monitored, and giving an indication of the distribution of the resulting products.
In this section, the generation of a reporter gene fusion of the *C.elegans* latrophilin gene, and the reporter green-florescent protein (GFP) is described.

3.10.1 Construction of an LPH: NLS: GFP: *lacZ* fusion vector

The promoterless reporter plasmid, pPD96.04, was selected from the Fire Lab Vector Kit, 1995 version (Andy Fire, personal communication). The vector encodes the SV40 nuclear localisation signal (NLS), S65C GFP mutant and *lac*Z with the *unc*-54 3' end. Using BamHI restriction digests (section 2.2.9) a 14.3Kb fragment of the *C.elegans* latrophilin gene (BO457.1) was inserted into the pPD96.04 vector using complementary BamHI digests. The BO457.1 cosmid (provided by *C.elegans* Knockout Consortium) has BamHI sites at 3984bp and 18383bp. The 18383bp site is in the middle of the exon 18329bp – 18441bp, this is the fusion site. This gives a 14399bp BamHI fragment for sub-cloning. This produced the LPH: NLS: GFP: lacZ fusion vector, which was named LPH: GFP, this plasmid is illustrated in Figure 3.34.

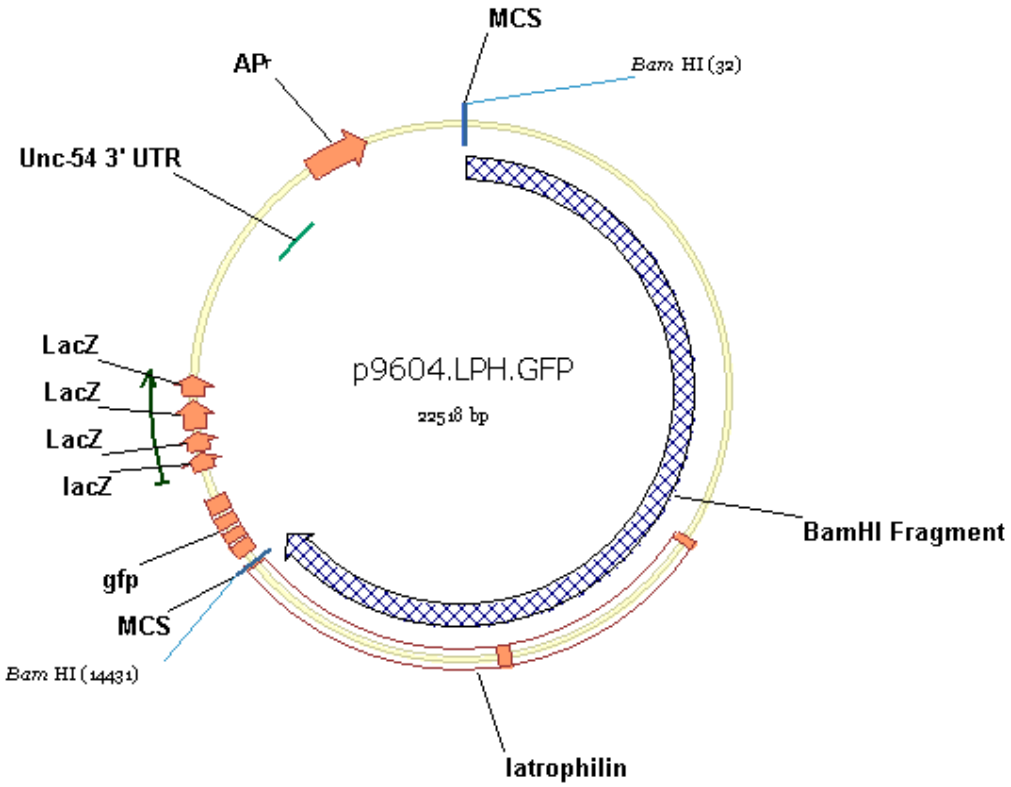


Figure 3.34 Plasmid map of LPH: GFP: NLS: *lacZ* fusion vector LPH: GFP. Plasmid was generated by subcloning of 14.3Kb fragment of BO457.1 into the GFP expression vector pPD96.04.

3.10.2 Transformation of *C.elegans* with LPH: GFP

Wild-type *C.elegans* were injected with the LPH: GFP fusion vector, at a concentration of 50µg/ml, along with the co-injection marker pRF4 also at a concentration of 50µg/ml as described in section 2.4.8.

pRF4 was used as the co-injection marker as it contains the semi-dominant right roller allele *rol-6* (Mello *et al*, 1991). The roller phenotype then allowed DNA transformed animals to be separated from the other worms in the F1 population.

Separate populations of *C.elegans* were also injected with UHP H_2O as a negative control and the vector pPD93.97 at a concentration of 50µg/ml plus 50µg/ml pRF4 as a positive control. pPD 93.97 is a body-wall muscle GFP fusion vector (L2370 *myo-3* N*gfp*) (Andy Fire, personal communication).

For each injection group (LPH: GFP, Negative & Positive controls), 30 wild-type *C.elegans* were injected.

After 3 days at 20°C, the numbers of roller phenotypes in the F1 generations were identified. This data is shown in table 3.2.

VECTOR INJECTED	n	Rolling F1 Progeny	% Transmission
None (UHP H_2O)	30	0	0
pPD93.97	30	40	5
LPH: GFP	30	47	6

Table 3.2 Transmission of rolling phenotype. Rolling F1 progeny present after microinjection of DNA expression vectors.

The resulting rolling F1 progeny from the originally injected adult *C.elegans* were transferred to new NGM plates in an attempt to generate F2 progeny that also carried the extrachromasomal arrays.

After a further 3 days at 20°C, the F2 progeny were analysed for the roller phenotype. This data is shown in table 3.3.

VECTOR INJECTED	Rolling F1	Rolling F2 Progeny	% Transmission
None (UHP H_2O)	0	0	0
pPD93.97	40	0	0
LPH: GFP	47	0	0

Table 3.3 Transmission of F1 rolling phenotype. Number of rolling F2 progeny present with the rolling phenotype transmitted from F1 progeny of adult *C.elegans* injected with different expression vectors.

As described in section 2.4.8, lines containing the selectable phenotype *rol-6* are required to have been transmitted through three generations before the worms can have the extrachromasomal array permanently integrated.

These experimental parameters were used to make sure that an integrated DNA transformation has occurred rather than just a transient mutation, which will be lost.

The data shows that the *rol-6* phenotype was only present in the F1 progeny and not in the F2 in both experimental injections (LPH: GFP) and the positive controls (pPD93.97). No roller phenotypes were seen in the negative controls as expected, this proves that the *rol-6* phenotypes seen in the LPH:GFP and pPD93.97 injections, was due to the integration of the injected DNA rather than a random mutation of artefact of the injection process.

The data shows 5% of pPD93.97 and 6% LPH: GFP F1 progeny with the roller phenotype. Usual % of F1 rollers are between 1-10% (Mello & Fire, 1995), so this data is within the expected frequency of transformed animals.

The fact that no F2 progeny retained the roller phenotype may be due to transient transformation, this has been shown to take place in DNA microinjections with the *rol-6* gene (Mello *et al*, 1991) rather than causing heritable transformants. Factors affecting the heritable transformation of DNA include the concentrations of DNA initially injected and the size of the vector being used (Mello *et al*, 1991). For this experiment to produce integrated transformants the number of injected animals and the concentration of DNA being injected would have to be optimised. This was not possible within this investigation due to the time constraints of the project.

3.11 RNAi of other genes in *C.elegans*

Further investigations were made using RNAi of a number of other genes in *C.elegans* which may have a neurotoxic effect in the nematode.

The gene families investigated were the glutamate receptor genes (GluR) and the genes for neuropathy target esterase (NTE).

