Characterisation of glutamate-gated chloride channels from Caenorhabditis elegans

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Characterisation of Glutamate-gated Chloride Channels from Caenorhabditis elegans

Submitted by Lucy Horoszok

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ABBREVIATIONS

Standard abbreviations used in text are as defined in "Instructions to Authors" of the Biochemical Journal 1996.

5-HT	5-hydroxytryptamine
ACh	acetylcholine
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid
APS	ammonium persulphate
ATP	adenosine triphosphate
avr-15	resistant to avermectin
BIDN	3,3-bis(trifluoromethyl)bicyclo[2,2,1]heptane-2,2-dicarbonitrile
B_{max}	maximal binding
bp	base pairs
BSA	bovine serum albumin
BZD	benzodiazepines
cDNA	complementary deoxyribonucleic acid
°C	degrees celcius
C. elegans	*Caenorhabditis elegans*
CPZ	chlorpromazine
Cys-loop	cysteine loop
d(d) ATP	2′(3′-di) deoxyadenosine 5′-phosphate
d(d) CTP	2′(3′-di) deoxycytidine 5′-phosphate
d(d) GTP	2′(3′-di) deoxyguanosine 5′-phosphate
d(d) TTP	2′(3′-di) deoxythymidine 5′-phosphate
d(d) NTP	2′(3′-di) deoxynucleotide 5′-phosphate
DNA	deoxyribonucleic acid
Dros	*Drosophila melanogaster*
EBOB	4-n-propyl-4'-ethynylbicycloorthobenzoate
EC_{50}	concentration producing a half-maximum response
EDTA	ethylenediaminotetra-acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPG	electropharyneogram
E_{rev}	reversal potential
FITC	fluorescein isothocyanate
g	gravitational force

GABA	γ aminobuteric acid
GBR	GABA receptor related
GFP	green fluorescent protein
GLC	glutamate chloride channel
GluCl	glutamate-gated chloride ion channel
HEPES	(N-(2-hydroxyethyl)piperazine-N'-2-ethane)sulphonic acid
HRP	horse-radish peroxidase
IC_{50}	concentration producing 50% inhibition
Ig	immunoglobulin
IGluR	inhibitory glutamate-gated chloride channel receptor
IP_3	insitol triphosphate
IPSP	inhibitory postsynaptic potentials
IPTG	isopropylthio-β-galactoside
IU	international units
Kb	kilobase
Kd	dissociation constant
kDa	kilodaltons
LB	(Luria-Bertani)-Broth
LGIC	ligand-gated ion channels
mA	milliampere
MBS	m-maleimidobenzoic acid N-hydrosuccinamide ester
MOPS	morpholinopropanesulfonic acid
mRNA	messenger ribonucleic acid
nACh	nicotinic acetylcholine
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
OD	optical density
PBS	phosphate buffered saline
PCP	phencyclidine
PCR	polymerase chain reaction
pH	$-\log_{10}$ (hydrogen ion concentration)
P_{2X}	P_2 purinoceptors
RACE	rapid amplification of cDNA ends
RDL	resistant to dieldrin
RMED	ring motor neurons, dorsal
RMEV	ring motor neurons, ventral
RNA	ribonucleic acid

RNase	ribonuclease
rpm	revolutions per minute
SCAM	scanning-cysteine-accessibility method
SDS	sodium dodecyl sulphate
SL	spliced leader sequence
SOS	standard oocyte saline
TBOB	1-phenyl-4-t-butyl-2,6,7-trioxabicyclo[2.2.2]octane
TBPS	t-butylbicyclophosphorothionate
TE	tris/Na_2EDTA buffer
M(1-4)	transmembrane (1-4)
U	units
unc-49	uncoordinated movement phenotype
UV	ultraviolet
V	volts
v/v	volume to volume
W	watts
w/v	weight per unit volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

GENE NOMENCLATURE

The uniform system for naming *C. elegans* genes consist of using three italicised letters and a hyphen, with the last digit of the name being numeral to describe the order of their discovery. The protein product of a gene can be referred to the relevant gene name. Some authors have decided not to use this nomenclature approach. Nomenclature for genes and gene product relevant to this are summarised below.

• Genes names:	subunit it encodes for:	alternative nomenclature
glc-1	GLC-1	GluClα_1
glc-2	GLC-2	GluClβ
glc-3	GLC-3	GLC-3
avr-15	AVR-15	GluClα_2
gbr-2/avr-14	AVR-14	Gbr2-A & B/GluClα_3

• Genes defined by mutation are assigned names containing three italicised letters and a Roman numeral referring to the detected mutant phenotype:

avr-14	resistant to avermectin
unc-49	uncoordinated movement phenotype

• For genes defined by cloning, on the basis of sequence similarity, the gene name refers to the predicted protein or RNA product:

glc	glutamate chloride channel
gbr	GABA receptor related

Where more than one protein product is predicted for a gene (e.g. as a result of alternative splicing), the different proteins are distinguished by additional capital letters, for example the *gbr-2/avr-14* gene encodes for two spliced products GBR-2A and GBR-2B.

• Sequences that are predicted to be genes (*Caenorhabditis elegans* sequencing Consortium, 1998) are named on the basis of the sequence cosmid plus a number, for example, ZC317.3.

SUMMARY

Glutamate-gated chloride ion channels (GluCl) are a family of ligand-gated ion channels structurally related to mammalian glycine and $GABA_A$ receptors, but pharmacologically distinct from all vertebrate ionotropic receptors. In nematodes and insects GluCls are the probable sites of action of the avermectin/milbemycin family of anthelmintics and insecticides, although the exact mode of action of the drugs, the anatomical localisation and native stoichiometry of the receptors remains unknown. This thesis provides a brief synopsis of our current understanding of the avermectin-sensitive channels in the free-living nematode *Caenorhabditis elegans* (*C. elegans*), in addition to addressing some of these fundamental questions.

The first aim of this study involved localising an existing GluCl clone (GBR-2) and examining the exact anatomical position in relation to other *C. elegans* subunits ($GluCl\alpha_2$ and β). A polyclonal antiserum raised against the GBR-2 receptor subunits showed a specific expression pattern in the extrapharyngeal nerve ring and within three neuronal cell bodies in the metacorpus, corpus and terminal bulb regions that represent the M3 pharyngeal neurons. The completion of the *C. elegans* genome-sequencing project revealed a novel gene, ZC317.3, predicted to encode a polypeptide with high sequence identity to the previously cloned $GluCl\alpha_1$ and α_2 subunits. Using a PCR-based strategy, the second objective of this thesis was the full-length amplification of this novel cDNA subunit. Successful isolation of a 1.4Kb cDNA subunit displayed 73-77% amino acid identity with other GluClα subunits and only 30-40% with GABA and glycine, indicating that this protein is also a member of the same amino-acid family of receptor proteins. The *Xenopus* oocytes expression system in conjunction with the two-electrode voltage clamp was used in the final objective of this study to investigate the functional expression of ZC317.3 cDNA. The pharmacology of these subunits resembled that of recombinant $GluCl\alpha_2$ receptors in a number of respects and as such is distinct from that of vertebrate $GABA_A$ and glycine receptors. As with all GluCl α subunits (with the exception of $GluCl\alpha_1$), when ZC317.3 cRNA was injected into *Xenopus* oocytes, a robust inward current in response to applied L-glutamate was observed. The agonist pharmacology of the ZC317.3 homo-oligomer produced robust, dose-dependent inward currents to L-glutamate yielding an EC_{50} of 1.9±0.03mM and a Hill coefficient of 1.46±0.1. Peak currents were approximately 1μA and desensitised rapidly. Application of 1mM GABA, glycine, NMDA or histamine all failed to activate the ZC317.3 homo-oligomers. The L-glutamate-gated analogue, ibotenic acid activated the homo-oligomer producing weak non-desensitising currents resembling the $GluCl\alpha_2$ and GluClβ homomers. As with all GluClα subunits cloned to date, the ZC31.7.3 channels were irreversibly gated by 100nM ivermectin, however, low concentrations of ivermectin failed to potentiate sub-maximal concentrations of L-

glutamate. The modes of action of convulsant antagonists (3,3-bis-trifluoromethyl-bicyclo[2,2,1]heptane-2,2-dicarbonitrile (BIDN), a novel bicyclic dinitrile, picrotoxinin and insecticide fipronil) were investigated on GLC-3 homomers recorded from oocytes. Picrotoxinin failed to inhibit L-glutamate agonist responses at concentrations up to 1mM. The present study provides the first evidence that a L-glutamate-gated chloride channel may be a molecular target for BIDN and the novel insecticide, fipronil, which reversibly inhibited glutamate-gated currents through the ZC317.3 receptor with an IC_{50} 0.22±0.07 and 11.53±0.11μM respectively. Further study of this receptor may provide insights into the molecular basis of non-competitive antagonism by these compounds. Based on the sequence of the polypeptide and functional expression data we conclude that this gene, designated *glc-3*, encodes a further GluCl subunit, GLC-3, from *C. elegans*.

This thesis is dedicated to my mother
and the memory of my father.

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I acknowledge with thanks the advice, support and guidance given throughout the course of this work by my supervisor Adrian Wolstenholme.

In addition I would also like to say 'thank you' to some people who have been particularly influential or just provided entertainment along the way:

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Many thanks to both past and present staff members in the biochemistry department, in particularly Chris Davey and Bill Bennett for introducing me to confocal microscopy, Sue Phillips for peptide synthesis and Sandra and Alison for all their technical help.

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Finally, but most importantly of all, I would like to thank all my family members, especially my mum and five sisters (Julie, Christine, Susan, Diane and Yvonne) who have unselfishly provided constant love, encouragement and financial support throughout my career.

PUBLICATIONS ARISING FROM WORK IN THIS THESIS

Suchitra Jagannathan, David L. Laughton, Catherine L. Critten, Thomas M. Skinner, Lucy Horoszok and Adrian Wolstenholme.

Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and *Ascaris suum* orthologues of the *Caenorhabditis elegans gbr-2* (*avr-14*) gene.

Molecular and Biochemical. Parasitology, (1999) 103, 129-140.

Lucy Horoszok, Valerie Raymond, David Sattelle and Adrian Wolstenholme.

GLC-3: A novel fipronil and BIDN-sensitive, but picrotoxinin-insensitive, L-glutamate-gated chloride channel subunit from *Caenorhabditis elegans.*

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Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and *Ascaris suum* orthologues of the *Caenorhabditis elegans gbr-2 (avr-14)* gene☆

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Abstract

The alternatively-spliced *Caenorhabditis elegans gbr-2/avr-14* gene encodes two subunits of the nematode ligand-gated chloride channel family which forms an important molecular target for the avermectin and related anthelminthics. We used reverse transcriptase–polymerase chain reaction (RT–PCR) techniques to isolate cDNAs encoding the products of the *gbr-2/avr-14* orthologues from the parasitic nematodes *Haemonchus contortus* and *Ascaris suum*. The predicted polypeptides possess all the characteristics of subunits of the ligand-gated chloride channels, sharing greater than 80% amino-acid identity with their counterparts in *C. elegans* and with partial sequences from the filarial species *Onchocerca volvulus* and *Dirofilaria immitis*. The pattern of alternative splicing of the *gbr-2/avr-14* gene observed in *C. elegans* is conserved in *H. contortus* but may not be in *A. suum*. Affinity-purified anti-GBR-2 antibodies were used to study the expression of these subunits in adult worms and they reacted specifically with the nerve ring, the ventral and dorsal nerve cords, the anterior portion of the dorsal sub-lateral cord and motor-neuron commissures in *H. contortus*. Specific immunofluorescence of the nerve cords was confirmed in *A. suum*; isolated muscle cells did not react with the antibody.

Keywords: Ivermectin; Glutamate receptor; Nervous system; cDNA cloning

Abbreviations: CSPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate; DIC, differential interference contrast; DIG, digoxigenin; Glu-Cl, glutamate-gated chloride channel; MOPS, 3-[*N*-morpholino]propanesulphonic acid; RACE, Rapid amplification of cDNA ends; RT–PCR, reverse transcriptase–polymerase chain reaction.

☆ *Note*: Nucleotide sequence data described in this paper have been deposited in the EMBL database under Accession numbers Y14233, Y14234 and Y18347.

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1. Introduction

The avermectins are a group of highly effective anthelminthics, insecticides and acaricides. The probable site of action of these compounds has been identified as a family of glutamate-gated chloride channels (Glu-Cl) [1], related to, but distinct from, the $GABA_A$ receptors, but which are not present in the vertebrate nervous system [2]. These channels have all the properties of the ionotropic receptor superfamily and are therefore presumably composed of five subunits. Each subunit possesses a characteristic structure consisting of a long N-terminal extracellular domain and a C-terminal domain with four membrane-spanning regions (TM 1–4) with an intracellular loop between the third and fourth of these membrane-spanning regions. To date, the majority of published studies on these receptors have concentrated on those from the free-living nematode, *Caenorhabditis elegans*. Given the importance of the avermectins for the control of animal and human parasites, it is clear that the receptors from these organisms also need to be well understood. The relative difficulty and expense of obtaining sufficient parasite material to allow pharmacological characterisation of the native receptors led us to adopt a molecular cloning approach with the ultimate aim of reconstituting these proteins in vitro.

Cully et al. [3] originally reported the cloning of two subunits of the Glu-Cl from *C. elegans;* an α-subunit which, when expressed in *Xenopus* oocytes formed chloride channels irreversibly gated by ivermectin, and a β-subunit which formed channels gated by glutamate. The sequences of the polypeptides showed all the characteristic motifs expected for inhibitory ligand-gated ion channels and possessed significant homology with vertebrate and invertebrate $GABA_A$- and glycine-receptor subunits. When expressed together, the two subunits formed an ivermectin-potentiated, glutamate-gated chloride channel. Subsequent work revealed that the α-subunit possessed a binding site for glutamate, but this was not coupled to opening of the ion channel [4]. Since then, the *avr-15* gene has been demonstrated to encode, via alternative splicing, two forms of an α2 subunit; the longer of the two polypeptides can be expressed to form ivermectin and glutamate-gated chloride channels [5,6]. The *avr-15* gene is expressed in the pharyngeal muscle cells pm4 and pm5, along with a number of neurons [5], though it is not clear which of the alternative gene products is present in these cells. The sequence of a putative α-subunit cDNA has been reported from *Haemonchus contortus* [7]; as yet no expression data are available. The *C. elegans* Glu-Cl β gene is also expressed in pm4 pharyngeal muscle cells [8]. A putative *H. contortus* orthologue of this gene was found to be expressed on motor neuron commissures and not in the adult pharynx [9]. This result suggests that the function of orthologous subunits may vary between nematode species and underlines the need for further characterisation of the Glu-Cl from parasites.

A further pair of cDNA clones encoding the alternatively spliced subunits GBR-2A and GBR-2B have also been isolated from *C. elegans* [10]. The pattern of alternative splicing probably results from a partial duplication of the original gene to produce two subunits with a common N-terminal extracellular domain that contains the agonist binding site. The differing C-terminal domains containing the transmembrane regions are likely to form the ion channel. Recently it has become that the *gbr-2* gene is identical to *avr-14*, a gene involved in avermectin-resistance that is expressed in some neurons of the nerve ring and a couple of neurons in the tail (J. Dent & L. Avery, personal communication). Closely related sequences have been cloned from the filarial species *Onchocerca volvulus* and *Dirofilaria immitis* [11], but it is not clear whether or not the partial gene duplication is conserved. We report here the cloning of cDNAs encoding what we believe to be the *H. contortus* and *Ascaris suum* orthologues of the GBR-2/AVR-14 subunits of *C. elegans*.

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tion of 100 ng/ml and applied to the blot at 68°C overnight. Following hybridization the blot was washed twice in 2 × wash buffer (2 × SSC, 0.1% (w/v) SDS) at room temperature. This was followed by two washes in 0.5 × wash buffer (0.5 × SSC, 0.1% (w/v) SDS) at 68°C for 15 min each. The bound probe was detected by washing in 100 mM maleic acid, 150 mM NaCl; 0.3%(v/v) Tween 20, pH 7.5 for 1 min. The membrane was incubated in blocking solution (Boehringer) by gentle agitation at room temperature for 1 h. This was then discarded and the membrane incubated at room temperature in blocking solution containing the Anti-Digoxigenin antibody diluted 1:10 000 for 30 min. This was followed by 2 × 15 min washes in 100 mM maleic acid, 150 mM NaCl; 0.3%(v/v) Tween 20, pH 7.5. Following removal of washing buffer the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH9.5) for 2 min at room temperature. Meanwhile, a 25 mM stock solution of CSPD was diluted 1:100 in detection buffer. The membrane was then placed between two plastic sheets and 0.5 ml of the chemiluminescence substrate scattered dropwise on the surface. The top sheet was then lowered and wiped with a damp tissue to remove bubbles and the bag was sealed and incubated at room temperature for 5 min. To decrease time taken for the chemiluminescence substrate to reach equilibrium the membrane was incubated at 37°C for 15 min. The membrane was then exposed to X-ray film overnight.

2.4. Preparation of anti-GBR-2 antibodies

Two 13 amino acids long peptides, LRTRMVLRREFSC (GBR-2A) and ARVVLRLRREYSC (GBR-2B), were synthesized on a Milligen 9050 Pep-synthesizer and purified by HPLC. The GBR-2B peptide was coupled to thyroglobulin by the *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) method [17] and New Zealand white rabbits subsequently immunized with the thyroglobulin-peptide conjugate as described [18]. Polyclonal anti-peptide antibodies were purified on an antigen column prepared by covalently coupling the peptide to cyanogen bromide-activated Sepharose 4B. The column was extensively washed with 20 mM phosphate buffer (pH 7.3) and specific antibodies eluted by 0.05 M diethylamine (pH 10.7). Antibody elution was monitored by measuring absorbance at 280 nm. 35 ml of eluant was dialysed overnight against PBS and concentrated to 1.6 ml using PEG-20 000.

2.5. Immunolocalisation of GBR-2

Adult *H. contortus* were fixed and permeabilised using the paraformaldehyde and 2-mercaptoethanol treatments described [18], followed by treatment with 115 collagenase digestion units/ml for 8 h. Affinity purified antibodies were diluted 1:5, 1:10 and 1:50 in 0.1% (w/v) BSA, 0.5% (v/v) Triton X-100, 0.05% (w/v) sodium azide and added to permeabilised worms for 24–72 h at 4°C. Unbound antibodies were removed by extensive washing with PBS and the worms treated with a 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG (Sigma) overnight at 4°C. This was followed by extensive washing with PBS/0.1% (v/v) Triton X-100 after which the worms were mounted as described [9] and viewed under a Zeiss LSM 510 confocal microscope. The immunostaining experiments were repeated on four separate occasions, each time using 10–12 worms.

Adult *A. suum* muscle cells and body wall tissue were prepared for immunolocalisation by collagenase digestion and fixed in 3.7% (w/v) paraformaldehyde [17]. Collagenase treatment was with 115 collagen digestion units/ml (Sigma Type 1A) in 1 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.5 for 8–16 h at 37°C. The exact length of time the collagenase treatment was allowed to continue varied from batch to batch of worms and enzyme: the reaction was terminated when the cuticle was largely digested and the worms became very fragile. The preparations were washed in PBS and incubated in 1:10 and 1:50 dilutions of anti-peptide antibody as described above. Washing and detection of bound antibody was as described for *H. contortus*.

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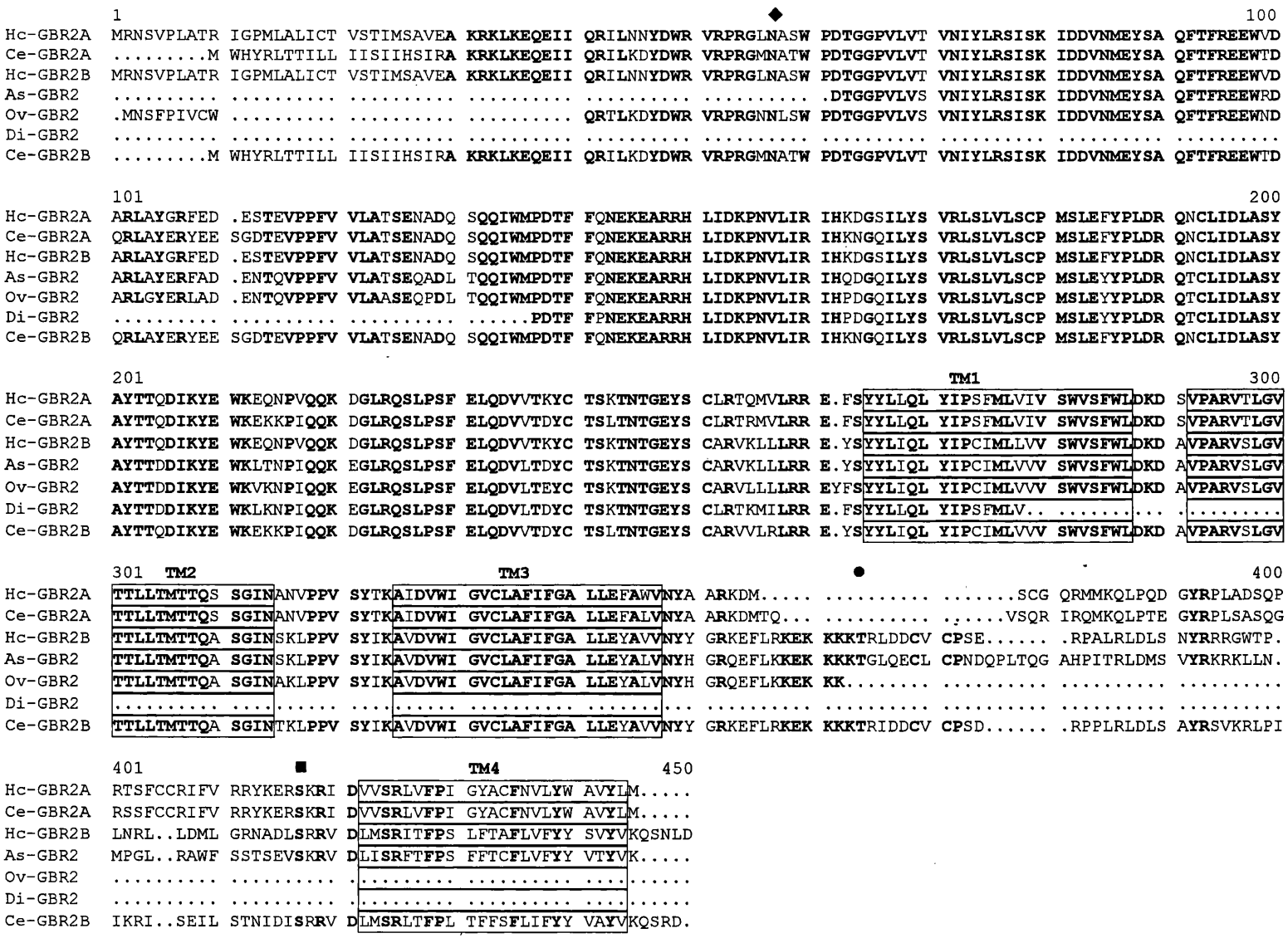
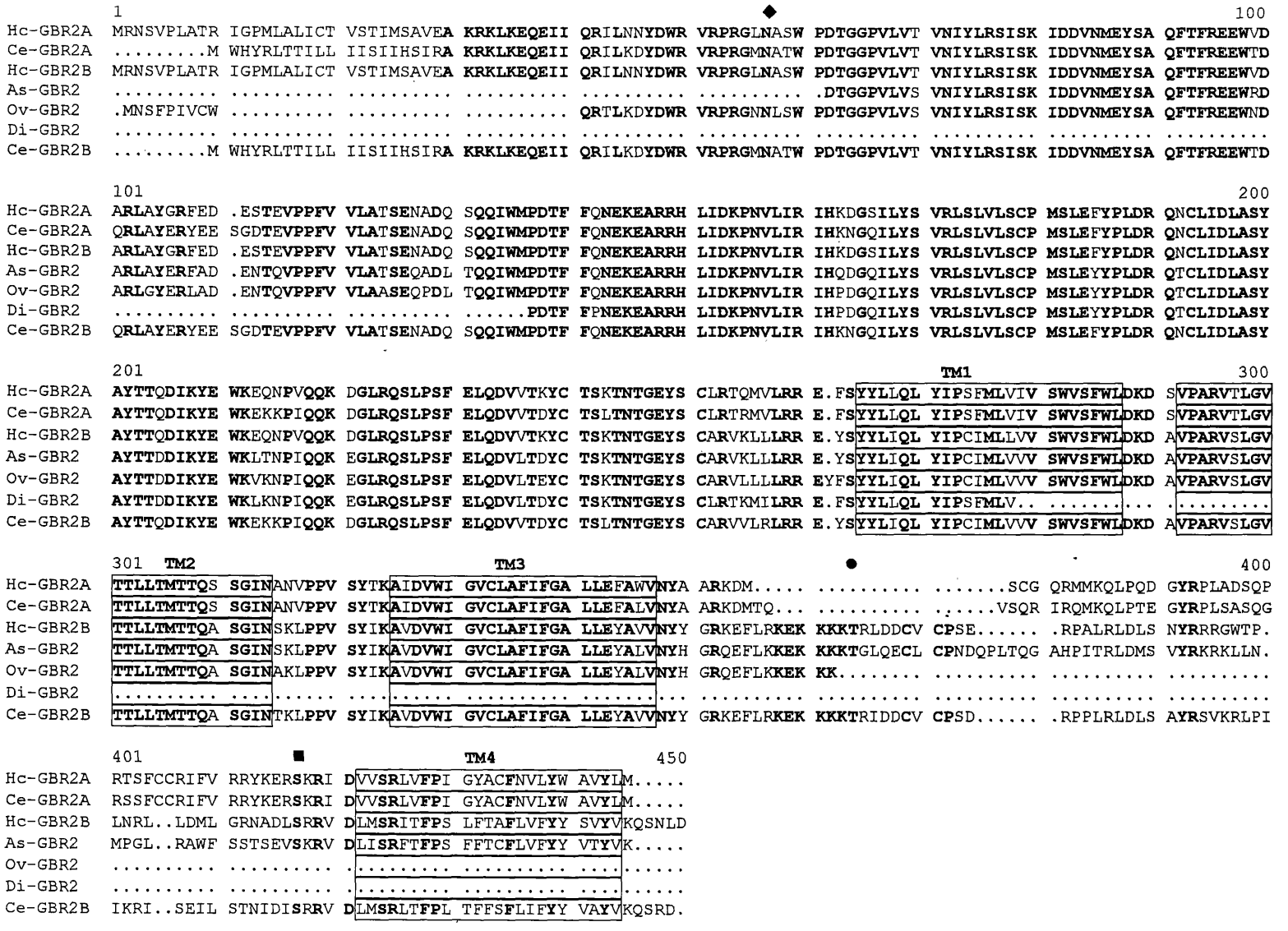


Fig. 2. Alignment of nematode GBR-2 subunit sequences. The *H. contortus* (Hc) and *A. suum* (As) sequences described in this manuscript are shown aligned with orthologous sequences from *C. elegans* (Ce) [10], *O. volvulus* (Ov) and *D. immitis* (Di) [11]. Conserved residues are in bold face and the predicted membrane-spanning regions boxed. The putative glycosylation site is indicated by (◆) and the conserved PKC (■) and cAMP-dependent protein kinase (●) consensus sites are shown.



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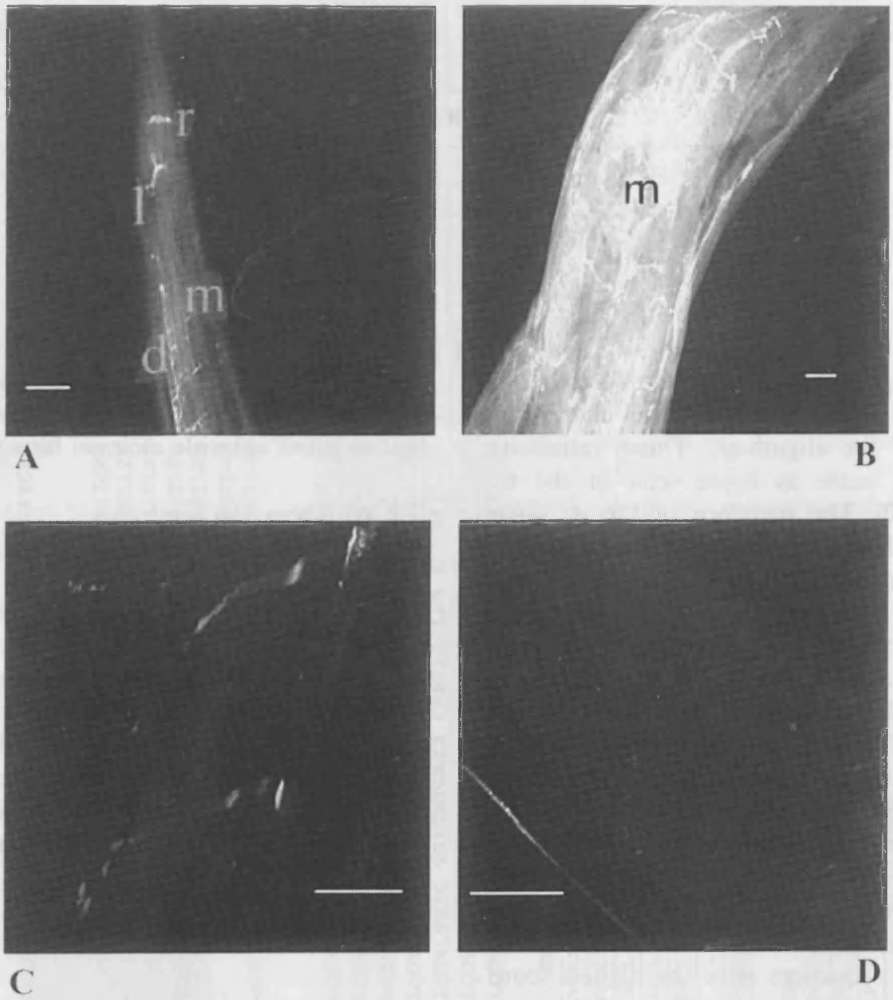
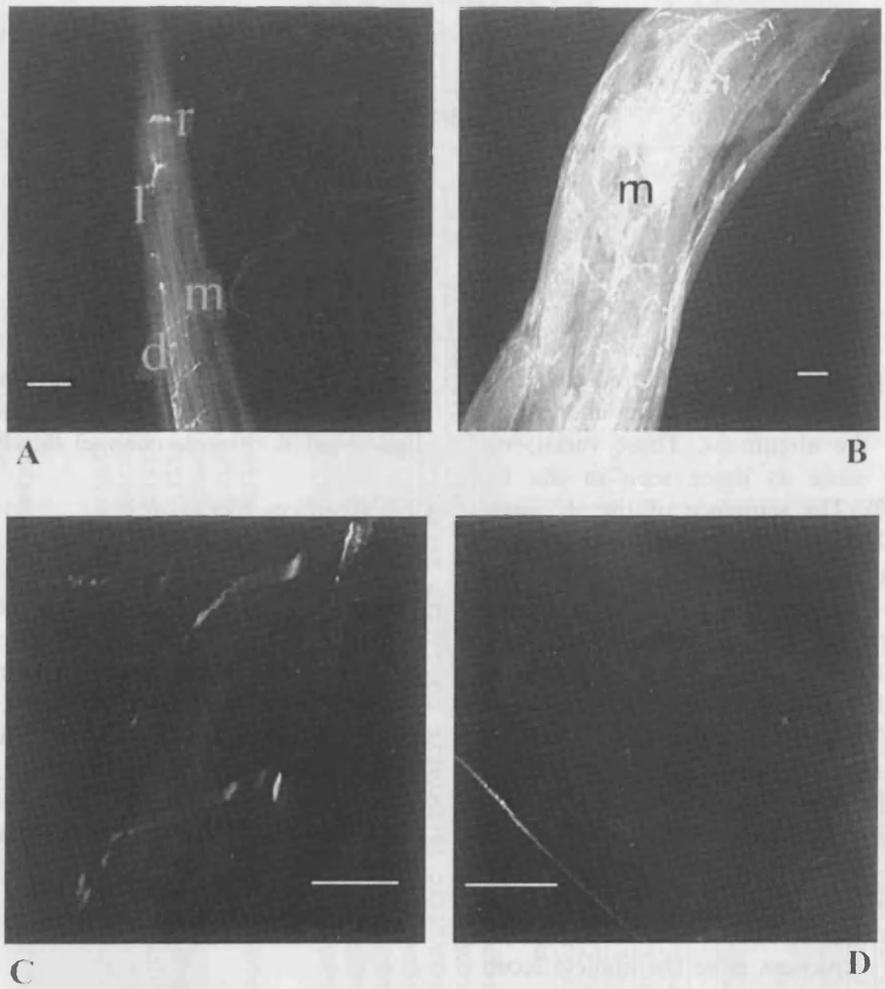


Fig. 4. Confocal images of anti-GBR2 antibody staining of whole mounts of adult *H. contortus*: (A) Staining of the nerve ring (r), motor neuron commissures (m) traversing the body to join the dorsal cord (d). Projecting towards the nerve ring is the structure discussed in the text (l); (B) Staining of the motor neuron commissures in the central region of the body; (C) High resolution image of motor neuron commissures; (D) Negative control using antibodies pre-adsorbed with 4 μM of the peptide. Affinity purified anti-GBR2 peptide antibodies were used at a 1:5 dilution. Scale Bar = 50 μm

form a similar analysis on mRNA from *A. suum* were frustrated by a low yield of RNA from the available tissue.

3.3. Localisation of GBR-2 expression

The GBR-2A and –2B subunits possess identical N-terminal amino-acid sequences until just prior to the first transmembrane domain. This made the design of synthetic peptides for the production of antibodies that could distinguish between the two subunits problematic. We synthesised a peptide that corresponded to amino-acid residues 243–254 of the *C. elegans* GBR-2A and -2B subunits and used the GBR-2B peptide to immunise rabbits. When the resulting antiserum was tested by ELISA it recognised both peptides equally well (data not shown) and was therefore unlikely to distinguish between the GBR-2A and –2B subunits in immunolocalisation experiments. In *C. elegans*, these antibodies showed that GBR-2 polypeptides were present in the head region (L. Horoszok and A.J. Wolstenholme, unpublished). Given the high level of sequence conservation



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4. Discussion

The sequences presented in this paper demonstrate that the *gbr-2/avr-14* gene and its products are conserved and expressed in a variety of parasitic nematodes. This suggests that the subunits encoded by this gene, and the receptors that they help to form, play an important role in the nervous system. The sequences of the *H. contortus gbr-2* cDNAs are very similar to those of *C. elegans*, as would be predicted by the molecular phylogeny [19]. The pattern of alternative splicing to produce the GBR-2A and -2B polypeptides is also conserved and two mRNAs were detected on Northern blots. By contrast we were unable to find any evidence for alternative splicing of the *A. suum gbr-2* gene and only a single cDNA could be amplified, though our inability to produce sufficient mRNA for Northern blot analysis in this organism prevents us making any definitive statement concerning the number of mRNAs transcribed from the *As-gbr-2* gene. This cDNA lacked the 5′ terminal region of the mRNA predicted by sequence alignments. The *A. suum gbr-2* cDNA is probably truncated, since no suitable initiation codon and signal peptide could be identified in the sequence. We believe the most likely reason for this truncated product is that the SL1 oligonucleotide primer was hybridising to similar internal sequences in the cDNA during the 5' RACE reaction. The absence of evidence for any alternative splicing of the *A. suum* gene might indicate that the partial gene duplication that is responsible for the two mRNAs in *C. elegans* and *H. contortus* took place fairly late in the evolution of the nematodes. It is also possible that our detection of only a single *gbr-2* mRNA in *A. suum* is a reflection of the tissue used in these experiments. Attempts to resolve this issue by isolating the gene using PCR experiments on genomic DNA were inconclusive. The single *A. suum* cDNA we did amplify was clearly more closely related to *gbr-2B* than *gbr-2A*, which might suggest that this was the original gene product. A comparison of the sequences of the GBR-2 subunits from the filarial species *O. volvulus* and *D. immitis* (Fig. 2) with those reported here showed that the *O. volvulus* subunit was more similar to GBR-2B. There is insufficient sequence of the *D. immitis* subunit to be certain, but the short sequence of TM1 that is present in the database is more similar to that found in the GBR-2A than the –2B subunits.

At present, there have been no reports of the successful expression of any GBR-2 subunit in an *in vitro* system. Based on sequence similarities and the avermectin resistance associated with mutations in the *C. elegans* gene [20] we believe that it is most likely that these subunits form part of the nematode Glu-Cl, but confirmation of this awaits successful expression studies.

We were able to produce antibodies against a synthetic peptide corresponding to a portion of the GBR-2B subunit that reacted specifically with neuronal structures in both parasitic species. ELISA experiments using peptides derived from the same part of the GBR-2A and –2B sequences indicated that the antibody was not able to distinguish between the two gene products. Given the level of sequence conservation between the orthologous subunits from all nematodes, we believe the antibodies are recognising the *H. contortus* and *A. suum* GBR-2 subunits in the immunofluorescence experiments. The results suggest that one or both subunits are expressed widely in the nematode nervous system, including the nerve ring, but are not present on pharyngeal or body-wall muscle cells. The localisation results are consistent with our ability to amplify *gbr-2* cDNAs from dissected muscle strips of *A. suum* that would include the nerve cords. Reporter gene experiments have shown that the *C. elegans gbr-2/avr-14* gene is expressed in neurons of the nerve ring and tail (J. Dent & L. Avery, personal communication), and these results are also consistent with our observations. Mutations in *avr-14* can, if combined with a mutation in a second subunit encoded by the *avr-15* gene, lead to a high level of ivermectin resistance in *C. elegans* [20]. AVR-15 is

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[19] Blaxter ML, De Ley P, Garey JR, et al. A molecular evolution framework for the phylum *Nematoda*. Nature 1998;392:71–5.

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Chapter 2 Materials and Methods

FIGURES

TABLES

Chapter 1

Introduction

1.1 Ligand-gated ion channel family

1.1.1 Synaptic transmission within the ligand-gated ion channel family

Chemical synaptic transmitters mediate a flow of information allowing cells to communicate with each other throughout the body. The first evidence that neurotransmitters served as chemicals released from nerve terminals was presented in 1921 when a substance was released from the vagus nerve it acted on the heart preparation below, to cause a reduction in the rate of its beating. This substance was later shown to be acetylcholine. With the later discovery of neurotransmitters, their antagonists, and precursors the underlying mechanisms in which these transmitters activated distinct classes of receptors was established. Since then neurotransmitter receptors have been divided into two classes: (1) ionotropic receptors, that elicit a rapid opening of an ion channel only when the receptor is occupied by an agonist; and (2) metabotropic receptors, which mediate a response indirectly via a G-protein and/or other intermediate secondary messengers.

In this and subsequent sections I explored the structure, pharmacological and functional classification on ionotropic receptors with particular relevance to the L-glutamate-gated anion channels.

