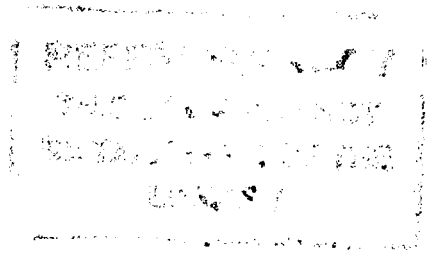
Characterization of Metabotropic Glutamate Receptors in *Caenorhabditis elegans*



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ABSTRACT

Glutamate is the principal neurotransmitter in mammals and its signaling is conserved between *Caenorhabditis elegans* and mammals. An important role of glutamate transmission in *C.elegans* has been established by genetic investigations of ionotropic glutamate receptors (*glr-1, nmr-1*), glutamate-gated chloride channels (*avr-15*) and vesicular glutamate transporters (*eat-4*). Metabotropic glutamate receptors are hypothesized to modulate the transmission properties of synapses in response to the magnitude and frequency of glutamate signaling. Three mGluR-like genes *(mgl-1, mgl-2* and *mlg-3)* have been identified in *C. elegans* accordingly. The aim of this MPhil/PhD project is to investigate the roles of metabotropic glutamate receptors (mGluRs) in regulating behaviour, using the nematode *C.elegans* as a model organism.

We obtained, backcrossed and characterized *mgl* mutants. Accordingly we assume that *mgl-1(1811), mgl-2(tm355)* and *mgl-3(tm1766)* are functional null mutants. We also made double mutants and triple mutants by outcrossing. Behavioural assays were carried out on the mutants, including longevity, growth rates, body bends, pharyngeal pumping, thrashing, forward and backward movement, locomotion towards bacteria, Osmotic Avoidance. The analysis on the mutant strains suggests that *mgl-1(tm1811)* mutants have a selectively disrupted switch between forward and backward locomotion. Backward movement is regulated during foraging. When food source is detected by worms, backward movement dramatically decreases. Locomotion towards bacteria is disrupted in *mgl-2(tm355)* mutants, they could not get to the food source as fast as the wild type. *mgl-3 (tm1766)* behaves normally in all the tested assays. Existing and self generated reporter strains have allowed us to study *mgl* expression patterns. An analysis of MGL-3::GFP suggests *mgl-3* is expressed in NSM and some other neurons, which would support further behavioral analysis.

This behavioural analysis of *mgl* mutants and expression pattern study of MGL provides an insight of neuromodulatory roles for these G-protein coupled receptors and suggest they play a broad role in defined circuits that integrate complex behaviour.

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List of Abbreviations

Amp	Ampicilin
AMPA	α-amino-3-hydrozy-5-methyl-4-isox azolepropionic acid
BHK	Baby hamster kidney
cAMP	Cyclic adenosine monophosphate
CASR	Extracellular calcium-sensing receptor precursor
C.elegans	*Caenorhabditis elegans*
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
DiI	1,1'-Dioctadecyl 3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (fluorescent carbocyanide dye)
DNA	Deoxyribose nucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EAAC	Excitatory amino acid carrier
EAAT	Excitatory amino acid transporter
EtBr	Ethidium Bromide
EtOH	Ethanol
GABA	γ-amino-butyric acid
GAP	GTPase-accelerating protein
GEF	Guanine nucleotide exchange factors
GFP	Green Fluorescent Protein
GLAST	Glial glutamate and aspartate transporter
GLT	Glial glutamate transporter
GluCl	Glutamate gated chloride channel
GPCR	G-protein coupled receptor
GTP	Guanine nucleotide triphosphate
GTPase	Guanine nucleotide triphosphatase
iGluR	Ionotropic glutamate receptor
IPTG	Isopropyl-ß-D-thiogalactopyranoside
Kainate	2-carbozy-3-carboxymethyl-4-iso propenylpyrrolidine
LB	Luria Broth
MGL	*mgl* encoded protein
mGluR	Metabotropic glutamate receptor
mRNA	messenger ribose nucleic acid
NGM	Nematode growth media