Glutamate is an excitatory neurotransmitter present in both vertebrate and invertebrate species (Kawano *et al*, 1996). In addition to its role in synaptic transmission, glutamate has also been implicated in the pathophysiology of a number of neurodegenerative syndromes (Donevan *et al*, 1998).

Neuropathy target esterase is an integral membrane protein present in all neurons and some non-neural cell types in vertebrates (Glynn 1999). The NTE protein is modified by exposure to organophosphorus esters (OP), causing inhibition of acetylcholinesterase (AchE), the resulting neurotoxicity can be fatal (Glynn 1999).

By using RNAi of these genes in *C.elegans* it may be possible to elucidate more information regarding the toxicity of these genes.

ESTs of both GluR and NTE genes were identified using the *C.elegans* Genome Database. The ESTs identified were obtained from Professor Y. Kohara (National Institute of Genetics, JAPAN) as phage clones.

Table 3.4 shows the clones identified. All clones were in pBluescript SK- (Stratagene).

GENE	Clone Name	Insert Size	Total Plasmid Size
GluR	yk87h12	2.32Kb	5.27Kb
	yk122f9	1.26Kb	4.21Kb
	yk420e7	2.25Kb	5.20Kb
	yk130h3	2.62Kb	5.57Kb
	yk250d3	2.16Kb	5.11Kb
	yk348g10	3.23Kb	6.18Kb
	yk440c12	1.04Kb	3.99Kb
NTE	yk10b10	2.17Kb	5.12Kb
	yk248e4	1.09Kb	4.04Kb
	yk232g6	2.45Kb	5.40Kb

Table 3.4 GluR and NTE ESTs. Identified from *C.elegans* database and obtained as phage clones from Y. Kohara (National Institute of Genetics, JAPAN).

Clones were subjected to phage excision (methods, section 2.3) to yield clones in pBluescript SK-.

Plasmids were linearised by restriction digest with PvuII as described in Methods (section 2.2.9). PvuII was used as it removed the cDNA insert from each plasmid and could be used for both T7 & T3 polymerase driven transcriptions (methods, section 2.2.15).

In vitro transcription was carried out on each plasmid and the cRNAs used to generate dsRNA complementary to the genes of interest.

10 N2 C.elegans were microinjected with 100μg/ml dsRNA for each clone being tested.

Injected animals were transferred to standard NGM plates and allowed to lay eggs for 5 hours at 20^{o}C to expel pre-injection fertilised eggs.

Worms were transferred to new NGM plates after incubation and the F1 progeny grown to the L4 larval stage.

Control worms were also injected with UHP H_2O as a negative control and *tra-2* dsRNA as a positive control.

F1 progeny were analysed for change in phenotype, this data is shown in Table 3.5.

dsRNA Injected	**n**	**F1 progeny with change in Phenotype**
UHP H_2O	10	0 (all remain with N2 morphology)
Tra-2	10	10 (all show male phenotype)
yk87h12	10	0 (all remain with N2 morphology)
yk122f9	10	0 (all remain with N2 morphology)
yk420e7	10	0 (all remain with N2 morphology)
yk130h3	10	0 (all remain with N2 morphology)
yk250d3	10	0 (all remain with N2 morphology)
yk348g10	10	0 (all remain with N2 morphology)
yk440c12	10	0 (all remain with N2 morphology)
yk10b10	10	0 (all remain with N2 morphology)
yk248e4	10	0 (all remain with N2 morphology)
yk232g6	10	0 (all remain with N2 morphology)

Table 3.5 Change of phenotype present in F1 progeny of *C.elegans* injected with dsRNA corresponding to a number of different genes.

Data obtained from the RNAi experiments show that UHP H_2O has no effect on the phenotype of injected worms F1 progeny. The positive control of *tra-2* dsRNA shows a 100% change in phenotype from hermaphrodite worms to all being male in sex; these data support that already shown in sections 3.6.3.1 & 3.6.3.2.

No change in physical phenotype was seen in any of the GluR or NTE dsRNA injected *C.elegans* progeny. This though does not mean that there is no change in phenotype

taking place, often phenotypic changes are subtler and can be elucidated by behavioural assays or electronmicroscopy of particular tissues.

Further investigations into possible assays for identifying phenotypes in GluR and NTE dsRNA injected *C.elegans* were not possible due to time limitations involved in this study.

Chapter 4. Discussion.

The nematode worm *Caenorhabditis elegans* has a simple, yet uniquely well described, nervous system. The complete cell linage and a "wiring" diagram from serial section electron micrographs that show the synapses between neurons (Bargmann, 1998) are two elements, which contribute toward the wealth of information available about the nematodes nervous system. One area in which *C.elegans*, and its nervous system, has been used as a model system, is in the studying of the mechanisms involved in neurotransmission. Much work has used *C.elegans* to study synaptic vesicle release (Iwasaki *et al*, 1997; Nonet *et al*, 1997; Nonet *et al*, 1998; Zhao & Nonet, 2001). One of the aims of this thesis is to examine the role of Black Widow Spider Venom (BWSV) when used as a tool for studying neurotransmission in *C.elegans*, as the toxins contained in the venom are known to stimulate the release of neurotransmitters (Rash and Hodgson, 2002).

4.1 Microinjection of black widow spider venom kills *C.elegans*

BWSV causes neurotransmitter release in vertebrate, insect and crustacean nerve terminals (Frontali *et al*, 1976) due to the presence of high molecular weight proteins, the latrotoxins (Krasnoperov *et al*, 1992). However, there are no published reports of the effects of BWSV on *C.elegans*. Figure 3.2 clearly shows the response of *C.elegans* to BWSV over a range of venom concentrations.

Over a million-fold concentration range, BWSV was acutely toxic to *C.elegans* after microinjection. The reason for injecting the venom into the worms are; previous unpublished data (Bell & Mee) show that steeping or soaking worms in venom does not

result in toxicity, suggesting that the venom does not penetrate the tough outer cuticle of the worm.

The toxic effects of the venom caused death in the injected worms, 100% lethality was seen in all worms at each concentration with control showing 0% toxicity. This data illustrates a potent action of the venom in the nematode. Therefore, over a million-fold dilution (0.12-1200μg/ml) of BWSV there is 100% lethality taking place. This potent killing effect does not permit the calculation of an LD_{50} for the venom in *C.elegans*. Lowering the concentrations of venom further in an attempt to calculate an LD_{50} was not possible because at lower concentrations the toxic response from the venom was highly variable. This may be due to instability of proteins at these concentrations or to non-specific absorption to plastic or glass containers during experimental procedures. Lack of an LD_{50} for *C.elegans* toxicity makes a comparison with BWSV toxicity in other animals somewhat difficult. The most potent toxic component of BWSV is the insect-specific toxin α-latroinsectotoxin which in the larvae of *Galleria mellonella* has an LD_{50}=15μg/kg (Grishin, 1998). The levels of toxicity seen in *C.elegans* show 100% lethality at concentrations as low as 1.2ng/ml, this shows that the toxicity of whole venom in *C.elegans* is many times higher than that seen for a purified toxic component injected into its particular species, making *C.elegans* a highly sensitive measure of BWSV toxicity.

This indicates that a component of black widow spider venom is acting on *C.elegans* and causing a toxic response. This may suggest that there is a common mechanism of action, as seen in vertebrates with α-LTX. However, it is necessary to characterise this toxic element to determine if there is any shared mechanism of action.