1.1.2 Structural relationship of ligand-gated ion channels

Initial biochemical and pharmacological studies on ligand-gated ion channels utilised highly enriched nicotinic receptor synapses isolated from the electric ray, *Torpedo.* Subsequent cloning of nicotinic acetylcholine and related receptor subunits (nAChR) (Changeux, 1993) have revealed an extensive family of ionotropic neurotransmitter receptor subunits, which are forever increasing in size. Comparison of these protein subunits to γ-aminobutyric acid ($GABA_{A\&C}$), glycine, 5-hydroxytryptamine ($5\text{-}HT_3$) and glutamate-gated cation channels revealed considerable sequence homology (sharing 20-40% identity) and protein similarities, indicating strong evolutionary conservation. Information obtained by hydrophobicity and membrane topology studies (Karlin and Akabas, 1995) has lead to the identification of four main structural families of ligand-gated ion channel; glutamate-gated cation channels, P_2 purinoceptors (P_{2X}), tyrosine-kinase-linked type and the "cys-loop" receptor family (which includes nACh, $5\text{-}HT_3$, $GABA_{A\&C}$, glycine and inhibitory glutamate-anionic channels (GluCl) (Fig. 1.1).

The cys-loop receptor family at present consists of five receptor types; $GABA_{(A+C)}$, glycine, $5HT_3$, GluCl and the archetypal example nACh. Structural studies have determined that these receptors are a multimeric complex composed of five cylindrical subunits that traverse the membrane (Brisson and Unwin, 1985; Unwin, 1995) creating a narrow central pore that acts as a pathway for specific ions, (Fig. 1.2, Miyazawa *et al.*, 1999). The membrane pore is lined by five α-helical M2 domains (one

from each subunit) (Changeux *et al.*, 1992) containing the gate of the channel in the absence of the ligand (Unwin, 1993).

Each subunit consists of a large extracellular N-terminal domain, which contains consensus sequences for N-linked glycosylation (Karlin and Akabas, 1995) and amino-terminal signal peptides. Signal peptides are cleaved co-translationally and after inserted into the membrane of the endoplasmic reticulum. A constant motif found in the amino-terminal of these receptors is two cysteine amino acids that form a disulphide bridge separated by 13 amino acids. The consensus sequence (CPMxLxxYPxDxQxC) for this feature has led to the designation "cys-loop" receptor family and is involved in assembly of the receptor subunit (Green and Wanamaker, 1997).

The carboxyl half of the subunits contains four extended hydrophobic domains, 19-27 amino acids in length, which form the transmembrane regions M1-M4 (Popot and Changeux, 1984). By traversing the plasma membrane they allow the receptor to be exposed to both internal and external chemical signals. Short regions of hydrophilic amino acids separate the first three transmembrane domains, with a longer cytoplasmic loop separating M3 and M4. The intracellular loop is of a mixed hydrophilicity and may contain consensus sequences for protein phosphorylation. Most ligand-gated ion channels are subjected to phosphorylation, which may have great importance for regulating receptor function (Moss *et al.*, 1995; Moss and Smart, 1996; Tanaka and Nishizuka, 1994).

Initial hydropathy studies (Karlin and Akabas, 1995) indicated that excitatory glutamate subunits were considered to transverse the membrane four times (Hollmann and Heinemann, 1994), as seen typically with the cys-loop receptor subunits. This allows the N-terminal and the carboxyl-terminus regions to face the extracellular space, with the predicted binding site on the N-terminal domain. Further observation of endogenous glycosylation sites attached to the intracellular loop inside the lumen of the glutamate receptor indicated this model was incorrect (Hollmann *et al.*, 1994; Taverna *et al.*, 1994).

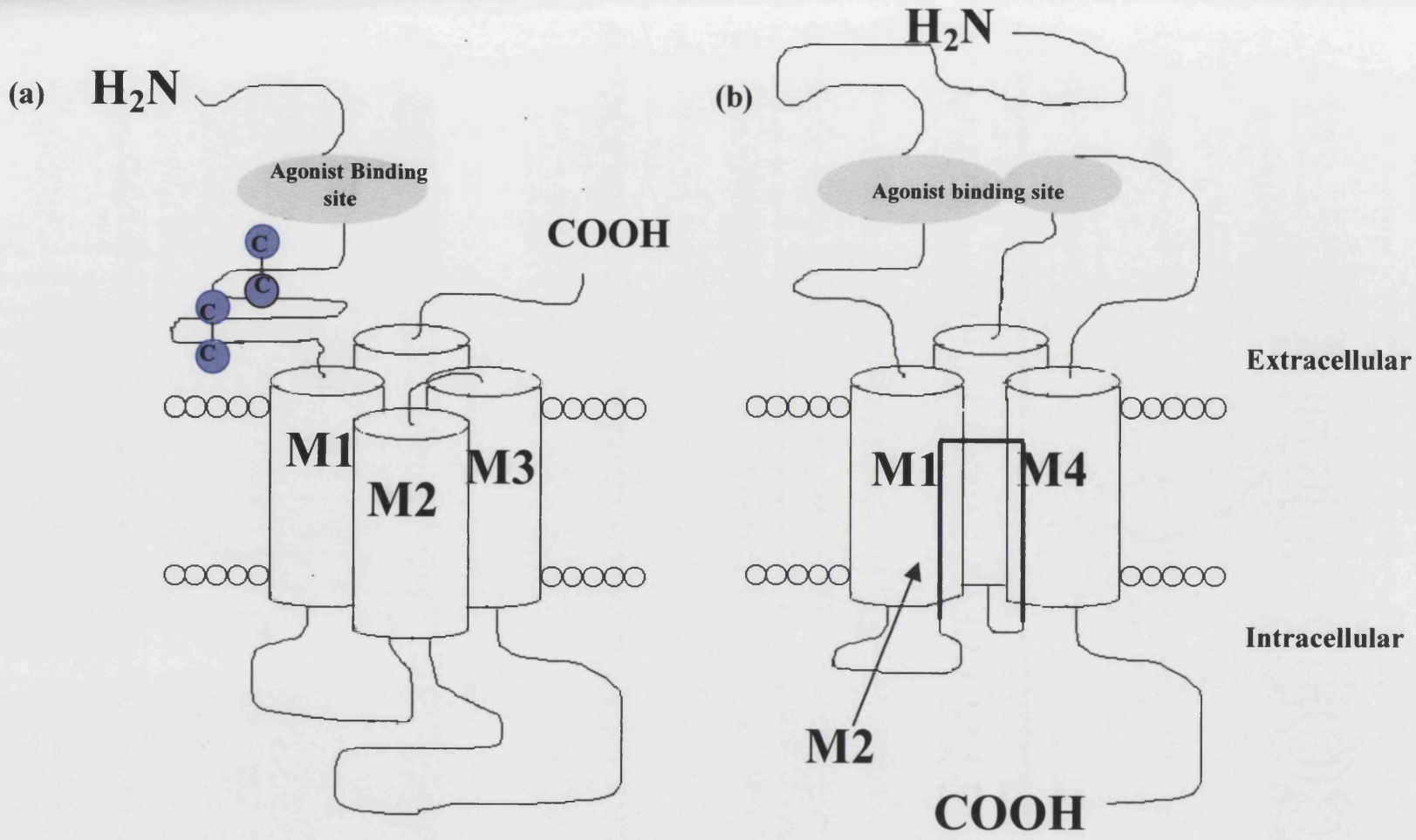


Figure 1.1 Schematic drawings of ligand-gated ion channels. (a) The cys-loop receptor family composed of four hydrophobic transmembrane regions (M1-4), with a long intracellular loop (M3-M4) and an extracellular amino-terminal. (b) Proposed structure of glutamate-cation receptor family, consisting of a large amino-terminal that combines with the extracellular M3-M4 loop to form an agonist-binding site. The C-terminus is intracellular and the M2 domain does not transverse the membrane. Drawings based on proposed electron diffraction imaging and hydropathy studies (Unwin, 1993; Karlin and Akabas, 1995).

Following much conflicting evidence (McGlade-McCulloh *et al.*, 1993; Yakel *et al.*, 1995), it has been currently accepted by most researchers (Bennet and Dingledine, 1995; Hollmann, 1997; Kuner *et al.*, 1996) (Fig. 1.1b) that the pore forming glutamate receptor subunits consist of three transmembrane regions (M1, M3 and M4), suggesting that the M2 sequence does not traverse the cell membrane but forms a re-entrant loop within it. Other dissimilarities from the cys-loop receptors soon emerged. The overall sequence homology of this class with the cys-loops is very low, especially within the M2 region. The amino-terminal domain of the glutamate-cation subunit is approximately twice as long (400 amino acids) (Barnard, 1992; Unwin, 1993) as the cys-loop receptor family and fails to contain the conserved cys-loop feature (Hollmann *et al.*, 1994b). The amino acid region separating the M3 and M4 domains is extracellular and appears to form part of the agonist binding site with the *N*-terminal domain (Hollmann, 1997), as opposed to the C-terminal which is intracellular (Fig. 1.1b). By comparison, the topology of the P_{2x} channels is substantially different from both the cys-loop and the glutamate-sensitive receptors. The P_{2X} receptors consist of two hydrophobic domains that transverse the membrane and are separated by a hydrophilic extracellular loop of approximately 270 amino acids (Brake *et al.*, 1994; Vallera *et al.*, 1994).

1.1.3. Structural-assembly of the cys-loop receptor subunits

Images derived from electron microscopy of the nAChR postsynaptic membranes from the *Torpedo marmorata* electroplax have outlined three dimensional structures for open and closed channel forms (Unwin, 1993, Unwin, 1995;) whilst retaining the receptor within its native environment. The experiment consists of spraying droplets of ACh whilst rapidly freezing the membranes in a low-salt solution trapping the structural response (Unwin, 1995). Comparison of the receptor open-channel structure with that of the resting state has allowed conclusions to be drawn concerning the mode of action.

Unwin's (1993, 1995) observations of the nAChR resolved the structure to be cylindrical with an overall length of 125Å and a diameter of 80Å (Fig. 1.3). The N-terminal region of the receptor reaches 60Å from the membrane surface and contains two cavities thought to be the acetylcholine (Ach) binding pockets. Furthermore, the cavities appear to be the correct size and shape to accommodate the acetylcholine molecule (Miyazawa *et al.*, 1999). In a recent study (Miyazawa *et al.*, 1999) narrow openings in the wall of the receptor from the vestibule to the extracellular acetylcholine binding sites have been observed and are proposed to be the primary route for acetylcholine molecules to gain access to the agonist binding site (Fig. 1.2).

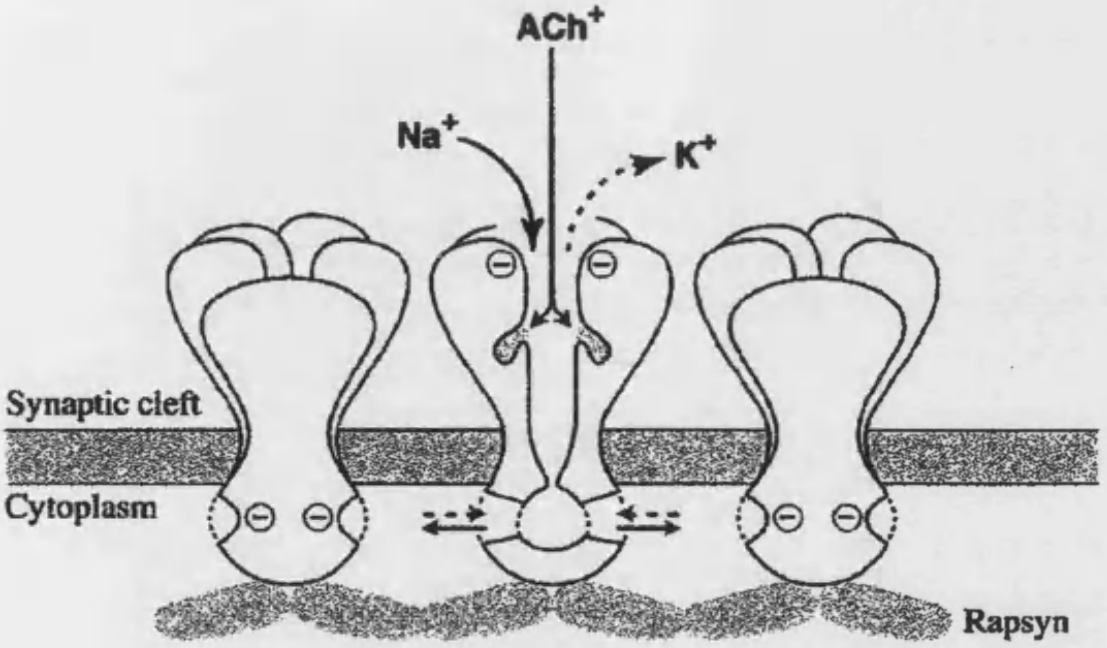


Figure 1.2 Schematic drawing summarising certain aspects of receptor structure (courtesy of Miyazawa *et al.* 1999).

1. Acetylcholine molecules enter the extracellular region of the vestibule before passing through narrow tunnels that lead towards binding pockets
2. Gate-forming amino acid residues line the M2 region two-thirds down the channel.
3. Upon gating the channel wall pores within the cytoplasmic domain act as ion filters regulating the movement of ions that enter or leave the cell. Rapsyn molecules and the cytoplasmic loop region cluster at the base of the subunit and discriminate between ion charges.

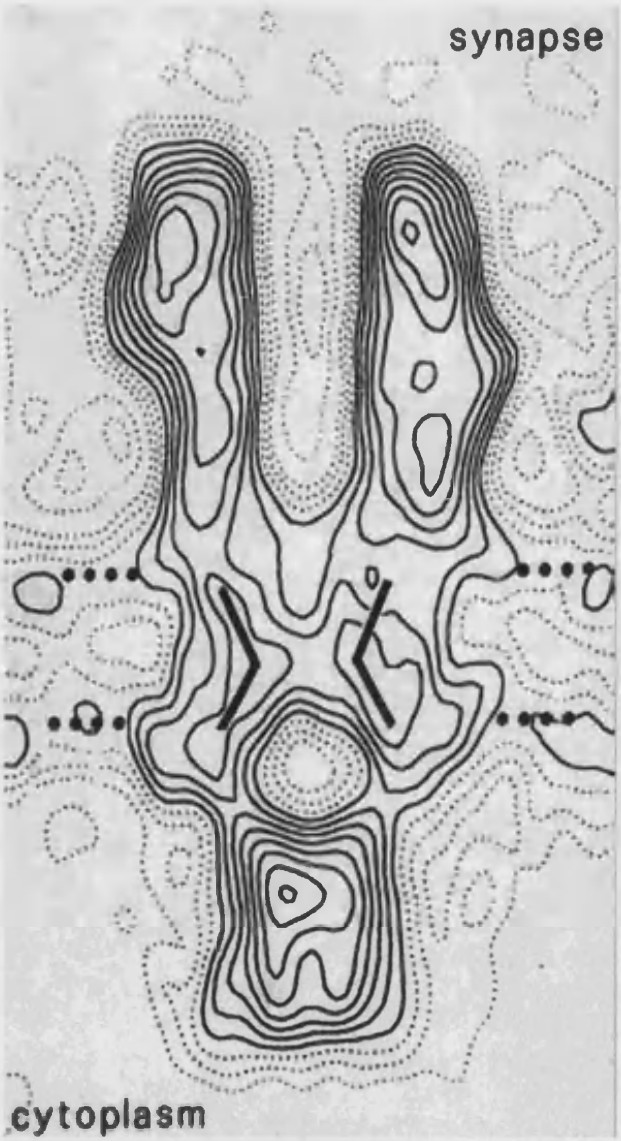


Figure 1.3 Electron diffraction imaging outlined a three-dimensional structure for the nAChR from the postsynaptic membranes of the *Torpedo marmorata* (Image courtesy of Karlin and Akabas, 1995). The cylindrical receptor was found to be 80Å in diameter and 125Å in length, with most of the protein mass (55%) on the synaptic side of the membrane and 20% on the cytoplasmic side. The extracellular portions of the nAChR molecule extends 60Å into the synapse, with the ACh binding site roughly 33Å above the membrane, while the intracellular domain extends 15Å into the cytoplasm (Unwin, 1995). At the synaptic face the pore is 20-25Å in diameter but narrows to 7Å where the receptor traverses the membrane bilayer. Image courtesy of Changeux, 1993).

The water-filled channel pore passes longitudinally extending the full length of the receptor. At the surface of the pore the diameter of the lumen is 20Å, but narrows rapidly to approximately 7Å two-thirds of the way through bilayer and then widens out as it approaches the cytoplasmic portion (Unwin, 1993). The narrow strip of density that bridges the pore in the closed state is thought to correspond to the receptor gate (Miyazawa *et al.*, 1999; Unwin, 1993). The pore lining is thought to be composed of five rod-shaped columns (corresponding to the M2 domains of each subunit), which form the pore lining. In a closed configuration the midpoints of the rods appear kinked and slope towards the centre of the channel. The pore is therefore exposed to ions in the extreme extracellular region but become closed off as the centre of the channel kinks. Upon gating small clockwise rotations of three rod-shaped proteins within the α-subunit binding domains produce conformational changes causing the disappearance of the α subunit-binding cavity (Miyazawa *et al.*, 1999). The disturbance becomes propagated down the shaft of the receptor resulting in distortion of all five rod-shaped columns that surrounds the central pore (Unwin, 1995). Clockwise rotation of these kinked sections lining the pore's lumen causes them to be swung sideways from the axis of the channel, opening the lumen by 9-10Å, whilst the cytoplasmic portion rotates inwards (Unwin, 1995).

The cytoplasmic base of the receptor in the closed formation also restricts the passage of ions by a central mass of clustering rapsyn protein (Miyazawa *et al.*, 1999). Ion flow from both directions has to traverse narrow openings (pores approximately 10Å in diameter protrude from each subunit) from inside the channel wall before entering or leaving the pore (Fig. 1.2) (Miyazawa *et al.*, 1999). It is plausible that the pores act as filters excluding anions and cations into the channel facilitating transport (Miyazawa *et al.*, 1999). In addition the cytoplasmic loop structure (M3-4) lies at the base of the subunit and has also been proposed to act as a filter (Popot and Changeux, 1984). The intracellular loop is made up of mixed hydrophilicity and is able to discriminate between ions of different charge. For cation conducting channels (ACh, 5-HT_3) the net charge of the loop would be negative, and for the anion channels (GABA, glycine, IgluCl) positive. This proposed structural model of the nACh receptor is thought to be similar to that of each of the five receptor types of the cys-loop family.

1.1.4. Ion selectivity within the pore

Unwin's structural data is in agreement with experimental data obtained from chemical labelling and mutagenesis (Unwin, 1995; Unwin, 1993). Early indications that the M2 domain may contribute to the lining of cys-loop receptors came from photoaffinity labelling studies. Non-competitive nACh blocking reagents such as the neuroleptic chlorpromazine, the anticonvulsant MK801, the dissociative hallucinogenic, anaesthetic phencyclidine (PCP) and the anaesthetic lidocaine derivative (Karlin and Akabas, 1995; Revah *et al.*, 1990) bound at various depths within the pore. At different levels five specific amino acids, one contributed by each subunit, identified key rings of charged or neutral amino acids (Fig. 1.4).

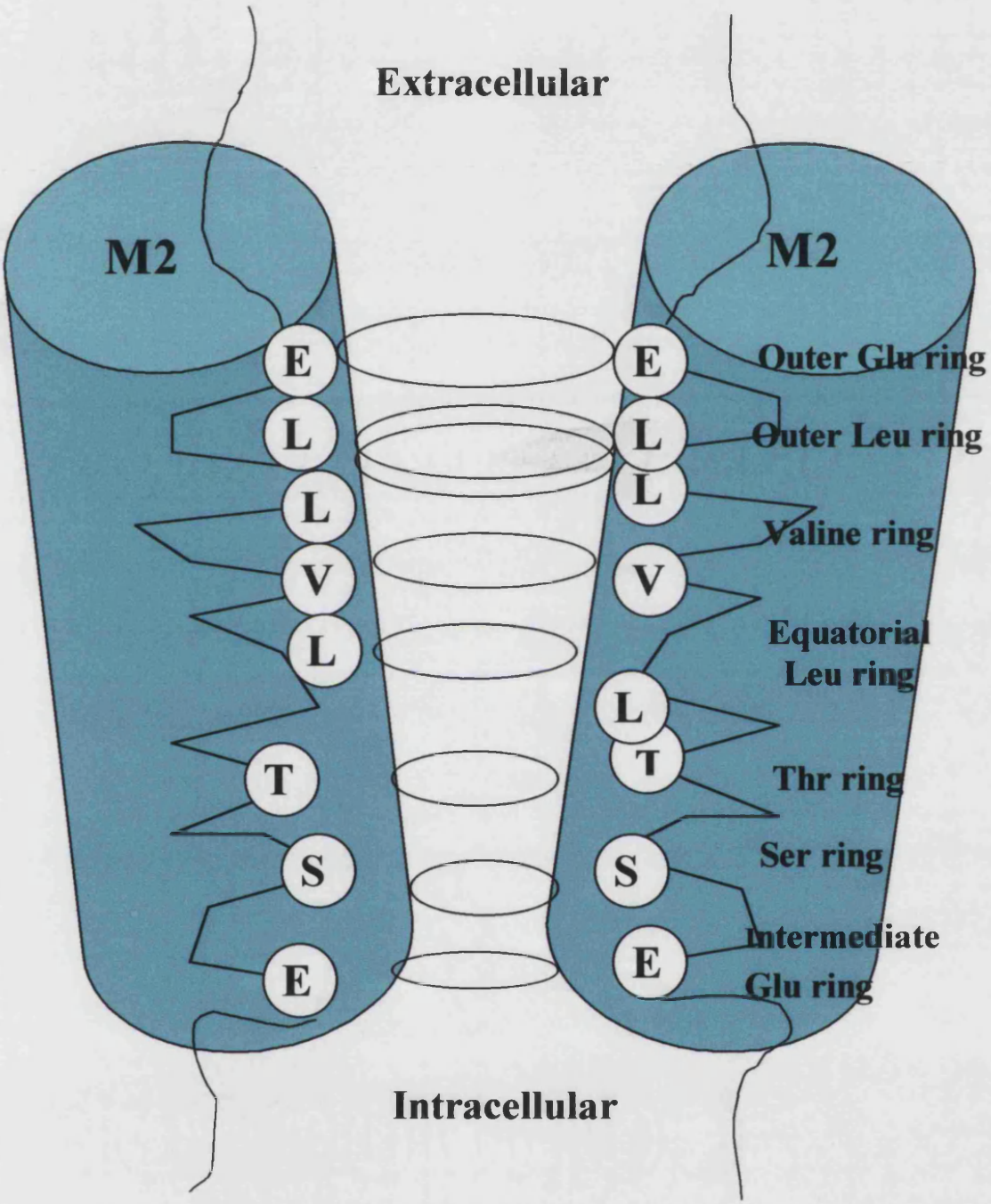


Figure 1.4 Model of the amino acid ring structure within the channel pore (adapted from Galzi and Changeux, 1995).

The seven rings identified govern the pharmacological and ion transport properties of the pore, three in particular have been classified as the major determinants of the rate of ion transport through the nAChR channel (Imoto *et al.*, 1988). The three most important ionic rings are composed of clusters of either negative or positive charged residues that are situated in between the transmembrane regions (Fig 1.5). The extracellular ring in the M2-M3 portion is located close to the external mouth of the channel and is thought sufficient to determine ion selectivity (Imoto *et al.*, 1988). The intermediate ring is positioned between the two other rings, towards the cytoplasm end. Anionic channels differ from cationic receptors in the charge of the intermediate ring (Fig. 1.5, Imoto *et al.*, 1988). The third, cytoplasmic ring in all nAChR and some GABA receptors contains a negative charge, which is based at the intracellular end (in the M1-M2 region) and has a weak effect on ion flow (Imoto *et al.*, 1988).

Positively charged groups (arginine and lysine residues) cluster at the mouth of the channel of the M2 domain of anion channels and determine ion selectivity into the cell (Barnard, 1992). Similarly cation channels (nACh and $5HT_3$) are occupied by negatively charged or neutral residues (Barnard, 1992; Unwin, 1993).

A)

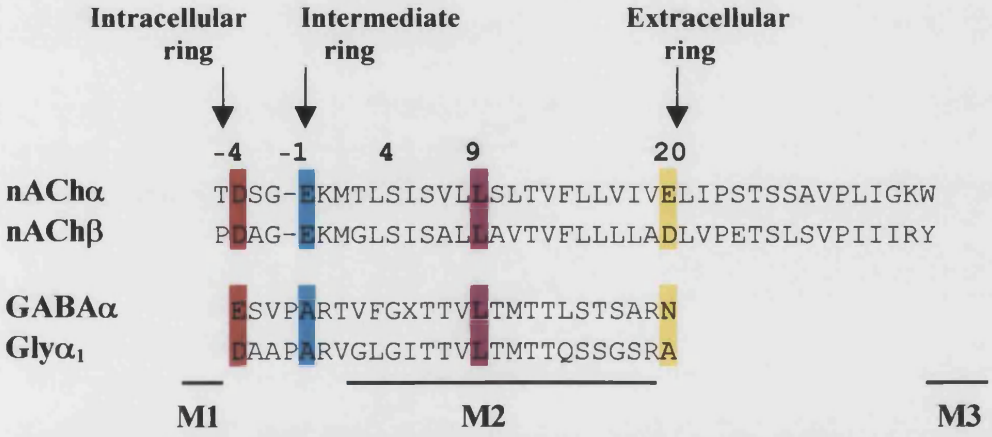


Figure 1.5. Alignment of M2 amino acid sequence from nACh, rat GABA α_1 and glycine α_1 receptor subunits indicating the locations of the three ionic rings, intracellular (, position –4), intermediate (, position –1) and extracellular (, position 20); (alignment taken from Noda *et al.*, 1982). The conserved equatorial ring of leucine residues (position 9), has been hypothesised to form the receptor gate (Unwin, 1993). This figure uses the terminology of Miller, which assigns position 1' to the amino terminus of the M2 domain (the cytoplasmic end, Miller, 1989).

1.1.5 The channel 'gate'

During resting or desensitised states the channel 'gate' prevents the flow of ions traversing the ion channel. To date there is some debate as to the location of the gate. Structural studies have suggested that conserved equatorial ring of leucine (Fig. 1.5, the number 9'leucine (▮)) side-chains projecting towards the lumen from the kink of each subunit may form a tight hydrophobic ring, occluding the channel to ions and therefore serving the purpose as a receptor gate (Unwin, 1993). In addition, leucine residues have been measured to project 7-8Å (Richmond and Richards, 1978) confirming that a barrier occluding the centre of the pore could be formed. Mutation of the leucine residue to amino acids of different size and polarity have been found to affect the functional properties of the channel (Bertrand *et al.*, 1992; Revah *et al.*, 1991) causing noticeable modifications to ion flow when responding to acetylcholine. Substitution of the leucine amino acid with a smaller, serine or alanine residue resulted in a receptor that still gates (Bertrand *et al.*, 1992; Labarca *et al.*, 1995; Revah *et al.*, 1991), suggesting the leucine residue may influence the ion transport of the receptor, but does not occlude the pore. In addition, residues deeper than the leucine were labelled in SCAM analysis in both GABA (Xu and Akabas, 1993) and nACh (Akabas *et al.*, 1994) channels in the closed formation, indicating the channel gate is more likely to be located on the intracellular end of the M2 (closer to the M1-M2 loop), rather than two-thirds down the M2 as originally proposed. This data indicates that the activation of a receptor is complex and the exact location of the channel gate is still clearly disputed by some authors.

1.2 Ligand-gated chloride channels

Despite the fact that vertebrate and invertebrate species fundamentally use the same neurotransmitters, distinct pharmacological differences in key target sites have been identified at the physiological and molecular level. In particular, the increasing knowledge in ligand-gated chloride channels seems to be particularly beneficial for attempts to exploit these sites for future drug development. The remaining components of this chapter explore a brief pharmacological profile of the ligand-gated chloride channels, which is relevant to inhibitory GluCls and experimental chapters to follow.

1.2.1 GABA and glycine receptors

The discovery of GABA was made in the early 1950s and the first report of GABA receptors was from invertebrate preparations of lobster and crayfish, where GABA exerted an inhibitory response that was later found to be blocked by picrotoxin (Bazemore *et al.*, 1957).

To date, vertebrate GABA receptors have been classified into three distinct subtypes: the ionotropic $GABA_A$ and $GABA_C$ receptors, which are members of the cys-loop ion channel family (Olsen and Tobin, 1990) and the metabotropic $GABA_B$ receptors which link to an effector channel via G-proteins (Andrade *et al.*, 1986) coupled to a second messenger system. The $GABA_A$ subtype forms a hetero-oligomeric protein composed of seven distinct polypeptide subtypes α_1-α_6, β_1-β_4 γ_1-γ_4, δ, π and θ (Sinkkonen *et al.*, 2000). The application of GABA leads to a rapid, reversible change in the chloride conductance of the cell membrane. Various combinations of these subunits are combined to form receptors of differing pharmacology and location within the nervous system (Betz, 1990; Macdonald and Olsen, 1994). Sequence comparisons of these polypeptides subtypes indicate they show 20-40% amino acid identity with each other, 10-20% amino acid identity with subunits of the nACh, glycine and IGluCl receptors and 60-80% amino acid identity amongst the individual members of the subunit family (Olsen and Tobin, 1990).

Pharmacologically the vertebrate $GABA_A$ and the $GABA_C$ receptor subunits can be distinguished by the antagonist bicuculline. The vertebrate $GABA_A$ receptors are competitively antagonised by bicuculline, whereas $GABA_C$ are insensitive (Feigenspan *et al.*, 1993; Sieghart, 1995). Conversely the majority of insect and nematode GABA receptors are insensitive to bicuculline, pharmacologically distinguishing them from their vertebrate counterparts (Sattelle, 1990). The $GABA_A$ receptor complex contains a large number of binding sites for drugs, notably the benzodiazepines (BZDs), barbiturates and steroids (Olsen and Tobin, 1990; Sieghart, 1995) which is outside the scope of this thesis, and only a few aspects of particular relevance to the experimental chapters that follow will be mentioned.

More extensive reviews on $GABA_A$ pharmacology can be found in: Sattelle, 1990; Sieghart, 1995; Krogsgaard-Larsen, 1994; Macdonald, 1994 and on glycine receptors in Becker, 1992; Vannier, 1997.

Recently the first full-length *Drosophila melanogaster* GABA-gated chloride channel RDL (resistant to dieldrin) was cloned (ffrench-Constant *et al.*, 1993a), which surprisingly showed higher overall sequence homology to vertebrate glycine receptors (31-32%) than $GABA_A$ or $GABA_C$ subunits (27-29%), revealing that RDL is a member of a novel class of GABA receptor subtypes (Zhang *et al.*, 1994). In addition, unlike most vertebrate $GABA_A$ receptor subunits, the RDL forms a highly functional homomeric receptor. To date, insect GABA-gated chloride channels are not readily classified as $GABA_A$ or $GABA_C$ type, but their antagonist profile is clearly distinct from their vertebrate counterparts (Sattelle, 1990) and potentially provides opportunities to exploit this receptor site for the development of selective, inhibitory neuromuscular drugs (Rauh *et al.*, 1990).

Vertebrate glycine-gated chloride channels tend to predominate in the spinal cord and brain stem regions, compared to GABA receptors, which are abundant in the cortex and cerebellum regions (Becker, 1992). Molecular cloning and affinity purified glycine receptor preparations have revealed four subtypes of the glycine receptor α subunits (denoted α1-4), but as yet no variations on the β subtype have been identified. Analysis of the amino acid sequence revealed these receptor subtypes to be highly homologous to inhibitory L-glutamate-gated anion channels found in invertebrates (approximately 40%), but was shown to differ in terms of both pharmacology and localisation. Glycine receptors are confined to vertebrate tissues and are gated by glycine, where as GluCls have only been identified in invertebrates and are mostly influenced by L-glutamate. Consistent with GluCls, the glycine receptors contain a second pair of cysteine residues within the N-terminal that does not exist in GABA or cation channel. Expression of α subunits form glycine-sensitive currents, which are blocked both by strychnine and picrotoxinin (Becker, 1992).

1.2.2 L-glutamate-gated chloride channels

The L-glutamate-gated chloride channels (GluCls) are an important group of ionotropic receptors, so far only known to be present on invertebrate nerve and muscle cells. The GluCls are closely related to $GABA_A$ and glycine receptors, and are presumed to possess the same pentameric structure (Section 1.1.2) (reviewed by Cleland, 1996), sharing about 40% identity with glycine receptor subunits and 30-35% identity with $GABA_A$ receptor subunits at the amino-acid level. Phylogenetic analysis of GluCl subunit sequences has led to the suggestion that they may be a distinct class of ionotropic receptors orthologous to vertebrate glycine receptors (Vassilatis *et al.*, 1997a).

Studies on the physiology and pharmacology of cloned and expressed GluCls has provided further insights into the properties and functions of inhibitory ionotropic receptors and opportunities for the

development of novel anti-parasitic drugs. There is now strong evidence that the avermectin/milbemycin family of endectocides and insecticides targets the GluCls (Arena *et al.*, 1995; Arena *et al.*, 1992a; Cully *et al.*, 1996a; Cully *et al.*, 1994), although not all GluCl subunits are sensitive to these compounds *in vitro*.

In the free-living nematode *C. elegans*, six cDNAs encoding two classes of putative GluCl subunits have been identified. The first two subunits to be cloned from *C. elegans* have been designated GluClα and GluClβ and were isolated using an expression cloning strategy (Cully *et al.*, 1994). The *glc-1* and *glc-2* genes encode these two subunits respectively. Later, Dent *et al.* (1997), reported the cloning of a subunit encoded by the avermectin resistance gene, *avr-15,* with a high level of sequence identity (75% at the amino-acid level) to GluClα but only 45% identity to GluClβ and it was therefore designated GluClα_2. Alternative splicing of *avr-15* resulted in two gene products – a short (GluClα_{2S}) and a long (GluClα_{2L}) form, that share ligand-binding and transmembrane domains (Dent *et al.*, 1997). The long form, GluClα_{2L}, included a long, 171 amino acid, N-terminal extension unrelated to any other ionotropic channel subunit sequence. In a later study Laughton *et al.*, (1997b) reported the cloning of two further *C. elegans* subunit cDNAs, GBR-2A and GBR-2B that were closely related in sequence to the known GluCl subunits. These two subunits are alternatively spliced products of a single gene, *gbr-2*. The GBR-2A cDNA contains the channel encoding sequence of GBR-2B and both subunits possess identical extracellular domains, suggesting that these nematodes will possess receptors with identical ligand binding sites but slightly different channel properties.

Dent *et al.*, (2000) demonstrated that the *avr-14* ivermectin resistant gene encodes the GBR-2 subunits, which have now been designated GluClα_{3A} and $_{-\alpha 3B}$. A more distantly related ionotropic receptor subunit cDNA, C27H5.8, was cloned using information from the genome sequence project (Cully *et al.*, 1996b), however, to date no functional data are available for this subunit. In the *Drosophila melanogaster* species a single GluCl subunit has been cloned from the insect heads (DrosGluClα) sharing ~48% sequence identity to the *C. elegans* GluClα subunits and 43% with the GluClβ subunit (Cully *et al.*, 1996a).

BLAST searches of the *C. elegans* genome sequence revealed a further predicted polypeptide, ZC317.3, with a high amino-acid identity to the cloned GluCl subunits. We report here the amplification and expression of the cDNA encoding this polypeptide. Based on the sequence of the polypeptide and functional expression data we conclude that this gene, designated *glc-3*, encodes a further GluCl subunit, GLC-3, from *C. elegans* (see chapter 4 and 5).

1.2.3 Ligand-binding sites of GluCl receptors

Studies of agonist binding sites to date have been particularly dependent upon photoaffinity labelling and mutagenesis studies. The formation of chimaeric homo-oligomers subunits has been advantageous in locating the essential agonist-binding site within the N-terminal domain. Evidence of discrete, homologous regions within the N-terminal of the cys-loop receptor and the extracellular loop between M2 and M3 have been found to influence the binding site in all ligand-gated ion channels. In a previous study (Etter *et al.*, 1996), chimaeras composed of the N-terminal of *C. elegans* GluClα receptor subunit (glutamate-insensitive subunit), with the channel domain of GluClβ (glutamate-sensitive subunit) yielded receptors with greater glutamate sensitivity than seen with the wild type GluClβ alone. This indicates that the GluClα homo-oligomer contains the necessary binding site within the N-terminal domain, but the C-terminal does not couple ligand binding to channel gating. Consequently, not only providing further evidence for the location of the agonist binding site in the N-terminal domain, but in addition, showing that the ligand binding region is coupled to channel gating within the C-terminal.

The GABA and nACh binding sites are formed by the interface of two adjacent subunits (Middleton and Cohen, 1991). If we assume similar protein folding occurs in all the cys-loop family it is possible that for the agonist binding sites of L-glutamate and ibotenic acid to exist it will require the co-assembly of two structurally distinct regions at the subunit interface. Thus, for the L-glutamate binding site to exist it will require the interface of two distinct α subunit classes (for example, two GluClα_2 subunits or one α_2 and one β subunit). The structural location of the amino acids from the *Torpedo* nACh receptor has led to the proposal of a multiple loop model-binding site. Three agonist-binding domains have been identified from the nAChα subunits (corresponding to A, B and C), (Kao *et al.*, 1984; Middleton and Cohen, 1991) and three from the non-α subunits, corresponding to D, E and F, (Czaijkowski and Karlin, 1995; Kao *et al.*, 1984; Middleton and Cohen, 1991).

Certain key amino acids that have been identified as potential binding sites in GABA receptor subunits have also been found to be conserved in GluCl receptor subunits (Wolstenholme, 1997). The residues that appear particularly conserved are at positions 212 (Q), 221-224 (YaxT) and 268-271 (TGxY), which correspond to 157-160 and 202-205 of the rat $GABA_A$ β_2 subunit (Fig. 1.6 and Fig. 1.7b). The conservation of these sites indicates their lack of participation in differentiating between amino acid ligands (Wolstenholme, 1997). A number of residues were also common to all cys-loop receptor subunits (WxDxxL and WxPD, Fig. 1.7a) and have been predicted to play some role in receptor assembly (Smith and Olsen, 1995).