NMDA	N-methyl-D-Aspartate
NSM	Neurosecretory material
PCR	Polymerase Chain Reaction
PLC	Phospholipase C
PTX	Pertussis toxin
RGS	Regulator of G-protein signaling
RNA	Ribose nucleic acid
TAE	Tris-acetate EDTA
TE	Tris-EDTA
TM	Transmembrane
⚥	Hermaphrodite
♂	Male

Amino Acids

A	Alanine	Ala
C	Cystine	Cys
D	Aspartate	Asp
E	Glutamate	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamatine	Gln
R	Argnine	Arg
S	Serine	Ser
T	Threonine	Thr
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

Nucleotides

a	Adenosine
c	Cytosine
g	Guanine
t	Thymine
u	Uracil

CHAPTER 1
INTRODUCTION

1 INTRODUCTION

1.1 Neuronal transmission

1.1.1 History

Previously it was thought that body worked according to mechanical or hydraulic principles. In 1791 Luigi Galvani showed nerves of frog legs conducted electricity, which demonstrated that muscles can be caused to twitch when nerves are stimulated electrically (Piccolino, 1997; Gazzaniga et al., 2002). In 1875 Camillo Golgi stained individual nerve cells. Using Golgi's method, Santiago Ramon y Cajal went on to find that the neurons were discrete entities (Gazzaniga et al., 2002).

1.1.2 The Neuron

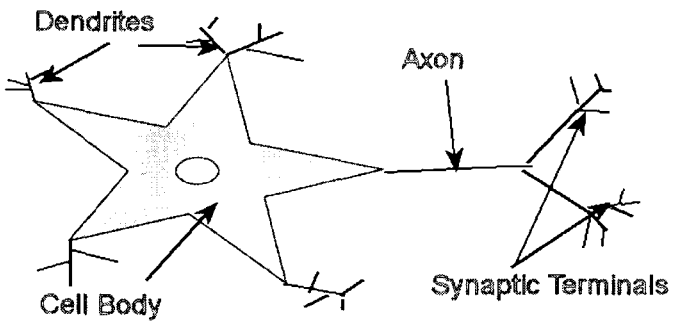


Figure 1.1 A diagram of a neuron. A neuron consists of dendrites, a cell body, axon and synaptic terminals. This structure reflects its functional subdivision into receiving (dendrites), integrating (cell body) and transmitting (axon) compartments (Gazzaniga et al., 2002).

Neurons are highly specialized cells that transmit impulses within animals. The cell body of a neuron, called the soma, contains the cell nucleus and the majority of the cytoplasmic inclusions and organelles. Radial extensions of the soma cell membrane, called dendrites, extend to other neurons and form the interface where impulses are transmitted from neuron to neuron. One long extension of the soma, called the axon, is the primary conduit through which the neuron transmits

impulses to neurons downstream in the signal chain. Axons branch into smaller extensions at their terminal end and eventually create presynapses with the target cell (e.g. neuron, muscle cell) (Holger; 1967).

1.1.3 Neuronal Transmission

In response to a chemical or mechanical stimulus, neurons generate an electrical impulse, conduct the impulse through its axon, and subsequently release a chemical signal, called a neurotransmitter. The neurotransmitters diffuse short and long distance through the extracellular and cerebrospinal fluid and reach receptors on neuronal cells (volume transmission; Benfenati and Agnati, 1991). Alternatively, neurotransmitters cross junctional spots named synapses, where the axons contact the dendrites of other neurons, to the membrane of the next neurone (synaptic transmission; Bradley, 1968). At synapses, neurotransmitters interact with specific receptors in the membrane. This depolarizes the membrane to generate a new action potential which continues the signal to their final targets.

1.1.3.1 Synaptic Transmission

Two types of synaptic transmission occur in the nervous system, electrical transmission mediated by electrical synapses and chemical transmission mediated by chemical synapses. In electrical transmission, synapses allow ions and small molecules to permeate between neighboring neurons (Eccles, 1982). Electrical synapses are formed at a narrow gap between the pre- and postsynaptic cells known as a gap junction. The majority of synaptic transmission is chemical transmission (Longstaff, 2000). The arrival of an action potential at the axon terminal results in the release of neurotransmitter from presynaptic vesicles. This process requires a rise in intracellular Ca^{2+} brought

by calcium entry into the axon terminal via voltage-dependent calcium channels. Then the neurotransmitter diffuses across the synaptic cleft and binds to specific receptors on the postsynaptic membrane. Subsequent binding of the neurotransmitter causes a change in the conformation of the receptor to transmit the signal (Longstaff, 2000).