BWSV presumably causes death in *C.elegans* by excitation of all neurons in the worm leading to massive neurotransmitter release, paralysis and then death. This is supported

by the observation that after BWSV injection, *C.elegans* develop a rigid straightening of the body before they die. They also show no response to touch, as if the muscles in the body wall have developed a type of "tetanus". This massive release of neurotransmitters is probably enough to cause death, the neurotransmitter acetylcholine is known to be the primary excitatory neurotransmitter in the worm and is also vital for life (Riddle *et al*, 1997) deficiencies in acetylcholine caused by pharmacological agents such as aldicarb cause paralysis and death in *C.elegans*. This is probably the same thing that happens with BWSV. Alternative scenarios to this method of BWSV induced killing include the possibility of the venom components inhibiting binding of the natural ligand to an important receptor in *C.elegans*, resulting in the prevention of an important physiological function taking place, which ultimately leads to the death of the worm. To investigate what the actual mechanism of BWSV induced killing is, electron microscope (EM) sections of synapses in *C.elegans* could be compared in worms that have been exposed to BWSV and those that have not. A classic response to BWSV in mammals is the lack of synaptic vesicles in the pre-synaptic membrane caused by the venom, which can be seen by EM section. If BWSV induced death is consistent in *C.elegans* as it is in mammals then worms, which were not exposed, to venom would have vesicles present in the pre-synaptic membrane whereas those exposed to BWSV would not.

4.2 BWSV toxicity in *C.elegans* is caused by a protein

The major constituents of spider venoms are protein, polypeptide and polyamine neurotoxins, enzymes, nucleic acids, free amino acids, monoamines and inorganic salts (Rash and Hodgson, 2002). In BWSV, separate components of the venom are active against mammals, insects and crustaceans, but each of the toxins is known to be a high molecular weight protein of which all are 100kDa or more in size. Indeed, even toxin

components of the same class vary in size, the insectotoxins range from 110kDa for δ & ε toxins to 140kDa for β insectotoxin, (Krasnoperov *et al*, 1992) though all of these components are high molecular weight proteins.

If the mechanism of action of BWSV was conserved, it would be predicted that the component of the venom, which is lethal to wild-type *C.elegans*, is also a high molecular weight protein. Therefore, venom was treated with denaturing agents as a preliminary determination of the role of the proteins in BWSV. The agents used were heat (Figure 3.3) and SDS at a concentration of 0.1% (Figure 3.4).

Both heat treatment and SDS treatment cause protein denaturation by breaking the bonds, which hold together the secondary and tertiary structure of the protein, and this change in structure causes a change in function. Both of these treatments caused the complete ablation of the toxicity seen in *C.elegans*, strongly suggesting that the active component, which caused the toxicity, is a protein. This component could therefore be a latrotoxin, though this experiment does not indicate whether the active component is a high or low molecular weight protein.

These data also show that denaturation by both chemical and thermal agents leads to venom denaturation. Proteins can be degraded by other methods; indeed, latrotoxins themselves are thought to be cleaved while in the venom gland of the spider by endoproteases (Sudhof, 2001) to give the mature 120kDa protein (Dulubova *et al*, 1996). An additional approach to reinforce this experimental question would involve using proteases, which could also be used to degrade BWSV, to see whether cleaving the high molecular weight protein has an effect on the potency of the venom in *C.elegans.*

Therefore, the component of venom, which causes toxicity in *C.elegans*, is a protein, but the data does not indicate if this is a high molecular weight protein such as a latrotoxin.

4.3 Purified α-latrotoxin has no toxic effect on *C.elegans*

BWSV contains a number of different components, which have already been identified (Krasnoperov *et al*, 1992). So far, seven separate active components of BWSV have been identified and isolated (Grishin, 1998) these are α-latrocrustatoxin (α-LCT), a crustacean specific protein, five insect specific proteins, α-latroinsectotoxin (α-LIT), β-latroinsectotoxin (β-LIT), γ-latroinsectotoxin (γ-LIT), ε-latroinsectotoxin (ε-LIT), δ-latroinsectotoxin (δ-LIT), and one vertebrate specific protein α-latrotoxin (α-LTX). The abundance of components in BWSV shows the specificity of the venom to a number of species. It would be expected that insect specific toxins would be most abundant as these are the main prey of the spiders but it is more surprising that the venom should contain crustacean and vertebrate specific toxins, though these may have evolved as defence mechanisms.

The specificity of α-LTX, which makes it the only venom protein toxic to vertebrates (Frontali *et al*, 1976), was examined with the injection of purified α-LTX into *C.elegans*. Figure 3.5 shows that while crude BWSV of a concentration of 1.2μg/ml causes 100% lethality in a population of wild-type *C.elegans*, injection of purified α-LTX at 240μg/ml causes no toxicity (0% lethality) in wild-type *C.elegans*. This data shows the specificity of α-LTX to vertebrates because it caused no response in the invertebrate worm, while total venom showed the high levels of toxicity seen in previous experiments contained in this thesis.

This data also shows that a different component of BWSV is responsible for the toxic effects of the venom seen in *C.elegans*, which may therefore be α-LCT, one of the insectotoxins or perhaps a new venom component that has yet to be separated or identified. The components of BWSV described already have been separated by

chromatographic fractionation. A similar method was used to identify the specific venom component, which causes the toxic effects that have been observed in *C.elegans.*

4.4 Identification of a component of BWSV toxic to *C.elegans*

Size exclusion chromatography separates the component proteins in BWSV by size. Eluted fractions from the column were tested for their toxic effects in *C.elegans* by microinjection of a sample of the fraction into wild-type worms. The response of *C.elegans* was analysed as in previous experiments into the toxicity of BWSV in the nematode. Five worms were tested for each separated fraction from the column and the % lethality plotted on the chromatograph for each fraction.

Peak toxic fragments correspond to eluted proteins of a size of >158kDa. This shows the presence of high molecular weight proteins, which are toxic to *C.elegans.* If, the mechanism of action of venom components is conserved then the venom component toxic to *C.elegans* should also be a high molecular weight protein. This data supports that theory; and validates the use of a size exclusion column to purify the venom and eliminate any low molecular weight contaminants, i.e., those of under 70kDa in size. The data in Figure 3.7 shows that fractions containing low molecular weight proteins have negligible toxicity in *C.elegans.* Analysis of the protein content of these fractions by electrophoresis shows that the early eluted fractions from the column, may contain high molecular weight proteins, but these show little toxicity in *C.elegans.* Those of a size of around 200-100kDa in fractions 9-12 which show the highest lethality correspond to when latrotoxins would be expected to be eluted from the column. These fractions do still have some low molecular weight contaminants, which could be associated with the toxicity seen in *C.elegans.* Yet fraction 14, which has no high molecular proteins, showed 0% lethality in *C.elegans* (data not shown), this reinforces the argument that high

molecular weight latrotoxins are responsible for BWSV toxicity in *C.elegans*. Though fractions 9 & 10 are those with peak toxicity, it is fractions 11 & 12 which have a molecular weight of ~100kDa. However, PAGE analysis of the fractions shows that the apparent protein sizes in fractions 9 & 10 are similar to those in 11 & 12. This can be explained by dimerisation of the latrotoxin monomers to form dimers of 200 – 280kDa, as is known for α-LTX (Ashton *et al*, 2000).

Pooled toxic fractions 9, 10, 11, 12 from SEC chromatography were separated on UnoQ column using the method of Krasnoperov (1992). Toxicity of eluted fractions from the UnoQ column was again tested by injection into wild-type *C.elegans*.

The method of Krasnoperov (1992) contains two purification steps by MonoQ column, whereas the approach used in this thesis uses SEC to eliminate contamination by low molecular weight proteins, followed by the initial MonoQ separation as per the Krasnoperov (1992) method. A comparison of the chromatograph produced in Figure 3.9, with the chromatograph produced in Krasnoperov (1992) allows the lethality peaks from the *C.elegans* injections to be compared with the positions of proteins eluted in the same order, and the same relative abundance as those in Krasnoperov (1992).