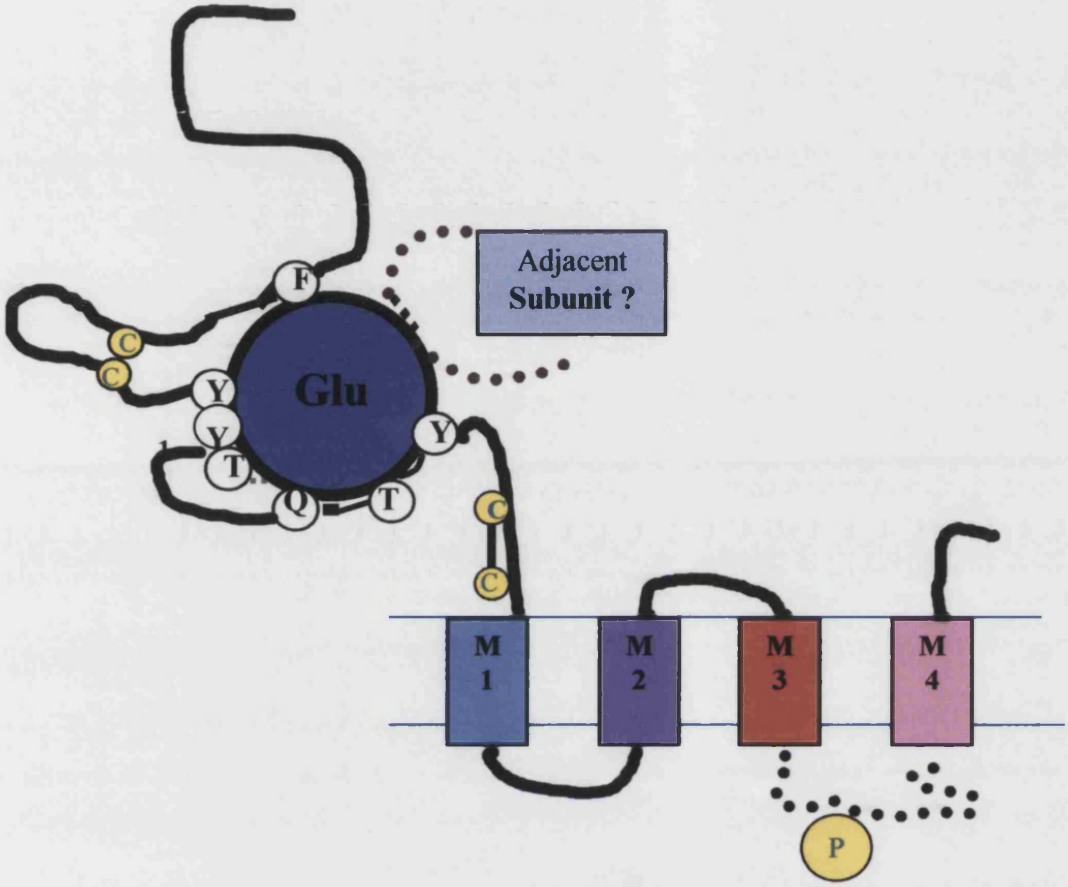


Figure 1.6 Potential amino acids involved in the glutamate-binding site of the GluCl receptor subunits. Key amino acids involved in the $GABA_A$ receptor binding sites (Olsen and Tobin, 1990) were also conserved in the glutamate-binding site of the GluCl receptor subunits (Wolstenholme, 1997). Schematic diagram courtesy of S. Wonnacott (adapted from a slide presentation). Key; C= cysteine loop, P= phosphorylation site, M (1 to 4)= transmembrane domains, F= phenylalanine, Y= Tyrosine, T= threonine, Q= Glutamine, not to scale.

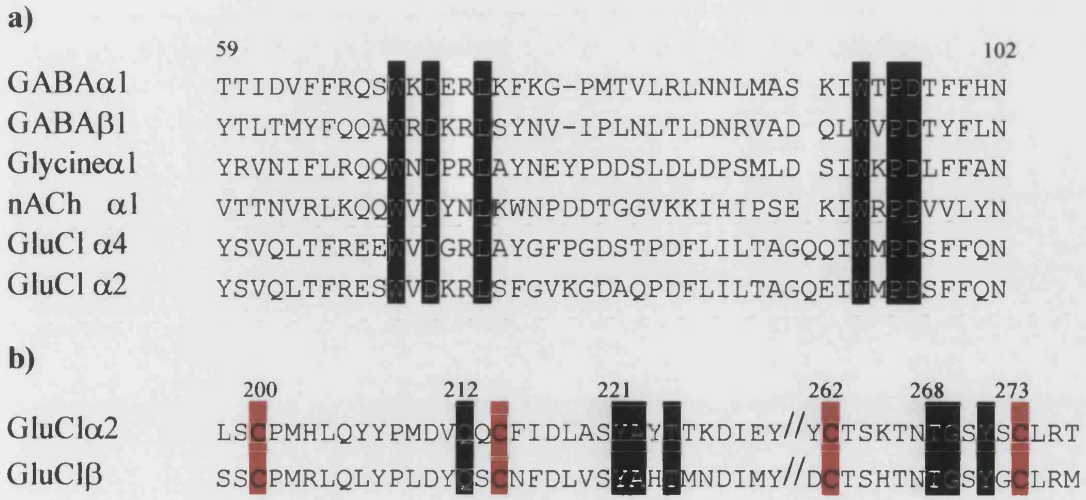


Figure 1.7 Ligand-gated ion channel receptor subunit alignment showing conservation about the GABA binding regions. a) The two highly conserved regions include WxDxxL and WxPD (high lighted in black) (reviewed in Smith and Olsen, 1995). GABA, glycine and nACh sequences are from rat, whereas the GluCl sequences are taken from *C. elegans*. b) Residues indicated for GABA agonist binding site are conserved in *C. elegans* GluCl subunits (highlighted in black) indicating they are not involved in discriminating between GABA and glutamate ligands (Wolstenholme, 1997).

1.3 Ligand-gated chloride channel pharmacology

1.3.1 L-glutamate and structurally related analogue, ibotenic acid

Recent studies have indicated that the classical excitatory ligand L-glutamate also now functions as an inhibitory ligand in invertebrates. The agonist profile of the L-glutamate-gated chloride channel is quite distinct from that of the excitatory channels. In general both classes of subunits are activated by L-glutamate and ibotenic acid, but the anionic receptors are insensitive to quisqualic acid, kainic acid and N-methyl-D-aspartate (NMDA) (Cull-Candy, 1976; Duce and Scott, 1985; Lea and Usherwood, 1973a). The excitatory glutamate agonists, kainate, NMDA, α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA), and quisqualate all failed to gate the inhibitory glutamate-gated chloride channel expressed from total *C. elegans* mRNA (Arena *et al.*, 1992a).

Ibotenic acid, a structural analogue of glutamate, gates the native glutamate-sensitive chloride channels present in locust muscle, locust neurons and lobster muscles (Cull-Candy, 1976; Lea and Usherwood, 1973b; Lea and Usherwood, 1973a; Wafford and Sattelle, 1989) in addition to expressed *in vitro* GluCl receptors (Cully *et al.*, 1992a, 1994; Dent *et al.*, 1997, 2000). However, ibotenate was found to be four times more potent than glutamate (EC_{50} of 70μM compared to 300μM for L-glutamate) in oocytes injected with total mRNA (Arena *et al.*, 1991; Cully *et al.*, 1994). In contrast, oocytes expressing homomeric GluCl channels yielded Cl^- currents of greater amplitude to L-glutamate than those observed with ibotenic acid. This observation has also been apparent in most arthropod and nematode native preparations, indicating that the probable *in vivo* subunit combinations have not been established with *in vitro* expression and additional subunits may be necessary to make up the wild-type channel.

1.3.2 Avermectins

Avermectin was first described as an anthelmintic in 1979, when nematode-infested mice were accidentally fed a fermentation broth generated from soil samples containing a bacterium, *Streptomyces avermitilis* (Campbell, 1981). The naturally occurring avermectins can be separated into four main components (A_{1A}, A_{2A}, B_{1a} and B_{2a}) of which only a synthetic derivative of the B_1 (22,23-dihydroavermectin, known as B_1 ivermectin) series will be described here. Ivermectin, a semi-synthetic mixture (consisting of 80% synthetic 22,23-dihydroavermectin B_{1a} and 20% natural B_{1b} products) of and is based on the natural product avermectin, produced by Streptomyces avermilitis (Campbell, 1981), with potential insecticidal and anthelmintic actions. These compounds have an excellent activity against nematodes, several ectoparasites, arthropods and plant pathogens (Putter *et al.*, 1981).

The success of these compounds has mostly been attributed to their ability to kill parasites without affecting the host organism. Vertebrate studies on rat brain observed that avermectin binding sites have approximately one-hundred-fold lower affinity than in *C. elegans* (Turner and Schaeffer, 1989) indicating that the concentration required to gate vertebrate channels would be far in excess of that required to kill the parasite. In contrast to this, studies have also indicated that in a similar manner to L-glutamate channels, ivermectin potentiated the response to low concentration of GABA in oocytes injected with rat brain mRNA (Rohrer *et al.*, 1995), consequently decreasing the EC_{50} from 42 to 3μM and reducing the effective concentration of ivermectin necessary to gate the mammalian $GABA_A$ receptor.

The anthelmintic mode of action of avermectins still remains unclear. *In vitro* experimental evidence indicates that avermectins act by disrupting neuromuscular transmission and increasing cellular permeability to chloride ions (Arena, 1994; Turner and Schaeffer, 1989). However, their behaviour in whole nematode preparations varies extensively. Incubation of the non-parasitic nematode, *C. elegans* on avermectin agar plates (20ng/ml), (Arena *et al.*, 1995) produced inhibition of pharyngeal pumping and a slow-onset of rigid paralysis. In contrast, its parasitic homologue *Haemonchus contortus* showed no obvious effects when submerged in the drug away from its natural environment (Avery, 1993; Turner and Schaeffer, 1989). Direct injection into the pig nematode *Ascaris suum* resulted in blocking signal transmission from excitatory motorneurons (Kass *et al.*, 1980) causing rapid paralysis that is neither rigid nor flaccid (Turner and Schaeffer, 1989). However, for blood sucking nematodes like *Haemonchus* the consumed blood containing the anthelmintic considerably contributes to the uptake of drug.

1.3.3 Ivermectin-sensitive chloride channels

When expressed in *Xenopus laevis* oocytes, the *C. elegans* α subunit formed functional homomeric channels, which are slowly activating and essentially irreversibly gated by ivermectin. The β subunit is not gated or potentiated by avermectin, but does form non-desensitizing channels gated by L-glutamate when expressed in the same system. When co-expressed in oocytes, the two subunits formed channels gated by L-glutamate and potentiated by very low (5nM) concentrations of ivermectin that have no direct effect on membrane current when applied alone. At higher concentrations, avermectin has the additional effect of directly activating the channel (Arena *et al.*, 1991). Furthermore, co-expression of the two subunits shifted the EC_{50} for glutamate from 380μM (for β) to 1360μM (for α & β), reducing the receptor sensitivity to the ligand, however, the EC_{50} values for ivermectin-induced currents were very similar (140nM for α and 190nM for α & β). These heteromeric channels resembled those formed following injection of total *C. elegans* mRNA into

Xenopus oocytes (Arena *et al.*, 1992a), however, there is no direct evidence that these subunits co-express in *C. elegans*.

The avermectin sensitive currents produced from oocytes injected with *C. elegans* total mRNA yielded irreversible, inward responses with an EC_{50} of 90nM and a reversal potential of –19.3± 1.3mV, strongly suggesting an avermectin-sensitive chloride channel (Arena *et al.*, 1992a). In addition, the L-glutamate-sensitive current is potentiated by submaximal concentrations of ivermectin, as demonstrated with GluClα_2 and heteromeric GluClα_1 and β subunits. All oocytes expressing either *C. elegans* mRNA or GluCl cDNA failed to be gated by GABA, benzodiazopines or bicuculline, which is consistent with data observed from binding assays (Schaeffer and Hains, 1989), however, avermectin current was antagonised by picrotoxin (Cully *et al.*, 1994; Cully *et al.*, 1996b). Later work demonstrated that the GluClα subunit possessed a binding site for L-glutamate but was unable to functionally gate the receptor when expressed alone in *Xenopus* oocytes (Etter *et al.*, 1996). This was initially discovered when a chimera between the *N*-terminal, extracellular region of GluClα and the C-terminal domain of GluClβ produced fully functional channels in oocytes that were gated by glutamate (Etter *et al.*, 1996). Secondly, when an engineered mutation was formed at position 308 in the M2 domain of GluClα, replacing a threonine with proline, glycine or alanine, homomeric channels became sensitive to glutamate (Etter *et al.*, 1996). However, the functional correlation of these binding sites in native tissues still remains to be determined.

Expression of the GluClα_{2L} subunit in oocytes formed homomeric channels gated both reversibly by L-glutamate and irreversibly by ivermectin (Vassilatis *et al.*, 1997b). The EC_{50} for L-glutamate was 208.3μM and 21.4μM for ivermectin, with Hill coefficients of 2.1 and 1.6 respectively. The Hill coefficient for most GluCl receptor subunits is approximately two, indicating that at least two molecules of glutamate must bind for the receptor to open. GluClα_2-induced avermectin currents were approximately 30-fold bigger than that observed with glutamate-sensitive currents (Dent *et al.*, 1997). Interestingly, co-expression of GluClα_2 and GluClβ resulted in channels that responded with a higher affinity to both L-glutamate and ivermectin, with EC_{50}'s of 62μM and 103nM respectively (Vassilatis *et al.*, 1997b) than when expressed alone. The increased sensitivity of the heteromeric combinations of GluClα_2 and GluClβ to L-glutamate and ivermectin may indicate they are naturally occurring (Vassilatis *et al.*, 1997b). Furthermore, the heteromeric channels were insensitive to GABA, glycine, aspartate, kainate and picrotoxin.

Recent data by Dent *et al.*, (2000) have shown that *avr-14* gene encodes an alternatively spliced GluCl related protein (previously called GBR-2A/2B) which has now been designated GluClα_{3A} & α_{3B}. When expressed in oocytes, GluClα_{3B} formed a ivermectin and glutamate-sensitive channel, however,

a response was only observed with homomeric GluClα_{3B} channels and not with GluClα_{3A} subunits (Dent *et al.*, 2000). Interestingly, the two subunits are products of alternative splicing indicating that they should contain identical ligand binding domains, with only two amino acid residue differences in their M2 regions, these are a threonine (α2A) to serine (α2B) substitution at positions 286 and a serine (α2A) to alanine (α2B) switch at 299. The amino acid substitution at position 286 is at the same position that is responsible for dieldrin and picrotoxin resistance in *Drosophila Rdl* subunits (ffrench-Constant *et al.*, 1993a).

A *Drosophila* glutamate-gated chloride channel was successfully expressed in oocytes forming homomeric glutamate- and ivermectin-sensitive currents. Unlike the GluClα_1, α_2 and β subunits from *C. elegans*, DrosGluClα exhibited robust, rapid, desensitised glutamate currents, which were not inhibited by picrotoxin or potentiated by avermectins (Cully *et al.*, 1996a). The EC_{50} for glutamate-gated currents was 23μM and 41nM for ivermectin-sensitive channels (Cully *et al.*, 1996a). It is therefore plausible that GluCl subunits represent targets of avermectins and may mediate some of the effects observed in whole nematode preparations.

1.3.4 Ivermectin binding studies

Specific, high affinity avermectin binding has now been established in at least two nematode species, *C. elegans* and *Haemonchus contortus*, and several insects (*Drosophila melanogaster, Schistocerca americana* and *Spodoptera frugiperda*). Surprisingly, the density of binding sites across the species range was quite variable. Both *Drosophila melanogaster* head preparations and *Schistocerca americana* metathoracic ganglia showed a 10- and 100-fold (respectively) difference in binding sites in comparison to mixed populations of *C. elegans*. In addition, specific avermectin binding sites were also observed in rat brain but were found to contain approximately one-hundred-fold lower affinity than identified in *C. elegans* (Turner and Schaeffer, 1989).

Presently, ivermectin resistance is presenting itself as an emerging problem, but to preserve its efficiency and to gain an understanding of the underlying mechanism of resistance a comparison of the target-binding site in resistant and wild type *Haemonchus* and *C. elegans* was examined. Studies conducted by Rohrer *et al.* (1994) and Hejmadi *et al.* (2000) demonstrated that membranes prepared from both sensitive and resistant *Haemonchus contortus* tissues exhibited high affinity ivermectin binding and the same number of binding sites per mg of tissue. The probable site of action of ivermectin are the L-glutamate chloride channels, suggesting resistance in the *Haemonchus* strain was unlikely to be due to affinity, alterations at the binding site or the number of available receptor binding sites (Hejmadi *et al.*, 2000; Rohrer *et al.*, 1994). Density of binding sites between *Haemonchus* and *C. elegans* nematodes appear to be quite similar, 0.43 and 0.4 pmol/mg, respectively

(Cully and Paress, 1991; Hejmadi *et al.*, 2000; Rohrer *et al.*, 1994). Competition binding experiments indicated the *C. elegans* [^{3}H]ivermectin binding sites were successfully inhibited by several avermectin derivatives, but not by the amino acid transmitter GABA (Cully and Paress, 1991).

1.3.5 Convulsant antagonists

Channel-blocking antagonists acting on GABA, glycine and GluCl channels are highly toxic convulsants that cause hyperexcitation in the nervous system of nematodes, insects and mammals. This section summarises the commercial, experimental and botanical toxins that antagonise the chloride ion channel and which are relevant to GluCls and the experimental chapters to follow.

All known functional GluCl-gated chloride channels, with the exception of *Drosophila melanogaster* and leech Retzius cells (Cully *et al.*, 1996a; Mat-Jais *et al.*, 1983), are blocked by picrotoxin. This plant-derived compound is composed of equimolar amounts of picrotin and picrotoxinin (Etter *et al.*, 1999), the former has little or no effect (Newland and Cull-Candy, 1992). Inhibition of anion and cation flux in vertebrate $GABA_A$ (Sieghart, 1995), $GABA_C$ (Feigenspan *et al.*, 1993) and glycine receptor (Pribilla *et al.*, 1992) as well as invertebrate GABA (Sattelle *et al.*, 1988), L-glutamate (Etter *et al.*, 1999) and nicotinic receptors (Benson, 1988; Sattelle *et al.*, 1988) has been attributed to the picrotoxinin component. However, invertebrate and vertebrate receptor tissues showed distinct variation in affinity for PTX (Hosie *et al.*, 1995a), which was later attributed to the onset and recovery of the picrotoxinin response subsequent to gating of the agonist. This implies that an open channel increased picrotoxinin affinity, suggesting the ligand-binding site is located within the channel pore (Inoue and Akaike, 1988). The functional state of the receptor would therefore reflect on the degree of functional block from the toxin (Inoue and Akaike, 1988). However, the site and mode of action of picrotoxinin is the subject of some debate and is discussed in more detail in chapter 5.

In *Drosophila*, a naturally occurring resistance to picrotoxin is caused by the RDL (resistance to dieldrin) mutation, which is a single amino acid substitution ($serine_{302}$ for alanine) in the M2 domain of a GABA-gated chloride channel (ffrench-Constant *et al.*, 1993a). An equivalent mutation was engineered into the *C. elegans* picrotoxin-sensitive GluClβ chloride channel, where the alanine in position 279 (the RDL equivalent site) was replaced with the threonine. The GluClβ (A279T) single amino acid substitution in the M2 domain reduced the picrotoxin sensitivity of homomeric channel at least 10,000 fold. The point mutation GluClβ (A279T) also altered gating properties to slow the rate of desensitisation (Etter *et al.*, 1999) but little effect on other channel properties was noted.

A number of convulsants were also found to block GABA currents at the related *Drosophila melanogaster* RDL ionotropic receptor, including picrodendrins (Hosie *et al.*, 1995a), (3,3-bis(trifluoromethyl)bicyclo[2,2,1]heptane-2,2-dicarbonitrile) BIDN (Hamon *et al.*, 1998; Rauh *et al.*,

1997b; Shirai *et al.*, 1995a) and fipronil (Cole *et al.*, 1993; Hosie *et al.*, 1995a). Fipronil and BIDN antagonism were also affected by the A302S channel mutation (Hosie *et al.*, 1995a; Shirai *et al.*, 1995b) that also makes the channel resistant to picrotoxinin and dieldrin (ffrench-Constant *et al.*, 1993a; Hosie *et al.*, 1995a). However, fipronil has not, so far, been reported to have any effect on any nematode ligand-gated anion channel and BIDN was found to have no effect on a picrotoxin-sensitive insect GluCl (Hamon *et al.*, 1998).

BIDN is a potent, key member of the polycyclic dinitriles class of insecticides (Buckingham *et al.*, 1995; Hosie *et al.*, 1995b), which display high potency at the GABA receptor convulsant site (Rauh *et al.*, 1997b). In a recent study BIDN failed to inhibit glycine responses from rat brain mRNA when expressed in *Xenopus* laevis (Hamon *et al.*, 1998), similarly nACh receptors currents from cockroach motor neuron (Df) were unaffected by the compound (Rauh *et al.*, 1997a). However, following the injection of mRNA from either rat brain or RDL GABA receptor subunits, BIDN blocked GABA-induced currents at concentrations of 1μM and above (Buckingham *et al.*, 1995; Shirai *et al.*, 1995b), clearly indicating that BIDN has the ability to antagonise both bicuculline-sensitive receptors from vertebrates and bicuculline-insensitive GABA receptors from insects. This suggests that bicuculline and BIDN probably act at different locations within the receptor complex (Hamon *et al.*, 1998). In comparative binding assays BIDN (100μM) bound to distinct sites from 4-n-propyl-4'-ethynylbicycloorthobenzoate (EBOB), t-butylbicyclophosphorothionate (TBPS), 1-phenyl-4-t-butyl-2,6,7-trioxabicyclo[2.2.2] octane (TBOB) and picrotoxin, also suggesting these sites of action within the GABA receptor complex are not identical (Rauh *et al.*, 1997b; Sattelle *et al.*, 1995).

In this study we examined the convulsant antagonist action on L-glutamate receptor subunits in *C. elegans*, where future advances of ligand interaction of both BIDN and fipronil may improve selectivity between insect and vertebrate receptors with the attempt of reducing organism toxicity levels within the host species. In summary, BIDN has previously been found to block only GABA-gated chloride channels of invertebrates and vertebrates, but in this study, BIDN was found to block recombinant L-glutamate-gated channels of *C. elegans*, therefore allowing BIDN to be used as a new pharmacological tool for L-glutamate receptors.

The phenylpyrazole, fipronil, is an outstanding insecticidal, miticidal (Yanese and Andoh, 1989) and herbicidal (Klis *et al.*, 1991b), which has been the first compound of its class to be commercialised. It has been observed to exhibit an excellent range of activity against insect pest (aphids, leafhopper, planthoppers, cockroaches, fleas, ticks and mites) (Colliot, 1992) and at low concentrations produces good selectivity between insects and mammals, allowing it to be used extensively in public and animal health. The phenylpyrazoles mode of action involves inhibiting the passage of chloride ions through the GABA-gated chloride channel and disrupting CNS electrical activity (Gant *et al.*, 1998).

Interestingly, fipronil has shown distinct differences between insect and vertebrate tissue binding affinities, which may indicate why the compound contains selective toxicity to insects over mammals. Essentially these differences include convulsant ligands TBPS, EBOB and BIDN binding at the same GABA receptor-binding site within vertebrate tissues, whilst fipronil binds with a much lower affinity. In addition, in insects, TBPS, EBOB and BIDN bind at three distinct sites, with fipronil acting only at the EBOB site (Cole *et al.*, 1993; Gant *et al.*, 1998). However further experiments to illustrate whether [^{3}H]EBOB and fipronil share the same binding site in invertebrates or if they just overlap needs to be determined.

In oocytes expressing the RDL-resistant GABA receptor, fipronil was also found to reduce the amplitude of GABA current. However, the RDL-mutant receptors containing the amino acid change A^{302}-S were found to be less sensitive to fipronil than wild type receptors, with corresponding IC_{50}'s of 100μM and 20μM respectively (Hosie *et al.*, 1995a). This indicates that the point mutation may be involved directly or indirectly affecting the binding site for fipronil and/or mechanisms involved in GABA-gated chloride currents (Hosie *et al.*, 1995a). Phenylpyrazoles have shown to have no activity at the AVM binding site when tested at a concentration of 100μM (Cole *et al.*, 1993).

The insect GABA-operated chloride channels appear to be important molecular targets of some insecticidal active molecules such as picrotoxin, BIDN, avermectins and fipronil. Chapter 5 reports a detailed antagonist profile of an expressed GluCl receptor, including for the first time a description of the pharmacological actions of convulsant $GABA_A$ antagonists bicyclic dinitrile, BIDN and insecticide, fipronil on novel *C. elegans* GluCl, GLC-3.

1.4 Caenorhabditis elegans

Nematodes were first identified in 384-322BC, and are the most numerous animals on earth. From around the world 29,500 species have been identified, of which approximately half are free-living and the rest are parasitic. Although, it is probable that many more species have yet to be formally identified (actual numbers may range from 40,000 to 10 million).

In this study we have used the free-living nematode *Caenorhabditis elegans* as a convenient, experimental model to investigate the pharmacological and molecular biology of anthelmintic receptor subunits. The nematode was initially proposed as a model organism by Sydney Brenner in 1974, and has since provided a wealth of knowledge in cell biology, neurobiology and genetics and serves as an excellent model system for the study of higher eukaryotes (Brenner, 1974). The nematode has several features that make it suitable for neurological investigations and in the last two decades has been the subject of extensive study, (reviewed by Wood, 1988). *C. elegans* is the first multi-cellular organism to have its entire genome sequenced, of which a detailed genetic map of the nematode can be accessed (*Caenorhabditis elegans* sequencing Consortium, 1998). The genome contains over a 100 million base pairs of DNA, approximately thirty times smaller than the human genome and encodes more than 19,000 genes.

The completion of the *Caenorhabditis elegans* genome-sequencing project (*Caenorhabditis elegans* sequencing Consortium, 1998) has permitted for the first time, a prediction of the total number and diversity of ionotropic receptor subunits present within a single organism. Analysis of the sequence predicts that the nematode genome encodes about ninety such receptor subunits (Bargmann, 1998), most of which are of unknown function. This includes 37 predicted ligand-gated anion channels subunits that will facilitate the cloning of homologous genes from parasitic nematodes. The availability of this database with recent advances in pharmacology and molecular biology will help provide an insight into new anthelmintic drug discovery.

The simplicity of *C. elegans* nervous system offers a chance to perform useful genetic and molecular manipulation that is attributed to its short life-cycle (generation time from egg to adult is complete within 3.5 days), self-fertilising, hermaphrodite form (approximately 300 offspring), small genome size and ease of obtaining variety mutant strains. The nervous system of the hermaphrodite consists of 302 neurons of which is represented by 118 varieties of different neuronal types (White *et al.*, 1986).

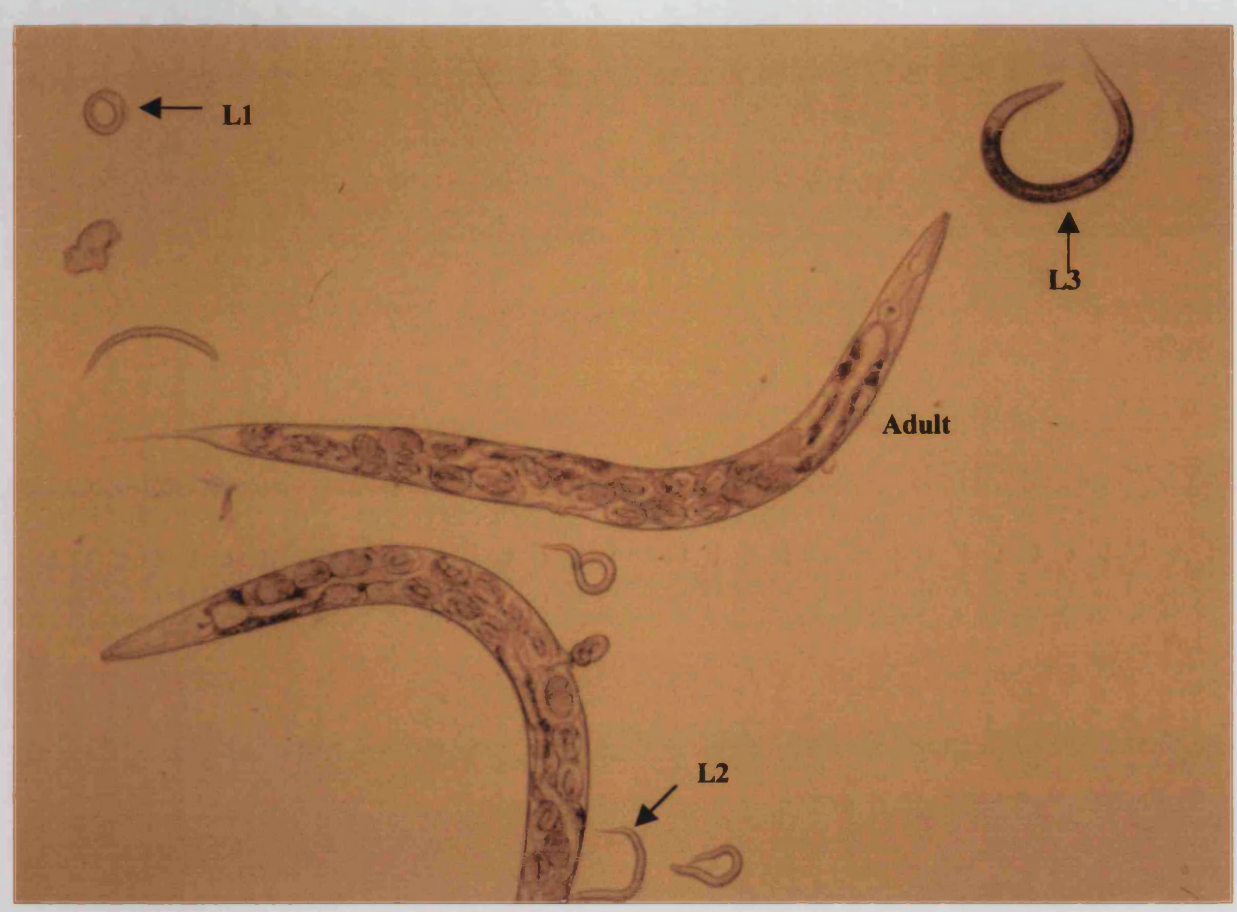


Figure 1.8 Stages of the *Caenorhabditis elegans* lifecycle. With adequate food supply and at standardized laboratory conditions (liquid culture, 20°C), *C. elegans* eggs develop through four larva molts before reaching adulthood; both hermaphrodites and males live for about 17 days. (Key: L1-3= Larvae stages 1-3). Photograph courtesy of Dave Laughton.

1.5. Project aims

Despite the GluCl proteins being well established as putative targets for avermectin compounds, the exact mode of action of the anthelmintic and the stoichiometry of the native receptor in any species is unknown. The completion of the *C. elegans* project provides the first opportunity to explore and identify a full complement of GluCl subunits within a single organism, facilitating advances in pharmacology and molecular biology.

Consequently, the first aim of this study was to map the expression of existing GluCl clones and examine the exact anatomical location in relation to other subunits, which may also determine native stoichiometry (Chapter 3). Second, was the isolation of novel cDNA (GLC-3) encoding a putative (α/β-like) GluCl subunit (Chapter 4) that will also facilitate future cloning of homologous ion channels from parasitic nematodes. Finally, pharmacological characterisation of the novel cDNA clone (GLC-3) will be expressed in *Xenopus* oocytes to investigate agonist and antagonist profiles that can be compared to existing GluCls (Chapter 5). Chapter 5 also describes the actions of novel GABA convulsant antagonists in an attempt to distinguish receptor subunit subtype-specific compounds to maximise the design of new anthelmintic compounds.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Media and solutions

2.1.1.1 Restriction endonucleases, their buffers and reaction conditions used

RESTRICTION ENDONUCLEASE	CONDITIONS	REACTION BUFFER (1x)	SEQUENCE
***Not* 1** ***Xba* 1** ***Pst* I**	Buffer 3 BSA (100µg/ml) 37°C	100mM NaCl 1mM Tris-HCl 10mM $MgCl_2$ 0.1mM DTT (pH 7.9 @ 25°C)	▼ GCGGCCGC CGCCGGCG ▲ ▼ TCTAGA AGATCT ▲ ▼ CTGCAG GACGTC ▲
NdeI	Buffer 4 BSA (100µg/ml) 37°C	50mM potassium acetate 20mM Tris-acetate 10mM magnesium acetate 1mM DTT (pH7.9 @ 25°C)	▼ CATATG GTATAC ▲
***Hind* III**	Buffer 2 37°C	50mM NaCl 10mM Tris-HCl 10mM $MgCl_2$ 1mM DTT (pH 7.9 @ 25°C)	▼ AAGCTT TTCGAA ▲
***Bam*H I**	Unique Buffer BSA (100µg/ml) 37°C	15mM NaCl 1mM Tris-HCl 1mM $MgCl_2$ 0.1mM DTT (pH 7.9 @ 25°C)	▼ GGATCC CCTAGG ▲

Restriction enzymes and associated buffers were supplied by New England Biolabs, Hitchin, U.K. All enzymes and buffers stored at -20°C as per manufacturer's recommendations. When necessary, 0.01 volumes of 100x BSA (10mg/ml) was also added to the digest.

2.1.1.2 DNA Modifying enzymes, polymerases and their reaction buffers

ENZYME	REACTION BUFFER (1x)	SUPPLIER
Calf Intestinal Phosphatase	100mM NaCl, 30mM Tris-HCl (pH 7.9), 10mM $MgCl_2$, 1mM DTT	New England Biolabs, Hitchin, U.K.
T4 DNA Ligase	50mM Tris-HCl (pH 7.2), 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 50mg/ml BSA (pH 7.8)	Gibco BRL, Paisley, U.K.
Taq DNA polymerase	50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% (v/v) Triton X-100	Promega Corporation, Madison, WI, U.S.A.
Expand™ High Fidelity PCR System	50mM Tris-HCl (pH 9.2), 16mM $(NH_4)_2SO_4$, 2.25mM $MgCl_2$	Boehringer Mannheim, Lewes, U.K.
T7 RNA Polymerase	400mM Tris-HCl (pH 7.5), 60mM $MgCl_2$, 20mM Spermadine HCl, 50mM NaCl.	Ambion Biotechnology, Oxon, U.K.
Superscript II	20mM Tris-HCl 100mM NaCl 0.1mM EDTA 1mM DTT 0.01% (v/v)	Boehringer Mannheim, Lewes, U.K.

Enzymes and buffers were stored at -20°C or as per manufacturer's recommendations.

2.1.1.3 General laboratory reagents

Chemical reagents (analytical grade) were obtained from Sigma-Aldrich Chemical Co. Ltd., Poole, U.K. and Fisons, Loughborough, U.K. except where stated otherwise.

REAGENTS	SUPPLIER
Water-saturated phenol	Rathburn, Walkerburn, U.K.
Absolute ethanol	Hayman Ltd., Witham, U.K.
Nunc plasticware	Life Technologies, Glasgow, U.K.
Bovine Serum Albumin	Sigma-Aldrich Chemical Company Ltd., Poole, U.K.
Diethylprocarbonate (DEPC)	Sigma-Aldrich Chemical Company Ltd., Poole, U.K.
Oligonucleotides	Perkin Elmer – Applied Biosystems, Warrington, U.K.
Falcon plasticware	Becton Dickinson Company, Plymouth, U.K.
Dimethylsulfoxide (DMSO)	BDH, Poole, U.K.
Dynabeads® Oligo$(dt)_{25}$	Dynal, Wirral, U.K.
Agarose (Hipure Low EEO)	Bio/gene Limited, Cambridge, U.K.

2.1.1.4 General laboratory solutions

SOLUTIONS	COMPONENTS
Phenol	Obtained distilled and stored in the dark at 4°C. Before use equilibrated with 2 volumes 50mM Tris-HCl (pH 8.0).
Chloroform	24:1 (v/v) Chloroform:isoamyl alcohol
Phenol/chloroform	50% (v/v) Phenol equilibrated with; 10mM Tris-HCl, pH 7.6, 49% (v/v) chloroform 1% (v/v) isoamyl alcohol Stored in the dark at 4°C
Ultrapure Deoxyribnucleoside triphosphates (dNTP's)	Each dNTP was dissolved in H_2O at a concentration of 100mM, pH adjusted to 7.0 with 0.5M Tris
1M Dithiothreitol (DDT)	3.09g DDT, 20ml sodium acetate (pH 5.2), filter sterilized and stored at –20°C
Ethidium Bromide	10mg/ml stock, stored in the dark at 4°C
0.5M Ethylenediaminetetra-acetate (EDTA) pH 8.0	186.1g disodium ethylenediaminetetra acetate.$2H_2O$, 800ml H_2O, pH adjusted to 8.0 with NaOH
Diethylpyrocarbonate (DEPC) H_2O	1% (v/v) DEPC added to dd H_2O, mixed vigorously for 10 minutes, left o/n at room temperature and autoclaved
10mg/ml RNase A	10mg RNase A is mixed in TE buffer, incubated at 95°C for 30 minutes and stored at –20°C
Sodium Azide	1% (w/v) sodium azide

2.1.1.5 General buffers

BUFFERS	COMPOSITION
Phosphate-buffered saline (PBS)	8g NaCl 0.2g KCl 1.44g Na_2HPO_4 0.24g KH_2PO_4 dissolve in H_2O, pH adjusted to 7.4 with HCl and made up to1 litre with H_2O
Sodium citrate/acetate	408.1g sodium acetate.$3H_2O$, pH to 5.2 with glacial acetic acid adjusted to1 litre with H_2O
Tris-borate (TBE) 5X	54g Tris-base 27.5g boric acid 20ml EDTA (pH 8.0), adjusted to 1 litre with H_2O
Tris-EDTA (TE) pH 7.6	10mM Tris-HCl (pH 7.6) 1mM EDTA (pH 8.0)
Morpholinopropanesulfonic acid (MOPS) 10X (pH 7.0)	0.2M MOPS buffer 0.05M Na acetate 0.01M Na_2 EDTA
6x DNA Loading Buffer	0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF 30% (v/v) glycerol in H_2O
6x RNA loading buffer	71µl formamide 16µl formaldehyde 4µl (100%) glycerol 8.6µl 10X MOPS buffer 0.24µl ethidium bromide (10mg/ml) 8µl of RNA loading buffer was mixed with 1µg of RNA
10x RNA electrophoresis buffer	20.93g MOPS (0.2M) 3.4g Na acetate (0.05M) 1.86g Na_2EDTA (0.01M) adjusted to 500ml with H_2O and stored in the dark at 4°C

2.1.2 *Escherichia coli* strains

Escherichia coli strains (XL1 and XL2 blues) were used routinely for cloning and general DNA manipulation, while TG1 was used for protein expression work.

STRAIN	GENOTYPE	SUPPLIER
XL1-Blue	*RecA1 endA1 gyr96 thi-1 hsdR17* ($r_k^- m_k^+$) *supE44 relA1 lac* [F' *proAB* $lac^q Z\Delta M15$ Tn*10* (Tet')]	Stratagene Ltd., Cambridge, U.K.
XL2-Blue	*RecA1 endA1 gyr96 thi-1 hsdR17* ($r_k^- m_k^+$) *supE44 relA1 lac* [F' *proAB* $lac^q Z\Delta M15$ Tn*10* (Tet') Amy Cam^r]	Ultracompetent Epicurian® XL-2s Stratagene Ltd., Cambridge, U.K.
TG1	F' *traD36 laql*^{q} Δ(*lac*)*M15 proA*$^+$*B*$^+$/ *supE*(*hsdM-mcrB*)5($r_k^- m_k^-$ *McrB*$^-$) thi Δ(*lac-proAB*)	Amersham International Plc, Little Chalfont, U.K.
OP50	*RecA1 endA1 gyr96 thi-1 hsdR17* ($r_k^- m_k^+$) *supE44 relA1 lac* [F' *proAB* $lac^q Z\Delta M15$ Tn*10*]	Stratagene Ltd., Cambridge, U.K.