1.2 Neurotransmitters

Neurotransmitters are the chemicals which account for the transmission of signals from one neuron to the next across synapses. Most neurotransmitters are synthesized in the cytosol of nerve terminals and are then stored into synaptic vesicles prior to exocytotic release. The classical neurotransmitters are Acetylcholine (ACh), Noradrenaline, Dopamine, γ -aminobutyrate (GABA), Glycine, Serotonin, Glutamate (Longstaff, 2000).

Neurotransmitters	Functions
Acetylcholine	Acetylcholine is critical in motor behavior, memory functioning, and cognitive performance (Burn, 1977; Biagioni *et la.*, 2000). There is a link between acetylcholine and Alzheimer's disease (AD): As AD progresses, the brain produces less and less acetylcholine (Richter *et al.*, 1980).
Noradrenaline	The central functions of norepinephrine are: regulation of alertness and of the wakefulness-sleep cycle, maintenance of attention, memory and learning, cerebral plasticity and neuro-protection (Januszewicz and Wocial, 1965; Randt *et al.*, 1971).
Dopamine	Dopamine is central to the reward system. Dopamine neurons are activated when an unexpected reward is presented. In nature, we learn to repeat behaviors that lead to unexpected rewards. Dopamine is therefore believed by many to provide a teaching signal to parts of the brain responsible for acquiring new motor sequences, i.e., behaviors (Meltzer and Stahl, 1976; Barbeau, 1968).
GABA	GABA inhibits the excitatory signals that result in feelings of anxiety and fear. GABA thus has a calming, tranquilizing effect on our emotions and prevents us from becoming overwhelmed in stressful situations. GABA is important in maintaining a normal level of firing for all kinds of different systems (Enna, 1984; Meldrum, 1975).
Serotonin	The functions of serotonin involve control of appetite, sleep, memory and learning, temperature regulation, mood, behavior, cardiovascular function, muscle contraction, endocrine regulation, and depression (Beckmann and Kasper, 1983; Leibowitz and Shor-Posner, 1986).
Glutamate	Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system and is critical for essentially all physiological processes ranging from control of motor and somatosensory function to information processing and storage. Glutamate is crucial for some forms of learning and memory. It has the capacity for destroying neurons when released in excessive amounts. It is involved in developmental, adult synaptic plasticity, neurogenesis, and neurodegeneration (Danbolt, 2001).

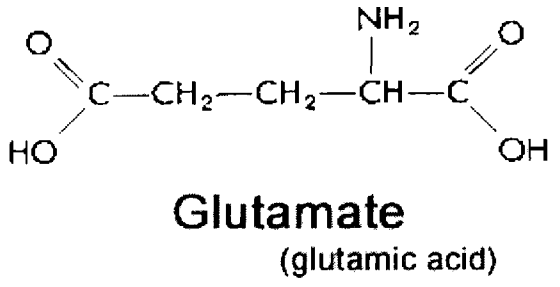
Table 1.1 Neurotransmitters and their known functions. The classical neurotransmitters are

Acetylcholine (ACh), Noradrenaline, Dopamine, γ-aminobutyrate (GABA), Serotonin, Glutamate. Their known functions are listed in the table.

Besides neurotransmitter, neurons also use neuropeptides to communicate information. Neurons very often make both a conventional neurotransmitter (such as glutamate, GABA or dopamine) and one or more neuropeptides. Neuropeptides can affect gene expression, local blood flow, synaptogenesis, glial cell morphology and behavior (reviewed by Pedrazzini et al., 2003).

1.2.1 Glutamate

Glutamate is the major excitatory neurotransmitter in mammalian nervous system and its concentration in brain is higher than any other amino acid (Fonnum, 1984). Its ability to depolarize and excite single neurones was first reported in the 1950s (Curtis and Watkins, 1960; Hayashi, 1952). During the following 20 years, several studies have provided support for the concept that glutamate is a neurotransmitter: it is presynaptically localized in specific neurones; it is specifically released by physiological stimuli in concentrations that is high enough to elicit postsynaptic response; mechanisms exist that would terminate neurotransmitter action rapidly (Curtis and Johnston, 1974; Fonnum, 1978; 1981; Roberts *et al.,* 1981; Dichiara and Gessa, 1981).