Ion exchange chromatography revealed the presence of multiple proteins of high molecular weight (~120kDa), which were toxic to *C.elegans*. Peak toxicity from UnoQ separation is in Fractions 16 & 22, with Fraction 16 having 80% lethality and Fraction 22 100% lethality. The order of eluted latrotoxins from Krasnoperov (1992) shows initial fractions containing latrocrustatoxins, followed by latroinesctotoxins and finally the vertebrate specific toxin α-latrotoxin, though the later fractions containing LTX has also δ-LIT present. Fractionation of SEC separated toxin on UnoQ would be expected to elute Latrotoxins in the same order with the same relative abundance as those in Krasnoperov (1992). Therefore, the proteins eluted early from the UnoQ column should be associated with LCT, toxicity data shows that early-eluted fractions show little

nematode lethality (20%, Fraction 10). Proteins eluted in the middle of the column run show the highest levels of toxicity and it would be interesting to determine if these fractions correspond to the insectotoxin fractions described by Krasnoperov (1992), although we have not during the scope of this thesis been able to determine this. It is unclear if the most toxic fraction (22) corresponds to the fraction containing α-LTX and δ-LIT. Testing of this fraction for vertebrate activity would show that the toxin had been separated in this fraction. However, direct testing of purified α-LTX showed it is not toxic to *C.elegans*, and so it may be that δ-LIT, or an unidentified protein, are responsible for toxicity to *C.elegans*. Analysis of the toxic fractions by PAGE shows an abundance of high molecular weight proteins though a number of the fractions also show the presence of low molecular weight contaminants, which were either not fully removed by SEC, or arise from proteolysis. As the eluted fractions from the UnoQ column contained multiple proteins, a further round of purification was undertaken to isolate the components of BWSV that showed toxicity in *C.elegans.*

Krasnoperov (1992), followed a program of initial MonoQ separation of venom at pH8, fraction B from this column fractionated toxins showing insect specific toxicity, by further purifications of fraction B, first using MonoQ at pH5.8 and then MonoS at pH6.2, Krasnoperov separated two insect specific toxins named γ & ε-LIT.

Identification of medium-salt fractions from our UnoQ column (Figure 3.9 & 3.10) allowed these fractions (14-16) to be pooled and separated using UnoS at pH6.2. Data generated in Figure 3.11 shows a significant reduction in the toxicity of the eluted fractions than those fractions, which were tested after separation on UnoQ. No fraction showed a higher lethality than 40% after a 1:500 dilution, and this activity was present principally in two fractions, 15 and 19. Fraction 15 when analysed by PAGE shows that separation has produced a high molecular weight protein with a molecular mass of

~115kDa, with no low molecular weight contaminants. The separation of γ and ε-LIT using a similar method show striking similarities (Krasnoperov *et al*, 1992). Both elute as a medium salt pool from an anion exchange column, and γ-LIT elutes just before ε-LIT on subsequent cation exchange chromatography at moderate salt concentration (ca. 0.2Molar) (Krasnoperov *et al*, 1992). γ-LIT is noticeably bigger than ε-LIT (120 Vs 110kDa), and ε-LIT is markedly more abundant than γ-LIT. These proteins are very similar to the proteins eluted from the UnoS at fractions ~12 and 15 respectively, they have a similar molecular mass and are eluted with the same relative abundance as the protein in fraction 15, though due to the later elution position of fraction 15, it would appear that the toxic component is probably the later eluted toxin ε-LIT which comes off the UnoS column at 0.26M NaCl compared to 0.2M NaCl for γ-LIT (Krasnoperov *et al*, 1992). Fraction 19 also shows 40% lethality upon microinjection into *C.elegans* this contrasts with other studies where, there are no toxic BWSV components purified this late in the salt gradient, toxicity in *C.elegans* from this fraction may be due to a latrotoxin which is not toxic to other organisms. Therefore, purification of BWSV using FPLC has identified a component of venom fraction 15, which is both toxic in *C.elegans* when microinjected, but also homogenous when analysed on PAGE (Figure 3.13), demonstrating that it is a high molecular weight latrotoxin which mediates toxicity in *C.elegans.*

Comparison of toxic fractions taken at each stage of the venom purification was further compared by LDS-PAGE. Unfractionated BWSV, venom after SEC, low and high salt kill peaks from UnoQ, and Fraction 15 from UnoS was compared. Neat BWSV shows the presence of many high and low molecular weight proteins as expected of this complex mixture. SEC shows the removal of low molecular weight contaminants leaving proteins of molecular weights in excess of 97kDa only, effectively leaving all the

latrotoxins. Low and high salt kill peaks show similar components, though there does appear to be some low molecular weight proteins still present in the high salt kill peak. UnoS yields a protein, which is 110kDa in size (Figure 3.13), which we have shown has similar chromatographic properties to ε-latroinsectotoxin (Figure 3.11).

It would seem that the fraction of BWSV toxic to *C.elegans* is an insectotoxin though we have not tested the fractions in insects, toxicity to *C.elegans* is ~1-2μg/kg which is much greater than the toxicity of ε-LIT in insects which has an LD_{50}~1000μg/kg. The toxicity of ε-LIT to *C.elegans* does not appear to be a feature common to all latroinsectotoxins, since a fraction (12) putatively containing α-latroinsectotoxin had only marginal toxicity to *C.elegans* (Figure 3.9), although Krasnoperov (1992) found it to be highly potent against insects with an LD_{50}~15μg/kg.

The data generated has allowed the separation of numerous latrotoxin venom components, which are not toxic to *C.elegans*, and, is consistent with the widely accepted view that latrotoxins show pronounced species-specificity, and more importantly shows that specific high molecular weight latrotoxins are responsible for the toxicity of BWSV to *C.elegans*. Purification of low salt peak fraction from UnoQ separation on UnoS column produced the homogenous component of BWSV toxic to *C.elegans*. Further analysis though could be made on the high salt peak fraction from the UnoQ column (Fraction 22, Figure 3.9). PAGE of this fraction (Figure 3.13) shows it still contains multiple high molecular weight proteins as well as low molecular weight proteins of ~70 & 44kDa. It is feasible that further purification of this fraction could lead to finding other components of BWSV with toxicity to *C.elegans*

Peptide sequence analysis of the *C.elegans* toxic fragment separated from BWSV gave a total of three peptide sequences, which are shown in Section 3.3.5.2. These peptide sequences are somewhat difficult to interpret, as they are not complete, and are

ambiguous in assigning e.g. Leu for Ile. Further MS/MS sequencing is required with a greater amount of protein to allow better peptide sequencing to take place, the resulting data would then be expected to determine the similarity of these peptides to those already known regarding latroinsectotoxins. The lack of a full primary sequence for ε-LIT is a significant handicap in attempting to assign the identity of the purified protein. Therefore, our data from studying the effects of BWSV on *C.elegans* strongly suggests that a latrotoxin is responsible for the death caused in the worm when injected with BWSV. This is because we have shown that a specific component causing lethality is a high molecular weight protein, which is both heat and chemical sensitive and has the same elution properties when separated using FPLC as that of latrotoxins, which have been described in separate publications. This protein also shows the same properties as other latrotoxins when analysed on PAGE and has visual, and, chromatographic similarity to the previously characterised latrotoxin, ε-latroinsectotoxin.

4.5 Latrophilin gene in *C.elegans*

Data generated in this thesis has shown that the venom of the black widow spider is toxic when injected in to the nematode *C.elegans*. FPLC separation of the proteins in the venom has shown that the component responsible for this lethality in the worm is a protein and that this protein is a high molecular weight latrotoxin.

This toxin is similar to a previously described insect-specific toxin with the name ε-latroinsectotoxin. *C.elegans* therefore responds to latrotoxins in a similar way to crustaceans, insects and mammals, which have already been shown to react to BWSV toxicity.