2.1.3 *Escherichia coli* plasmids

Plasmid vectors used in cloning and expression studies.

PLASMID	GENOTYPE AND SIZE	SUPPLIER
PBluescript™	Amp^r, 2.96Kb	Stratagene Ltd., Cambridge, U.K.
pCDNA3.1	Amp^r, 5.4Kb	Stratagene Ltd., Cambridge, U.K.
pCR®2.1	Amp^r, 3.9Kb	Stratagene Ltd., Cambridge, U.K.
PGEM®-T Easy	Amp^r, 3.01Kb	Promega, Southampton, U.K.

2.1.4 Reagents used in the cultivation of C. elegans and bacterial strains

MEDIUM	COMPONENTS (g/L)
LB (Luria-Bertani)-Broth	10g bacto-tryptone, 5g yeast extract, 10g NaCl
DYT	20g bacto-tryptone, 10g yeast extract, 10g NaCl
LB and DYT agar	LB-broth or DYT broth 1.5% (w/v) agar
NGM agar	2.5g peptone, 5mg cholesterol, 3g NaCl, 17g agar, 1mM $MgSO_4$, 5mg K_2PO_4
M9 buffer	6g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.25g $MgSO_4 7H_2O$
S buffer	5.8g NaCl, 6.8g KH_2PO_4, (pH 6.0)
S medium	1Litre S buffer, 1ml ethanol-saturated cholesterol, 0.01M $C_6H_5O_7K_3$, 0.03M $CaCl_2$, 0.03M $MgCl_2$, 0.05mM EDTA, 0.025mM $FeSO_4$, 0.1mM $ZnSO_4$, 0.001mM $CuSO_4$
Freezing medium	0.1M NaCl, 0.01M KH_2PO_4, 6% (v/v) glycerol, 0.06mM $MgCl_2$

Solutions were sterilised either by autoclaving at 120°C, 1.41KPa for 20 minutes, or by filtering using Millipore 0.22μM syringe filters. All solutions were stored at room temperature in colourless glass or plastic bottles, unless otherwise stated.

Salts were obtained from BDH or Sigma-Aldrich Chemical Company Ltd., Poole, U.K. and culture media from Difco laboratories, East Molesey, Surrey, U.K.

2.1.5 Antibiotic solutions

ANTIBIOTIC SOLUTION	STOCK CONCENTRATION	WORKING CONCENTRATION
Ampicillin	50mg/ml in H_2O	60μg/ml
Carbenicillin	50mg/ml in H_2O	60μg/ml
Chloramphenicol	34mg/ml in ethanol	170μg/ml
Kanamycin	10mg/ml in H_2O	50μg/ml

2.1.6 Buffers used in plasmid DNA preparation and analysis

Wizard™ Minipreps DNA Purification System kits were purchased from Promega and Sephaglas™ Bandprep kit from Pharmacia Biotech, St Albans, Herts. U.K..

BUFFER	COMPOSITION
Resuspension Buffer	50mM Tris-HCl (pH 8.0) 10mM EDTA 100μM RNase A
Standard Lysis Buffer (made up prior to use)	0.2M NaOH 1% (w/v) SDS
Potassium Acetate Solution/Neutralisation buffer	3M potassium acetate 11.5% (v/v) acetic acid, pH 4.6
Equilibration Buffer	0.75M NaCl 50mM MOPS (pH 7.0) 15% (v/v) ethanol 0.15% (v/v) Triton X-100
Wash Buffer	1M NaCl 50mM MOPS (pH 7.0) 15% (v/v) ethanol
Elution Buffer	1.25M NaCl 50mM Tris-HCl (pH 8.5) 15% (v/v) ethanol
Tetra-EDTA (TE) pH 7.6	10mM Tris-HCl (pH 7.4) 1mM EDTA (pH 8.0)
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)	20mg X-gal 20ml formamide
10x Tris-Borate (TBE) pH 7.5	0.89M Tris-HCl 0.89M Boric acid (H_3BO_3) 20mM EDTA, pH 8.0

2.1.6.1 Buffers used in DNA purified with Sephaglas Bandprep™ Kit

REAGENT	COMPONENTS
Gel solubiliser	Buffered NaI
Sephaglas	20% (w/v) Sephaglas in aqueous solution
Wash buffer	60% (v/v) ethanol 20mM Tris-HCl (pH 8.0) 1mM EDTA 0.1mM NaCl
Elution buffer	10mM Tris-HCl 1mM EDTA

2.1.7 Materials for antiserum production and characterisation

Peroxidase conjugated goat anti-rabbit serum, thryroglobulin, tetramethylbenzidine, dimethylsulphoxide, dithiothreitol and *m*-maleimidobenzoic acid *N*-hydrosuccinamide ester (MBS) were purchased from Sigma-Aldrich Chemical Company Ltd., Poole, U.K. Biorad Ltd., Hemel Hempstead, U.K. supplied BIOGEL P-2 and Cyanogen Bromide activated Sepharose™ was obtained from Pharmacia, St Albans, Herts., U.K.

2.1.7.1 ELISA (Enzyme- Linked Immunosorbent Assay) reagents

REAGENT	COMPOSITION
Coating buffer	15mM Na_2CO_3, 35mM $NaHCO_3$, 0.01% NaN_3 (pH 9.6)
Washing buffer	PBS, 0.1% Tween™
Stock acetate/citrate buffer	1M Sodium acetate (NaOAC), 30mM Trisodium citrate ($Na_3C_6H_5O_7$) pH 6.0
Stock Tetramethyl benzidine solution	10mgml^{-1} TMB in DMSO
Substrate solution	5% stock acetate citrate buffer, 1% stock TMB solution, 0.006% hydrogen peroxide
Stop solution	1.84M H_2SO_4 (1:10 dilution of 98% stock)

2.1.7.2 Immunocytochemistry reagents

REAGENT	SOLUTIONS
Antibody buffer	5ml Triton-X, 2ml 0.5M EDTA, 1g BSA, 0.3ml sodium azide pH 7.2 with HCl, adjusted to 100mls with 10x PBS
Antibody dilution solution	1% BSA (w/v), 1% Triton-X (v/v), 0.5% sodium azide (w/v)
BME solution	5% (v/v) β-mercaptoethanol, 1% Triton-X (v/v)
Collagenase buffer	1mM $CaCl_2$, 100mM Tris-HCl pH 7.5
Coupling buffer	0.2M $NaHCO_3$, 0.5M NaCl, pH 8.5
Inject Alum	Insoluble aluminum hydroxide suspension
PBS/Triton-X	1% PBS/Triton-X (v/v)
Poly L-lysine slides	200-400mg poly L-lysine (Sigma P1524) 200mls dd H_2O 0.2g Sodium azide (0.1%) Mix together and store at 4°C Wipe slides very clean and place back to back in slide holder, dry in 60°C oven >5 minutes. Shake in poly L-lysine for 5 minutes and dry in 60°C oven >1 hour.

2.1.8 Reagents used in cloning of the ZC317.3 cDNA receptor subunit

2.1.8.1 Reagents for isolation and purification of RNA and cDNA synthesis

REAGENTS	COMPOSITION
Genomic DNA synthesis preparation	
TEN9 buffer	50mM Tris-Cl (pH 9.0)
	100mM EDTA
	200mM NaCl
	100g/ml DNase free-RNase A
Protein digestion	20% SDS (w/v)
	10mg/ml proteinase K
Separation of phases	20ml equilibrated phenol (pH 8.0)
TE buffer (pH 7.6)	10mM Tris-HCl (pH 7.6)
	1mM EDTA (pH 8.0)
DNA precipitation	0.1 volume of 3M NaOAc (pH 5.5)
Total RNA Extraction	
Tripure (Boehringer)	Monophasic solution of phenol and guanidine thiocyanate for RNA, DNA and protein isolation.
Glass Beads (Sigma)	425-600 microns, acid washed and autoclaved
Synthesis of cDNA	
DTT	2µl (0.1M) stock
RNasin	1µl RNasin (40units)
Superscript II buffer	4µl (5x)
dNTPs	1µl of a 10mM stock
Superscript II enzyme	1µl (200 units)
RNase H	1µl (2 units)
Primers	
RoRidt	$^{5'}$GACTACGTTAGCATCTAGAATTCTCGAG$[T]_{17}{}^{3'}$
Random hexomer	$(N)_6$

RNase-free solutions were prepared in 0.1% (v/v) DEPC treated water and sterilised at 1.41 Kpa for 20 min. SDS solution alone was prepared by addition of RNase-free SDS to sterilised DEPC water. Baked glassware was used for all purposes. cDNA encoding the *C. elegans* GluClα_2 subunit was provided by Dr. Joe Dent, Department of Molecular Biology and Oncology, University of Texas Southwestern Medical Centre at Dallas, 5323 Harry Hines Blvd, Dallas, TX 75235-9146, U.S.A. All other reagents were of Analar grade, obtained from the Sigma-Aldrich Chemical Company Ltd.,

Poole, U.K. Drummond glass capillaries were obtained from Laser Laboratories Systems Ltd., Southampton, U.K., while borosilicate glass capillaries were obtained from Clark Electromedical Instruments, Reading, U.K.

2.1.9 Solutions used in Xenopus oocyte preparation and electrophysiology

2.1.9.1 Standard oocyte solutions

SOLUTIONS	COMPOSITION
Standard Oocyte Saline (SOS)	96mM NaCl, 12mM KCl, 108mM $MgCl_2$, 5mM HEPES (pH 7.6)
Incubation Solution	2.5mM sodium pyruvate, 1μg/ml streptomycin, 100units/ml penicillin, 50μg/ml Gentamycin made up in SOS solution
Collagenase	100mg collagenase, 50ml SOS

2.1.9.2 Sources of chemicals

GABA, histamine, Glycine, L-Glutamate, ibotenate, NMDA and Picrotoxinin were obtained from Sigma-Aldrich Chemical Company Ltd., Poole, U.K., whilst fipronil and BIDN were obtained from RP Agro-chemicals, North Carolina U.S.A.

2.2 Methods

2.2.1 Biochemical techniques used in the purification of DNA

2.2.1.1 Agarose gel electrophoresis of DNA

Gels were prepared by dissolving 1-4% (w/v) agarose in electrophoresis buffer by boiling and then allowing to cool to 50-60°C. Ethidium bromide was added to a concentration of 0.5μg/ml and the mixture poured into a horizontal perspex gel apparatus. Once set, the comb was removed and gel submerged in a tank of 1xTBE. DNA samples (0.1-2μg) were taken up in gel loading buffer (Table 2.1.1.5) and electrophoresed at 120V until the marker dyes had migrated to the end of the gel. Phage λ DNA, cut with the restriction enzyme *Pst* 1, was used as a size marker. The DNA was visualised by fluorescent ultraviolet light (UV).

2.2.1.2 Purification of DNA from agarose gel using Sephaglas™

The band of interest was cut out using a clean razor blade and any excess agarose was trimmed away. The gel slice was weighed and 250μl (or 1μl for each mg of agarose, whichever was the greater) of gel solubiliser (2.1.6.1) was added. Incubation at 60°C for 10 minutes dissolved the agarose. Sephaglas BP (5μl, 20% (w/v) Sephaglas in aqueous suspension) was added (or 5μl per estimated μg of DNA present) and the contents of the tube mixed by vortexing. Incubation was continued on the bench for a further 5 minutes with additional periodic vortexing. The Sephaglas was spun down at top speed in a microcentrifuge for 1 minute and the supernatant aspirated. Wash buffer (40μl or 8x the volume of sephaglas used) was added and the pellet resuspended by vortexing. The Sephaglas was spun down again, and the supernatant aspirated. This step was repeated twice and the pellet was air dried for 10 minutes. DNA was eluted by adding a suitable volume of elution buffer (2.1.6.1), not less than half the volume of Sephaglas used. The sample was vortexed briefly to resuspend the pellet and incubated at room temperature for 5 minutes, then centrifuged again for 1 minute at top speed before the supernatant was carefully removed and retained.

2.2.1.3 Concentration of DNA by ethanol precipitation

DNA was concentrated by precipitation with 0.1 volumes 3M sodium acetate (pH 5.2) and 2.5 volumes absolute ethanol at -70°C for 20 minutes or -20°C overnight, centrifuged at high speed for 20 minutes, washed in 70% (v/v) ethanol and resuspended in sterile water.

2.2.1.4 Purity of DNA preparations

DNA solution (5µl) was diluted to 1ml with water. Spectrophotometer (Perkin Elmer UV/VIS Lambda 11) readings were taken at A_{260} and A_{280}. The A_{260} reading was adjusted and used to estimate the concentration of the DNA using the following equations:

$1A_{260}$ = 50µg/ml double stranded DNA

$1A_{260}$ = 33µg/ml single stranded DNA

The ratio (A_{260}/A_{280}) provided estimation for the purity of the DNA preparation. A ratio of 1.8 indicated high purity. A ratio greater than 1.8 suggested RNA contamination while a lower figure indicated protein contamination.

2.2.2 DNA modifying techniques

2.2.2.1 Restriction digest techniques

Restriction digests were performed generally in a total volume of 100µl or less. Plasmid DNA (1-5µg) was incubated for 1-3 hours at 37°C with 10units of restriction enzymes (2.1.1.1) and 0.1 volume of the associated 10x reaction buffer. Enzyme reactions were stopped by the addition of 0.5M EDTA (pH 8.0) to a final concentration of 10mM.

2.2.2.2 Removal of 5' phosphate group

The 5' phosphate group was removed from DNA fragments by treatment with alkaline phosphatase. The DNA and associated reaction buffer (Table 2.1.1.2) was incubated in a total reaction volume of 50µl. After 30 minutes incubation at 37°C the reaction was stopped by the addition of 5µl 0.5M EDTA and the enzyme inactivated by incubation at 65°C for 10 minutes.

2.2.2.3 Ligation of sticky and blunt ended DNA

In a standard ligation of sticky or blunt-ended DNA, approximately 50ng of vector was used with a two-fold molar excess of insert to vector. This was incubated with 2 units of T4 DNA ligase and associated buffer (Table 2.1.1.2) in a final reaction volume of 20µl. The reaction was incubated at 15°C for 4 to 15 hours prior to transformation into competent cells.

2.2.2.4 Single stranded DNA sequencing

Clones were sequenced directly on an automated Perkin Elmer ABI PRISM™ 377 DNA Sequencer. Sequencing was performed in a 10µl reaction volume and set up in a 0.2ml thin-walled microcentrifuge tubes. Prior to the addition DNA polymerase enzyme the reaction mix is heated to 94°C to denature the DNA template.

2.2.3 Standard Microbiological Techniques

2.2.3.1 Bacterial strains, plasmids and antibiotics

All bacterial strains, plasmids and antibiotics used in this report are listed in Table 2.1.2, 2.1.3 and 2.1.5 respectively.

2.2 3.2 Maintenance of bacterial strains and plasmids

Stocks of bacterial strains and plasmids in the appropriate host cell were prepared for long term storage at -70°C by mixing 0.5ml of a fresh overnight culture with 0.5ml of 100% glycerol. For short-term storage, strains were plated out onto LB-agar, sealed with nescofilm and stored at 4°C.

2.2.3.3 Isolation of plasmid DNA from E. coli using Promega "Wizard"™ miniprep method

For small-scale preparation, an isolated colony was picked and used to inoculate 5-10ml liquid medium containing antibiotics (Table 2.1.6). After overnight growth in an orbital incubator at 37°C, 210rpm, a 1.5ml sample of the culture was transferred to a micro-centrifuge tube and centrifuged at 10,000g for 5 minutes. The pellet was resuspended by vortexing in 100µl of Cell Resuspension Solution. Cell Lysis Solution (200µl) was added and the tube's contents mixed by inverting several times. After the addition of 200µl of Neutralisation Solution the tube was again inverted several times and spun at 10,000g in a microcentrifuge for 5 minutes. The clear supernatant was retained and the pellet of bacterial debris discarded.

A Wizard™ Minicolumn was prepared by attaching the barrel of a 3ml syringe to the Minicolumn and 1ml of resuspended Wizard™ miniprep DNA Purification Resin was added to the Minicolumn/syringe assembly. The clear supernatant was transferred to the barrel of the Minicolumn/syringe assembly and the plunger pushed gently, after which the syringe was removed from the column and the plunger withdrawn. The syringe barrel was then reattached and 2ml of Column Wash Solution passed through the Minicolumn. The syringe was again removed and the Minicolumn transferred to a clean 1.5ml microcentrifuge tube. The Minicolumn was centrifuged at 10,000g for 2 minutes to dry the resin. Following this 50µl of water was added and the column left to stand for 1 minute, centrifuging at 10,000g for 30 seconds eluted the plasmid DNA.

2.2.3.4 Preparation of competent cells (strains XL1, XL2 and TG1)

Competent cells were made using the calcium chloride (method adapted from Mandel and Higa, 1970). A 10ml culture of the desired cell strain was prepared. A 100µl sample of the culture was used to inoculate 50ml LB medium. The culture was grown 37°C, 210rpm, until an A_{550} of 0.3 was reached. The cells were cooled briefly on ice (5 minutes) before pelleting at 3000-4000rpm in a Sorval H-1000 rotor at 4°C. The cells were resuspended gently in 10mls of sterile, ice-cold, 0.1M $CaCl_2$ and incubated on ice for at least 1 hour. The cells were pelleted and resuspended in 2ml of the 0.1M $CaCl_2$ solution prior to transformation.

2.2.3.5 Transformation of competent cells with plasmid DNA

Competent cells (100µl) were mixed with the transforming DNA (up to 50µg) in a pre-cooled microcentrifuged tube. The mixture was incubated on ice for 30 minutes before incubating at 42°C for 45 seconds (heat shocking). After 2 minutes on ice, the volume was increased to 1ml with LB broth and incubated in the orbital shaker at 37°C, 210rpm for 1 hour. Up to 200µl of the transformed mixture was plated out onto LB plates containing antibiotic and incubated, inverted, at 37°C.

2.2.3.6 Denaturing agarose gel electrophoresis

The formaldehyde gel was prepared by melting 1.5% (w/v) agarose in RNase-free water. The agarose was allowed to cool to 55°C before the addition of 5x MOPS buffer and formaldehyde to give 1x and 2.2M final concentrations, respectively. The agarose mixture was poured into an RNase-free perspex gel cast and allowed to set in a chemical fume hood. RNA samples were then prepared by mixing 8µl of RNA loading buffer (Table 2.1.1.5) in a sterile microcentrifuge tube with 0.5µg RNA. The RNA sample was then incubated at 60°C for 15 minutes prior to loading on the gel and electrophoresised at 70V in MOPS buffer (1x MOPS buffer, Table 2.1.1.5). Restriction fragments of DNA or RNA ladder were convenient molecular weight markers. When the RNA had reached the end of the gel the ethidium bromide stained RNA was examined and photographed under ultraviolet light.

2.2.4. Maintenance of Caenorhabditis elegans

The Bristol N2 strain of *C. elegans* was routinely maintained on NGM agar plates seeded with OP50, a uracil-requiring mutant of *E. coli*. Plates were incubated at 15°C and sub-cultured every 10 days. *C. elegans* worms were grown in large scale liquid cultures on dense suspensions of *E. coli* OP50. An overnight culture of OP50 in DYT medium was spun at 300g for 5 minutes at room temperature and resuspended in complete S basal medium at a concentration of 20g wet weight per litre. Two litre flasks with 500ml of the bacterial suspension were inoculated with $2x10^4$ worms washed off plates, and cultures vigorously aerated at 20°C for 7-10 days until the culture started to clear. The suspension was transferred to a 1litre glass cylinder and nematodes allowed to settle for 1 hour at 16°C. The complete S medium suspension was carefully poured away and worms were washed in M9 buffer until remaining bacteria was removed. Worms were resuspended in cold 35% (w/v) sucrose and spun in a swinging bucket rotor at 700g for 5 minutes. *C. elegans* worms that floated to the top were removed and washed twice with 0.1M NaCl . The yield was ~3-5g wet weight of nematode per litre of culture.

2.2.5 Antiserum Production and Characterisation

2.2.5.1 Peptide design and synthesis

Several features of the polypeptide were considered prior to peptide synthesis including:

- Peptides containing hydrophilic amino acids were more likely to be exposed on the surface of the native protein.
- Hydrophilic peptides are more likely to be soluble for coupling reactions.
- Presence of proline residues in β-turns are thought to form portions of known epitopes (Lane, 1988).
- Carboxy or N-terminal sequences are often exposed.
- Stretches of amino acids that are more flexible are more likely to be epitopes.
- An N-terminal cysteine was added during peptide synthesis to allow conjugation of the peptide to thyroglobulin using the m-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) method.

Ideal antisera possess high titre coupled with specificity (Lane, 1988). High titre can be influenced by the use of adjuvant, which allows continued presence of immunogen to allow extensive polyclonal proliferation. Avidity and specificity depend critically upon the purity of the immunogen administered and the initial design of the peptide. All peptide sequences were automatically synthesised using a fmoc synthesis on a Milligen 9050 Pepsynthesizer by Sue Phillips (University of Bath). The quality of the peptide was checked by reverse-phase HPLC.

2.2.5.2 Peptide coupling and carrier proteins

A 20mg/ml solution of bovine thyroglobulin was dissolved in 10mM potassium phosphate (pH 7.2), dialysed overnight at 4°C against 10mM potassium phosphate (pH 7.2) and the final concentration adjusted to 16mg/ml. Thyroglobulin was then activated by adding 250µl of the dialysed thyroglobulin to 85µl m-maleidobenzoic acid N-hydroxysuccinimide (MBS) and mixed end over for 30 minutes at room temperature. The thyroglobulin was then desalted by passing it through a Pharmacia Bio-Gel P-30 column, equilibrated with 50mM potassium phosphate (pH 6.0) and collected as three fractions. The cysteine residue of the peptide was reduced (enabling the carrier protein to bind) by dissolving in 10mM potassium phosphate (pH 7.2) and incubated overnight at room temperature with a final concentration of 200mM dithioreitol. The peptide was then desalted by passing it through a Bio-gel P2 (Bio-rad, Laboratories, California, U.S.A.) column. The peptide fractions were collected, pooled and freezed-dried overnight. Peptide was dissolved in 10mM potassium phosphate (pH 7.2) to a final concentration of 5mg/ml.

The three fractions of thyroglobulin were combined with 1ml of peptide, conjugation was allowed to proceed allowing the compound to oxidize and produce disulfide bonds. The pH was adjusted to 7.4 and mixed at room temperature for 3 hours. Solid NaCl was added to a final concentration of 0.9% (w/v) to adjust the compound to physiological osmotic potential, prior to injection. The peptide-conjugate was used at a concentration of 1mg/ml (pH to 7.4).

2.2.5.3 Immunisation

New Zealand white rabbits were immunised with thyroglobulin-peptide conjugate. A typical schedule was as follows: for each rabbit 0.25ml (45mg/ml) of adjuvant (Imject Alum), was added dropwise to 0.25ml of immunogen solution (1mg /1ml). This was mixed thoroughly for 30 minutes, prior to injection into two intraperitoneal sites. Additional injections were made every ten days. ELISA tests done on trial bleeds after each injection and pre-immune control allowed the monitoring of antibody titre. Exsanguination was performed on anaesthetised rabbits; approximately 150-200mls of blood was obtained per rabbit.

2.2.5.4 Harvesting serum

Between 5-10mls of blood was collected from each rabbit and allowed to clot for 1 hour at 37°C. The clot was then freed from the walls of the container and left overnight for the clot to retract. The serum was centrifuged at 4000 r.p.m. for 10 minutes to remove the red blood cells. For long term storage at –20°C, 0.1% sodium azide was added at this stage. Preservatives are unnecessary for sera stored at -70°C.

2.2.5 5 ELISA protocol

An ELISA test for the specificity of the antisera to the nematode antigen was performed. The plate was coated with 100µl of antigen solution (10µg/ml diluted in coating buffer) and left overnight at 4°C. Plates were then washed 3 times with PBS/Tween (1% (v/v)) and left for 2 hours at room temperature with a casein-blocking agent. Following 2 washes with PBS/Tween, the test antiserum was applied in doubling dilutions (starting at 1:50) and left overnight at 4°C. Three washes of PBS/Tween removed the unbound primary antibody before applying the (HRP)-conjugated goat anti-rabbit IgG peroxidase enzyme (Amersham NA934) diluted 1:1000 in PBS/Tween. The plates were then incubated for 2 hours at room temperature before three washes with PBS/Tween and two washes with PBS. The hydrogen peroxide substrate was then applied and incubated for 5-15 minutes at room temperature. The reaction was stopped with 50µl of 1.84M H_2SO_4 and the O.D. read at 450nM.

2.2.5.6 Coupling of thyroglobulin to CNBr activated Sepharose

Severe contamination was removed from 3g CNBr activated sepharose by 3 series of repeated washes with 300mls of 1mM HCl (pH 2-3), 200mls distilled H_2O and 200mls of 0.1M $NaHCO_3$ (pH 8.3) on a sintered funnel. The ligand (5mg) was dissolved in 5mls of coupling solution and excess ligand washed away with 3 bed volumes of 0.1M $NaHCO_3$ mixed with 0.5M NaCl. Remaining active groups on the gel were blocked with 0.1M Tris-HCl (pH 8.0). The mixture was rotated end over for 12 hours at 4°C. Three wash cycles in alternating pH (0.1M sodium acetate (pH 4.0) mixed with 0.5M NaCl, 0.1M Tris-HCl (pH 8.0) and 0.5M NaCl), removed excess uncoupled ligand that remained after coupling.

2.2.5.7 Affinity purification of GBR-2B antiserum

Gel slurry was poured into a 10ml column and washed profusely with column buffer via a peristaltic pump at 1ml per minute. The serum was re-cycled through the column overnight at 4°C. Excess ligand was washed off with 20mM Na_2HPO_4 (pH 7.3) prior to blocking excess active groups which remain on the gel after coupling. Diethylamine (pH 11.5) eluted the antibody that was measured using UV photospectrophotometer linked up to a chart recorder. Antibody containing fractions were dialysed in PBS overnight and then concentrated in polyethylene glycol 20,000 for 2-3 hours. The column was regenerated by washing once with 20mM Na_2HPO_4 (pH 7.3) and three times with 0.1M Tris HCl (including 0.5M NaCl pH 8.3) and 0.1M sodium acetate (including 0.5M NaCl pH4.0).

2.2.6 Immunocytochemistry

2.2.6.1 Preparation of C. elegans whole worm tissue preparations

Using the N2 strain of *C. elegans*, adult worms were grown on several plates and then washed off the agar with M9 buffer (2.1.4). Nematodes were then pelleted by centrifugation at 1500rpm for 5 minutes at 4°C. Worms were incubated overnight at 4°C in 4% paraformaldehyde and 1% Glutaraldehyde fixative. Fixed worms were then permeabilised by a 1% Triton-X, 5% 2β-mercaptoethanol (2β-ME)/collagenase (pH 6.9) treatment and incubated overnight at 37°C with gentle shaking. The reduction step of 2 β-ME reduced the disulphide linkages between cuticular collagens thereby enhancing the effects of the collagenase in the next step. Following this overnight incubation the worms were gently washed five times in PBS (pH 7.2), resuspended in 0.4ml of collagenase buffer and incubated at 37°C to allow permeabilisation of the highly cross-linked nematode cuticle and penetration of antibodies. Polyclonal serum raised in rabbit against the synthetic peptide was applied to the fixed, permeabilised worm at the dilution of 1:10 and 1:100, in antibody buffer. Primary antibody was incubated at 4°C for three days. Three 15-minute washes in PBS/Triton-X removed unbound primary antibody. The fluorescently tagged secondary antibody (goat anti-rabbit-TRITC) was diluted 1:50 in antibody buffer and incubated for five hours at room temperature with gentle shaking. Following 6 washes, the stained nematodes were transferred to microscope slides, mounted in Vectrashield (Vector Laboratories Inc., Burlingame, CA, U.S.A.) and viewed on the confocal microscope (Zeiss LSM 510) under fluorescence.

2.2.6.2 Pre-absorption of primary antiserum for negative controls

Purified antisera were incubated with excess, pure peptide to a final concentration of $20mg/ml^{-1}$ and incubated at 37°C for a minimum of one hour. The "adsorbed" antibody/antigen complex was used as a negative control.

2.2.6.3 Freeze-crack immunocytochemistry

Nematodes were prepared for freeze-crack immunocytochemistry in the same way as described in section 2.2.6.1. Poly-Lysine slides were labelled with a diamond glass pen (2.1.7.2. for poly-lysine preparation) and placed on the bench-frosted side up. Using a glass pipette, worms were spread on the slides and sandwiched carefully by placing an unlabelled poly-lysine slide (frosted side down) on top. Slides were then placed on dry ice for at least 20 minutes. At this point, slides could be saved at -80°C. Prior to use (following -80°C incubation), slides were warmed up on dry ice for 20 minutes before cracking.

2.2.6.4 Cracking, fixing and staining of nematodes

Frozen slides were cracked by swiftly pulling them apart. The unlabelled top slide was discarded and the wormless sides placed back-to-back and placed into a cold slide holder. Worms were fixed by immersing them in ice-cold methanol for two minutes, followed by ice-cold acetone for four minutes. The slides were air dried for less than a minute and placed into a block for 1 hour at room temperature. Immunocytochemistry was carried out as described in section 2.2.6.1. All fluorescence microscopy of nematodes was examined using a Zeiss LMS 510 confocal microscope, with a helium neon laser and rhodamine filter.

2.2.7 Cloning of the ZC317.3 cDNA receptor subunit from C. elegans using the Polymerase Chain Reaction (PCR)

2.2.7.1 Small-scale worm RNA extraction

Worms were washed off 4 plates (9cm), that were just clearing, using M9 buffer (Table 2.1.4) and spun down at 150g. Approximately 200µl of "packed" *C. elegans* worms were transferred into 2ml microcentrifuge tubes and resuspended with 0.8ml of TRIzole®Reagent (GIBCOBRL) and 0.5ml glassbeads (425-600 microns, acid washed, Sigma-Aldridge) and vortexed at high speed for 1 minute. The samples were left to stand at room temperature for 10-15 minutes with occasional vortexing. The homogenised worms were then centrifuged briefly and the supernatant drawn off the beads and placed into clean 1.5ml microcentrifuge tubes. The beads were rinsed with a further 200µl Trizole, vortexed briefly and the supernatants combined. Chloroform (200µl) was added to the sample and vortexed for 30 seconds prior to being incubated at room temperature for 5 minutes. The mixture was microcentrifuged at 12,000g for 15 minutes (at 2-8°C), allowing the phases to separate. The upper aqueous phase was removed, taking care not to remove any of the interface. Precipitation of the RNA was achieved by the addition of isopropanol (0.5ml), vortexing and incubation at room temperature for 10 minutes. The RNA was recovered by centrifugation at 12,000g for 30 minutes at 4°C. Once pelleted the aqueous layer was removed and the RNA washed with 1ml 75% ethanol, briefly mixed and microcentrifuged at 7,500g for 5 minutes at 4°C. Ethanol supernatants were removed and the pellets allowed to dry for 5-10 minutes, prior to resuspending in 200µl nuclease free water and stored at –70°C. RNA (5µl) was diluted in 1000µl H_2O and the O.D. measured at 260 and 280 to determine the concentration and purity. (Note a 260/280 ratio of <1.6 indicates partially dissolved RNA). The concentration of the RNA was determined using the following equation:

$$\text{RNA concentration } (\mu g/ml) = 100/5 \text{ (dilution factor)} \times O.D._{260} \times 40$$

Approximately one 9cm petri-dish of healthy worms (0.2ml of worms) yielded about 100-150µg of total RNA.

2.2.7.2 Synthesis of cDNA from total RNA

Total RNA was reversed transcribed into cDNA using Superscript™ reverse transcriptase enzyme. Random hexomer primer (4µl, Fig. 4.1) was added to 11µl of dH_2O and 1µg of eluted total RNA, and heated to 70°C for 2 minutes to disrupt any secondary structure. The reaction was immediately chilled on ice prior to the addition of the following; 2µl of 0.1M DTT, 1µl RNasin (40units, Promega), 4µl of superscript (II) buffer (SK) and 1µl 10mM dNTP's. The reaction was heated to 42°C for 2 minutes prior to the addition of 1µl of Superscript™ reverse transcriptase enzyme (200U, Promega) and the incubation continued for a further 50 minutes. The reaction was heated to 70°C for 15 minutes to inactivate the enzyme. RNase H (2units, GIBCO BRL) was added and the reaction incubated for 20 minutes at 37°C. The reaction volume was increased to 100µl with dH_2O before a phenol:chloroform extraction was performed. cDNA was precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and incubated at -20°C for a minimum of 1 hour. The cDNA was pelleted and washed in 70% ethanol before being air-dried and resuspended in H_2O (usually 100µl).

2.2.7.3 Isolation of genomic DNA from C. elegans

Following the method of Herman and Frischauf [1987 #238], nematodes were ground to a powder under liquid nitrogen with a pestle and mortar. The powder was quickly resuspended in 20ml of TEN9 buffer (2.1.8.1.). The solution was transferred to a 50ml tube and shaken to give an homogenous suspension. SDS (1ml of a 20% solution) and 1ml of proteinase K (10mg/ml) were added and mixed by inverting the tube. Digestion was allowed to continue overnight at 55°C. The solution was transferred to a large flat-sided glass bottle (500ml), and 20ml of equilibrated phenol (pH 8.0) was added. The bottle was placed flat on a gently rocking platform (30rpm) for 1-3hours. The phases were separated by centrifugation at 3000rpm for 10minutes and the aqueous phase drawn off with a wide-bore pipette into a fresh bottle. The phenol extraction was repeated twice more, until the aqueous phase was clear. Remaining debris was removed by centrifugation at 9000rpm for 20 minutes at room temperature. The supernatant was dialysed against a 1000-fold volume of TE (pH 7.6), first at room temperature, to avoid SDS precipitation, then at 4°C overnight. DNA was precipitated by the addition of 0.1vol of 3M sodium acetate (pH 5.5) and 0.8 volumes of 2-propanol. The solution was mixed gently by inverting the tube several times. The DNA was spooled out with a glass rod and placed into TE (1ml per g of tissue) in a microcentrifuge tube. The tube was placed on a rotating wheel (10rpm) and the DNA allowed to dissolve overnight at 4°C.

2.2.7.4 Design of oligonucleotide primers

Blast searches of the *C. elegans* genome sequence revealed a predicted polypeptide, ZC317.3 with high amino-acid sequence identity to the other cloned GluCl subunits. Based on the sequence of the polypeptide we were able to design gene specific oligonucleotides primers corresponding to the 5' (*) and 3' (†) ends of the predicted gene. All oligonucleotides used were custom made by Perkin-Elmer and had been HPLC purified and lyophilised.

(*) ZC317.3	$^{5'}$CTTGATGAGTCTCCGTTCACTTC$^{3'}$
(†)Zc317.3	$^{5'}$CAATTTCATTTGGCTTCCGGTGCG$^{3'}$
SLI	$^{5'}$GGTTTAATTACCCAAGTTTGAG$^{3'}$

2.2.7.5 Calculation of primer annealing temperature

Predicted melting temperatures (T_m) of primers were estimated using the equation:

$$T_m=4(GC)+2(AT)$$

2.2.7.6 The Polymerase Chain Reaction

All PCR's were performed in the Perkin-Elmer, Model PTC-100™ (MJ Research, Inc) thermo-cycling machine. Expand High Fidelity (Boehringer Mannheim) was used that ensures high 3'-5' exonuclease activity for proofreading of the extension product.

The PCR reaction was performed in a 50µl reaction volume and set up in a 0.2ml thin-walled microcentrifuge tube by adding:

5µl	10X Expand High Fidelity DNA polymerase buffer, 15mM $MgCl_2$ included
7.5µl	4X dNTP's (2mM)
1µl	2X oligonucleotide primer (20µM)
1µl	10ng cDNA template
0.75µl	Expand High Fidelity polymerase enzyme (2.6units/0.75µl)

H_2O (dd) to 50µl total volume.

2.2.7.7 Temperature cycling conditions

Hot start	2min	94°C	
Denaturation	2mins	94°C	
Annealing	1.5mins	55°C	typically 40 cycles
Extension time	2mins	72°C	

The final step involved heating to 72°C and maintaining this temperature for 5 minutes to ensure all extension products were double stranded.

2.2.7.8 Subcloning PCR products

PCR products were analysed on 1-3% agarose gels and bands of the predicted size were excised and purified with Sephaglas. Primers incorporating unique restriction sites resulted in PCR products that were digested with the appropriate restriction enzyme, re-purified and ligated into a suitable digestion vector, either pGEM®-T Easy or pBluescript™. PCR primers that did not incorporate restriction sites resulted in PCR products that were cloned into a pCR® 2.1 vector (Invitrogen cloning kit®). Freshly amplified PCR products (50ng) generated using *Taq* DNA polymerase have a 5' A overhang due to the terminal transferase activity of this enzyme. The purified, amplified DNA was used for a 1:1 (vector:insert) ligation reaction as follows:

For a 10µl ligation reaction:

Fresh PCR product	Xµl
10X Ligation buffer	1µl
pCR® 2.1 vector (25ng/µl)	2µl
Sterile water to total of	9µl
T4 Ligase (4.0 units)	1µl

The reaction mixture was incubated at 15°C overnight, after which time the reaction was cooled on ice or stored at -20°C until ready for transformation. Transformation and subsequent sequencing were performed as above. The cDNA was subcloned into pGEM®-T Easy (Promega, Southampton, U.K.). DNA sequencing reactions were carried out by DNASHEF, Edinburgh, U.K. and analysed using the GCG package mounted on a Silicon Graphic Unix workstation.

2.2.8 Functional expression of glutamate- chloride channels in Xenopus oocytes

2.2.8.1 Preparation of cRNA for In vitro transcription

The cloning of *C. elegans* GLC-3 receptor subunit was described in section 2.2.7. Plasmid DNA encoding *C. elegans* glutamate receptor subunits were prepared from glycerol stocks as described in section 2.2.3. The DNA encoding each subunit (11μg) was linearised with the restriction enzyme *Not1* at 37°C overnight as described in section 2.2.2.1. The resulting linearised template was precipitated once with 0.1 volumes sterile 3M sodium acetate and 3 volumes sterile absolute ethanol at –20°C, overnight. Linearised template was centrifuged at high speed for 30 minutes, washed with sterile 70% ethanol, dried and resuspended in 20μl RNase-free DEPC treated sterile water. The linearised plasmid was diluted to a final concentration of 1μg/μl with RNase-free water.