Glutamate
(glutamic acid)

Figure 1.2 Chemical structure of glutamate. Glutamate is a simple molecule which has two carboxyl groups and one amino group.

Although glutamate is a simple molecule (figure 1.2), it is the major excitatory transmitter in the

central nervous system. More than 90% of nerve cells in the cat spinal cord respond to the application of low doses of glutamate by microiontophoresis (MacDonald and Nistri, 1977). Most of the major sensory pathways and some motor pathways are glutamatergic. All pyramidal cells in the cerebral cortex and granule cells in the cerebellar cortex, which is the most abundant neuron in the mammalian brain, release glutamate (Fonnum, 1978).

1.2.1.1 Glutamate Release

Glutamate release occurs by exocytosis. In response to excitation of the axon terminal by action potentials, the glutamate-loaded synaptic vesicles fuse with the presynaptic membrane so that the contents of the vesicle are liberated into the synaptic cleft. It works by a Ca^{2+}- dependent mechanism that involves N- and P/Q-type voltage-dependent Ca^{2+} channels (Birnbaumer et al., 1994). After release of glutamate, the vesicle is recycled from the inside surface of the presynaptic membrane to form new vesicles by endocytosis. The vesicles are subsequently loaded with transmitters via active transporters in the vesicle membrane. Glutamate synthesis and release pathways are highly conserved between invertebrate and vertebrate including the major experimental model (eg. Drosophila, *C.elegans*, rat) (Bargmann, 1998).

1.2.1.2 Glutamate and Neurodevelopment

Glutamate plays an important role in neuronal differentiation, migration and survival in the developing brain (H. Komuro and P. Rakic, 1993; Hack and Balázs, 1994; Yano *et al.*, 1998). The N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor can induce apoptosis in vulnerable neurons (Ikonomidou et al. 1999).

1.2.1.3 Glutamate and Neurodegeneration

Neurodegeneration induced by excitatory neurotransmitter glutamate is considered to be of particular relevance in several types of acute and chronic neurological impairments ranging from cerebral ischaemia to neuropathological conditions such as motor neuron disease, Alzheimer's, Parkinson's disease and epilepsy. An imbalance between glutamate and GABA neurotransmitter systems may lead to hyperexcitability (Taylor and Meldrum, 1995).

1.3 Glutamate as a Neuromodulator

Neurotransmitters convey information between adjacent nerve cells, and neuromodulators alter neuronal activity by amplifying or dampening synaptic activity. Neuromodulation can occur in two ways. A neuromodulator might be released from neurons, glia, or secretory cells to change the tone of local synaptic activity by altering the effectiveness of a neurotransmitter. Or it might affect neurotransmitter synthesis, release, receptor interactions, reuptake or metabolism. It could also be released from either within the brain or other parts of the body to directly act on far distant neurons, sometimes with long-lasting effects (Vogt, 1954; Werman, 1966; McClennan, 1970; Phillis, 1970).

There is evidence that glutamate acts as a neuromodulator of slow neurotransmission. Stimulus-evoked slow excitatory postsynaptic potentials (EPSPs) in submucous neurones are suppressed while the amplitudes of slow inhibitory postsynaptic potentials (IPSPs) are enhanced by glutamate (Ren et al., 1999).

1.4 Neurotransmitter Receptors

A neurotransmitter receptor is a protein on the surface of a cell that binds to a specific ligand, such

as a neurotransmitter, antagonist, etc. and involved in signaling to the neuron. Receptors were classified on the basis of drug agonist effects and compounds that antagonized those effects. Four main families of receptors have been revealed by cloning and structural studies (reviewed by Humphrey, 1997).

1.4.1 Ligand Gated Ion Channels

Several neurotransmitters convey their signals by directly opening ion channels and changing the cell membrane potential or ionic composition. They are multi-subunit receptors in which all subunits traverse the membrane (Humphrey, 1997). Modulation of channel gating by the binding of ligands to a variety of sites on the receptor complex can significantly affect function; the benzodiazepine enhancement of chloride ion transport via the $GABA_A$ receptor being an important example. Indeed the $GABA_A$ receptor may have 11 or more modulatory sites, making its pharmacology one of the most complex. There may be several variants of each subunit, which, if incorporated into the receptor, can alter affinity for the neurotransmitter and its modulators (reviewed by Humphrey, 1997).