The mammalian specific toxin, α-latrotoxin is the most studied component of BWSV due to its ability to cause massive neurotransmitter release at synapses (though this is true for both crustacean and insect toxins also), the toxin has been shown to act by a number of mechanisms, which are all still not fully understood. Either way, α-latrotoxin will bind to mammalian receptors called latrophilin/CIRL and neurexin. Therefore homologues of these receptors may be responsible for the mediation of latrotoxin-induced toxicity. Database analysis of the *C.elegans* genome identified a homologue for the latrotoxin receptor latrophilin. This homologue, named BO457.1, was identified as one of the secretin family of serpentine receptors. Of this family, five sub-family branches with homologues in *C.elegans* are known, they are, flamingo-cefla (F15B9.7), latrophilin-1 (BO457.1) and 2 (BO286.2), calcitonin receptor (C13B9.4), corticotrophin releasing factor (ZK643.3) and secretin receptor (C18B12.2) (Mastwal & Hedgecock, personal communication). All of these receptors are characterised by being 7-transmembrane repeat and G-protein coupled receptors. Both BO457.1 and BO286.2 have similarity with the rat latrophilin receptor, yet BO457.1 has the greater similarity of the two. Overall sequence comparison of the rat latrophilin gene and BO457.1 shows identity is low at only ~30%, but identity to rat latrophilin at the domain defined for the binding of α-latrotoxin (Krasnoperov *et al*, 1999) is significantly higher at ~39%, this shows that sequence similarity is greater in domains which are associated with the binding of latrotoxins. The cDNA (3.045Kb) and protein structures of BO457.1 are shown in Figure 3.14, the cDNA encodes a 1014 amino acid peptide with a predicted p*I* of 7.2. Further comparisons of sequence identity between the rat and *C.elegans* latrophilin homologues were carried out by Pfam 6.6 alignment (Figure 3.15). These alignments showed further conservation between the two proteins with previously identified domains remaining conserved, these include a galactose-binding lectin domain and a hormone receptor motif at 181-240 amino-acids which contains four conserved cysteine

residues with the rat. A G-protein receptor Protease cleavage Site (GPS) also with four cysteines conserved with the rat latrophilin and a 7-transmembrane domain of the secretin family are present. The BLAST analysis also identified a novel sequence motif immediately C-terminal to the 7-transmembrane domain, containing four conserved cysteine residues. This domain is only 55 amino acids in length, but shows marked conservation, ~50% identity, with a number of higher mammalian proteins of the G-protein coupled receptor superfamily. The function of this domain is unclear, but the two pairs of conserved cysteines may well be used to form a higher order structure.

4.6 RNAi of the latrophilin gene in *C.elegans*

RNAi or RNA interference is the phenomenon of gene silencing by exposure to double-stranded RNA complementary to the gene of interest. This phenomenon was initially observed in *C.elegans* (Fire *et al*, 1998) but has now been observed in fungi, plants, invertebrates (*C.elegans*, *Drosophilia*) and vertebrates (Zebrafish, Mice) (Bosher *et al*, 2000). The reverse genetic technique allows the analysis of the gene function of the *C.elegans* latrophilin gene to take place by identification of a phenotype, using the identified gene sequence.

The ~3Kb cDNA inserted into pGEM-T was used to generate dsRNA complementary to the latrophilin gene by *in vitro* transcription. Injection of the dsRNA into *C.elegans* generated a phenotype that was characterised by a number of behaviours.

F1 progeny of the dsRNA injected worms showed a visual phenotype consisting of an extended gut peristalsis cycle and apparent constipation. This constipated phenotype corresponds to phenotypes observed in genomic knockout worms, which are associated with synaptic transmission and presynaptic assembly. These genes include, *aex-3*, *dec-4*, *egl-2*, *8*, *10*, *19*, *30*, *36* and *exp-2*, in total 25 different genes when knocked out in *C.elegans*

show the constipated phenotype. These genes are responsible for many nervous system functions, for example, *aex-3* is a regulator of presynaptic activity related to the defecation cycle, and is a putative ortholog of human MAP-kinase activating death domain protein, MADD (Iwaski *et al*, 1997), *egl-10* is a member of the G-protein gamma subunit protein family. The fact that all of these are genes associated with synaptic activity in *C.elegans* suggests that the gene for latrophilin in *C.elegans* is also responsible for some aspect of the nervous system; this is an interesting novel finding as, though latrophilin has been shown to be responsible for triggering uncontrolled synaptic activity in mammals it has not, as far as we know, been implicated in the synaptic activity of *C.elegans* until now. Further characterisation of the phenotype shows that the silencing of the latrophilin gene has affected the defecation cycle in *C.elegans*. Defecation in the nematode *C.elegans* is achieved by a cyclical stereotyped motor program, the first stage being the contraction of the posterior muscles (pboc), contraction of the anterior muscles (aboc) and then the expulsion (exp) of the gut contents (Thomas, 1990). The standard *C.elegans* defecation motor program occurs approximately every 45-50 seconds and is an example of a circadian rhythm in the worm (Fred Kippert, personal communication), the defecation oscillator consists of the aboc, pboc and exp periods, which were all present in the LPH RNAi worms but there was a significant increase in the average cycle span from ~45-50 to ~75 seconds.

This change in defecation cycle time may be due to LPH RNAi interfering with some aspect of the defecation cycle which also has an effect on the ability of the worm to expel food from its gut. *Aex-3* is related to the defecation cycle because it is a regulator of presynaptic activity involved in the cycle. It may be that the latrophilin gene has a normal function in *C.elegans* associated with the defecation cycle and its regulation. Any change in the cycles regulation could then be affecting the worms capacity to remove food from it gut. Conversely, silencing the latrophilin gene may cause an accumulation of food due to

an effect on another aspect of the worms gut regulation this food accumulation may then influence the length of the defecation cycle.

4.7 Latrophilin RNAi affects *C.elegans* sensitivity to neuroactive compounds

Another aspect of characterising phenotypes involved with the nervous system is that more information regarding the type of mutation can be elucidated by exposing the worms to a number of pharmacologically active agents and then comparing them to known responses seen in wild-type worms.

Three substances were selected to test on *C.elegans* and LPH RNAi *C.elegans*. Aldicarb, an acetylcholine esterase inhibitor, is primarily used in behaviour studies as an indicator of whether a mutation is post or presynaptic in nature (Riddle *et al*, 1997). This is possible because the mutation can be classified as pre or postsynaptic in nature by two principle criteria. Firstly, levels of acetylcholine are usually raised in mutant worms with a presynaptic defect, due to the accumulation of unreleased transmitter, but will be normal in mutants with defects, which are postsynaptic. Mutants with postsynaptic alterations will be resistant to the acetylcholine receptor agonist levamisole while presynaptic mutants will have a normal or even raised sensitivity to levamisole.

The normal response of wild-type worms to aldicarb or levamisole is paralysis, as any interference with acetylcholine, the only neurotransmitter essential for worm viability (Riddle *et al*, 1997), will at high enough concentrations induce death.

Experiments with aldicarb and levamisole on wild type and LPH RNAi *C.elegans* show a normal response of the wild-type worms to both drugs, as concentrations increase the % paralysis increases until saturation occurs and all the worms are paralysed (Figure 3.25). LPH RNAi worms exposed to aldicarb do not show an altered sensitivity to the drug

compared to wild type. Worms that have a presynaptic mutation usually show an increased level of paralysis than that seen in wild-type, this would be expected in LPH RNAi worms as the receptor latrophilin is a presynaptic receptor in mammals (Sudhof, 2001), any mutation in *C.elegans* would therefore be expected to confer some increased sensitivity to aldicarb. Exposure to levamisole shows a reduced sensitivity of LPH RNAi worms to the drug, resistance of a mutation to levamisole (such as *Lev-1*) is usually used as criteria for identifying mutations, which are postsynaptic.