Maintaining RNase-free conditions, 20-40μg of 7-methylguanosine capped cRNA was synthesised with T7 RNA polymerase transcription kit (mMessage mMachine, Ambion, Austin, TX, U.S.A.) using the following protocol:
The following were mixed:

5μl	10x Transcription buffer
5μl	100mM DTT
5μl	of each; ATP, CTP, UTP, and GTP (5mM)
0.5μl	10mg/ml BSA
2.5μl	cap analogue ($M^7G(5')ppp(5')G$)
10μl	linearised plasmid DNA (10μg)
2μl	40U/μl RNase Inhibitor
2μl	T7 Polymerase

and the volume made up to 50μl with nuclease-free water and incubated at 40°C for 1 hour. Following the incubation time the template was digested with 2 units DNase for 15 minutes at 37°C to minimise DNA degradation. The resulting transcripts were treated with phenol, phenol-chloroform and chloroform extraction prior to ethanol precipitation and resuspended in nuclease-free water to a final concentration of 1μg/μl. At this point, appropriate volumes (approximately 10-20μl) of transcript from each subunit were mixed, centrifuged at high speed for 40 minutes, washed in 70% ethanol, dried and re-suspended in 10-20μl (depending on average concentration of transcripts) of sterile

RNase free water. The concentration and integrity of cRNA produced from *in vitro* transcription was determined by spectophotometry, and on a denaturing gel as described in section 2.2.3.6.

2.2.8.2 Xenopus oocytes preparation and injection

Xenopus laevis females were anaesthetised by submersion in 0.5% Tricaine (3-aminobenzoic acid ethyl ester) at room temperature for approximately 40 minutes until leg retraction ceased. Ovarian lobes were extracted through an incision made in the skin and abdominal body wall in the posterior ventral side of the frog. The incision was sutured using Mersilk™ 4.0 (Ethicon Ltd., U.K.) and the frog was left to recover in water before returning to its original colony. The ovarian lobes were sliced into smaller clumps of approximately 50 oocytes, where stage V and VI were defolliculated manually after a 5-30 min incubation with collagenase (type 1A; 2mg/ml), in a calcium-free version of standard oocyte saline SOS (Table 2.1.9.2). Oocytes were then rinsed in several washes of SOS and transferred and stored for up to 3 days in incubation medium (Table 2.1.9.2).

Injection needles were pulled from 3.5 Drummond glass capillary tubing (Clark Electromedical, U.K.) on a stationary coil Narishige microelectrode puller (Optical Instruments Services Ltd., Croyden, U.K.) to produce a tip of approximately 1μM which was then gently broken to give a tip with a slightly larger diameter. Needles were back filled with sterile mineral oil and attached to the plunger of a Drummond "Nanoject" microinjector (Broomhall, PA, U.S.A.). With the aid of a binocular microscope oocytes were injected cytoplasmically with either 50ng of *in vitro* transcripts in a total volume of 50nl or for control eggs 50nl of nuclease-free H_2O. Oocytes were incubated at 16°C for 2-7 days in changes of incubation medium (Table 2.1.9.2).

2.2.8.3 Electrophysiological recordings

Microelectrodes were pulled from borosilicate glass capillaries on a microelectrode puller (Scientific and Research Instruments, Ltd., U.K.) to produce a resistance, when filled with 3M KCl, of between 0.5 and 5 MΩ. Each oocyte was positioned in a perspex-recording chamber, secured with steel pins, impaled with two microelectrodes and perfused continuously with SOS (5ml/minute) by gravity fed system (5ml/min). Electrophysiological recordings were performed using a two-electrode voltage clamp amplifier OC-725C (Warner, Warner Instrument, Corp., U.S.A.) on oocytes with a resting membrane potential of greater than –25mV and maintained at a holding potential of E_h= –80mV unless otherwise stated. In each case, the change in current (or lack of) across the membrane of the oocyte required to keep the oocyte clamped at –80mV was recorded on a Hewlett-Packard chart recorder. Data was analysed using the program graph pad prism (Graphpad Software) and presented as the mean +/- the standard error of the mean. Hill coefficients were calculated using the following equation:

$$\frac{I}{I\max} = \frac{I\min}{I\max} + \frac{I\max - I\min}{[1+10^{(\log EC_{50} - [ag]*nH}]I\max}$$

This equation was used to fit a sigmoid curve of variable slope to the normalised data. $\%I_{max}$ and I_{min} represent the maximal and minimal currents induced by a particular agonist. The EC_{50} is the concentration of agonist necessary to elicit half the maximum response and n_H is the slope (Hill) coefficient.

2.2.8.4 Data analysis

In each case, experiments were repeated at least 4 times ($n \geq 4$). All values are quoted as mean ± standard error of the mean. Concentration response curves were fitted using non-linear regression in Graphpad Prism (version 3, San Diego, U.S.A.). Data on graphs are shown as a percentage of the maximum response.

2.2.8.5 Ligand application protocol

Saline insoluble ligands (IVM, fipronil, BIDN and Ibotenate) were dissolved in DMSO (<1% v/v final concentration in saline); this solvent concentration was shown to have no undesirable effects either on drug-induced responses or on uninjected oocyte membranes when cells were clamped at E_h =80–mV. Only those oocytes which gave stable responses to at least 2 applications of 3mM L-glutamate were used, this allowed us to assess the quality of the oocyte and RNA expression. An acceptable inward current for 3mM L-glutamate would be between 150 and 250nA. A dose-dependent relationship for L-glutamate was generated by challenging the expressed receptor first with 3mM applications of L-glutamate followed by increasing concentrations of L-glutamate (10μM to 100mM) at 5 minute intervals, preventing desensitisation. The response to each concentration of agonist was expressed as a percentage of the peak response (%Imax). When investigating the antagonist action of compounds, oocytes were pre-incubated for 2 minutes in the antagonist prior to each co-application of L-glutamate and the antagonist. The process was repeated with higher concentrations of antagonist following 5 minutes recovery period in normal saline. All ligands and standard oocyte saline was applied using a gravity fed system at a constant rate of 5mls/minute, unless otherwise stated. The amplitude of the response to co-application of antagonist and L-glutamate was subtracted from the values obtained in the presence of 3mM L-glutamate in physiological saline.

2.2.8.6 Current-voltage relationship of L-glutamate chloride channels

The L-glutamate induced current was determined by subtracting the current required to clamp the oocyte membrane at a given potential in saline, from that required to clamp the membrane at the same potential in the presence of a non-desensitising L-glutamate concentration (3mM). To check the ion channels selectivity to chloride ions a reduction of external NaCl concentration to 54.9mM and its replacement by 45.1mM monosodium gluconate was examined and compared to the predicted chloride equilibrium potential E_{Cl} given by the Nernst equation for chloride ions.

$$E_S = \frac{RT}{F} \ln \frac{[Cl]_o}{[Cl]_I} \ln \frac{[K]_o}{[K]_I} \ln \frac{[Na]_o}{[Na]_I} \ln \frac{[Ca]_o}{[Ca]_I}$$

Here R is the gas constant (8.314 JK^{-1} mol^{-1}), T is the absolute temperature (K), F is the Faraday constant (96,500 C mol^{-1}) and E_S is the equilibrium potential. The subscripts O and I stand for outside and inside, respectively. The Nernst equation allows us to derive ionic equilibrium potential (E_S) for one or all the permeable ions present within a cell. According to the Nernst equation, how large the equilibrium potentials for a living cell will vary linearly with absolute temperature and logarithmically with ionic concentration ratio. Equilibrium potentials change sign if the charge of the ion is reversed or if the direction gradient is reversed, and they fall to zero when there is no gradient.

Values of *RT/F*:

Temperature (°C)	*RT/F*
0	23.54
5	23.97
10	24.2
15	24.83
20	25.26
25	25.69
30	26.12

Chapter 3

Immunocytochemical localisation of GBR-2 subunits from Caenorhabditis elegans

3.1 Introduction

In both free-living and parasitic species of nematodes (Avery and Horvitz, 1990; Geary *et al.*, 1993) the mode of action of ivermectin and related analogues is to selectively paralyse the parasite by increasing muscle Cl^- permeability. However, the identity of the channel target in whole nematode preparations has been controversial (Arena, 1994). Observed behaviour in whole nematode preparations varies extensively. Incubation of *C. elegans*, on avermectin plates (20ng/ml) produced inhibition of pharyngeal pumping and slow onset of rigid paralysis. In contrast *Haemonchus contortus* showed no obvious effects when submerged in the drug at similar concentrations (Avery, 1993; Turner and Schaeffer, 1989). Furthermore, direct injection into the nematode *Ascaris suum* resulted in rapid paralysis that was neither rigid nor flaccid (Bernt *et al.*, 1998; Kass *et al.*, 1980). But nematodes examined at pharmacologically relevant concentrations of avermectin (0.1-1nM) showed no visible effect on motility in any susceptible nematode species (Geary *et al.*, 1992). In contrast, pharyngeal pumping in *C. elegans* (Avery and Horvitz, 1990) and *Haemonchus contortus* (Geary *et al.*, 1993) appeared extremely sensitive to low concentration of avermectin, where paralysis is produced at 10-100 fold lower concentrations than those required to inhibit motility (Geary *et al.*, 1992; Geary *et al.*, 1993; Martin, 1997).

3.1.2. Anatomy of the C. elegans pharynx

In order for *C. elegans* to eat, rhythmical contraction and relaxation of the pharynx must occur. The pharynx forms a self-contained muscular organ that is composed of three function parts, the corpus, the isthmus and the terminal bulb, containing 20 muscle cells of eight anatomical types and 20 neurons (Fig. 3.1), (Albertson and Thompson, 1975). The eight muscle types, denoted pm1-8, line the pharynx and are innervated by motor neurons M1-5 (Fig. 3.1). Bacterial food enters the pharynx through the buccal cavity situated at the anterior end of the animal and becomes accumulated within the two sub-divisions of the corpus, the procorpus and the meta-corpus. Peristaltic action of the isthmus combined with intermittent pumping action of corpus, anterior isthmus and terminal bulb delivers the food to the terminal bulb. Contraction of the terminal bulb muscles grinds the bacterial food plug, which then passes into the intestines. Pharyngeal relaxation returns the grinder to its relaxed position, closes the lumen and expels any remaining liquid leaving the bacteria trapped within the corpus (Albertson and Thompson, 1975). The I5 motor neuron synapses onto the M3 and M4 motor neurons that innervate the pm4 and pm5 muscle cells (of the corpus and isthmus), in addition to sensory endings found in the terminal bulb. The neuronal endings of the I5 senses when the terminal bulb is becoming full and responds by inhibiting the M3 and M4 motor neurons (Avery, 1993).

Electropharyngeogram (EPGs) recordings have observed inhibitory postsynaptic potentials (IPSPs) generated by the M3 and M4 motor neurons which synapse directly onto the pm4 and to a lesser extent pm5 muscles cell that are directly responsible for regulating pharyngeal muscle relaxation (Dent *et al.*, 1997). The IPSPs generated by the M3 motor neuron have been hypothesised to be of an inhibitory glutamatergic transmission that can be influenced by avermectin analogues (Dent *et al.*, 1997). Furthermore, removal of the two M3 motor neurons from the pharynx lead to an absence of IPSPs and consequently increases the duration of muscle contraction. In view of such observations, it is plausible that for ivermectin to be effective in *C. elegans*, the compound must irreversibly bind to ivermectin sensitive channels, causing lethal increases of chloride conductance that inhibit essential cells of the pharyngeal pump (Dent *et al.*, 1997).

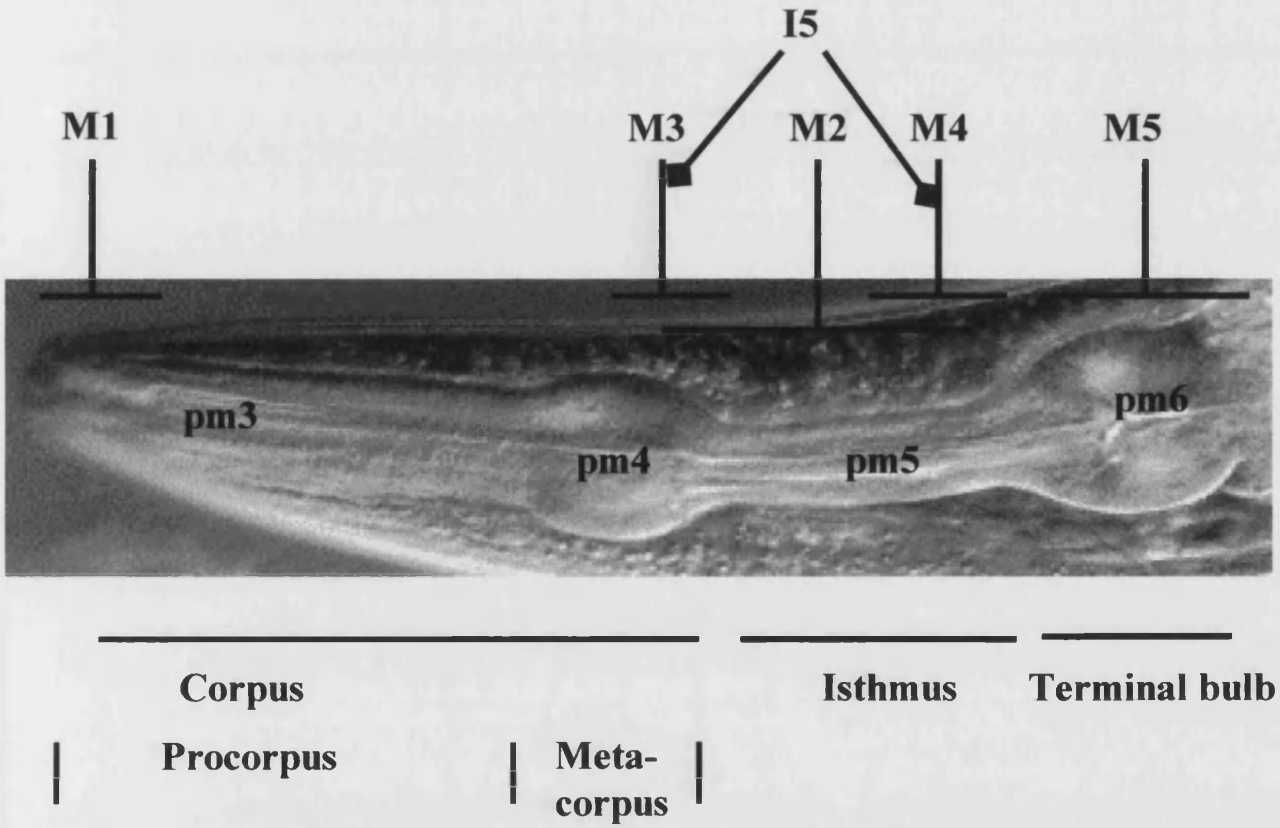


Figure 3.1 Anatomy of the *C. elegans* pharynx.

The pharynx is divided into three functional parts: the corpus, the isthmus and the terminal bulb. The corpus is further subdivided into the procorpus and metacorpus. Eight muscle types denoted pm 1 to 8 line the pharynx, four (pm3 to 6) of which are individually innervated by five motor neurons (M1-M5) and one sensory neuron (I5). Three types of small muscle pm1, pm2 and pm8 are not shown (Albertson and Thompson, 1975). Photograph courtesy of Leon Avery PhD taken from http://eatworms.swmed.edu/worm_labs/Avery/.

To date only two glutamate-gated chloride channel receptor subunits of *C. elegans* have been localised to specific cells. In both studies, GluClβ (Laughton *et al.*, 1997a) and GluClα_2 (Dent *et al.*, 1997) were localised using reporter gene constructs. Using a LacZ reporter gene construct, GluClβ was found to be expressed in the three pm4 pharyngeal muscle cells of the metacorpus (Fig. 3.1), with additional pattern of expression in nuclei of the terminal bulb in the earlier stages of development (egg to L3 stages) (Laughton *et al.*, 1997a). The *avr-15* gene which encodes protein, GluClα_2, was found to be expressed in all the muscles of the metacorpus (pm4) and the isthmus throughout larval and adult stages. Expression was also observed in a few neurons in the head, which include RMED (ring motor neurons, dorsal) and RMEV (ring motor neurons, ventral). Weak staining was also demonstrated in ventral cord neurons near the anus, namely DA9 and VA12, indicating that the GluClα_2 promoter is active in pharyngeal muscle as well as some extrapharyngeal motor neurons that may be involved in movement (Dent *et al.*, 1997). The GluClα_2:GFP transformed animals exhibited a green fluorescence, due to a 6kb DNA fragment encoding the first three exons of GluClα_2 fused to green fluorescent protein (Dent *et al.*, 1997). Expression of the GluClα_2 receptor subunits within the pm4 muscles cells was found to have very similar expression patterns to the GluClβ subunits, indicating the two may form a heteromeric pharyngeal receptor onto which the M3 neuron synapses (Fig. 3.1) (Dent *et al.*, 1997). Expression of the *C. elegans* GluClα_1 subunit is not known. The location of an avermectin-sensitive GluCl channel, has also been identified using a two microelectrode current clamp technique in the parasitic nematode *Acaris suum* (Martin, 1996). Pharyngeal muscle of this parasite possessed glutamate receptors that gate chloride channels and that are sensitive to the avermectin analogue milbemycin D (Martin, 1996). Although it appears that avermectins may have more than one site of action in nematodes, these results provide further evidence that points to the inhibition of the pharyngeal pumping as a major mode of action.

In view of such observations the first aim of the study was to map the patterns of an existing *C. elegans* GluCl clone, GBR-2 and examine the anatomical localisation in relation to GluClβ and GluClα_2. The alternatively spliced gene *avr-14* encode two proteins (Figure 3.2), GBR-2A and -2B from *C. elegans* which is also conserved in the parasitic species *Haemonchus contortus* (Jagannathan *et al.*, 1999). Sequence analysis of the GBR-2 from *Haemonchus* indicates that, like *C. elegans*, the gene encodes two receptor subunits, Hc-GBR2A and Hc-GBR-2B. Orthologues of this receptor subunit have also been observed in *Ascaris suum* (Jagannathan *et al.*, 1999) and *Dirofilaria immitis* (Yates, 2000).

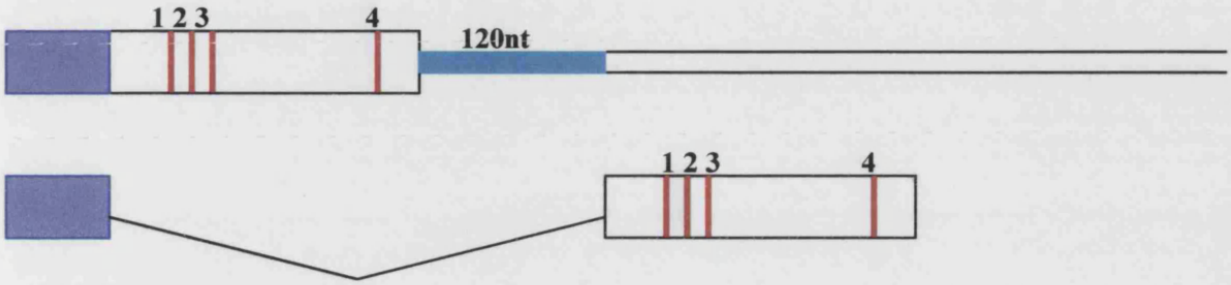


Figure 3.2 Schematic diagram of the alternatively spliced products of GBR-2A and –2B derived from the gene *avr-14*. Both subunits possess a common N-terminal indicated by ■ and transmembrane domains are labelled 1-4. The GBR-2A product contains the channel encoding sequence of the GBR-2B cDNA, 120nt downstream from the GBR-2A termination codon (not to scale).

3.2 Results

3.2.1 Peptide design and synthesis

The *C. elegans avr14* gene encodes alternatively spliced products corresponding to the GBR-2A and GBR-2B subunit cDNAs. The cDNA-spliced products possess high homology within the N-terminal domains making the design of synthetic peptides in this region troublesome. Several key features were considered to check the suitability of the region prior to peptide synthesis. Structural analysis of the sequence was examined using GCG program PEPTIDESTRUCTURE (Fig. 3.4). From the most prominent region of the N-terminal sequence (amino acids 240-270) several candidate domains were examined for specificity, antigenicity and sequence homology to other nematode sequences. Using the Genbank database an alignment of all known nematode sequences were compiled allowing us to discriminate between proteins regions that share high homology with the peptide sequence. From these results peptides (GBR-2A and GBR-2B) were designed from equivalent regions of the putative extracellular domain just upstream from M1, where heterogeneity of the two N-terminal sequences was greatest (50%). Alignment of 13 consecutive amino acids appeared highly conserved with two orthologue nematode sequences, *Haemonchus contortus* (Hc-GBR-2) and *Ascaris suum* (As-GBR-2) (Fig. 3.3). Peptide sequences were automatically synthesised using a fmoc synthesis on a milligen 9050 Pepsynthesizer by Sue Phillips at the University of Bath. The quality of the peptide was checked by reverse-phase HPLC.

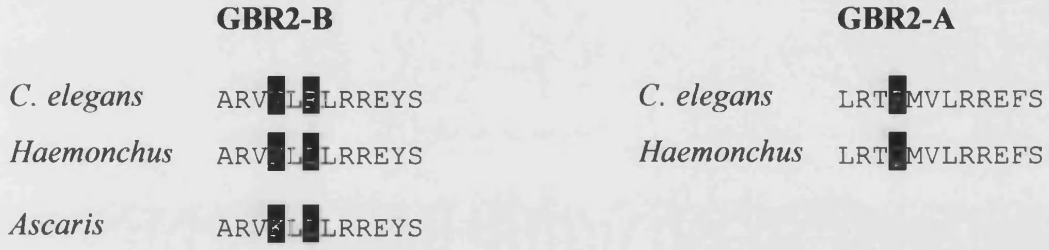
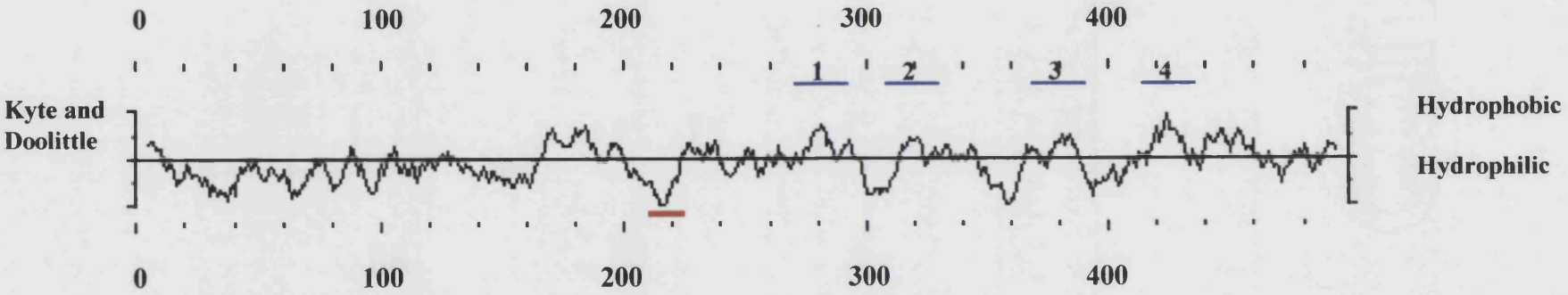


Figure 3.3 Alignment of the alternatively spliced GBR-2A and GBR-2B receptor subunits indicates sequence conservation from *C. elegans*, *Haemonchus* and *Ascaris suum* nematodes. Peptides designed to *C. elegans* GBR-2A and GBR-2B N-terminal domains show high homology to parasitic orthologues. Residues where sequences are not conserved are indicated in black. Peptides correspond to amino acid residues 243-254 of the *C. elegans* GBR-2A and –2B subunits.

Figure 3.4 Predicted hydrophilicity of GBR-2B.

Hydrophilicity plot displaying the N-terminal domain followed by four transmembrane regions (marked by solid blue lines). A red solid line indicates the GBR-2B sequence chosen for peptide synthesis. This profile was constructed using the GCG computer program PEPTIDESTRUCTURE.



3.2.2 Polyclonal serum analysis

Throughout immunisation the titre of the absorbed antiserum was monitored against the free peptide using the ELISA method (Section 2.2.5.5.) to ensure specificity remained and that the maximum titre had been obtained prior to purification (Fig. 3.5). Affinity purification of polyclonal serum against the free peptide sepharose column was then performed to remove unwanted antibodies against the thyroglobulin carrier protein and increase overall specificity of the serum. Addition of the antiserum to the column allowed specific antibodies to bind non-covalently to the antigen, whilst non-specific antibodies are washed away with phosphate buffer (pH 7.3). Serum eluted from the column under high alkaline (pH 10.7) was monitored through an optical ultraviolet unit, calibrated initially with 10mg/ml bovine serum albumin (BSA). The eluent was collected as absorbance peaked at 280nM.

An ELISA experiment to examine cross-reactivity between peptides was also performed. This involved titre measurements of anti-GBR-2A polyclonal serum against the GBR-2B free peptide and vice versa. At various dilution factors and in both experiments the antisera against both subunits could not distinguish between the GBR-2A and -2B antigens, indicating cross-reactivity between the peptides. Polyclonal antibodies made to the original peptide GBR-2B obtained the highest titre and was therefore used for all subsequent localisation experiments to study *gbr-2* expression patterns.

3.2.3. C. elegans Immunocytochemistry

Nematodes were prepared for freeze-crack immunocytochemistry as described in section 2.2.6.3. Following the cracking of the *C. elegans* slides, nematodes were fixed in either methanol/acetone or paraformaldehyde/glutaraldehyde and permeabilised using acetone (Section 2.2.6.4). In all cases 10% goat serum was used as blocking agent and FITC-conjugated goat anti-rabbit IgG was used as the secondary antibody. *C. elegans* treated with rabbit polyclonal antibodies raised against the N-terminal of the GBR2 subunit at 1:10 and 1:100 dilutions showed bright, specific staining of four sets of cells in the region of the pharyngeal nerve ring (Fig. 3.7). No other specific staining of internal organs or muscular tissue was identified. Identical patterns of localisation were observed in the isolated pharyngeal preparations as with the whole mount preparations, with the additional specific staining of 2 neuronal cell bodies observed in the corpus and terminal bulb regions of the pharynx (Fig. 3.8 and 3.9). Both neuronal cell bodies observed surrounding the pharyngeal muscle cells appear to have anterior and posterior processes extending outwardly. Nematodes exposed to "absorbed" antibody/antigen complex or pre-immune polyclonal serum, showed no specific fluorescence, only small amounts of non-specific staining of external body wall, buccal cavity and intestinal contents due to bacterial cross-reactivity with FITC secondary antibody (Fig. 3.10). In general polyclonal serum dilutions at 1:10 and below showed very low levels of background fluorescence.

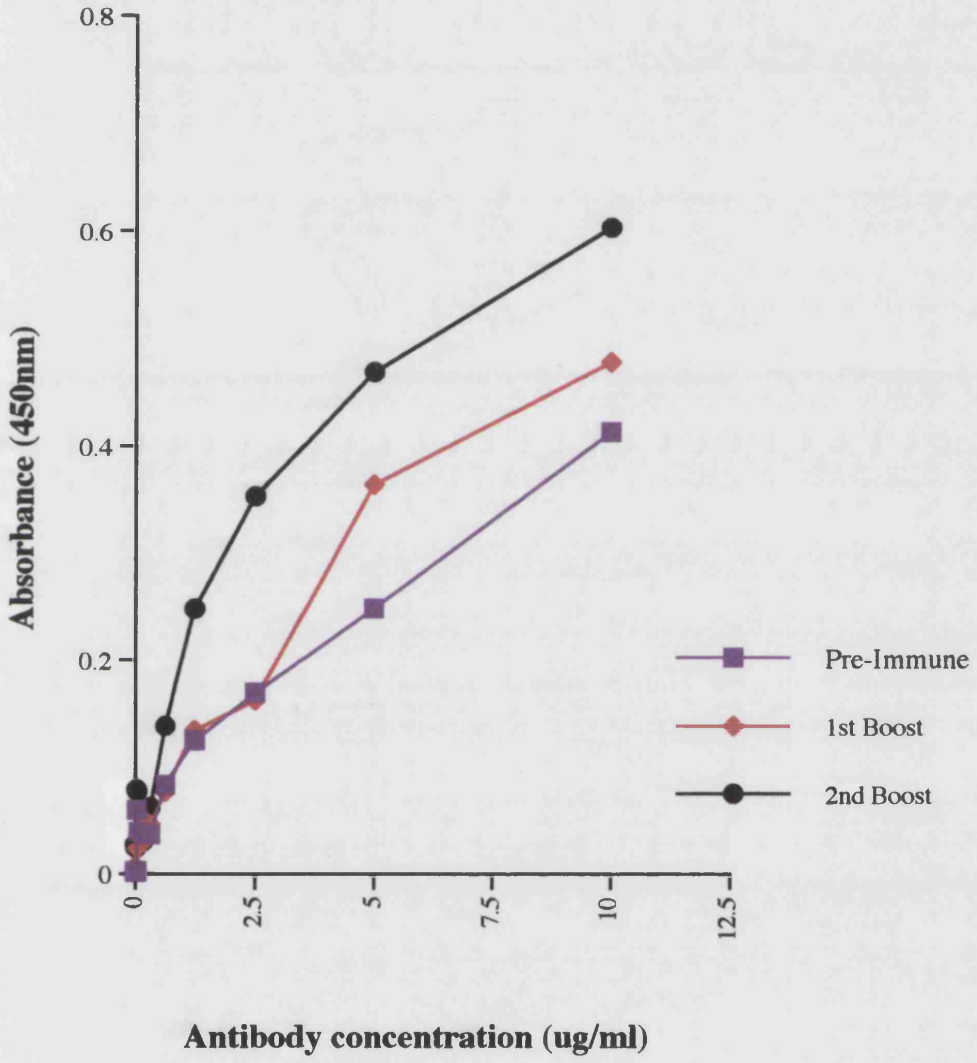


Figure 3.5 ELISA analysis of pre-immune and GBR-2B antiserum titres against free GBR-2B peptide.

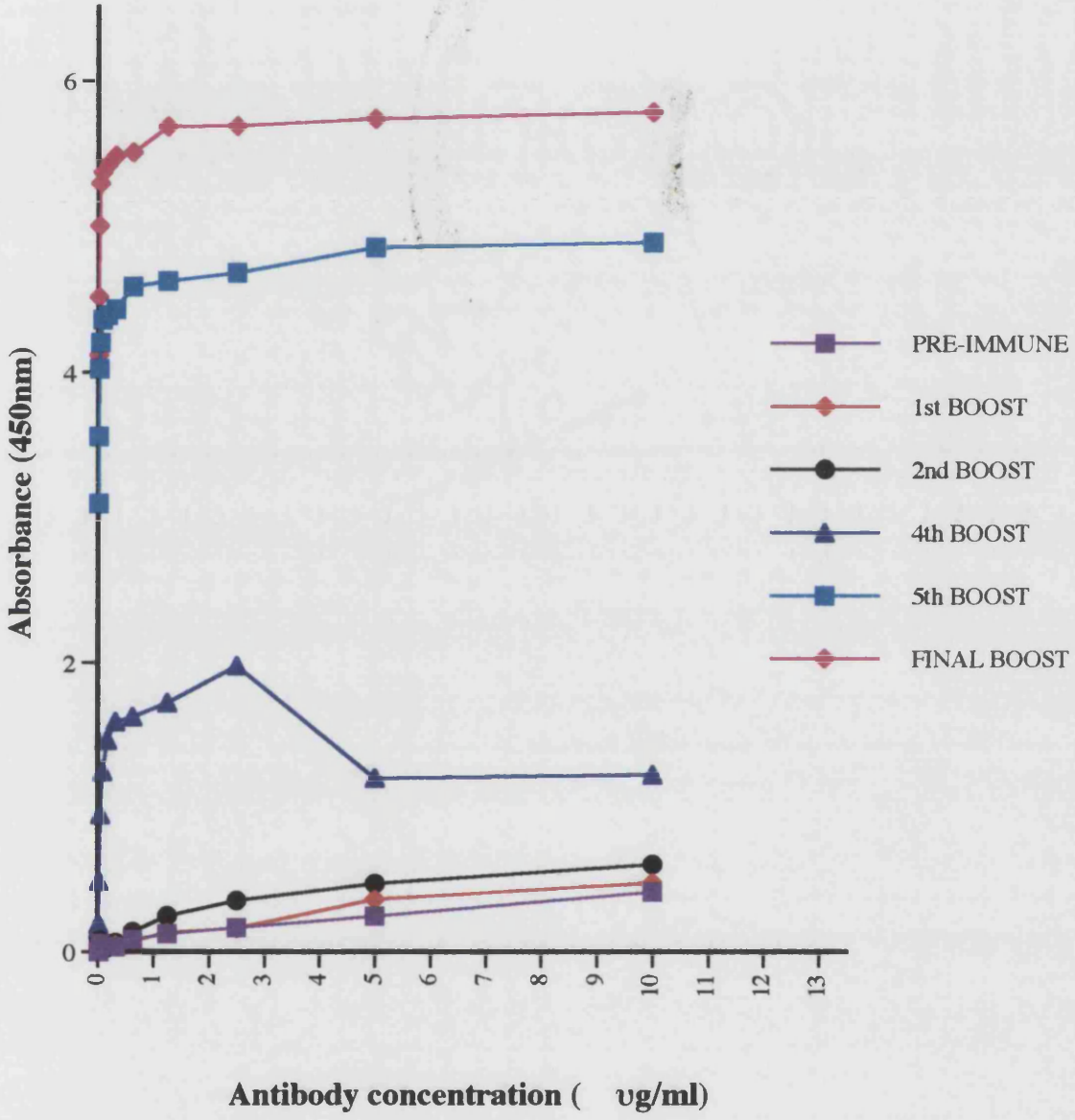


Figure 3.6 ELISA analysis of pre-immune and CNBR purified GBR-2B serum.

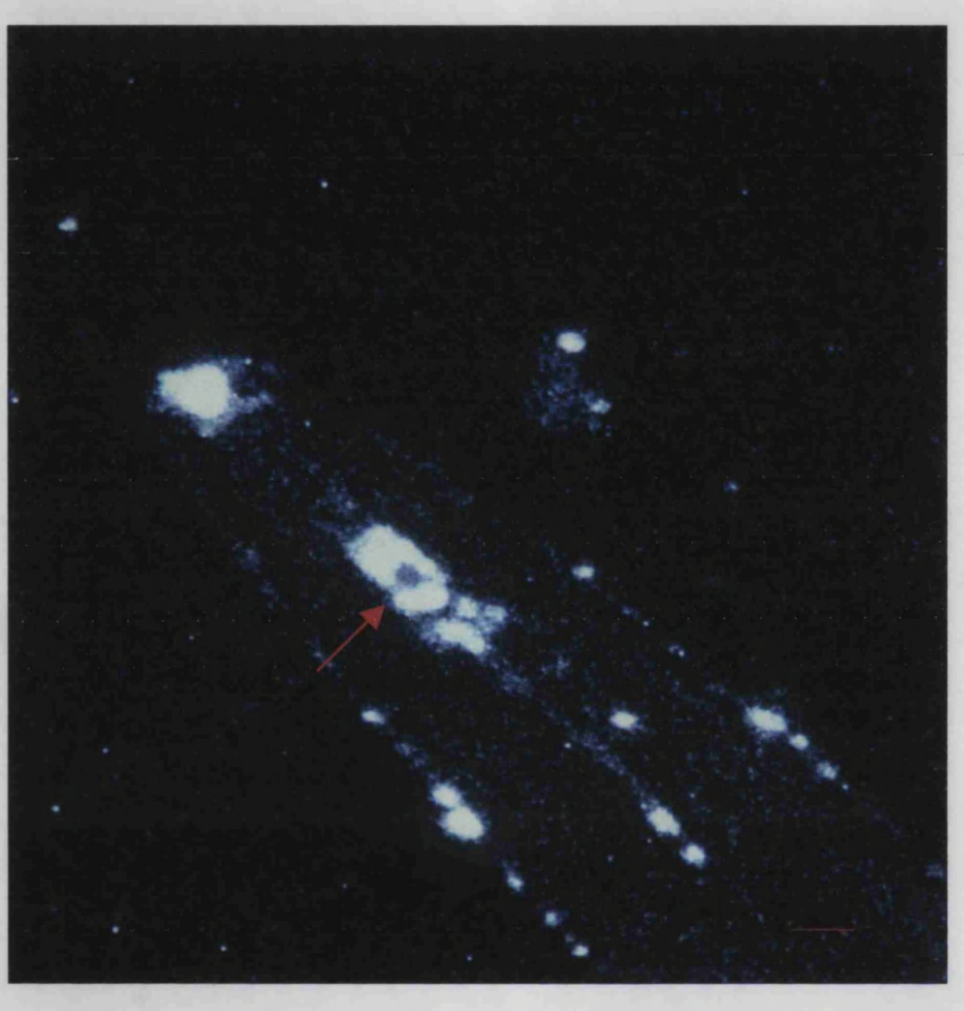


Figure 3.7 Confocal images of *C. elegans*, anterior head sections. Red arrow indicates pronounced, immunofluorescent staining of pharyngeal nerve ring using rabbit anti-GBR2 polyclonal serum at 1:10 dilution. Four distinct sets of cells surrounding the pharynx was observed, no other specific staining fluorescence of internal organs or muscle tissue was identified Scale bar indicates 100μM.

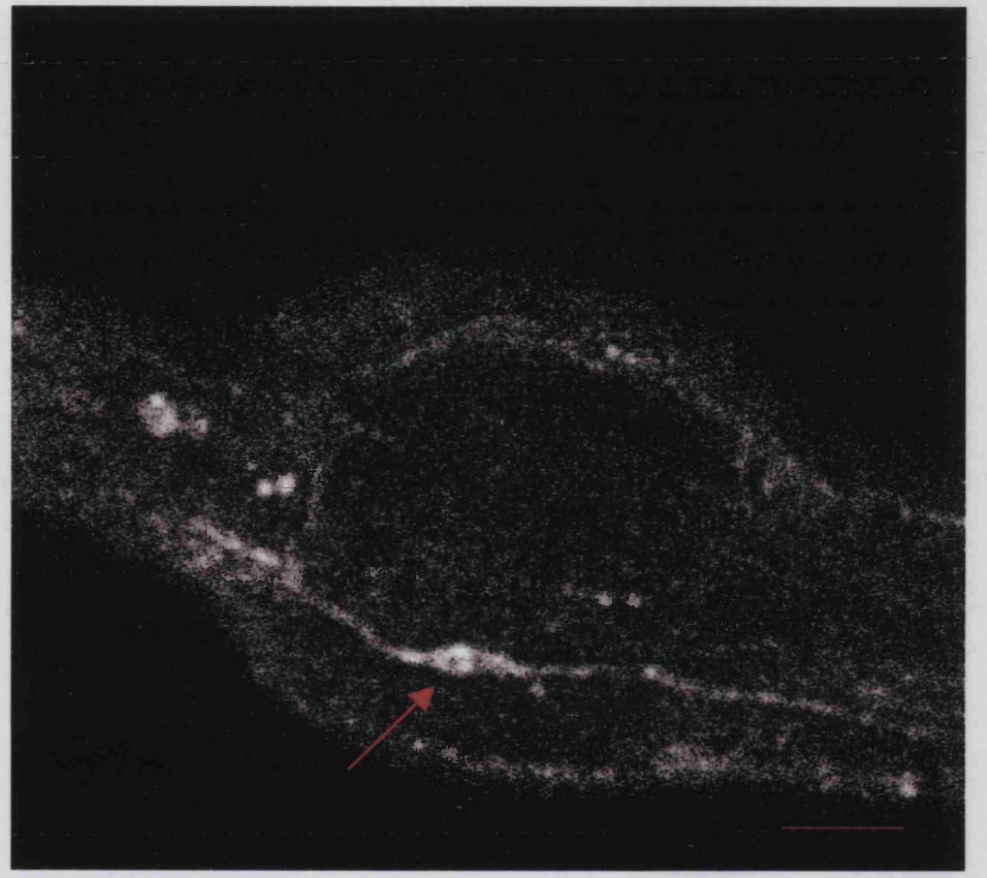


Figure 3.8 Confocal laser micrograph image of anti-GBR-2 antibody staining within isolated dissected pharynx. The anterior section of the worm is pointing towards the right hand side of the picture. The arrow indicates the staining of an individual neuronal cell body within the corpus of the pharynx and the scale bar indicates 10μM. Purified antibody was used at a dilution concentrations of 1:20. Scale bar indicates 100μM.