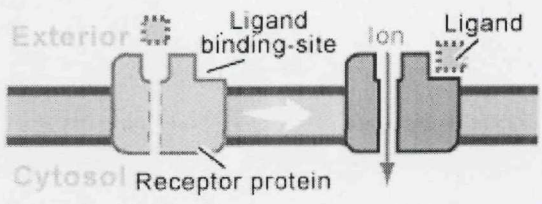


Figure 1.3 Ligand-gated ion channels. When a specific chemical messenger binds to the binding site on the ion channel, this allows it to open up and allows the ions to pass down their concentration gradient.

1.4.2 G-protein-coupled receptors

Receptors linked to guanine nucleotide-binding regulatory proteins or G-proteins, make up the largest proportion of the membrane-bound receptors. The receptor consists of a single polypeptide chain with 300–500 amino acids, arranged as seven connected a-helices that traverse the membrane forming a bundle. The connections between these helices form three extracellular and three intracellular loops. The extracellular amino terminal and the intracellular carboxy terminal vary greatly in sequence and length as does the long third cytoplasmic loop that provides the coupling with the G-proteins. The binding sites for agonists are often buried in pockets within the bundle of a-helices (reviewed by Humphrey, 1997).

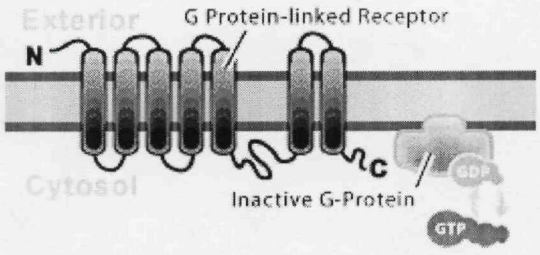


Figure 1.4 G-protein coupled receptor. It consists of 7 transmembrane domains, sites for ligand binding and for coupling to G proteins. When not stimulated, the a subunit is coupled to guanosine diphosphate (GDP). Activation of the G-protein coupled receptor by an agonist results in dissociation of the heterotrimeric G-protein into an a subunit coupled to guanosine triphosphate (GTP) and a ßγ dimer.

1.4.2.1 G-protein Signalling

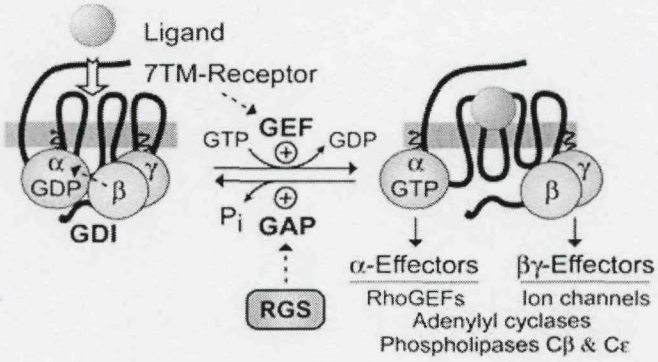


Figure 1.5 Model of the guanine nucleotide cycle governing 7TM receptor-mediated activation of heterotrimeric G protein-coupled signaling. The Gßγ heterodimer serves to couple Ga to the receptor and also to inhibit its spontaneous release of GDP. Ligand-occupied, 7TM cell-surface receptors stimulate signal onset by acting as guanine nucleotide exchange factors (GEFs) for Ga subunits, facilitating GDP release, subsequent binding of GTP, and release of the Gßγ dimer. Both the GTP-bound Ga and liberated Gßγ moieties are then able to modulate the activity of various enzymes, ion channels, and other effectors. Regulator of G-protein signaling (RGS) proteins stimulate signal termination by acting as GTPase-accelerating proteins (GAPs) for Ga, dramatically enhancing their intrinsic rate of GTP hydrolysis (Siderovski and Willard, 2005).