Therefore, the pharmacology of the latrophilin knockout worms suggests that latrophilin in *C.elegans* is involved in a postsynaptic process, whereas α-latrotoxin works on receptors such as latrophilin at the presynaptic nerve terminal. This is a somewhat confusing situation, as previous studies have used the sensitivity of mutated worms to aldicarb/levamisole as a method of identifying the type (post or pre) of synaptic defect present. It should be noted that the latrophilin gene in *C.elegans* is being silenced by RNAi, and is not a complete knockout. Specifically, RNAi is characterised as not working in the nervous system completely. It may therefore be that these represent actions in peripheral nervous tissue. Therefore, the 3045bp cDNA is being silenced, but as this is not a complete genomic knockout there may still be some residual activity of the receptors involved, and this could have an effect on the worm's response to the drugs. What this data shows, is that several neuroactive agents have different effects in LPH RNAi worms, although it is not clear whether this is due to a direct effect on the nervous system. A third pharmacological agent was used on LPH RNAi worms to examine effects to the worm's nervous system of silencing the latrophilin gene. Imipramine is a serotonin re-uptake inhibitor. Serotonin stimulates egg-laying and pharyngeal pumping in *C.elegans* (hence the use of counting pharyngeal pumps as a measure of the effects of imipramine) (Riddle *et al*, 1997). Therefore, the prevention of serotonin re-uptake will cause the number of pharyngeal pumps to increase with an

increase in drug concentration, as a greater concentration of serotonin is available in the synaptic cleft due to its inability to be recycled, this will therefore increase stimulation of the pharynx. Exogenous serotonin can also inhibit locomotion and defecation in *C.elegans.*

Data in Figure 3.25 (C) shows the number of pharyngeal pumps made per minute by wild type and LPH RNAi *C.elegans* when exposed to imipramine concentrations of 0-2mg/ml. Wild type *C.elegans* show a drop in the number of pharyngeal pumps taking place per minute from ~95 for worms exposed to no drug, to ~40 for worms given 2mg/ml, higher concentrations of imipramine were not used as complete pharyngeal shut-down occurs in the presence of concentrations of 5mg/ml. In LPH RNAi *C.elegans* worms show a lowering in response to imipramine, at 0mg/ml worms have a rate of ~110 pumps per minute and at 2mg/ml a rate of ~85 per minute. The first comparison to make is that resting pharyngeal rates are different in the two worm populations. Like the defecation cycle, the pumping of the pharynx is a highly regular occurrence with the number of pumps per minute changing only when stimulated by the presence of excess food. In these experiments worms were examined on plates with no food, as this would not then influence the rate of pumping. LPH RNAi worms having a pharynx that pumps ~15 times more per minute than wild type. This is significant as the pharynx is the area where most of the neurons in *C.elegans* are present. Changes in resting function here seem to indicate that silencing of latrophilin in *C.elegans* has a greater effect on the worms nervous system and its function than first thought. Over the range of imipramine concentrations LPH RNAi worms show a less resistance to the effects of imipramine than those seen in the wild type. The level of pumping falls in total only ~25 per minute over the drug range compared to ~55 in wild type, a difference of over 50%.

The most suprising conclusion made from this experiment is that in wild type *C.elegans* you would expect to see an increase in pharyngeal pumping as this is the classical

response of the worm to imipramine. Instead we see a drop in pharyngeal pumping over the drug concentration range, perturbation of the nervous system of LPH RNAi *C.elegans* is an obvious explanation for the drop in pharyngeal pumping seen in those worms, but the nervous system of the wild type worms should be functioning normally.

Impiramine does though have a complex pharmacology in mammals were it is an acetylcholine antagonist and also has effects on other transmitter systems such as acetylcholine and dopamine. It is not known whether these functions are also applicable to *C.elegans,* and whether some of these ancillary functions are having an effect on the worms is not known. What is clear though is that the silencing of the latrophilin gene in *C.elegans* causes a change in the sensitivity of these worms to a number of pharmacological substances, which helps to further characterise the mutation and phenotype of the latrophilin knockout *C.elegans.*

As previously mentioned, serotonin inhibits locomotion and defecation in *C.elegans*; therefore imipramine should cause a decrease in both of these functions. Further experiments could be carried out to test this theory, as LPH RNAi worms have defecation cycle defects it would be interesting to see whether imipramine could have an effect on the cycle in these worms. These experiments could not be undertaken during this thesis due to the constraints of time.

4.8 LPH RNAi *C.elegans* are resistant to BWSV

Further characterisation of the LPH RNAi phenotype was undertaken by investigating the effect of BWSV on these worms. Our previous work has shown that BWSV is highly toxic to wild type *C.elegans* upon microinjection over a million-fold range of dilutions (Figure 3.2). Therefore, the effect of BWSV over the same dilution range in LPH RNAi *C.elegans* was examined.

LPH RNAi worms showed complete resistance to BWSV over a concentration range of 0-1200µg/ml (Figure 3.26). The toxicity of BWSV was completely ablated by the absence of the latrophilin gene in *C.elegans* supporting our theory that latrophilin is responsible for the mediation of BWSV toxicity in the nematode. An interesting challenge to the argument that latrophilin meditates BWSV toxicity in *C.elegans* is the evidence that RNAi has a low level of penetrance in nervous tissues (Fire *et al*, 1998). We have shown that latrophilin has a role in the nervous system as silencing this gene results in changes in sensitivity to pharmacological agents and some behaviours controlled by the nervous system, if there is low penetrance of dsRNA into these tissues then you would expect some resistance to the effects of venom in some worms due to the latrophilin gene not being fully silenced. This is not the case in LPH RNAi *C.elegans* as they all show resistance to BWSV toxicity; therefore there may be some element involved in controlling the worm's response to venom. Co-suppression of another gene with similar homology to latrophilin, for example in the transmembrane region of the receptor, may be responsible for BWSV mediation, the only way to test this theory would be to use a truncated form of the latrophilin gene without the transmembrane region and see if this affected sensitivity to venom in the worm.

This further characterises the LPH RNAi phenotype in *C.elegans.* Worms are therefore, resistant to BWSV, and its active component in *C.elegans,* have a physical phenotype characterised by an expanded gut lumen, increase in defecation motor program cycle time, an increase in resting pharyngeal pumping and a change in sensitivity to neuroactive compounds which cause a known response in wild type worms. A further implication of the data generated by the silencing of the latrophilin gene in *C.elegans* shows that the knockout of the gene has significant effects on the normal phenotype of the worm. This implies that the normal function of the latrophilin receptor is required for these effects. Therefore, this data shows that the signalling activity of the latrophilin receptor is

important, and in turn, this suggests that the receptor may have a role to play in mediating BWSV toxicity through its signalling function in addition to the pore-forming ability of BWSV.

All of these factors have been illustrated by the experiments carried out, yet the assumption that latrophilin is the sole regulating factor in mediating BWSV (and it components) toxicity in *C.elegans* has not been fully justified. We have also not shown how the latrophilin gene regulates BWSV; we have only shown that its absence results in a resistance of *C.elegans* to the previously toxic effects of the venom. Important additional experiments that need to be carried out include finding if BWSV binds to the latrophilin protein in *C.elegans* and also showing that it is a functional protein when expressed in cells.

4.8.1 The process of dsRNA injection does not effect the sensitivity of *C.elegans* to BWSV

To examine whether the process of microinjection affects the sensitivity of *C.elegans* to BWSV a gene non-related to latrophilin and BWSV was used to study BWSV toxicity. The gene chosen was the cytochrome P450, CYP37A1, as far as we know this gene has no association with latrophilin or other components of BWSV and its toxicity. Cytochrome P450s are enzymes responsible for detoxification of xenobiotic compounds; in mammals these enzymes are mostly found in the liver, the main site for chemical detoxification in humans (Venkatakrishnan *et al*, 2001).

An EST of putative CYP37A1 was obtained from Professor Y. Kohara and this cDNA used as a template for double-stranded RNA production. CYP37A1 RNAi *C.elegans* were generated, close examination of these worms showed no physical phenotype, which could help characterise what kind of mutation had taken place. The lack of a visual phenotype does mean that there is little evidence that RNAi of the CYP37A1 gene in

C.elegans has taken place. During all dsRNA injection procedures though, a positive control group of *tra-2* gene dsRNA injections were made in parallel to the experimental dsRNA injections. If any of the offspring of the *tra-2* dsRNA injected adults did not show a *tra-2* knockout phenotype (see Figure 3.22) then the experimental injections were discarded and not used for BWSV injection. Groups injected with CYP37A1 dsRNA in parallel with *tra-2* groups showing 100% *tra-2* phenotypes were used for BWSV injections CYP37A1 RNAi worms were then injected with BWSV and the toxicity in these worms compared to wild type worms injected with the same concentration of venom (Figure 3.28).