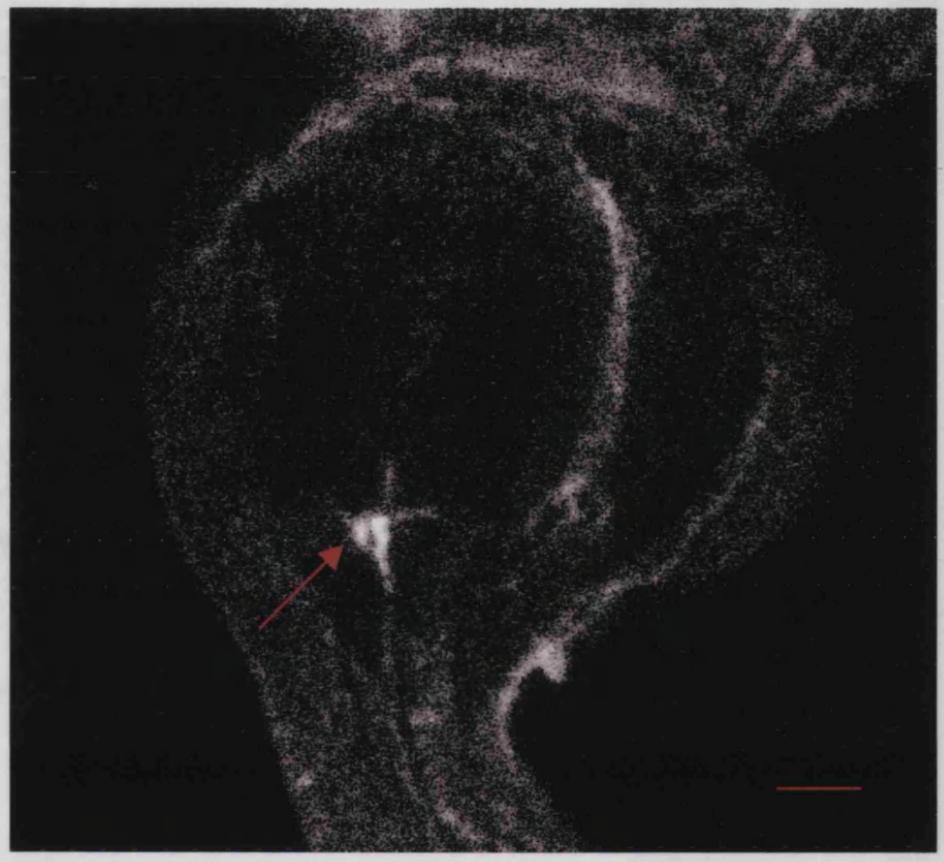


Figure 3.9 Neuronal cell body immunofluorescence observed in the terminal bulb of the isolated pharyngeal preparation (red arrow). The *C. elegans* pharyngeal preparation is orientated with the anterior end of the nematode at the bottom of the micrograph. Affinity purified GBR-2 antibody, was used at 1:20 dilution, scale bar indicates 10μM.

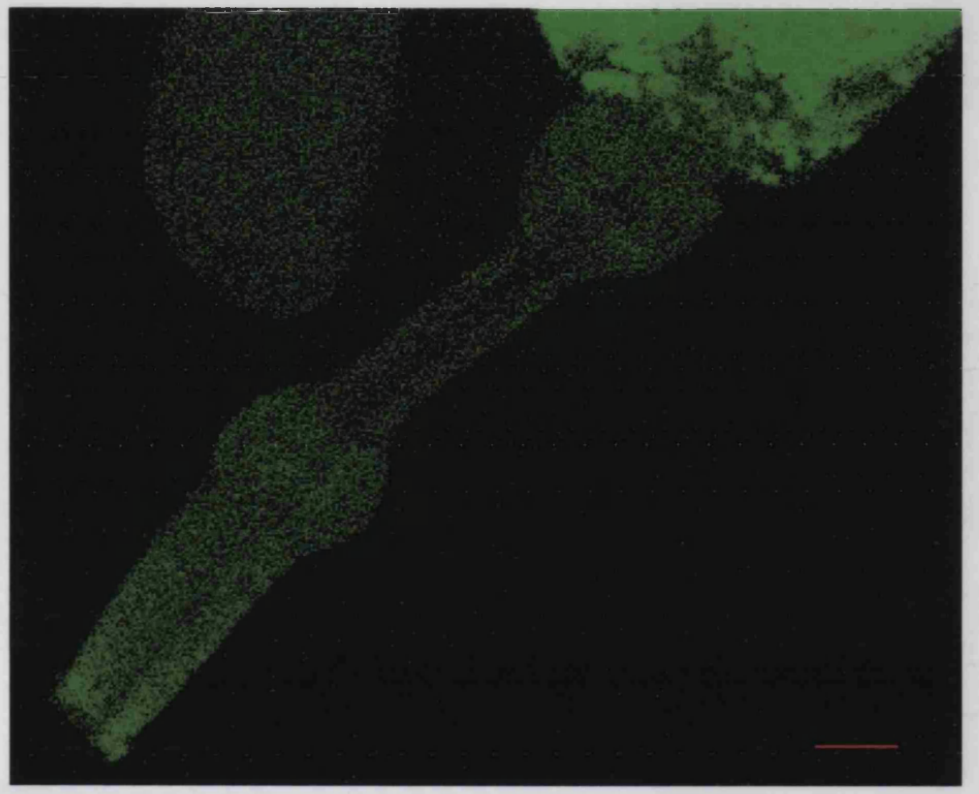


Figure 3.10 Isolated pharyngeal preparation obtained with secondary antibody only, showing an absence of punctate staining. The fluorescence on the right of the image is gut autofluorescence. Scale bar indicates 100μM.

3.3 Discussion

The *C. elegans avr-14* gene encodes two subunits, which are members of the glutamate-gated chloride channel ion family. Both subunits possess a common N-terminal domain but have two different, though closely related, channel transmembrane regions (Laughton *et al.*, 1997b), indicating the presence of very similar binding sites coupled to different chloride channels. The *C. elegans* GBR-2A cDNA contains the channel encoding sequence of the GBR-2B cDNA approximately 120 nucleotides downstream from GBR-2A termination codon (Fig. 3.2). Parasitic orthologues of the GBR-2A and GBR-2B subunits have been found to exist in *Haemonchus contortus*, sharing approximately 88% and 83% amino acid identity respectively. However, the pattern of alternative splicing may not be conserved in *Ascaris suum* (Jagannathan *et al.*, 1999) or *Dirofilaria immitis* (Yates, 2000). *C. elegans* GBR-2A and GBR-2B receptor subunits share 82% identity at the amino acid level. In a recent study RNAs corresponding to each of the two spliced variants encoded by *avr-14* gene were injected into oocytes. Interestingly, oocytes only expressing the GBR-2B protein responded to both L-glutamate and ivermectin, transcript 2A did not respond to ivermectin or L-glutamate (Dent *et al.*, 2000).

Despite the inability of the anti-GBR-2 antiserum to distinguish between the *C. elegans* GBR-2A and -2B subunits, immunohistological labelling has proven successful, producing distinct, extrapharyngeal immunoreactive cells, indicating that one or both subunits are expressed (Fig. 3.5). In pre-absorbed (antigen:anti-GBR-2 serum) and pre-immune controls, no specific staining was examined, although non-specific staining of buccal cavity, anus and external body wall was observed. In view of such observations the GBR-2B antibodies were used to locate the subunits in isolated pharyngeal preparations (Figure 3.8, 3.9 and 3.10). This technique allows lower concentration of anti-GBR-2B anti-serum to be used, reducing background fluorescence and allowing more specific detail to be observed. In addition, the pharynx can be exposed to the antibodies solutions without the aid of permeabilisation techniques, which can often lead to increased background fluorescence. Identical patterns of localisation were observed in the isolated pharyngeal as with the whole mount preparations, with the additional specific staining of 2 neuronal cell bodies observed in the corpus and terminal bulb regions of the pharynx (Fig. 3.8 and 3.9, Holden-Dye, 2000). Both neuronal cell bodies observed surrounding the pharyngeal muscle cells appears to have anterior and posterior processes extending outwardly. From this pattern of immunoreactive neurons it is reasonable to assume that the GBR-2 subunits exists on the M3 motor neurons that innervate pm4 and pm5 muscle cells. Previous studies indicate IPSPs generated by the M3 motor neuron have been of an inhibitory glutamatergic transmission (Dent *et al.*, 1997). I can therefore hypothesise that GBR-2 channels operate as pre-synaptic autoreceptors on the M3 neuron and are maybe indirectly involved in regulating glutamate release. Furthermore, Dent *et al.*, (2000) generated a fusion protein between the *avr-14* gene and a GFP fusion protein, which was found to be expressed within a group of 40 extrapharyngeal ring

neurons of the head, clearly confirming GBR-2 existence within the nerve ring of *C. elegans*. The GBR-2 protein displayed additional activity in the ventral nerve cord and mechanosensory neurons. As reporter gene fusions do not always faithfully reflect the expression pattern of the intact gene, perhaps because of the absence of enhancer elements located downstream or within introns, two methods of localisation (GFP-fusion proteins and immunocytochemistry) should be conducted to give greater confidence in the work. Here I provide additional immunocytochemistry data, which supports the conclusion that this subunit is expressed on extrapharyngeal cells and on pharyngeal neurons.

The anti-GBR-2 antibodies were also used to study the pattern of localisation in both male and female species of *Haemonchus contortus* and *Ascaris suum* (Jagannathan *et al.*, 1999). Specific immunofluorescence in motor neuron commissures, nerve cords and the extrapharyngeal region was demonstrated. Once again, non-specific binding was observed in samples incubated only with secondary antibody and anti-GBR-2 pre-absorbed with a large excess of synthetic peptide. Differences in GBR-2 distribution between *Haemonchus* and *C. elegans* were observed. In *C. elegans* the extrapharyngeal staining appears to be confined to four subsets of cells and two specific cell bodies within the corpus and terminal bulb, whereas in *Haemonchus* immunofluorescence appears to be diffuse across the whole nerve ring region. In addition GBR-2 expression was observed from adult *Ascaris* and *Haemonchus* dorsal cord and motor neuron commissures, which was absent in *C. elegans* immunofluorescence preparations. This may reflect differences in the expression of GBR-2 orthologues between the two species. Comparing GBR-2 experiments with that of GluClβ and α_2 receptor subunits, it is plausible these subunits are also involved in pharyngeal pumping, but are unlikely to co-assemble with GluClα_2 and GluClβ to form a native receptor.

In summary, GBR-2 receptor subunits formed specific expression patterns over three nematode species. This is the first demonstration of inhibitory glutamatergic synapses found in the extrapharyngeal nerve ring in any nematode species. In *C. elegans* and *Haemonchus* species fluorescence in the extrapharyngeal nerve cord region is observed which has also been verified in *C. elegans* GFP:*avr-14* fusion experiments (Dent *et al.*, 2000). In addition, GBR-2 may have specific roles in dorsal and ventral nerve cords of *Ascaris suum* and *Haemonchus* species and pharyngeal pumping in *C. elegans*. However GBR-2 physiological roles (if any) in pharyngeal action and in location still remain to be determined. To distinguish clearly between the expression of GBR-2A and GBR-2B polypeptides in *C. elegans* and *Haemonchus* additional subunit-specific antisera should be raised to unique intracellular regions indicating which subunits exist in the extrapharyngeal region and if co-expression of GBR-2A and GBR-2B is observed.

Chapter 4

Cloning of a novel glutamate-gated cDNA receptor subunit (ZC317.3) from Caenorhabditis elegans

4.1. Introduction

When I commenced this Ph.D. the only cloned and fully-functionally expressed GluCl subunits known to exist in *C. elegans* were GluClα and GluClβ (Cully, Vassilatis *et al.* 1994). Although other predicted GluCls had been cloned (Chapter 1) and sequence analysis indicated high homology to GluClβ and GluClα, the absence of expression studies prevented confirmation of this function. The GluClα and GluClβ subunits were isolated by Cully *et al.* (1994) using an expression cloning strategy. The *glc*-1 and *glc*-2 genes encode these two subunits respectively and, when expressed in *Xenopus* oocytes, the α subunit formed functional ivermectin-sensitive homomeric channels. The GluClβ subunit formed non-desensitising channels gated by L-glutamate but was ivermectin-insensitive. When co-expressed in oocytes, the two subunits formed channels gated by L-glutamate and potentiated by very low (5nM) concentrations of ivermectin. Shortly after, Dent *et al.* (1997) reported the cloning of a subunit encoded by the avermectin resistant gene, *avr-15*. When expressed in *Xenopus* oocytes, GluClα_2 subunits were gated both reversibly by L-glutamate and irreversibly by ivermectin. Recent data by Dent *et al.* (2000) have shown that *avr-14* gene encodes an alternatively spliced GluCl (previously called GBR-2A/B) which has now been designated GluClα_{3A} and α_{3B}. When expressed in oocytes, GluClα_{3B} formed ivermectin and glutamate sensitive channels, however a response was not observed with GluCα_{3A} subunits. Consequently, these studies provided strong evidence that GluCls are targeted by the avermectin/milbemycin family of endectocides and insecticides (Arena *et al.*, 1995; Arena *et al.*, 1992a; Cully *et al.*, 1994; Cully *et al.*, 1996b). Subsequently, studies on the physiology and pharmacology of cloned and expressed GluCls may provide an insight into other properties and functions of inhibitory ionotropic receptors and opportunities for the development of novel anti-parasitic drugs.

The completion of the *C. elegans* genome sequence (*Caenorhabditis elegans* sequencing consortium, 1998) has permitted for the first time prediction of the total number of ionotropic receptor subunits present within an organism. The sequence predicted 19,000 genes of which 37 seem to encode ligand-gated anion channels most of which are of unknown function. Blast searches of sequence database revealed a predicted polypeptide, ZC317.3, with high amino acid identity to the previously cloned GluCl subunits. The generation of a full-length ZC317.3 cDNA clone by using PCR-based strategies was attempted. This strategy was preferred, as library screening can often be time consuming and can depend upon higher expression levels of the gene of interest. Initial amplification attempts were made using an alternative PCR technique, RACE (rapid amplification of cDNA ends), (Frohman *et al.*, 1988), where unknown initiation and termination codons can be obtained after an internal known stretch of sequence has been obtained. In numerous free-living and parasitic nematodes a common 5'

stretch of nucleotide sequence known as the spliced leader (SL) sequence has been demonstrated to exist (Nilsen 1993). Many mRNAs from nematodes contain the same 22nt leader sequence at the 5' ends which have been precisely acquired from a separately transcribed (100nt) RNA known as the SL RNA via *trans*-splicing (Bektesh, Doren *et al.* 1988; Nilsen 1993). In all cases examined the SL sequence has been perfectly conserved and is invariably located 0-38nt upstream of the ATG start codon, allowing a specific 5' exon region to be used for the initial design of a sense strand primer. Once the computer-predicted ends of the reading frame has been established, gene specific oligonucleotide primers can be designed to yield the desired product.

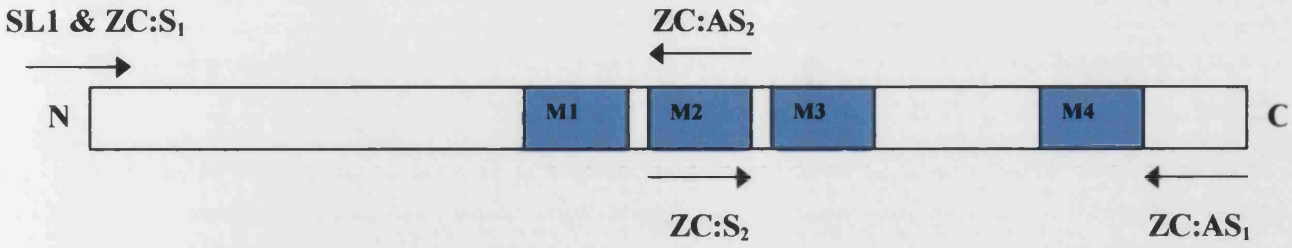
4.2 Results and Discussion

4.2.1 Amplification of 3' and 5' ends of ZC317.3 cDNA using RACE PCR

Using the EMBL database, all known nematode and *Drosophila* GluCl sequences were extracted and aligned (using the GCG program "pile-up") allowing us to search for high amino acid conservation to aid the design of oligonucleotides. A minimum of seven consecutive amino acids was chosen to allow for specific amplification. This was used in combination with the predicted ZC317.3 sequence from the *C. elegans* genome-sequencing database. From the sequence alignments an octopeptide motif TTLLTMTT in M2 appeared highly conserved amongst most available GluCls, $GABA_A$ and glycine receptor sequences. Both an antisense (termed $ZC:AS_2$) and a sense (termed $ZC:S_2$) primer were designed and synthesised to ZC317.3 M2 region (Fig. 4.1). Our initial attempt to detect mRNA transcribed from ZC317.3 gene were made using RACE PCR based strategies (Frohman, *et. al.*, 1988). Primer designed to the 5' end spliced leader sequence (SL1) was then used in conjunction with the $ZC:AS_2$ M2 octopeptide and a predicted 3' antisense primer ($ZC:AS_1$) was used with the sense octopeptide M2 primer ($ZC:S_2$).

A modified method from the standard PCR technique, "Touchdown" was used to enhance specificity during amplification. This involves a stepwise decrease in annealing temperature, starting at or above the expected annealing temperature. The amplification of both 5' and 3' terminal regions of ZC317.3 was achieved by performing PCR on single stranded cDNA at an initial annealing temperature of 65°C for 1.5 minutes, which was then step-wise decreased to 63 and finally 54°C. The ZC317.3 5' RACE reaction produced a single band of 800bp at the 5' end, which was re-amplified, subcloned and sequenced. The 3' RACE PCR reaction produced several bands, one of which was 550bp. Both products matched convincingly with the predicted ZC317.3 sequence obtained from the *C. elegans* genome database. Many structural features, for example M2, M3 and M4 motifs were clearly visible, suggesting the computer-predicted ends of the open-reading frame were accurate and allowed us to synthesise oligonucleotides that included the predicted initiation and termination codons (Fig. 4.1).

(a)



(b)

Primers	Sequence (5' to 3' direction)
SL1	GGTTTAATTACCCAAGTTTGAG
External ZC:S_1	CTTGATGAGTCTCCGTTCACTTC
External ZC:AS_1	CAATTTCATTTGGCTTCCGGTGCG
Internal ZC:S_2	CTACTCACAATGACCACACAGG
Internal ZC:AS_2	CCTGTGTGGTCATTGTGAGTAG

Table 4.1 Oligonucleotide primer sequences and locations used for the amplification of full-length ZC317.3 receptor cDNA (arrows not to scale). (a) Schematic representation of ZC317.3 subunit, indicating four hydrophobic segments, which are assumed to span the membrane in blue and approximate primer locations, represented with an arrow (not to scale). (b) Gene specific primers for ZC317.3 are labelled ZC accordingly and given suffix/S to denote a sense primer and /AS to denote an antisense primer.

Although theoretically a complete sequence had been obtained for ZC317.3, the sequence data was derived from two separate PCR products. To enable the clone to be used for expression studies and to ensure the sequence originated from a single receptor subunit it was necessary to amplify a full-length ZC317.3 cDNA from a single PCR reaction. The full-length sequence was obtained with a second PCR amplification using both the gene specific primers designed to the 5' and 3' regions and first strand cDNA as the template. After an initial melting at 94°C for 2 minutes, Expand™ High Fidelity was added and 40 cycles of amplification performed at 94°C for 2 minutes, 55°C for 1.5 minutes and 72°C for 2 minutes, with a final extension time of 5 minutes. The PCR reaction produced a single cDNA band of approximately 1400bp (Fig. 4.2), which was re-amplified, purified and ligated into pGEM®-T-EASY vector using TA overhangs which are produced by the Expand enzyme during the PCR reaction. The sequence has been submitted to the EMBL database (Accession number AJ2243914) and is shown in Fig. 4.3.

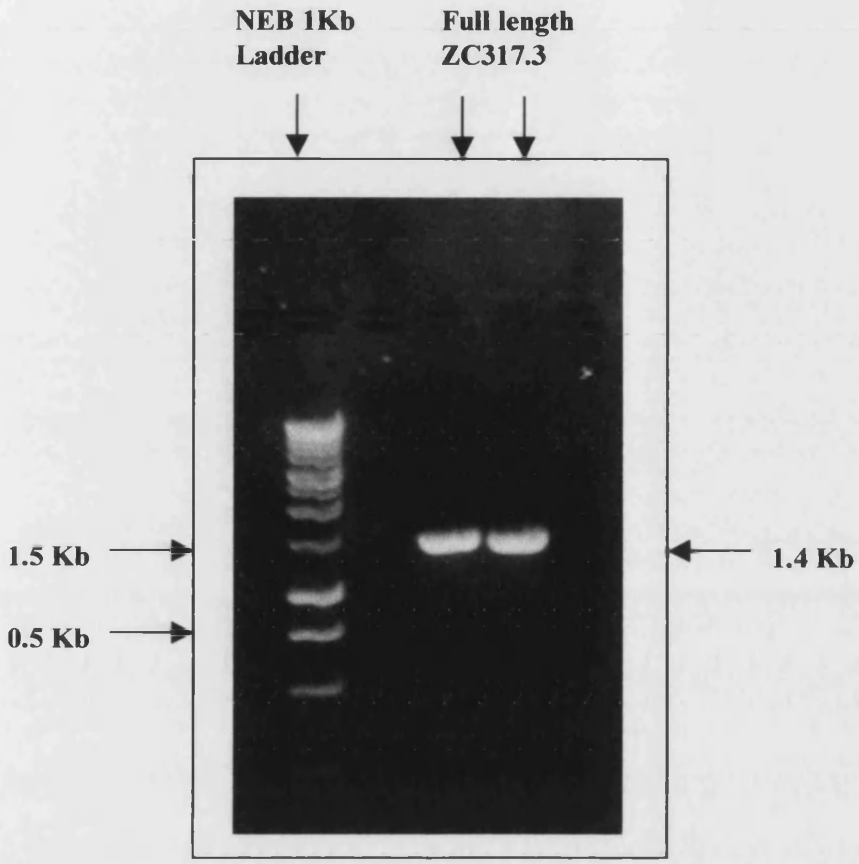


Figure 4.1 Agarose gel electrophoresis of a PCR amplification product predicted to encode the full length protein coding sequence of ZC317.3.

4.2.2 Sequence Analysis

The ZC317.3 cDNA shares many sequence characteristics with other invertebrate L-glutamate-gated chloride channel subunits (Section 1.2.3), which are presumed to be hetero-oligomeric complexes of five subunits, each of which span the membrane four times. The predicted mature gene product after signal peptide cleavage is a 484 amino acids polypeptide. The 5'end of the open reading frame includes a large N-terminal extracellular domain of 222 amino acids that contains a disulphide bridge consisting of a pair of cysteines separated by 13 amino acids (Stephenson 1988), leading the description of this receptor class to be called " cys-loop receptors" (Karlin and Akabas 1995). A characteristic 2nd pair of cysteine residues at positions 199 and 210 in addition to the dicysteine loop (positions 138 and 152) distinguishes the L-glutamate and the vertebrate glycine-gated chloride channels from the $GABA_A$, $GABA_C$ and nACh receptors (Fig. 4.3).

```
          1                                                  50.51                                                 100
ZC317.3   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
GluClα1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
GluClα2   MIGRLRRGFI LFPQLFLLFV SISSIFLFVL VECKPPKSLN RMRSKGTYNA AAFNSPSMIN NGLLLAGFEN NDTSRETRES YEEYKDDIDH LLEGGDLTFY
 GluClβ   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 Drosα1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 Gly α1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 GABAα1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          101                                                150.151                                               200
ZC317.3   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
GluClα1   .......... .......... .......... .......... .......... .......... .......... .........M ATWIVGKLII ASLILGI.QA
GluClα2   DEGADTAAGD VVITSETPVE KHKEVHFKNE PEEEEIGKED DGGGEAEEGE YEEENGSDAE EEEESPEKVE PATSTITTEA QTTTTPEEVT QDVSDNIEDD
 GluClβ   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 Drosα1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 Gly α1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
 GABAα1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          201                                                250.251                                               300
ZC317.3   .......... .......... .......MSL RSLLNILLIV AFWIVGGNCD A..S.SD... ..TEIIKKLL GK.G.YDWRV RPPGINLTIP GTHGAVIVYV
GluClα1   QQARTKSQ.. .........D IFEDDNDNGT TTLESLARLT SPIHIPIEQP Q..T.SD... ..SKILAHLF TS.G.YDFRV RPPTDN.... G..GPVVVSV
GluClα2   EDARPKSEEH SPKHAASSSD LFEDDDQ..S TTLESIARLS APAHVPIEQP Q..T.SD... ..TEILEHLL TR.G.YDHRV RPPGEDGTIH G..GPVVVSV
 GluClβ   .......... .......... .......MTT PSSFSILLLL LLMPVVTNGE YSMQ.SE... ..QEILNALL KN...YDMRV RPPPAN...S STEGAVNVRV
 Drosα1   .......... .......... ...MGSGHYF WAILYFASLC SASLANNAKV NFRE.KE... ..KKVLDQIL GA.GKYDARI RPSGIN.... GTDGPAIVRI
 Gly α1   .......... ....MAHVRH FRTLLSGFYF WEAALLLSLV ATKETNSARS RSAPMSP... ..SDFLDKLM GRTSGYDARI RPNFKG.... ...PPVNVTC
 GABAα1   .......... .......... .MKKSRGLSD YLWAWTLILS TLSGRSYGQP SQDELKDNTT VFTRILDRLL ...DGYDNRL RPGLGER... ....VTEVKT
```


301 350.351 400
ZC317.3 NMLIRSISKI DDVNMEYSVQ LTFREEWVDG RLAYG.FPGD STPDFLILTA GQQ.IWMPDS FFQNEKQAHK HDIDKPNVL. IRIHRDGRIL YSVRISMVLS
GluClα1 NMLLRTISKI DVVNMEYSAQ LTLRESWIDK RLSYG.VKGD GQPDFVILTV GHQ.IWMPDT FFPNEKQAYK HTIDKPNVL. IRIHNDGTVL YSVRISLVLS
GluClα2 NMLLRSISKI DNVNMEYSVQ LTFRESWVDK RLSFG.VKGD AQPDFLILTA GQE.IWMPDS FFQNENQAYK HMIDKPNVLI IRVHKDGTIL YSVRISLVLS
GluClβ NIMIRMLSKI DVVNMEYSIQ LTFREQWIDP RLAYENLGFY NPPAFLTVPH VKKSLWIPDT FFPTEKAAHR HLIDMENMF. LRIYPDGKIL YSSRISLTSS
Drosα1 NLFVRSIMTI SDIKMEYSVQ LTFREQWTDE RLKFD..DIQ GRLKYLTLTE ANR.VWMPDL FFSNEKEGHF HNIIMPNVY. IRIFPNGSVL YSIRISLTLA
Gly α1 NIFINSFGSI AETTMDYRVN IFLRQKWNDP RLAYSEYPDD S...LDLDPS MLDSIWKPDL FFANEKGANF HEVTTDNKL. LRIFKNGNVL YSIRLTLTLS
GABAα1 DIFVTSFGPV SDHDMEYTID VFFRQSWKDE RLKFK....G PMTVLRLNNL MASKIWTPDT FFHNGKKSVA HNMTMPNKL. LRITEDGTLL YTMRLTVRAE
TM1
401 450.451 500
ZC317.3 CPMHLQYYPM DVQTCLIDLA SYAYTENDIE YRW.KKTDPV QLKKGLHSSL PSFELNNVDT TL.CTSKTNT GTYSCLRTVL ELRRQFSYYL LQLYIPSTML
GluClα1 CPMYLQYYPM DVQQCSIDLA SYAYTTKDIE YLW.KEHSPL QLKVGLSSSL PSFQLTNTST TY.CTSVTNT GIYSCLRTTI QLKREFSFYL LQLYIPSCML
GluClα2 CPMHLQYYPM DVQQCFIDLA SYAYTTKDIE YVW.KEETPV QLKAGLSSSL PSFQLTNTST TY.CTSKTNT GSYSCLRTII QLRRQFSYYL LQLYIPSCML
GluClβ CPMRLQLYPL DYQSCNFDLV SYAHTMNDIM YEW.DPSTPV QLKPGVGSDL PNFILKNYTT NADCTSHTNT GSYGCLRMQL LFKRQFSYYL VQLYAPTTMI
Drosα1 CPMNLKLYPL DRQICSLRMA SYGWTTNDLV FLW.KEGDPV QVVKNLH..L PRFTLEKFLT DY.CNSKTNT GEYSCLKVDL LFRREFSYYL IQIYIPCCML
Gly α1 CPMDLKNFPM DVQTCIMQLE SFGYTMNDLI FEW.QDEAPV QVAEGL..TL PQFLLKEEKD LRYCTKHYNT GKFTCIEVRF HLERQMGYYL IQMYIPSLLI
GABAα1 CPMHLEDFPM DAHACPLKFG SYAYTRAEVV YEWTREPARS VVVAEDGSRL NQYDLLG.QT VDSGIVQSST GEYVVMTTHF HLKRKIGYFV IQTYLPCIMT
TM2
TM3
501 550.551 600
ZC317.3 VIVSWVSFWL DRGAVPARVT LGVTTLLTMT TQASGINAKL PPVSYTKAID VWIGACLTFI FGALLE.FAW VTYISSRSFY KRNKNCSSRN SLLIETKQAL
GluClα1 VIVSWVSFWF DRTAIPARVT LGVTTLLTMT AQSAGINSQL PPVSYIKAID VWIGACMTFI FCALLE.FAL VNHIAN.... KQGVERKAR. ....
GluClα2 VIVSWVSFWI DRTAVPARVT LGVTTLLTMT TQSSGINAKL PPVAYIKAID VWIGACMTFI FCALLEFFAW VTYIANK... KQDANKRAR. ....
GluClβ VIVSWVSFWI DLHSTAGRVA LGVTTLLTMT TMQSAINAKL PPVSYVKVVD VWLGACQTFV FGALLE.YAF VSYQDSVRQN DRSREKAARK A.........
Drosα1 VIVSWVSFWL DQGAVPARVS LGVTTLLTMA TQTSGINASL PPVSYTKAID VWTGVCLTFV FGALLE.FAL VNYASRSGSN KANMHKENMK ....
Gly α1 VILSWVSFWI NMDAAPARVA LGITTVLTMT TQSSGSRASL PKVSYVKAID IWMAVCLLFV FSALLE.YAA VNFVSRQ... ....
GABAα1 VILSQVSFWL NRESVPARTV FGVTTVLTMT TLSISARNSL PKVAYATAMD WFIAVCYAFV FSALIE.FAT VNYFTKRGYA WDGKS..... ....

```
          601                                                650.651                                                700
ZC317.3   IIPNTVVAQF PEHPEEVEMG LAQAPDVWIR QSGNGKTVSR VNGHINHNND EAAELIIFDA KHKNRRFVWW TNFRNIRLIR WIRNRLNVDD NAKRADLISR
GluClα1   .......... .TEREKAEIP LLQ....... ...NLHND.. VPTKVFNQEE KVRTVPL... ...NRR.... ...QMNSFLN LLETKTEWND ISKRVDLISR
GluClα2   .......... .TEREKAELP FLQ....... ...NSHNDVW VPREVAEQER EVMTVRM... ...NRR.... ...QTNSVWK WIKTKTEWND KSKRADLISR
 GluClβ   .......... QRRREKLEM. .......... .......... VDAEVYQPPC TCHTFEARET F......... ....RDKVRR YFTKP...DY LPAKIDFYAR
 Drosα1   .......... .KKRRDLEQA SLDAASDLLD TDSNA...TF AMKPLVRHPG DPLALEKRLQ CEVHMQAPKR PNCCKTWLSK FPTRQCSR.. .SKRIDVISR
 Gly α1   ...HKELLRF RRKRKNKTEA FALEKFYRFS DTDDEVRESR LSFTAYGMGP CLQAKDGVVP KGPNHAVQVM PKSADEMRKV FI.......D RAKKIDTISR
 GABAα1   ........VV PEKPKKVKDP LIK......K NNTYAPTATS YTPNLARGDP GLATIAKSAT IEPKEVKPET KPPEPKKTFN SV........ ..SKIDRLSR

                   TM4
          701                                                750
ZC317.3   VLFPTLFVCF NFVYWTKYSQ YHAPEAK
GluClα1   ALFPVLFFVF NILYWSRFGQ QNVLF
GluClα2   VMFPVLFLTF NISYWTHYGQ YGVAIST
 GluClβ   FVVPLAFLAF NVIYWVSCLI MSANASTPES LV
 Drosα1   ITFPLVFALF NLVYWSTYLF REEED
 Gly α1   ACFPLAFLIF NIFYWVIYKI LRHEDIHHQQ D
 GABAα1   IAFPLLFGIF NLVYWATYLN REPQLKAPTP HQ
```

Figure 4.3 Alignment of *C. elegans* receptor subunit ZC317.3 with invertebrate ligand-gated chloride channels.

Representative members of the ligand-gated chloride channel subunit family from invertebrate rat ($GABA_A$, and glycine α_1), *C. elegans* (GluCls α_1, α_2, β) and Drosophila (α) receptor subunits aligned with the full-length receptor subunit, ZC317.3. The putative transmembrane regions (M1-4) are underlined and for the ZC317.3 sequence only, the N-linked glycosylation site is indicated by ■. The four cysteine residues in the N-terminal domain is signified by C, and the 2 x S residues (consensus site for PKC phosphorylation) highlighted in blue (S)

Homologous regions within the cys-loop (^{218}CPMxLxxYPxDxQxC^{232}, Fig. 4.3) appear to be, conserved across invertebrate species to mammals. The cys-loop appears to be essential for events during subunit assembly, including subunit folding, oligomerisation and producing correct oligomeric stoichiometry (Green and Millar, 1995). Part of the assembly also appears critical for the formation of binding sites for agonist, allosteric modulators and antagonists, (for reviews see Galzi and Changeux, 1995; Karlin and Akabas, 1995; Smith and Olsen, 1995).

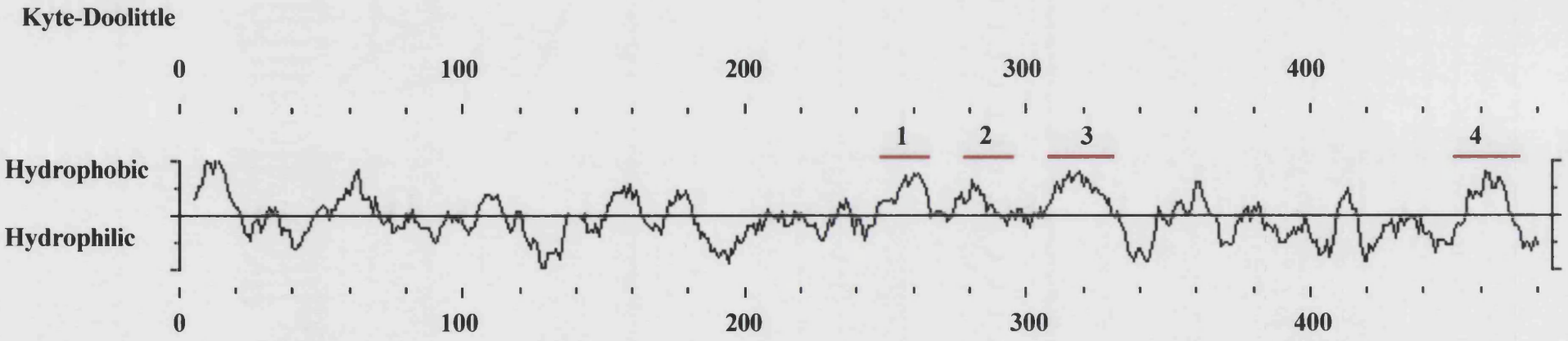
Studies with homo-oligomers composed of chimaeric subunits have provided further evidence for the location of the agonist-binding site in the N-terminal domain. In a recent study (Etter, Cully *et al.* 1996) a homo-oligomer composed of chimaeric subunits provided evidence not only for the location of the agonist binding site in the N-terminal domain, but in addition that the ligand binding region is coupled to channel gating within the C-terminal domain. A chimaera composed of the N-terminal domain of *C. elegans* GluClα (a glutamate-insensitive channel) with the C-terminal of GluClβ subunit (glutamate-sensitive subunit), yielded a receptor with greater glutamate sensitivity (EC_{50} 530±90nM) than seen with wild-type GluClβ homomers (EC_{50} 380μM). This data indicates wild-type GluClα contains the necessary binding site within the N-terminal extracellular domain, but the C-terminal part of GluCl does not couple ligand binding to channel gating.

The motif WxDxxL (corresponding to residues ^{137}**WVDGRL**^{142} in ZC317.3 protein, Fig. 4.3) has been found to be conserved in all cys-loop receptor subunits and may contribute to channel gating (Smith and Olsen, 1995). It has been reported that most ligand-gated channels need the interface between two adjacent subunits to be co-expressed before the agonist will bind, as yet it has not been ascertained whether this also holds true in *C. elegans* GluCls. If we assume that similar protein folding occurs in all the cys-loop family, it is possible that the co-assembly of two structurally distinct homologous regions at the subunit interfaces may form the agonist binding sites of L-glutamate and ibotenate. As most GluCls are capable of forming homo-oligomers and therefore have all the necessary amino acid residues within the binding site, for glutamate or ibotenate binding site to exist it may require the interface of two distinct α subunit classes or one subunit from an α class and one from β.

The cytoplasmic domain between M3 and M4 is the most divergent region of ligand-gated chloride channels. Most ligand-gated chloride channels are subjected to protein phosphorylation modification and the conservation of these residues may have great importance for regulating receptor function (Raymond, Blackstone *et al.* 1993; Tanaka and Nishizuka 1994), although this role is still poorly understood (Vijayaraghavan, Schmid *et al.* 1990).

Figure 4.4 Hydrophilicity analysis of GluClα_4.

The predicted Kyte and Doolittle hydropathy plot of *C. elegans* receptor cDNA; ZC317.3 was constructed using the GCG program PEPTIDESTRUCTURE. The hydropathy plot predicts a proposed extracellular N-terminal, followed by four membrane-spanning domains (denoted by a solid red line, M1-4). The predominantly hydrophilic intracellular loop separates the M3 and 4 domains.



Nicotinic acetylcholine, $GABA_A$ (Raymond, Blackstone *et al.*, 1993; Krishek, Xie *et al.*, 1994; Moss, Gorrie *et al.*, 1995) and glycine (Song and Huang 1990; Swope, Moss *et al.*, 1992) receptor function can all be regulated by phosphorylation of defined serine, tyrosine and threonine cytoplasmic residues. Biochemical approaches have demonstrated that phosphorylation of serine and threonine residues by PKC and PKA on $GABA_A$ receptors decreases chloride conductance (Sigel 1995; Moss and Smart 1996) inhibiting receptor function. However, others have indicated that PKC (Lin, Browning *et al.*, 1994) and PKA (Cheun and Yeh, 1992) potentiate a GABA-gated response. Alternatively, tyrosine phosphorylation has been considered to be involved in numerous processes, including growth, differentiation (Wan, Man *et al.*, 1997), and ischemia (Kindy 1993). In all of these cellular processes tyrosine phosphorylation is know to inhibit $GABA_A$ receptor function and potentiation. Studies indicate that protein kinase catalyse the transfer of a charged phosphate molecule from ATP to a key serine, tyrosine or threonine within the M2 and M3 intracellular loop (Swope, Moss *et al.*, 1992; Moss and Smart, 1996). However, neither the N-linked glycosylation site nor the protein kinase C sites is conserved between ZC317.3 and other *C. elegans* α subunits, though a glycosylation site is present at a homologous position on the $GluCl\alpha_3$ subunits. The significance of the putative phosphorylation sites in GluCl is poorly understood. A candidate site for N-linked glycosylation is located in the extracellular domain at residue 26 of the mature polypeptide.

The hydropathy profile (Kyte and Doolittle, 1982) of the predicted gene was constructed using the GCG protein analysis software (Fig. 4.4) and displays four extended hydrophobic domains corresponding to putative α helices spanning the membrane domains. Both the position and amino acid sequence of the four membrane domains are conserved with respect to other known members of the ligand-gated chloride family. The plot also indicates a potential intracellular loop of 127 amino acids, which separates the M3 and M4 domains. When a comparison of the ZC317.3 cDNA sequence was made with the genomic sequence predicted by Genefinder, this revealed that one of the intron/exon boundaries was not as predicted, resulting in a longer (43 amino acids) intracellular loop between the M3 and M4 domains.

Database searches of the ZC317.3 sequence exhibited a ≥40% amino-acid identity to other invertebrate GluCl subunits (Table 4.1). An alignment illustrating the relationship between the GluClα-like subunits is shown in Fig. 4.3. Compared to GluClα and α2, ZC317.3 is truncated by about 30 amino acids at the N-terminus, producing an extracellular domain similar in size to GBR-2, and has a slightly longer intracellular loop than any of the other GluCl subunits. All four-membrane domains are highly conserved with respect to other GluClα subunits (Fig. 4.3). The M2 domain is especially homologous with the exceptions of threonine 335 in ZC317.3 which is substituted for serine in GBR2B, alanine 348 in ZC317.3 is replaced with serine in $GluCl\alpha_1$ and α_2 and finally, serine 349 in ZC317.3, α2 and GBR2b is an alanine in $GluCl\alpha_1$.

	GluClα	*GluClβ*	*GluClα$_{2S}$*	*GluClα$_{2L}$*	*GBR2A*	*GBR2B*	*C27H5.8*	*DROSα*	*GABAα$_1$*	*GLYα$_1$*
ZC317.3	73%	63%	77%	77%	60%	58%	40%	48%	32%	40%
GluClα		45%	77%	77%	57%	54%	38%	46%	33%	40%
GluClβ			47%	47%	48%	47%	39%	44%	33%	40%
GluClα$_{2S}$				96%	57%	54%	38%	48%	33%	39%
GluClα$_{2L}$					57%	55%	38%	47%	33%	38%
GBR2A						79%	41%	52%	31%	37%
GBR2B							40%	50%	31%	37%
C27H5.8								37%	32%	38%
DrosGluClα									37%	43%
GABAα$_1$										35%

Table 4.5 Compilation of the percentage amino acid identities of vertebrate and invertebrate $GABA_A$, glycine and L-glutamate receptor subunits. Receptors are given the suffix GluCl to denote glutamate-gated receptor subunit and *Dros* indicates a *Drosophila melanogaster* subunit. A more distantly related ionotropic receptor subunit cDNA, C27H5.8 was cloned using information form the genome sequencing project.

C. elegans ***Drosophila melanogaster*** **Rat**

4.3. Summary

We report here the amplification of a full-length, novel subunit from *C. elegans*. Overall, sequence resembles in size, sequence and predicted secondary structure subunits of other cloned invertebrate GluCls. However, the ZC317.3 only shows approximately 30% homology with GABA and 40% homology with Glycine cDNAs indicating they are not species homologues of the same polypeptide. To date, no homologue of this subunit has yet been identified in other nematode species. The *C. elegans* ZC317.3 gene is therefore designated *glc-3* and the subunit it encodes GLC-3 (see nomenclature page IV). GLC-3 appears to be most closely related to the previously characterised GluClα and -α2 subunits where the homology is highest within the transmembrane regions M1-M3.

Future studies will include direct *in vitro* expression of the gene in *Xenopus* oocytes, this will not only provide further evidence that GLC-3 is part of the GluCl family, but also offers an opportunity in identifying the *in vivo* subunit combinations. With their known sequence and subunit components, recombinant receptor studies offer an important tool in identifying the mode of action of insecticides and the structural elements involved in toxicity and selectivity.

Chapter 5

Agonist and antagonist pharmacology of the GLC-3 homo-oligomers

5.1 Introduction

The first report identifying the existence of the IGluCls was by Lea and Usherwood (1973), on *Schistocerca gregaria* (locust) leg muscle. Since then, subsequent reports have emerged identifying their existence in crustaceans, molluscs and nematodes. Inhibitory ionotropic glutamate receptors appear to be confined to invertebrate species and have been demonstrated to be the targets of a wide range of avermectin and related anthelmintic.

Electrophysiological studies on *Xenopus* oocytes injected with mRNA from the *C. elegans* GluClα (α_{2s}, α_{2L} and α_{3B}) subunits have produced undisputed evidence that ivermectin directly induces chloride currents and was able to potentiate (GluClα and GluClβ heteromers) glutamate-induced chloride currents at nanomolar concentrations. In addition, all of the functional α subunits (with the exception of GluClα_1) have also been observed to produce reversible L-glutamate- and ibotenate-gated chloride currents at micromolar concentrations (Cully *et al.*, 1994; Dent *et al.*, 1997; Dent *et al.*, 2000). The GluClβ homomer forms reversible, non-desensitising, L-glutamate-sensitive channels that are non-responsive to ivermectin (Cully *et al.*, 1994). On co-expression of GluClα_1 and β receptor subunits, an ivermectin potentiated chloride channel is formed.

Although the primary structure of the *Drosophila* GluClα is ~50% identical to the *C. elegans* GluCls, some differences have been identified in their antagonist pharmacology. In contrast to the functional *C. elegans* GluClβ, α_2 and α_3 channels, *Drosophila* GluClα exhibited rapid, robust inward currents in response to L-glutamate and ibotenate which were not inhibited by picrotoxin or potentiated by avermectin (Cully *et al.*, 1996a). Interestingly, oocytes expressing either DrosGluClα subunits, *C. elegans* total RNA or *C. elegans* native pharyngeal GluCls produced channels that were significantly more sensitive to ibotenate than L-glutamate. This appears contradictory with oocytes expressing *C. elegans* recombinant GluCls (α_2, β, and α_3), which produced full agonist responses to L-glutamate, but only weak, small chloride currents to ibotenate. However, until more is known of the composition of native GluCls of *C. elegans*, relating pharmacological differences to underlying receptor properties will remain difficult. Although the completion of the *C. elegans* genome sequence (*Caenorhabditis elegans* sequencing Consortium, 1998) has permitted for the first time a prediction on the total number of IGluCls present, the functional subunit combination is not known for any nematode muscle or neuronal GluCl. Future pharmacological investigation of cloned heteromer-forming GluCls offers a better understanding of the anthelmintic drug-receptor interactions in *C. elegans* and orthologous parasitic species.

The polycyclic dinitriles (for example BIDN), phenylpyrazoles (fipronil) and picrotoxin are convulsant antagonists that inhibit GABA-mediated chloride currents. The binding site where these convulsant drugs interact is still unknown, although some authors (Gant *et al.*, 1998; Hosie *et al.*, 1995a) have speculated that these compounds act deep within the channel pore that is lined by the M2 domain. Both fipronil and BIDN have shown effective commercial, insecticidal and pesticidal action, which have been discussed further in Chapter 1. In view of the structural similarities between GABA and IGluCl receptors, BIDN and fipronil were specifically chosen, as their effects on GluCls have not been previously characterised. Furthermore, the pharmacological success of fipronil (as with the avermectin class of compounds) has mostly been attributed to their ability to exhibit a range of activity against insect pest and mites without affecting the host organism, allowing it to be used extensively in public and animal health. The plant derived compound picrotoxin is composed of equimolar amounts of picrotin and picrotoxinin (Etter *et al.*, 1999), the former has little or no effect (Newland and Cull-Candy, 1992). The site and mode of action of picrotoxinin is the subject of some debate and is discussed in detail in the discussion of this chapter.

To date little else is known of the pharmacology of GluCl receptors expressed *in vitro*, which would be necessary to understand the molecular basis of anthelmintic action for which no vertebrate counterpart has been described. This chapter reports a detailed description of the agonist and antagonist profile of *C. elegans* GLC-3 receptor subunit providing the first demonstration that GluCl RECEPTORSare a molecular target for convulsant antagonists BIDN and fipronil. In addition, the actions of L-glutamate, ibotenate and ivermectin are also described, as well as the lack of effects from picrotoxinin and several amino acid ligands. The chemical structures of ivermectin, fipronil, BIDN and picrotoxinin are shown in Fig 5.1.

The *Xenopus* oocyte expression system in conjunction with the two electrode voltage clamp recording is a convenient means of expressing and recording current across the membrane from exogenous LGICs. This system was therefore used for the potential characterization of the GLC-3 receptor subunits. Oocytes from the South Africa clawed frog offer a convenient lipid membrane used as a heterologous receptor expression system for several reasons. Late staged (V & VI) oocytes are large cells (1-2mm in diameter) simplifying both microinjection of cDNA into the nucleus and also the insertion of microelectrodes for voltage clamping. Although they possess certain voltage-dependent ion channels and G-protein coupled receptors (Snutch, 1988), these offer little interference to electrophysiological studies on GluCl ionotropic receptors. Oocytes injected with foreign mRNA into the cytoplasm or cDNA into the nucleus, assemble fully functional hetero-oligomers with post-translational modifications such as phosphorylation and glycosylation (Snutch, 1988). However, there are some disadvantages to the oocyte expression system. These include the short expression period compared with stably transfected cell lines (at most, two weeks of expression before oocyte

deterioration) and the small number of oocytes that can be assessed for function during one experiment. Furthermore, oocyte expression systems are often seasonally dependent (oocytes tend to be more reliable between the months March and October, Snutch, 1988) and can produce erratic, non-functional responses.

5.1.1 Aims

The final aims of this study are as follows:

(i) Using electrophysiological techniques, investigate the agonist profile of endogenous neurotransmitters (L-glutamate, GABA and glycine) on the GLC-3 homo-oligomer.

(ii) Examine the potential of the anthelmintic ivermectin as an agonist at the receptor.

(iii) Using the current-voltage relationship, determine the ion selectivity of GLC-3 receptors.

(iv) Due to GluCls sequence similarity to $GABA_A$ receptor subunits, examine the potential effects of known $GABA_A$ antagonists and insecticide compounds.

5.2 Results

5.2.1 Actions of L-glutamate and Ibotenate at the GLC-3 homo-oligomer

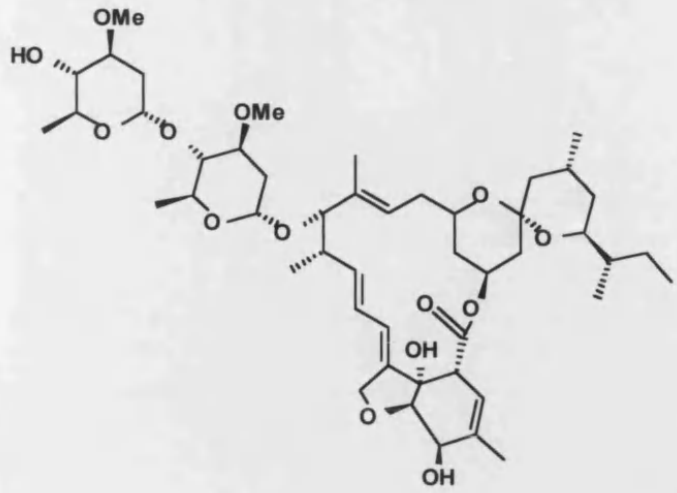
Xenopus oocytes injected with *in vitro*-transcribed cRNA encoding the GLC-3 polypeptide responded to L-glutamate, yielding robust, dose-dependent inward currents at a holding potential of E_h=–80mV (Fig. 5.2). Dose-dependent currents elicited by L-glutamate (Fig. 5.2b) generated an EC_{50} value of 1.9 ± 0.03mM and an estimated peak plateau at concentrations of 8mM and above. The Hill coefficient of 1.46 ± 0.1 suggested more than one L-glutamate molecule was necessary to gate the channel. Control oocytes injected with the same volume of deionised water and non-injected oocytes failed to respond to L-glutamate when it was applied at concentrations from 3μM to 10mM (n=6, Fig. 5.2b). Applications of 3mM L-glutamate were used thereafter as the standardising agonist, allowing reproducible responses to subsequent drug applications. The conformationally constrained L-glutamate analogue, ibotenic acid, activated the GLC-3 receptor at 1mM, but produced non-desensitising currents of a smaller amplitude and slower onset than L-glutamate (Fig. 5.3b). The amplitude and shape of the ibotenate response were similar to those obtained from *C. elegans* GluClβ and α_2 homomers (Cully *et al.*, 1994; Dent *et al.*, 1997).

5.2.2 Negative effects of GABA, glycine, histamine and NMDA

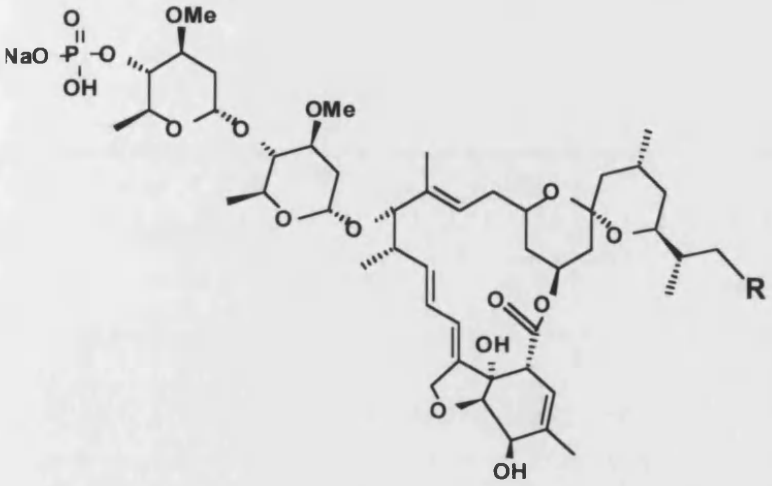
GLC-3 homo-oligomers were insensitive to NMDA, glycine, histamine and GABA at 1mM, however, a robust inward current was elicited by 1mM L-glutamate (Fig. 5.3a). The application of 3mM L-glutamate was used prior to the application of the above ligands, to standardise the agonist responses to changes seen in both oocytes and mRNA expression. The negative responses seen here appear to be consistent with data obtained from other GluCls (Cully *et al.*, 1994; Dent *et al.*, 1997).

5.2.3 Current-voltage relationship determined for GLC-3 homomers

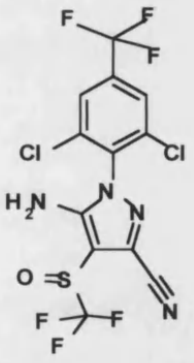
The current-voltage relationship was determined using L-glutamate (3mM) to ascertain if the GLC-3 homo-oligomer was selective for chloride ions. The inwardly evoked L-glutamate-gated responses decreased in size as the membrane potential was increased step-wise from –80mV to –20mV. As the membrane potential was increased to become more positive (increasing from 0 to +40mV) the L-glutamate-gated responses increased in size and changed to an outward direction. L-glutamate induced currents were found to reverse near the predicted chloride reversal potential for oocytes (≅ -20mV, Fig. 5.4) (Dascal, 1987). In order to ascertain that the GLC-3 homo-oligomer was selective to chloride ions, the external chloride concentration was lowered (from 107.6mM to 54.9mM) and replaced with mono-sodium gluconate, allowing equilibrium of Cl^- ions.



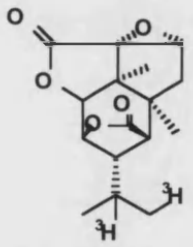
$_{22,\ 23}$-Dihydroavermectin B_1a



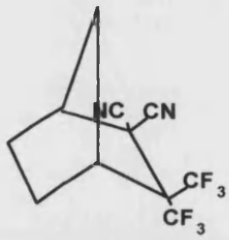
Ivermectin-$_4$"-OPO_3"



Fipronil



picrotoxinin



BIDN

Figure 5.1 Chemical structures of the agonist and antagonist compounds used in this study and/or referred to in the text. Ivermectin is composed primarily of 22,23-dihydroavermectin B1a, where R=CH_3, but also contains some 22,23-dihydroavernectin B1b, where R=C_2H_5. Chemical structures were prepared using Chem 4-D Draw, (ChemInnovation Software, U.S.A).

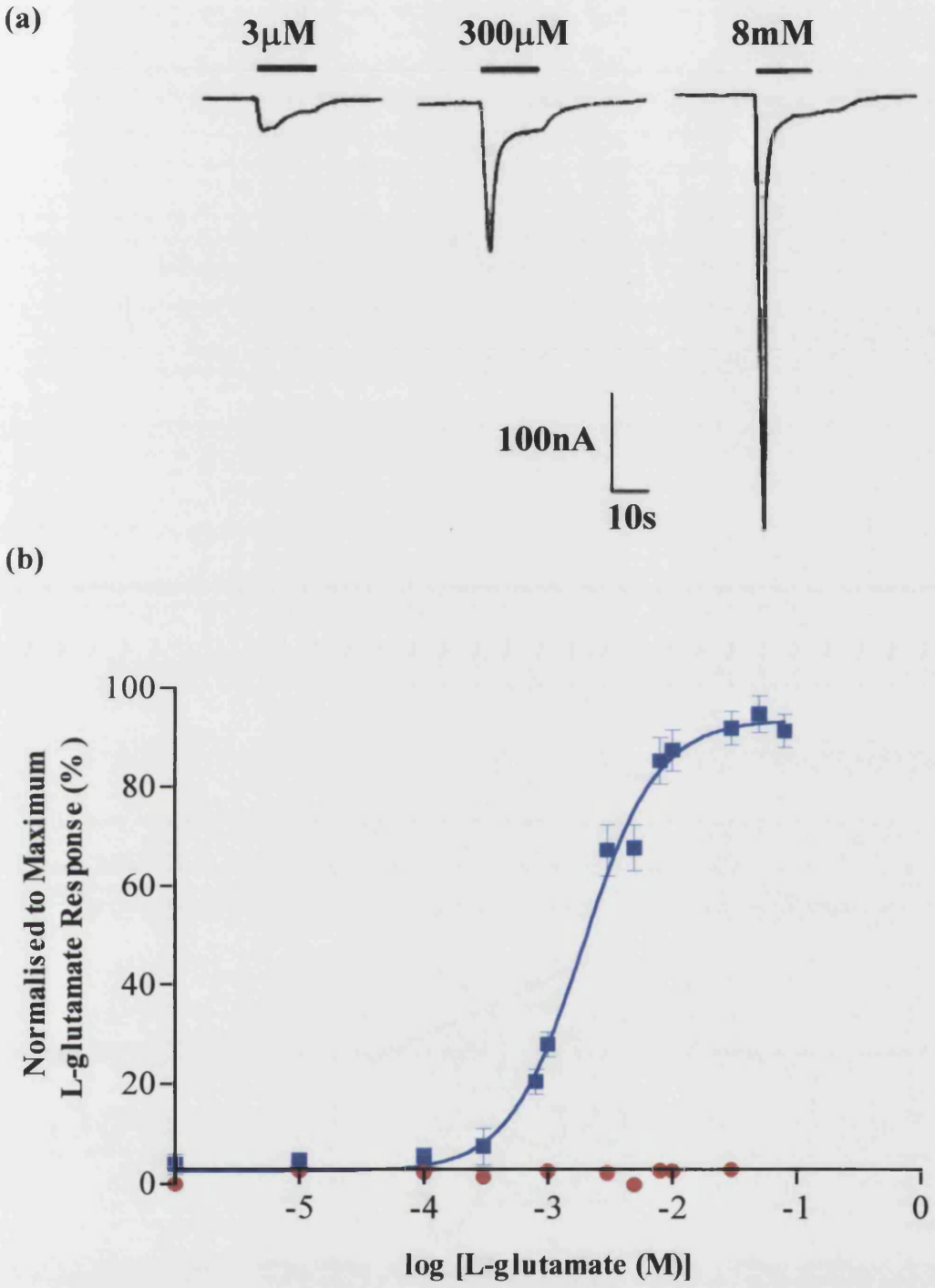
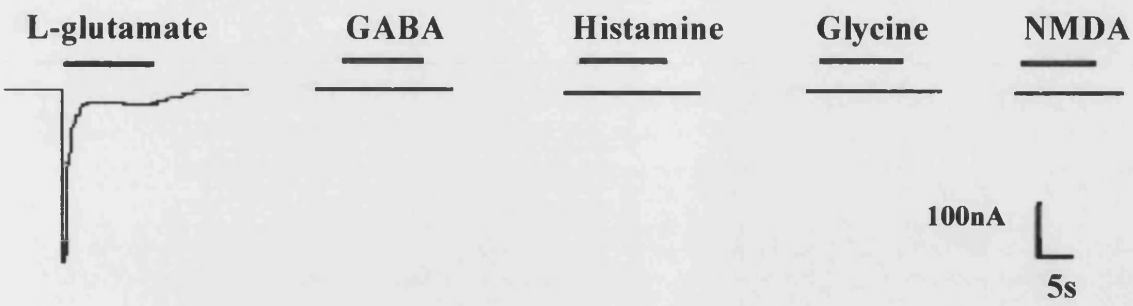


Figure 5.2 Dose-dependent L-glutamate-gated currents in oocytes injected with GLC-3 cDNA.

(a) L-glutamate induced dose-dependent inward currents from *Xenopus* oocytes injected with GLC-3 cRNA and voltage clamped at –80mV. Desensitisation was observed with higher concentrations of L-glutamate, approximately 10μM and above. Horizontal bars indicate the duration of agonist application and downward deflection denotes an inward current.

(b) L-glutamate concentration-response curves of oocytes injected with *in vitro* cRNA encoding the GLC-3 subunit (■ n≥6), and water-injected control oocytes (● n≥6), clamped at –80mV.

(a)



(b)

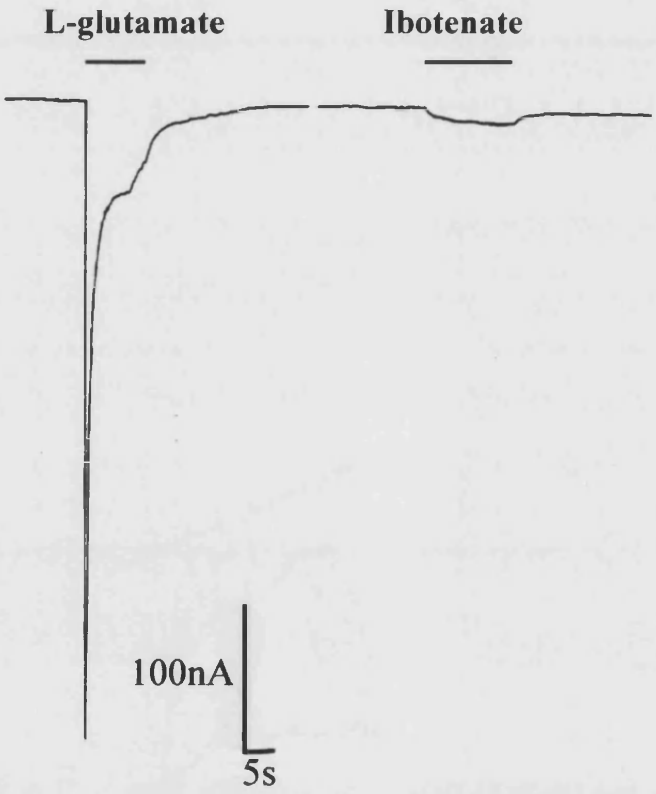


Figure 5.3 Comparison of inward currents induced by 1mM L-glutamate, ibotenate and the negative effects of NMDA, GABA, glycine, and histamine on the GLC-3 homo-oligomer.

Horizontal bars indicate the duration of agonist application and downward deflection denotes an inward current.

(a) Currents were not observed for the following 1mM applications of GABA, glycine, histamine and NMDA, although inward responses were induced by 1mM L-glutamate.

(b) Chloride currents induced by 1mM ibotenic acid and 1mM L-glutamate.

The reversal potential was estimated to be (E_{Cl})=–16.6mV for oocytes in the presence of NaCl (normal SOS, Table 2.1.9.2) and (E_{Cl})=–7.3mV for oocytes in the reduced extra-cellular Cl^- saline, shifting the reversal potential (E_{rev}=9.3mV) for the L-glutamate-sensitive currents. The glutamate-induced current was determined by subtracting the current required to clamp the oocyte membrane at a given potential in saline, from that required to clamp the membrane at the same potential in the presence of 3mM L-glutamate.

5.2.4 Agonist actions of ivermectin on GLC-3 homo-oligomers

Oocytes expressing the GLC-3 protein exhibited slowly activating, irreversible, inward current in response to ivermectin, that were dose-dependent and not observed in control uninjected oocytes or in oocytes injected with distilled water. Large non-desensitising ivermectin-sensitive currents were observed following the application of 0.1μM and 1μM ivermectin, whereas 0.01μM ivermectin failed to gate the channel (Fig. 5.5a, n=3). The time course for maximal activation of ivermectin-sensitive currents was 28±18.5 seconds for GLC-3, 10 seconds slower than observed for GluClα_1 receptors (Cully *et. al.*, 1994). A two-phase agonist response was observed following the co-application of ivermectin (1μM) and glutamate (3mM). An initial rapid, desensitising response to L-glutamate was followed by a slower, non-desensitising, irreversible response characteristic of ivermectin (Fig. 5.5b). There was no apparent increase in the amplitude of either the L-glutamate or ivermectin peaks when co-applied than observed when applied alone.

5.2.5 Actions of convulsant antagonists on GLC-3 receptors

Picrotoxinin is a potent antagonist of many native and recombinant vertebrate and invertebrate $GABA_A$ receptors (Becker, 1992; Cull-Candy, 1976; Olsen and Tobin, 1990; Pribilla *et al.*, 1992), and of some native and recombinant GluCls (Arena *et al.*, 1992a; Cleland, 1996; Cully *et al.*, 1994; Etter *et al.*, 1999; Vassilatis *et al.*, 1997a). However, at concentrations of 0.01 to 1000μM, it failed to inhibit the robust L-glutamate (3mM) agonist response in oocytes expressing the GLC-3 receptor (Fig. 5.6, n=6).

The bicyclic dinitrile convulsant, BIDN and the phenylpyrazole insecticide, fipronil (10μM), had no direct effect on either un-injected oocytes, or oocytes injected with GLC-3 cRNA all of which were clamped at E_h=–80mV. However, when L-glutamate was co-applied with either fipronil or BIDN the L-glutamate response was significantly reduced. Incubation of the oocytes for 2 minutes in saline containing one of the L-glutamate antagonists, fipronil or BIDN, at concentrations from 0.1μM to 100μM, suppressed the amplitude of the L-glutamate response in a dose-dependent manner (n≥5, Fig. 5.6). The amplitude of the L-glutamate responses was restored after 10 min rebathing in normal saline, though at antagonist concentrations at and above 10μM recovery was slower and often

incomplete. The estimated IC_{50}'s for the co-application of L-glutamate with BIDN and fipronil were $0.22 \pm 0.07\mu M$ and $11.53 \pm 0.11\mu M$, respectively (Fig. 5.7).

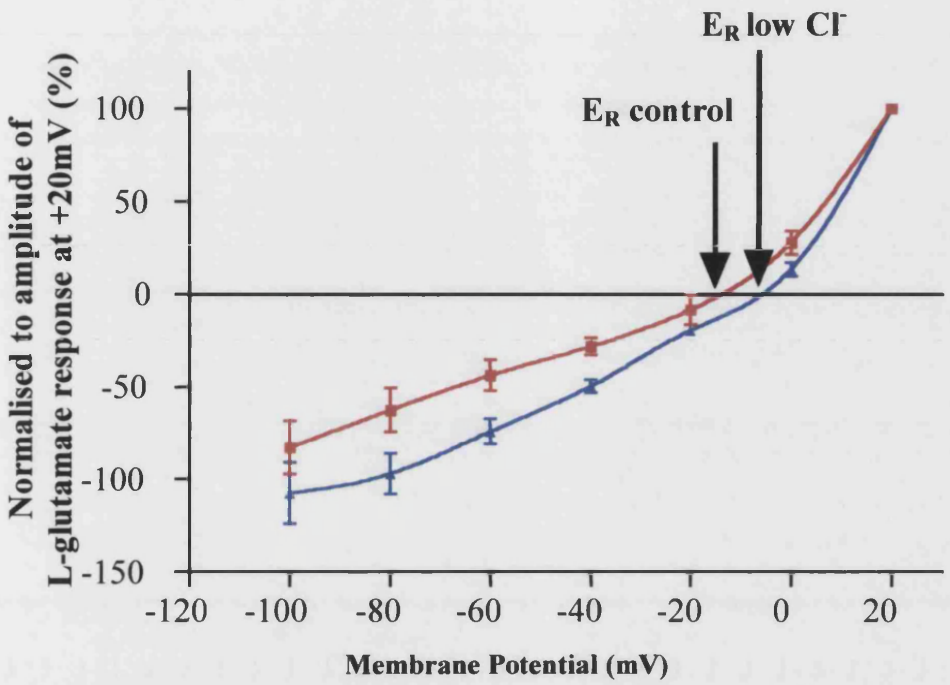


Figure 5.4 Current-voltage relationship of L-glutamate-sensitive GLC-3 channels.

The L-glutamate-induced currents mediated by GLC-3 homo-oligomers reversed at −16.6mV in normal oocyte saline (■) and 7.3mV in reduced Cl^- saline (▲) indicating the receptor was selective to chloride ions. The L-glutamate responses were normalised to the peak current amplitude at +20mV.

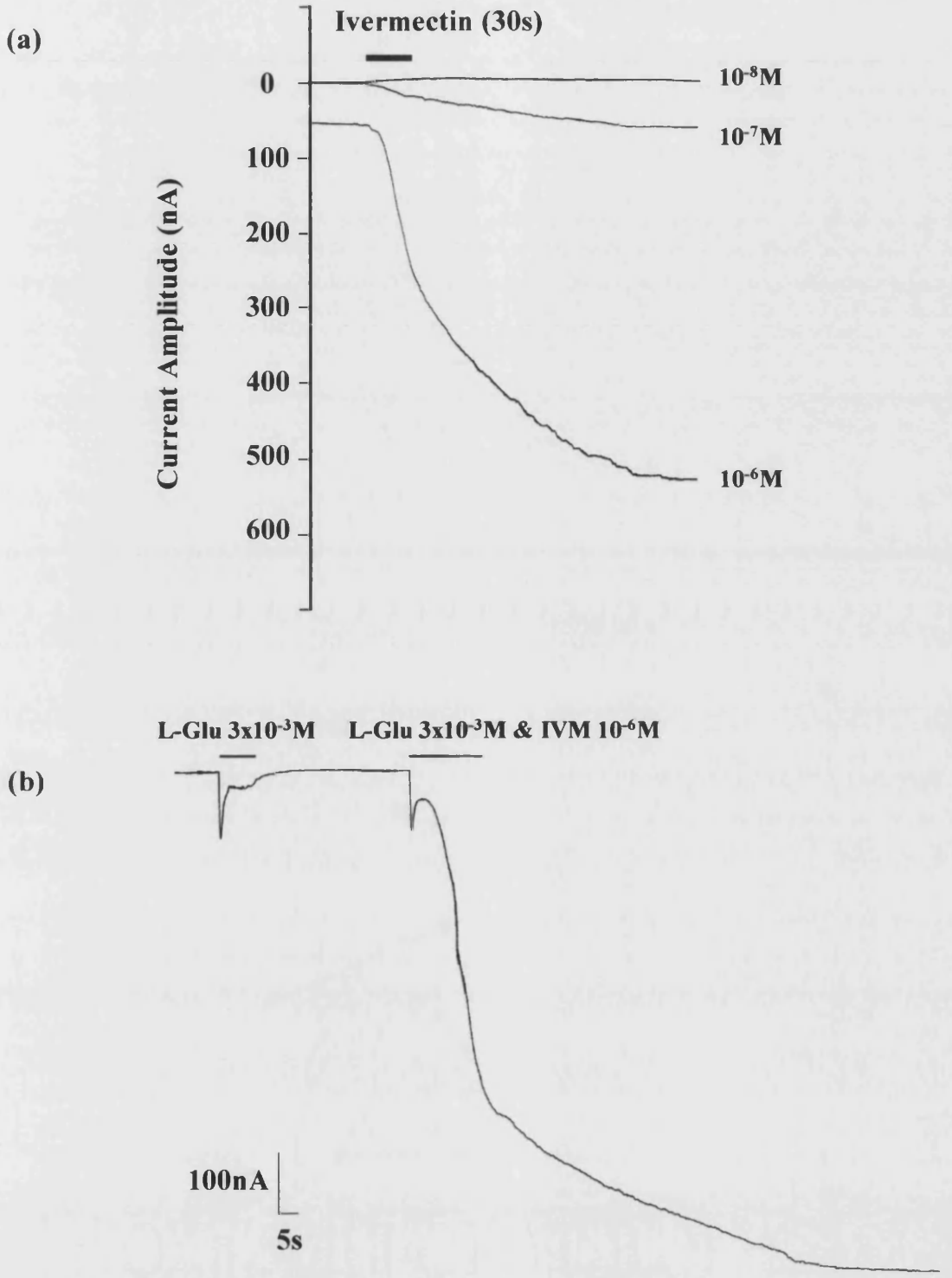


Figure 5.5 Effects of ivermectin on GLC-3 homo-oligomers.

(a) Ivermectin-induced, dose-dependent inward currents from one *Xenopus* oocyte expressing GLC-3 protein. Horizontal bars above the traces indicate a 30-second application of ivermectin and downward deflection denotes an inward current.

(b) Inwardly activating, biphasic current in response to the co-application of L-glutamate ($3x10^{-3}M$) and ivermectin ($10^{-6}M$)

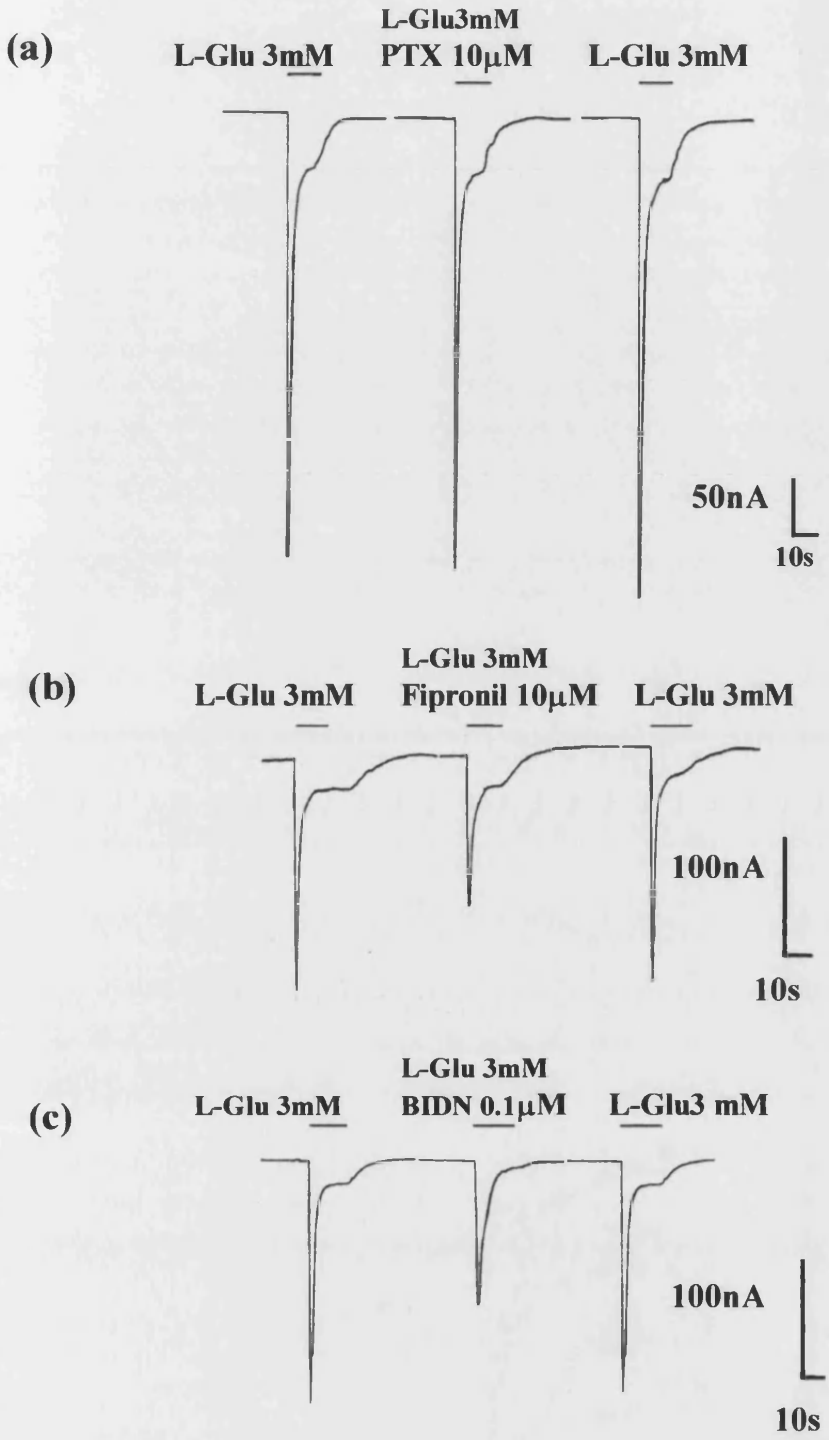


Figure 5.6 Effects of GABA-channel antagonists picrotoxinin, fipronil and BIDN on L-glutamate-elicited (3mM) responses recorded from GLC-3 homo-oligomers. Horizontal bar indicates duration of agonist and antagonist ligands.