Data generated showed that CYP37A1 RNAi *C.elegans* show the identical response to BWSV injection as that seen in wild type worms. 100% of injected animals were killed by venom injection. Therefore, the silencing of a gene in *C.elegans* using dsRNA injection does not affect the sensitivity of the gene-silenced worms to injected BWSV. This eliminates the argument that the actual process of RNAi or an artefact caused by it can have any affect on BWSV toxicity in *C.elegans.* This further supports our evidence for latrophilin being the gene and receptor responsible for BWSV mediated toxicity in *C.elegans.* Further experiments were carried out to examine the possibility of either the second identified gene associated with latrophilin (BO286.2) or the gene for the neurexin 1α homologue in *C.elegans* having a role in controlling BWSV toxicity in *C.elegans.*

4.8.2 RNAi of Neurexin 1α homologue has no effect on BWSV toxicity

In mammals both latrophilin and neurexin 1α can mediate the effects of BWSV toxicity in cells (Sugita *et al*, 1999). Recent experiments with neurexin 1α knockout mice have shown that the absence of the neurexin gene causes a decrease in the effects of BWSV,

when measured as glutamate release from synaptosomes (Geppert *et al*, 1998). This suggests a need for neurexin 1α and in the mediation of BWSV in mammals.

By isolating an EST for the neurexin 1α homologue in *C.elegans* from Y. Kohara's EST database we generated dsRNA complementary to the neurexin 1α gene and used this to generate Neurexin 1α RNAi *C.elegans.* These worms were then injected with BWSV to test the sensitivity of these worms to the venom (Figure 3.30). The lack of a visual phenotype for neurexin 1α RNAi *C.elegans* does mean that there is little evidence that RNAi of the neurexin 1α gene in *C.elegans* has taken place. During all dsRNA injection procedures though, a positive control group of *tra-2* gene dsRNA injections were made in parallel to the experimental dsRNA injections. If any of the offspring of the *tra-2* dsRNA injected adults did not show a *tra-2* knockout phenotype (see Figure 3.22) then the experimental injections were discarded and not used for BWSV injection. Groups injected with neurexin 1α dsRNA in parallel with *tra-2* groups showing 100% *tra-2* phenotypes were used for BWSV injections

Data produced shows that the silencing of the gene for neurexin 1α in *C.elegans* has no effect of the worms sensitivity to BWSV, the worms behaved as wild type worms upon injection, 100% of the Neurexin 1α RNAi worms were killed by the venom.

This further illustrates that the latrophilin gene is solely responsible for BWSV toxicity in *C.elegans*, as neurexin 1α has no effect. This is in contrast to the responses seen in mammals where both receptors appear to be responsible, and required for the regulation of BWSV toxicity (Sugita *et al*, 1999).

This lack of regulation may be due to the simplicity of the worm's nervous system. Though there is a neurexin 1α homologue present it may not be involved with BWSV toxicity. The structure of neurexin is that of a cell-surface receptor, *C.elegans*, having a simple nervous system may have a different role for the cell-surface receptor, which has

homology with neurexin. With little scope for redundancy in the worm's nervous system, the receptor may have a different role than BWSV toxicity. Nematodes are an ancient organism, and at some point the neurexin gene may have become redundant in the worm, conservation of signalling molecules in the organism though would mean that the homologous neurexin gene would still be present in the genome, even though it is not active in regulating BWSV toxicity in the worm.

It is important to remember that homology between two genes does not guarantee homology of function. We have previously stated that *C.elegans* has a gene, which is related to BO457.1, this being BO286.2. It is possible that the phenomenon of BWSV toxicity is controlled by BO286.2 or by co-operation between BO457.1 and BO286.2. To test this possibility we examined the effects of BWSV on *C.elegans* lacking the gene BO286.2.

4.8.3 BO286.2 has no mediatory effect on BWSV toxicity in *C.elegans*

The gene BO286.2 is a member of the secretin family of serpentine receptors, of which BO457.1 is also a member. BO286.2 shows some similarity with BO457.1 and as it is also a member of the same gene family, it may well have some role to play in the regulation of BWSV toxicity in *C.elegans.* A deletion mutant lacking the gene BO286.2 was obtained from the *C.elegans* Knockout Consortium, and was injected with BWSV to see if the deletion of this gene had any effect on BWSV sensitivity (Figure 3.31). BO286.2 deletion mutants showed no difference in BWSV toxicity than seen in wild type worms with 100% of the mutant worms showing death when injected with venom. This data shows that closely related genes of BO457.1 have no mediatory effect on BWSV toxicity. This would be expected as in mammals, only the receptors latrophilin and neurexin have been identified as responsible for BWSV binding and mediation, we

have shown that neurexin 1α has no role in BWSV toxicity in worms, it would therefore be highly unlikely to find a second latrophilin receptor.

The G-protein coupled receptor BO286.2 has no effect on BWSV, again, it may have some homology with BO457.1 and latrophilin but it is more likely that this receptor has a different role in the nervous system than mediation of spider venom.

Our data shows that latrophilin plays the primary role in mediating the effects of BWSV in *C.elegans*, it also suggests that latrophilin may have the principal role in mediating the effects of BWSV in mammals. There may also be some specialisation-taking place within the latrophilin family in mammals. In mammals, there have been three latrophilins identified, latrophilin-1, 2, and 3 (Sugita *et al*, 1998 & Ichtchenko *et al*, 1999). Each of these is differentially expressed, latrophilin-1 is found in all tissues (Sugita *et al*, 1998), latrophilin-2 in tissues outside of the brain (Sugita *et al*, 1998) and latrophilin-3 only in the brain (Ichtchenko *et al*, 1999). Latrophilins-1 & 2 bind the toxin α-LTX whereas latrophilin-3 does not (Ichtchenko *et al*, 1999). Therefore, *C.elegans* may have the ancestral member of the latrophilin gene family (1, 2 & 3), which has a variety of functions, but these functions may, over time, have become specialised to individual proteins in mammals. Further studies into this could include the analysis of the distribution of latrophilin in *C.elegans* (which we have already began for latrophilin) and what their functions are in relation to the known distributions and functions of the mammalian latrophilins.

Our data shows that the receptors neurexin 1α and latrophilin-2 (BO286.2) do not have a role in the mediation of BWSV in the nematode *C.elegans*. We have yet to extensively prove that it is latrophilin-1 (BO457.1) that is solely required for BWSV toxicity. There are other genes, which may have similar homologies to specific sequences within latrophilin that may ultimately play a role in controlling the response to venom. In the mammalian model there are still aspects of the second messenger system that has not be

elucidated which may have a greater influence on BWSV toxicity that the receptors which may only be tethers for the toxin at plasma membranes. If it is the case that latrophilin only "recruits" the toxin to the plasma membrane and it is the toxin itself that then mediates an unknown intracellular mechanism then it would then appear that the receptors themselves might not been mediating the observed toxicities. However, my data shows that the latrophilin gene has an important role in *C.elegans*, as judged by its noticeable phenotype, and demonstrates that the signalling role of this gene is important. It is possible to address the role of the latrophilin gene as a receptor in *C.elegans* by making transgenic *C.elegans* lacking the endogenous gene, but expressing various mutants of the normal latrophilin gene.

4.9 RNAi by dsRNA feeding shows the same effects in *C.elegans* as dsRNA injections

Studies involving RNAi have shown that dsRNA interference can function across cell boundaries therefore making the site of injection non-essential for successful gene inactivation (Fire *et al*, 1998).

This makes it possible to use RNAi either by soaking worms in a dsRNA solution (Tabara *et al*, 1998), or by feeding worms with *E.coli* expressing target gene dsRNA, as the dsRNA can be absorbed through the gut and sent to somatic tissues and the germ line (Timmons & Fire, 1998). We have therefore elected to examine the effects of inducing latrophilin RNAi on *C.elegans* by dsRNA feeding.

A fragment of the latrophilin homologue in *C.elegans* was isolated by PCR (Figure 3.32); this 1.1Kb fragment has 39% identity with the latrotoxin-binding domain of latrophilin (Krasnoperov *et al*, 1999). This fragment was chosen because it lacks the 7-transmembrane domain, removing any possibility of non-specific cross-reaction with

other *C.elegans* 7-transmembrane receptors. Feeding of the 1.1Kb fragment of LPH dsRNA caused 100% phenotype penetrance (Table 3.1), which is the same level of phenotype penetrance seen in microinjection of dsRNA (Chris Mee, data not shown). The phenotype observed was the same as that seen in worms injected with full-length latrophilin dsRNA. Though we did not test these worms for other characteristics known to belong to the worms such as, defecation cycle time, sensitivity to drugs and pharyngeal pumping.

We did though test the effects of BWSV injection on *C.elegans* fed with bacteria containing the 1.1Kb dsRNA fragment of the latrophilin gene (Figure 3.33). Worms showing the LPH RNAi phenotype showed 100% resistance to the toxic effects of BWSV over a million fold dilution ranges. This shows that worms which have undergone gene silencing by either microinjection of full length LPH dsRNA or feeding of dsRNA complementary to the latrotoxin binding site of latrophilin both have complete resistance to the toxic effects of BWSV in *C.elegans* over a million fold dilution.

Therefore we have shown that BWSV is acutely toxic to *C.elegans* and that multiple high molecular weight latrotoxins of ~110kDa are responsible for this toxicity in the nematode. We have also shown that a homologue of the gene for latrophilin, BO457.1, can be silenced in *C.elegans* and causes the worm to become resistant to the toxic effects of BWSV, this effect was shown to be specific to latrophilin and is not seen with a neurexin 1α homologue or genomic deletion mutant of the G-protein coupled receptor BO286.2. To add to this, worms affected by dsRNA specific to the latrotoxin-binding site of the latrophilin gene show the exact same response to BWSV as those worms with the full-length gene silenced. Moreover, RNA interference of the latrophilin gene results in a marked constipated phenotype, associated with an elongated defecation cycle time, and altered reaction to a number of neuroactive substances.

4.10 GFP expression studies of the latrophilin gene in *C.elegans*

To study the expression of the latrophilin gene in *C.elegans* we used the reporter green-fluorescent protein (GFP) as a way of visualising the areas in which the latrophilin protein is expressed. The gene fusion vector made contained a nuclear localised expression signal allowing the cell in which latrophilin is expressed to be pinpointed. The fusion vector made is shown in Figure 3.34. For the vector to be used to study expression patterns it has to be integrated into the DNA of the nematode. This was performed by microinjection into the gonads of the nematode; the DNA is then transformed into the germline of the progeny of the worm and identified by the presence of a dominant genetic marker (*rol-6*), which is transformed into the germline at the same time as the fusion vector.

Transmission of this rolling phenotype is shown in Table 3.2. Initial % transmission is usually in the range of 1-10% (Mello & Fire, 1995). Both the experimental and control DNA injections have been transmitted at a frequency of 6 and 5% respectively, which is within the expected range for an optimised DNA injection experiment. It is necessary to transfer the phenotype through three generations to remove any transient transmission of the DNA and to establish a permanent line of mutated worms. Therefore, F1 progeny with the rolling phenotype were selected and their progeny (F2) analysed for the continued presence of the phenotype, this data is illustrated in Table 3.3. 0% transmission of the roller phenotype occurred in the F2 progeny preventing the continuation of the experiment and the opportunity to study the expression pattern of latrophilin in *C.elegans.*

This loss of phenotype is a common phenomenon as DNA integration into *C.elegans* occurs at low copy numbers, with only a small percentage of animals fully integrating the cloned DNA over a number of generations.

Mello (1991) outlines a method for the efficient gene transfer of extrachromasomal sequences in *C.elegans* and the maintenance of these germ lines. Two major factors are responsible for generating an efficient gene transfer technique. The transformation of transient expression and heritable extrachromasomal arrays are dependent on the concentration of the DNA initially injected. By increasing the concentration of injected DNA from 12.5μg/ml to 50μg/ml, Mello saw a 4-fold increase in the % heritable expression in F1 progeny. Though DNA concentrations in excess of 50μg/ml show little increase in heritable expression, indicating the possibility that saturation has taken place. In the latrophilin expression injections a total of 100μg/ml of DNA was injected giving 6% transformation, this indicates that it is not the concentration of DNA being used preventing the generation of heritable lines.

The second possibility for the lack of integrated transformants is the size of the DNA. A large number of plasmid molecules must assemble to form a single array (Mello *et al*, 1991) with around 110-165 plasmid molecules required, this may not be occurring in our experiments. With this in mind it should be noted that raising the initial injection population could therefore increase the possibility of producing a heritable transformant. By doubling the number of injected adults you double the possibility of generating long continuous plasmid molecules, which can form heritable arrays.

This would be the next study to undertake to optimise the conditions for the formation of a heritable population of worms containing the LPH expression vector, but was not possible due to the lack of time at the end of the thesis.

4.11 RNA interference of other nervous system genes in *C.elegans*

Further gene homologues in *C.elegans* were investigated using RNAi, to examine whether the function of other genes associated with the nervous system homologous to mammalian genes, which may cause toxicity. Using reverse genetics we hope to elucidate some of the functions involved with these genes using *C.elegans* as a model system. The phenotypes obtained from the RNAi injections of these genes are shown in Table 3.5. None of the glutamate receptor or neuropathy target esterase genes caused any change in visual phenotype in *C.elegans.* Though there is no apparent visual phenotype this doesn't mean that there is no gene silencing taking place in the worms injected with the dsRNA. As has been shown in this thesis, phenotypes may be far more subtle, with characteristics such as variations in body functions such as pharyngeal pumping, defecation cycle, egg laying and male-mating behaviour, all of these functions often are affected when the worms nervous system is disrupted. More subtle changes than these may have taken place as well, changes in the worms responses to pharmacological agents along with microscopic changes in the nervous system that can only be seen under electron microscopy. Other changes in the nervous system could be analysed by using electrophysiological tests such as the use of an electropharyngeogram. Any of these techniques could be used; the essence of the work is that even though a phenotype cannot be seen, this doesn't mean that there has been no disruption of the genes function by RNAi.

4.12 Further studies

Further work that could help elucidate more information regarding the subjects studied in this thesis includes; finishing the GFP expression studies, and therefore finding the expression pattern of the latrophilin protein in *C.elegans.*

Further examination of the latrophilin receptor could be done on a genomic knockout, which would be a total gene knockout instead of the gene-silenced knockdown caused by RNAi. This would also reduce the time consuming nature of generating LPH RNAi *C.elegans.* Further characterisation of the latrophilin RNAi *C.elegans* phenotype could be done by using methods not involving BWSV, such as the use of Western blotting and immunocytochemistry along with antibodies raised to latrophilin in *C.elegans*, this would show if the protein was indeed down regulated in the worm. Electron microscopy would show any gross morphological changes in the worm's nervous system, indeed a classical response of BWSV toxicity is the absence of synaptic vesicles in the terminal button. Examination of the nerves/muscles could also be done by electrophysiological tests such as electropharyngeograms using extracellular recordings of pharyngeal activity, especially as there does seem to be some change in the pharynx in LPH RNAi *C.elegans*, though further pharyngeal pumping studies are necessary.

Chapter 5. References.

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