(a) Picrotoxinin (10μM) failed to block 3mM L-glutamate-induced currents.

(b) Fipronil (10μM) reproducibly reduced the amplitude of the 3mM L-glutamate response, producing a total block at approximately 100μM.

(c) BIDN (100nM) inhibited 3mM L-glutamate currents at concentrations from 0.01-100 μM.

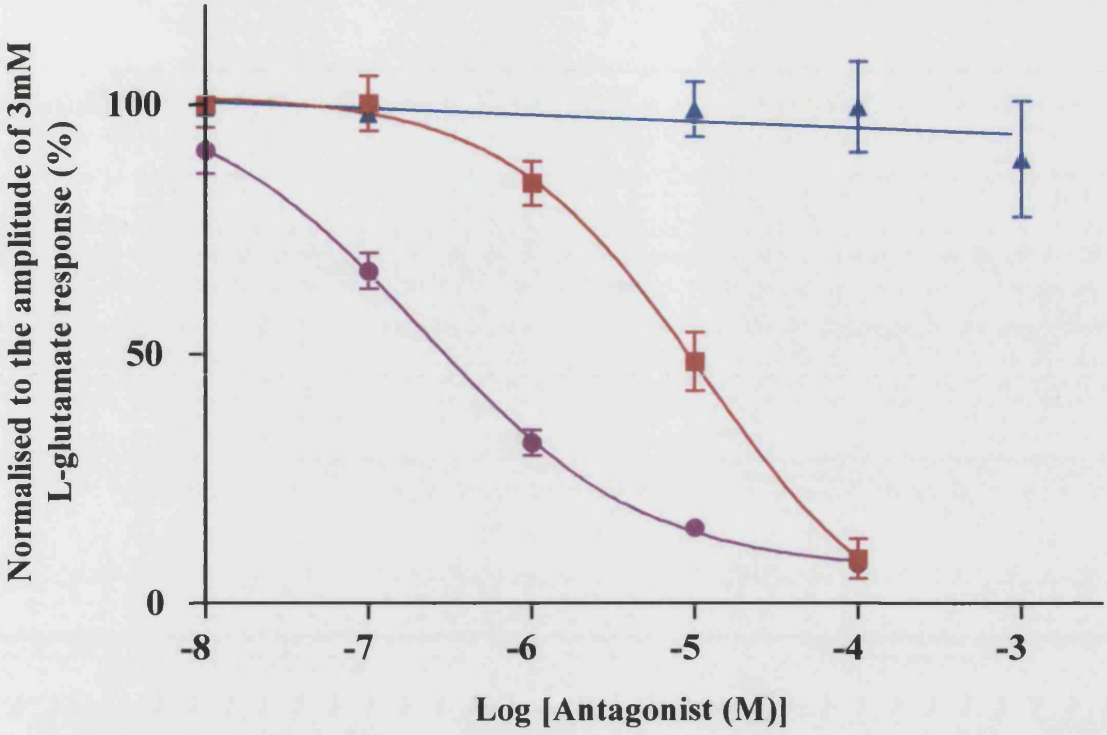


Figure 5.7 Dose-inhibition curves for the GABA-channel antagonists, picrotoxinin (▲), fipronil (■) and BIDN (●) expressed in *Xenopus* oocytes injected with GLC-3 cRNA.

5.3 Discussion

5.3.1 Agonist effects of L-glutamate and ibotenic acid

Inhibitory glutamate-gated chloride channels have been identified in crustaceans, molluscs, insects and nematodes, where only a small degree of pharmacological variation has occurred across the invertebrate phyla. The amino acid agonist L-glutamate and its structurally related analogue ibotenate, gate both native, locust leg muscle, (Cull-Candy, 1976; Duce and Scott, 1985); as well as *C. elegans* pharyngeal preparations, (Holden-Dye, 1998) and recombinant expressed GluCl receptor subunits (*C. elegans* GluClβ, GluCl α & GluClβ, (Cully *et al.*, 1994); DrosGluClα, (Cully *et al.*, 1996a)).

The high level of sequence identity between GLC-3 and other *C. elegans* and *Drosophila melanogaster* GluCl subunits suggested that the novel polypeptide might have a similar function. When expressed in *Xenopus* oocytes the agonist pharmacology of *C. elegans* GLC-3 homo-oligomers mimics a number of aspects of that of the native and *in-vitro* insect and nematode receptors. The EC_{50} for L-glutamate of 1.9 ± 0.03 mM at the GLC-3 receptor was very similar to the reported values of 1.36 ± 0.05 mM for the GluCl α + β receptor (Cully *et al.*, 1994) and 2.0 ± 0.3 mM for the GluClα2 receptor (Dent *et al.*, 1997). The estimated Hill coefficient for GLC-3 channels was greater than 1, indicating that at least 1 molecule of L-glutamate was necessary to gate the channel, in agreement with all other expressed *C. elegans* GluCls (Cully *et al.*, 1994; Dent *et al.*, 1997; Vassilatis *et al.*, 1997a). A striking feature of the currents activated by L-glutamate on GLC-3 homomers is the rapid desensitisation of current back to base-line levels, seen at high concentration of the agonist. Similarly, the *Drosophila melanogaster* (Cully *et al.*, 1996a) L-Glu-sensitive currents desensitised rapidly and completely in the continued presence of L-glutamate, however, these effects on other *C. elegans* GluCls (β, α_{2L} and α_{2S}) have not been so noticeable.

Ibotenic acid, a glutamate analog closely related to muscimol, activates all known *C. elegans in vitro* and *in vivo* GluCls (Cleland, 1996), with the exception of recombinant *C. elegans* GluClα. In most nematode native preparations, ibotenic acid (Holden-Dye, 1998; Martin, 1996) is known to maximally activate chloride currents with greater desensitisation than L-glutamate. However, the effect of ibotenate on homomeric GLC-3 channels was similar to that on *C. elegans* GluClα_2 and GluClβ homo-oligomers, activating the GLC-3 channels at 1mM, but producing weak, non-desensitising currents of a smaller amplitude and slower onset than glutamate.

5.3.2 GLC-3 channels are chloride selective

The permeability properties of the expressed channels (GLC-3) were selective for chloride ions, as indicated by the shift in reversal potential for L-glutamate when a percentage of the external chloride ions were substituted for monosodium gluconate. Reduction of the external NaCl concentration to 54.9mM and its partial replacement by 45.1mM monosodium gluconate shifted the reversal potential (E_{rev}) for L-glutamate-sensitive currents from −16.6mV to 7.3mV, which is in the appropriate direction predicted by the Nernst equation. The current-voltage relationship for the GLC-3 channels also appear to be consistent with other *C. elegans* heteromeric (α & β) and the homomeric channels, which exhibited outward rectification whether activated by L-glutamate or ivermectin (Cully *et al.*, 1994; Dent *et al.*, 1997).

5.3.3. GLC-3 homomers are ivermectin-sensitive

The avermectins are capable of acting as potent agonists on invertebrate receptors of many different species (Cully *et al.*, 1996a). The ability to irreversibly bind ivermectin, leading to channel opening, may be diagnostic for α subunits of the *C. elegans* GluCl, as the GluClβ subunit is not activated by these compounds (Cully *et al.*, 1994). The complex pharmacology avermectins have on chloride ion channels across the invertebrate phyla has led some authors to propose that avermectins act at multiple sites (Scott and Duce, 1985). When applied at varying concentration, avermectins have 3 general effects, potentiation, direct gating and blockade.

Firstly, avermectins directly gate ligand-gated chloride channels. In this study, ivermectin increases chloride permeability in GLC-3 homo-oligomers, as with other *C. elegans* α subunits (Table 5.1.) as GluClβ subunits are not activated by these compounds (Cully *et al.*, 1994). What appears to be characteristic among all expressed insect and nematode channels (GLC-3, GluClα_1, GluClα_2 and DrosGluClα) and perhaps some native insect channels, is that the agonistic effect of ivermectin often appears to gate the channel very slowly and yet typically irreversibly. The time for maximal activation of ivermectin-sensitive currents was 28±18 seconds for GLC-3 homo-oligomers, compared to 18±1 seconds for GluClα. The time course for GluClα and GluClβ heteromers and poly $(A)^+$ RNA was 42±2 and 36±3 seconds respectively, which is much slower than that observed for homomeric channels (Cully *et al.*, 1994). After the ivermectin-sensitive current was irreversibly activated, the glutamate response was markedly reduced in comparison to the control L-glutamate response seen in expressing GLC-3 oligomers.

	L-glutamate EC_{50}(mM)	Hill Coefficient L-glutamate	IVM EC_{50} (nM)	Hill Coefficient IVM	Max. Activation IVM-current (s)
Poly (A) RNA	—	—	104	1.5	36±3
GluClα	—	—	140±15	1.5±0.2	18±1
GluClβ	0.038±20	1.9±0.2	—	—	—
Gluα & β	1.360±50	1.7±0.1	190±7	2.5±0.2	42±2
GluClα$_2$	2.0±0.3	1.5	—	—	—
GILC-3	1.9±0.03	1.46±0.1	—	—	28±18
DrosGluClα	0.023	2.0	41	1.2	—

Table 5.1 EC_{50}, Hill coefficients and activation times for ligands acting on *C. elegans* and *Drosophila melanogaster* subunits expressed in *Xenopus* oocytes (Cully *et. al.*, 1992; 1994; 1996; Dent *et. al.*, 1997; 2000).

In support of the electrophysiological data, binding studies have identified high affinity avermectin binding sites from at least two insect species (*Drosophila melanogaster* and the locust *Schistocerca americana)* and two nematode species (*C. elegans* and *Haemonchus contortus*) (Table 5.2, Rohrer *et al.*, 1995). Interestingly, there appears to be vast differences in the species and tissue specific variation of avermectin binding sites. Locust metathoracic ganglia and *Drosophila* adult head tissue possess an immensely enriched source of avermectin binding sites (Bmax = 42pmol/mg) in comparison with *C. elegans* (Bmax = 0.46pmol/mg).

	Kd (nM)	Bmax (pmol/mg)
Drosophila melanogaster		
Adult heads	0.20	3.50
Adult bodies	0.34	0.22
Scistocerca americana		
Metathoracic ganglia	0.16	42.00
Heads	0.27	3.50
Leg muscle	0.15	0.18
Caenorhabditis elegans		
Mixed stages	0.24	0.40
Haemonchus contortus		
L3 larvae	0.13	0.43

Table 5.2 Binding assay data of [^{3}H] ivermectin (represented in: Cully & Paress, 1991; Rohrer *et al.*, 1992).

Interestingly, fipronil (a GABA-gated chloride channel blocker, (Cole *et al.*, 1993), did not compete for the [^{3}H]ivermectin binding sites in either locust *S. americana* metathoracic ganglia or *Drosophila melanogaster* head preparations (Moffatt, 1993). Similarly, glutamate also did not compete with [^{3}H]ivermectin binding to nematode membranes (Cully and Paress, 1991; Schaeffer and Hains, 1989). However, Bermudez (1991) reported that both picrotoxin and ivermectin could reverse some of the binding effects of [^{35}S] TBPS on locust GABA receptor membranes (Bermudez *et al.*, 1991). This indicates that the binding site of avermectins is physically dissimilar to fipronil and glutamate binding sites and the blocking action may act near the picrotoxin-binding site within the M2 region of the channel.

The second effect of avermectins is the ability to block ligand-gated chloride channels. At higher concentrations (micromolar) avermectins have been shown to inhibit GABA-activated chloride currents in locust neuronal tissue (Bermudez *et al.*, 1991), locust leg muscle (Duce and Scott, 1985), and GABA and IGluCl currents in *Acaris suum* preparations (Holden-Dye *et al.*, 1988; Martin and Pennington, 1989). In this study, ivermectin showed no indication at any concentration to block glutamate-induced currents, which appears to be consistent for all the *C. elegans* IGluCl receptors expressed in oocytes.

The third effect of avermectins was observed from electrophysiological studies on *Xenopus* oocytes injected with cRNA. It was observed that oocytes injected with *C. elegans* recombinant RNA α_2 and α_1 & β heteromers, low concentration of avermectins (5nM) was found to potentiate glutamate-sensitive currents approximately 4-5 times the control response. In addition, in oocytes injected with total RNA, submaximal concentrations of avermectin slowed the rate of desensitisation of the glutamate response and shifted the Hill coefficient from 1.7 to 1 (Arena *et al.*, 1992a). In contrast to this, the application of low concentration ivermectin (5nM) co-applied with submaximal concentration of glutamate, did not appear to substantially increase the glutamate-sensitive current in oocytes injected with *C. elegans* GLC-3 cRNA. Interestingly, the DrosGluClα subunit also failed to observe any significant potentiation of the glutamate-response (Cully *et al.*, 1996a) following pretreatment with ($IVMPO_4$) ivermectin phosphate (1nM). A logical explanation for why avermectins potentiate some GluCls and not others is not known. It has been predicted (Cleland, 1996) that avermectin site of action is at or near the picrotoxin-binding site, since both picrotoxin and ivermectin inhibit [^{35}S] TBPS (Bermudez *et al.*, 1991) binding to locust GABA receptors and picrotoxin can reverse some of the effects of ivermectin (Fritz *et al.*, 1979). Interestingly, two subunits DrosGluClα and GLC-3 that were not potentiated by ivermectin also appeared to be picrotoxin-resistant, providing additional evidence that ivermectin and picrotoxin may act at a similar site of action.

It might be that GLC-3 is only a minor component of the ivermectin receptors found in *C. elegans*, since Vassilatis *et al.* (1997b) concluded that the majority of [^{3}H]-ivermectin binding sites present in membrane preparations could be accounted for by the GluClα_1 and GluClα2 subunits. There is, however, a great deal that is unknown about the effects of ivermectin and its related compounds and it seems likely that further study of GLC-3 may be instructive.

5.3.4 The GLC-3 homomers are picrotoxinin-insensitive

Picrotoxin is a potent convulsant agonist, produced by plants of the *Menispermaceae* family (Klunk *et al.*, 1982) and is composed of equimolar amounts of picrotin and picrotoxinin (Etter *et al.*, 1999). Only picrotoxinin is thought to be active as an antagonist (Curtis and Johnston, 1974) at the $GABA_A$ receptor (Lynch *et al.*, 1995), where it has been described to be occasionally 50 times more potent than picrotin (Jarboe *et al.*, 1968). However, in glycine receptors (Lynch *et al.*, 1995) the two compounds have been found to be equipotent. In contrast, we have found both picrotoxinin and picrotin (data not shown) to be equally insensitive at antagonising *C. elegans* GLC-3 homo-oligomers at concentrations (1mM) sufficient to block other *C. elegans* GluCls (Cully *et al.*, 1994). The only other picrotoxinin-resistant GluCls observed to date are in Leech Retzius cells (Mat-Jais *et al.*, 1983) and *Drosophila melanogaster*, DrosGluClα, (Cully *et al.*, 1996a). Picrotin showed minor antagonist activity against the homomeric *C. elegans* GluClβ and GluClα channels, but picrotoxinin appears to be the active antagonist (Cully *et al.*, 1994).

The mechanism of action of picrotoxinin is thought to involve complex channel blocking effects, which may be related to desensitisation and interaction with the M2 channel pore segment. In *Drosophila* RDL subunits a naturally occurring mutation in the residue of the second membrane spanning region (M2) ($serine_{302}$ for alanine, Fig. 5.8) confers picrotoxinin, dieldrin and fipronil resistance (ffrench-Constant *et al.*, 1993a; Pribilla *et al.*, 1992; Zhang *et al.*, 1994). Residue 302 of *Drosophila* Rdl corresponds to the same residue that is altered in rat glycineρ_1, glycineα, (Lynch *et al.*, 1995; Pribilla *et al.*, 1992), GluClβ in *C. elegans* (position 279T) (Etter *et al.*, 1999) and rat $GABA_A\alpha$, β_2, γ_1 or γ_2 (Gurley *et al.*, 1995; Wang *et al.*, 1995). Figure 5.8 shows an alignment of the transmembrane II domains, from the ligand-gated anion channels, which indicate both naturally occurring and synthetic mutations of picrotoxin-insensitivity. These mutations are predicted to lie deep within the receptor pore (Olsen and Tobin, 1990; Smith and Olsen, 1995; Xu *et al.*, 1995). However, the mechanism by which their mutations cause picrotoxin-insensitivity is not completely understood. Most authors are in agreement that the M2 mutation causes a block to the flow of chloride current through the channel pore (Etter *et al.*, 1999; Gurley *et al.*, 1995; Inoue and Akaike, 1988). In addition, this proposal is consistent with the observation from Gurley *et al.*, (1995); Etter *et al.*, (1999); Newland and Cull-Candy, (1992); that blocking of the current by picrotoxin is facilitated by

ligand binding. However, the mutation could be inducing this picrotoxin-sensitive block in one of many ways.

1. It is possible that picrotoxin could bind within the channel pore and physically obstruct the flow of ions (Zhang *et al.*, 1994). This block of chloride ions could involve the drug physically blocking the channel pore or by inducing a conformational change that physically blocks conduction, alternatively picrotoxin could bind within the channel pore and prevent a conformational change from occurring, thus blocking conduction (Gurley *et al.*, 1995; Zhang *et al.*, 1994).
2. Second, some authors propose that changing an amino acid away from the picrotoxin binding-site, but within the channel (ffrench-Constant *et al.*, 1993a; Newland and Cull-Candy, 1992; Revah *et al.*, 1991; Zhang *et al.*, 1994) allows picrotoxin to act by stabilising the desensitised state of the $GABA_A$ receptor (i.e. keeping the channel in a closed state) from a site located within the pore of the channel. Interestingly, in support of this proposal Zhang *et al.*, (1994) found that desensitisation appeared to be dramatically slower and often incomplete in the Rdl mutant.
3. A third possibility is that the channel pore mutation could disrupt the picrotoxin-binding site or prevent access of picrotoxin to its site of action (Gurley *et al.*, 1995). Switching amino acids near the picrotoxin binding site may destabilise the conformation of the protein that binds the drug more tightly and therefore causing an alteration to the antagonist binding site (Zhang *et al.*, 1994). However, despite a plethora of evidence from many different sources we cannot determine if picrotoxin blocks chloride-gated channels by physical occlusion of the pore or by some other means.

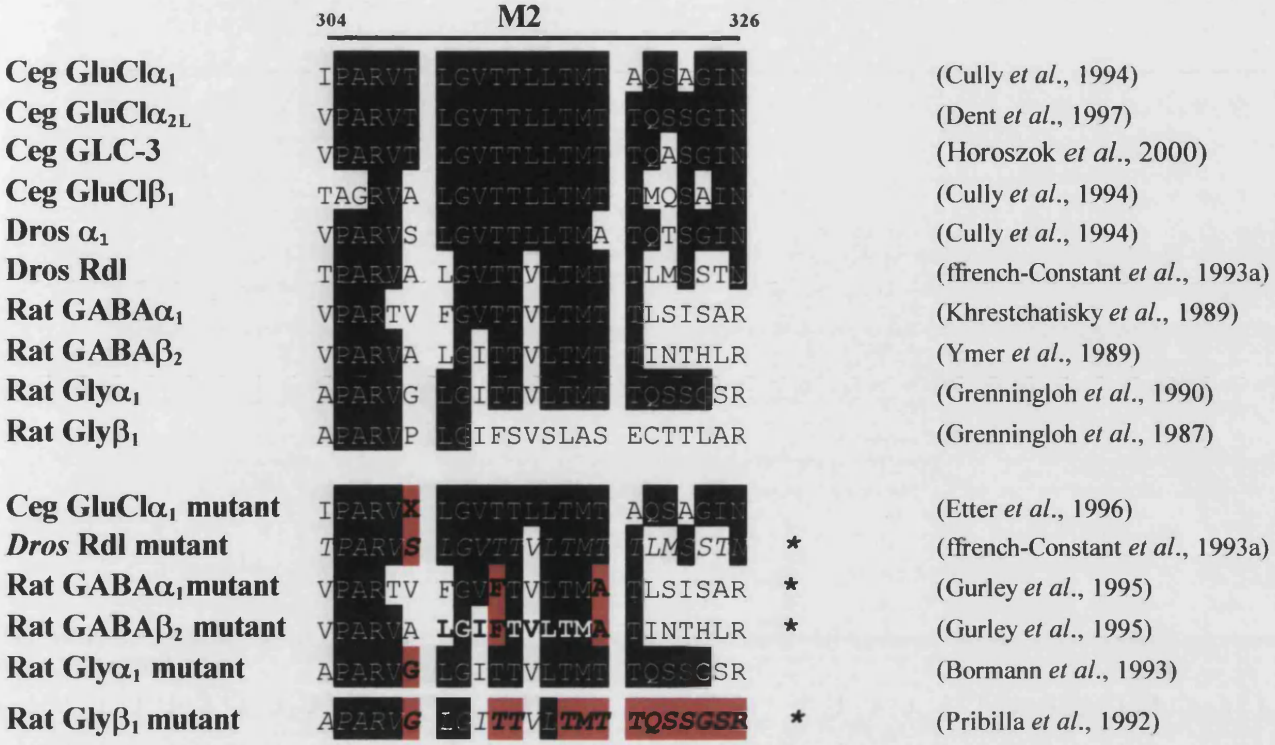


Figure 5.8 Alignment of various *C. elegans* and *Drosophila Melanogaster* L-glutamate receptor subunits with Rat $GABA_A$ and glycine α sequences indicating the high degree of homology in the M2 region. Consensus sequence is boxed in black and channel mutations referred to in the text are highlighted in red. An asterisk denotes subunits conferring picrotoxin-insensitivity. Red shaded boxes of the sequence rat glycineβ_1 are residues mutated to match the corresponding sequence of rat glycineα_1. The naturally occurring amino acid threonine was substituted for Glu, Ala, Ser, Cys, Pro and Val producing mutations of GluClα_1 M2 region (indicated by X).

5.3.5 Convulsant antagonist effects on GLC-3 homo-oligomers

Experiments with non-competitive antagonists of ionotropic chloride channels revealed some novel features of the homomeric GLC-3 receptor. The GLC-3 channels are inhibited by BIDN and fipronil with IC_{50}s of 0.22 and 11μM respectively; this is the first report that any GluCl is sensitive to the actions of BIDN or a phenylpyrazole. The insecticidal activity of these compounds is thought to target the convulsant binding site of both bicuculline-sensitive and bicuculline-insensitive GABA-gated chloride channels (Deng *et al.*, 1993; Hosie *et al.*, 1995b; Rauh *et al.*, 1997b). This indicates that BIDN and fipronil may therefore share a common mechanism of action, but act at quite different sites on the receptor channel complex. Naturally occurring mutations in residues of the second membrane-spanning region (M2) of RDL receptor subunits ($serine_{302}$ for alanine) confers both picrotoxinin, BIDN, dieldrin and fipronil resistance (ffrench-Constant *et al.*, 1993a; Gurley *et al.*, 1995; Hosie *et*

al., 1995a; Pribilla *et al.*, 1992; Wang *et al.*, 1995; Zhang *et al.*, 1995; Zhang *et al.*, 1994). However, the sequences of the M2 regions of the *C. elegans* GluCl subunits (Fig. 5.8) are almost identical to each other and do not suggest any reason for the observed differences in fipronil and BIDN sensitivity. It is possible that residues outside the ion channel affect the antagonist sensitivity and specificity of the GluCl. Lynch *et al.*, (1995) showed that mutations at the extracellular end of the M2 of the mammalian glycine receptor α subunit, notably alterations in lysine-274, transformed picrotoxin from an allosteric competitive antagonist to an allosteric potentiator at low (0.01 – 3 μM) concentrations and to a non-competitive antagonist at higher (>3μM) concentrations.

Again, the sequence of this region of GLC-3 does not reveal any unusual features; in particular, the basic residue (K274) is conserved. In insect membranes [^{3}H]-BIDN was displaced competitively by dieldrin but not by fipronil, picrotoxin, EBOB, TBPS or 1-phenyl-4-t-butyl-2,6,7-trioxabicyclo[2.2.2]octane (TBOB) (Rauh *et al.*, 1997b; Sattelle *et al.*, 1995), indicating that BIDN probes a different site from the convulsant ligands (as represented by Fig. 5.9). In cockroach motor neuron preparations, (Sattelle *et al.*, 1988) BIDN failed to inhibit nAChR or $GABA_B$-like receptors indicating a preference for vertebrate and insect $GABA_A$ and *C. elegans* L-glutamate channel.

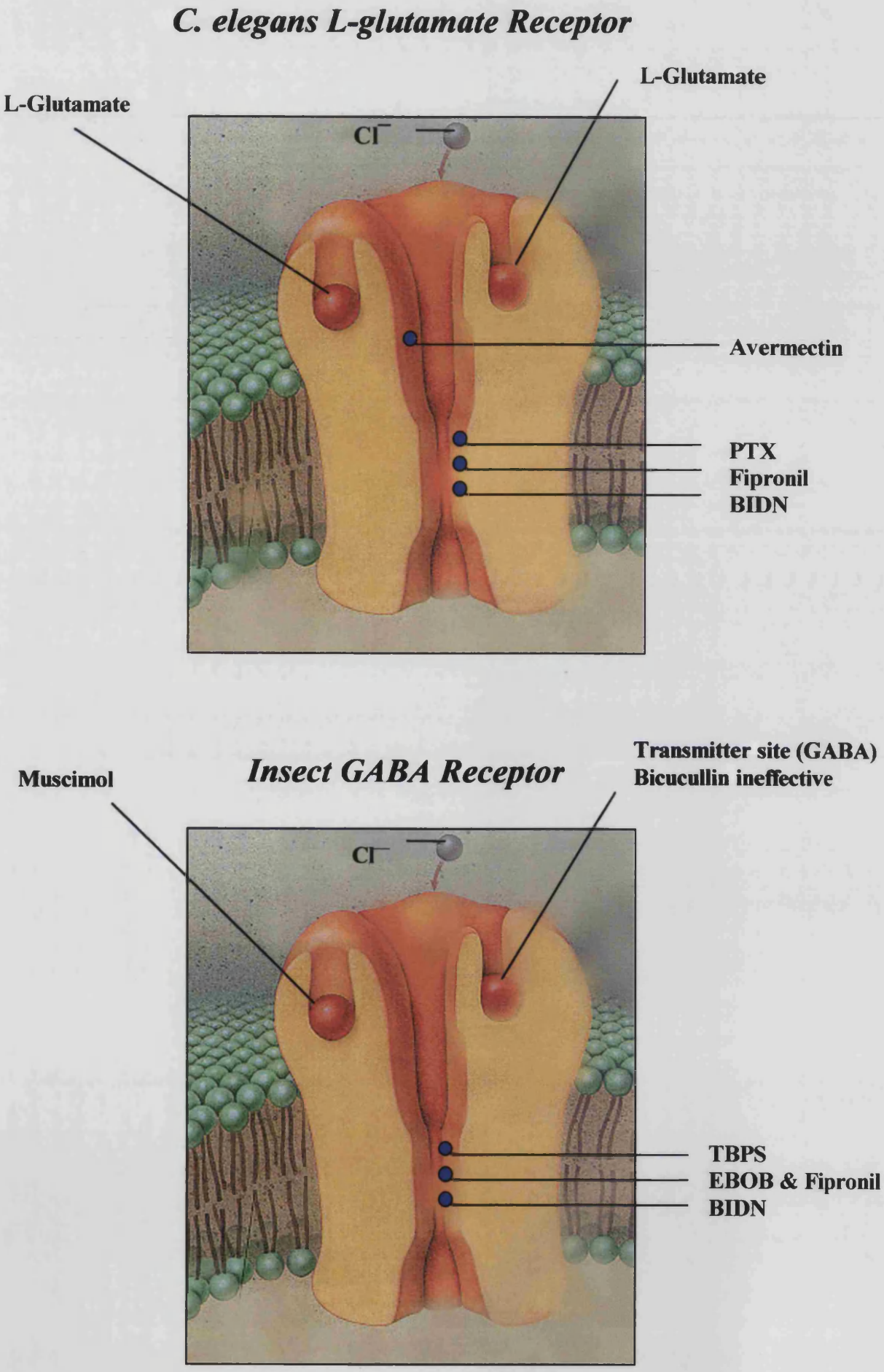


Figure 5.9 Proposed models of *C. elegans* L-glutamate (Horoszok *et. al.*, 200) and insect GABA receptors (Cole *et al.*, 1993; Hosie *et al.*, 1995a).

At very low concentrations fipronil has been very effective at controlling insects (Colliot, 1992), but at the same time displaying good selectivity between invertebrates and mammals (Cole *et al.*, 1993; Tomlin, 1994)). Fipronil selective toxicity is also indicated in radioligand binding assays, where the insecticide binds with a higher affinity to insect GABA receptors than vertebrate components. In rat brain membrane binding assays fipronil had no significant effects on [^{3}H] muscimol (Abalis & Elderfrawi, 1986), although it did displace [^{35}S] TBPS, [^{3}H]BIDN and [^{3}H]EBOB with a low affinity (Cole *et al.*, 1993). However, using membrane preparations from housefly head, fipronil failed to compete for [^{35}S] TBPS (Olsen *et al.*, 1989), [^{3}H] BIDN and [^{3}H] muscimol (Lunt *et al.*, 1985). Clearly, however, fipronil did bind with a high affinity to the EBOB binding site (Gant *et al.*, 1998), indicating that in insects, fipronil acts only at the EBOB binding site, which may be close to, but distinct, from the other two cyclodiene binding sites (TBPS and BIDN). Whereas in the vertebrate GABA-chloride channel, fipronil displaces all three cyclodienes (TBPS, EBOB and BIDN) at low affinities, suggesting they act at the same site. Clearly, however, the potencies of all 4 compounds (picrotoxin, BIDN, fipronil and dieldrin) are affected by the same mutation (A302S) in the RDL channel, suggesting that they either recognise a common feature of GABA receptor, or share a common mechanism of action (Hosie *et al.*, 1995a).

The interesting pharmacology observed for GLC-3 indicates that this and other *C. elegans* GluCls may prove to be a valuable tool for studying the detailed interaction of non-competitive antagonists with ligand-gated chloride channels. In conclusion, we have identified two novel convulsant compounds acting at the L-glutamate receptor that show a similar potency for the L-glutamate-gated channels as the GABA-gated convulsant site. In particular, it will be interesting to determine whether or not other native and other recombinant GluCls are sensitive to fipronil, BIDN and related compounds. Detailed investigation of this latest member of the GluCl gene family using the advanced molecular genetic techniques available for *C. elegans* will also enhance our overall understanding of the nematode nervous system.

Chapter 6

Conclusions and future perspectives

6.1. Conclusions

Anthelmintics are an integral part of the agricultural industry, and the need to develop more efficient and selective compounds is ongoing. Most of the current anthelmintics have been found to target their actions on neurotransmitters involved in neuronal signalling of ion channels within the nervous system. In order to study the actions of these compounds, the identification of the neurotransmitters involved in neuronal signalling and the mechanism of interaction with the receptor subunit needs to be examined at the molecular level. The work described in this thesis has tried to address some of these fundamental issues.

The glutamate-gated chloride receptor has become established as the probable target for avermectin compounds, although the exact mode of action and the stoichiometry of the native GluCl receptors remain unknown. However, the successful completion of the *C. elegans* genome project allowed us to predict the total number of L-glutamate receptor subunits present within this organism. Using the polymerase chain reaction allowed direct amplification of a 1.4kb *C. elegans* GLC-3 receptor subunit cDNA, which displayed high amino acid identity (73-77%) with other GluClα subunits from nematodes, confirming that GLC-3 is also a member of the same amino-acid superfamily of receptor proteins.

Following the successful cloning of GLC-3, one of the principal aims of this project was to ascertain if GLC-3 homo-oligomers exhibited the same characteristic pharmacology as other GluCl receptor subunits. The GLC-3 homo-oligomers did resemble the pharmacological profile of other *in vitro* expressed GluClα subunits, and was distinctly dissimilar from the invertebrate $GABA_A$ and glycine receptors. Clearly, oocytes expressing GLC-3 homo-oligomers displayed robust, dose-dependent currents that were initiated by L-glutamate and smaller, non-desensitising currents from the L-glutamate analogue ibotenic acid, in addition to insensitivity to GABA, histamine, NMDA and glycine ligands. However, the *in vitro* pharmacology of the GluCls is quite distinct from that of the native channel. In general, L-glutamate, ibotenate and ivermectin activate both sets of receptors, but the apparent sensitivity of the recombinant homomers is far less than observed in native tissues. This suggests that native nematode receptors form heteromers of the GluCl receptors and it is plausible that unidentified subunit combinations exist.

The GLC-3 receptor subunits are striking in their similarity to other GluClα homomers, especially in their sensitivity to L-glutamate and ivermectin compounds, however, in earlier studies the only antagonist observed to inhibit either the ivermectin or the glutamate-gated currents have been picrotoxin and flufenamic acid ($GABA_B$ coupled antagonist). Of particular interest, was the finding

that two $GABA_A$ channel antagonists blocked, dose-dependently, L-glutamate-induced chloride currents from GLC-3 homo-oligomers, namely BIDN and the insecticide fipronil. The activity of these channel blockers has not been explored on native or other recombinant L-glutamate receptors, representing the first electrophysiological data on these antagonists.

All other known *C. elegans* GluCl receptor subunits expressed in oocytes (with the exception of GluClα) have either their L-glutamate or ivermectin currents blocked by picrotoxinin. In contrast, we have found picrotoxinin to be ineffective at antagonising L-glutamate-gated currents in GLC-3 homo-oligomers. An equivalent point mutation within the M2 domain at both GluClβ from *C. elegans* and RDL (A301S) homo-oligomers from *Drosophila* have been shown to reduce picrotoxinin potency. However, the M2 sequence of the *C. elegans* GLC-3 subunit is almost identical to other GluClα subunits and do not suggest any reason for observed differences in picrotoxinin sensitivity. The GLC-3 homo-oligomer may prove to be a good subject for site-directed mutagenesis studies of the M2 domain.

In this study immunocytochemical methods were used to characterise a member of the glutamate-gated chloride channels in nematodes. Polyclonal antibodies raised against GBR-2 GluCl receptor subunits to localised specific cells in the free-living species *C. elegans*. The *avr-14* gene from *C. elegans* and *Haemonchus contortus* is alternatively spliced and encodes for two subunits, GBR-2A and GBR-2B. Despite the sequence conservation between *Haemonchus* and *C. elegans* GBR-2A and -2B subunits (88% and 83% respectively) the distribution of localisation was not completely conserved, clearly however, such high identity suggests that *Haemonchus* GBR-2 is the parasitic orthologue of *C. elegans* GBR-2 receptor subunits. The GBR-2 receptor subunits formed specific expression patterns (Fig. 3.5) in extrapharyngeal tissue in *C. elegans* and *Haemonchus contortus*, and subsequently motor nerve cords in *Ascaris suum* and *Haemonchus contortus* preparations. Subunits were also observed on 3 neuronal cell bodies observed in the corpus, metacorpus and terminal bulb regions of the *C. elegans* pharynx. From this pattern of immunoreactive neurons it is reasonable to assume that the GBR-2 subunits exist on the M3 motor neurons that innervate pm4 and pm5 muscle cells. Previous studies indicate IPSPs generated by the M3 motor neuron have been of an inhibitory glutamatergic transmission (Dent *et al.*, 1997). I can, therefore, hypothesise that GBR-2 channels operate as pre-synaptic autoreceptors on the M3 neuron and are maybe indirectly involved in regulating glutamate release. In correlation to *C. elegans* GluClβ and α_2 receptor subunits, it is plausible that GBR-2 are also involved in pharyngeal pumping, but do not co-assemble with GluClα_2 and β to form a native receptor. Previous studies have indicated the pharyngeal muscle and surrounding neurons to be the major site of action of ivermectin in free-living and parasitic species (Avery *et al.*, 1997; Li *et al.*, 1997; Raizen *et al.*, 1995).

Using cloning, immunocytochemistry and electrophysiology techniques, this thesis attempts to provide a brief synopsis of our current understanding of the ivermectin-gated chloride channels and allows us to examine subunit configuration and anatomical localisation of the receptor as well as pharmacological characterisation. This study will also facilitate cloning of homologous ion channels from insects and parasitic nematodes allowing their genes to be used to establish a screening system for novel anthelmintic and insecticidal compounds and to identify addition drug binding distinct from the ivermectin binding-site.

6.2 Future work

To distinguish clearly between the expression of GBR-2A and -2B polypeptides, subunit-specific antisera should be raised to unique intracellular loop regions. These results should indicate which subunits are present in the extrapharyngeal region and if the co-expression of GBR2-A and -2B exists. Future studies will also involve the immunolocalisation of GLC-3 receptor subunits in *C. elegans*, using polyclonal antibodies raised against the N-terminal subunit domain and reporter gene studies. This may provide some clues as to which subunits (if any) co-assemble in the native receptors.

In this study pharmacological characterisation of GLC-3 homo-oligomers to ivermectin and GABA convulsant antagonists was shown. However, this study involves just one α-subunit, it will be constructive to see if subsequent co-expression studies of GLC-3 with other subunits such as GluClα_2 and α_1 may lead to the assembly of more functional hetero-oligomeric receptors which may mimic the pharmacology of the native receptors. Although, as yet, there is no evidence that GLC-3, α_2 and α_1 in any combination are hetero-oligomerically expressed *in vivo*. Clearly, a much more thorough *in vivo* and *ex-vivo* pharmacological investigation should be undertaken to identify the development of a new generation of anthelmintics, such as BIDN and fipronil which have been found to exert suitable toxicity properties on GLC-3 receptor subunits in recombinant expression studies.

With the aid of known sequence and subunit components and comprehensive physical maps of the *C. elegans* genome (Coulson *et al.*, 1986), these studies will offer an important tool in determining anthelmintic-receptor interactions. Site-directed mutagenesis experiments of this functional homomer (GLC-3) will hopefully prove valuable in identifying key residues responsible for agonist and antagonist binding sites and improve on selectivity at the target site.

Appendices 7

Appendix 1

7.1 Escherichia coli genetic markers

MARKER	DESCRIPTION
Amy	Amylase
Ara	Mutation destroys ability to use arabinose
Cam^r	Chloramphenicol resistance
EndA1	DNA specific endonuclease 1, mutation improves quality and quantity of miniprep
F	Contains the F plasmid
GyrA46	DNA gyrase subunit A
HsdR($r_k^- m_k^+$)	Ablates type 1 restriction but not methylation of *E.coli* strain K
Lac	Unable to utilise lactose
Laclq	Overproduces the *lac* repressor protein
LacZ	β-Galactosidase
LacZM15	Specific N-terminal deletion permitting α-complementation
ProAB	Requires proline for growth
RecA1	Recombination deficient
RelA1	Permits RNA synthesis in absence of protein synthesis
RpsL	30S ribosomal subunit S12: mutation confers streptomycin resistance
SupE44	Suppressor of amber (UAG) mutations
Thi	Requires thiamin (vitamin B1) for growth
Tn10(*tetR*)	Contains the TN10 transposon, conferring tetracycline resistance

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