The existence of G-proteins was demonstrated when it was observed that stimulation of second messenger systems such as adenylyl cyclase required not only the receptor agonist but also the presence of guanosine triphosphate (GTP) (reviewed by Humphrey, 1997). The G-protein was eventually isolated and found to be a heterotrimer with subunits a, ß and γ, in order of decreasing molecular weight. In its resting state the G-protein complex is not associated with any particular receptor and is freely diffusible in the plane of the membrane and can interact with several different receptors and effectors. Specificity for a particular agonist-receptor complex, always producing the same end biochemical change in the cell, is guaranteed by the variability of the structure of the G-protein a subunit. Many variants of the subunits have been described and the number is still growing (reviewed by Humphrey, 1997). Two bacterial toxins (pertussis and cholera toxins) have been particularly useful in helping to distinguish which type of G-protein is involved in a particular

situation. Cholera toxin causes persistent activation of the G-protein that stimulates adenylyl cyclase (Gs), thus causing the excessive secretion of fluid from the gastrointestinal epithelium that is characteristic of cholera. Pertussis toxin has no effect on Gs but prevents the actions of other G-proteins such as Gi that inhibits adenylyl cyclase activity. Several G-proteins are inhibited by pertussis toxin and thus its functional effects are less obviously explicable in terms of G-protein inhibition. The agonist-receptor complex causes a conformation change in the intracellular domain to a form that has high affinity for the G-protein. The process of binding the receptor to the G-protein catalyses the disassociation of guanosine diphosphate from the a subunit of the G-protein and it is exchanged for intracellular GTP. This, in turn, causes the dissociation of the a subunit from the ß and γ subunits. The latter two proteins appear to be involved in anchoring the protein to the membrane. They also have a signal transduction role since some isoenzymes of adenylyl cyclase are modulated by the combined ß and γ subunits of the G-protein and not by the a subunit (reviewed by Humphrey, 1997).

1.4.3 Direct enzyme-linked receptors

These receptors possess large intracellular and extracellular domains joined by a single membrane-spanning helix. The extracellular domain provides the binding site for peptide agonists, usually hormones, that regulate differentiation, development and growth (reviewed by Humphrey, 1997). The intracellular domain is often a protein kinase that phosphorylates amino acid residues both of its own cytoplasmic domain and of target proteins. Many phosphorylate tyrosine, and the group has been called the tyrosine kinase-linked receptors, of which receptors for insulin are a typical example. However, some receptors are serine/threonine kinase linked and others are linked to guanylyl cyclase (e.g. the atrial natriuretic peptide receptor). The phosphorylated residues

provide binding sites for other intracellular proteins and eventually lead to altered gene transcription.

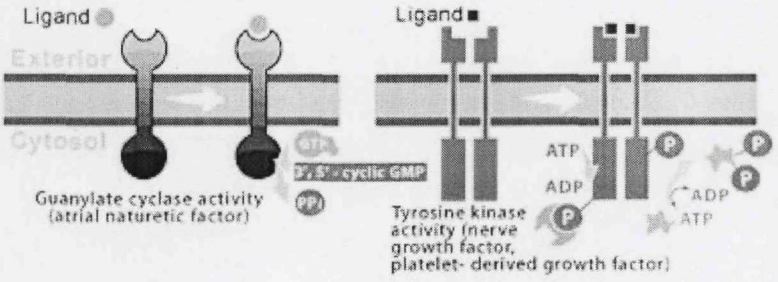


Figure 1.6 Direct Enzyme-linked receptor. It only spans the membrane once. The internal side of the receptor acts as an enzyme, which is activated when the appropriate ligand binds to the external portion of the receptor. The largest class of receptors with this family acts as tyrosine protein kinases, which phosphorylate tyrosine side chain residues on selected intracellular proteins.

1.4.4 Intracellular receptors

These non-membrane-bound receptors may be nuclear (e.g. thyroid hormone receptor), predominantly cytosolic, or form high molecular weight complexes with heat shock proteins (e.g. glucocorticoid receptor). In the latter, the presence of a ligand leads to the dissociation of the receptor from the complex and the movement of the receptor-ligand complex into the nucleus. It is obvious from the intracellular location of these receptors that ligands for these receptors must be lipid soluble and pass into the cell. The binding of the ligand to the receptor results in an uncurling of the receptor protein to reveal the DNA binding domain. The DNA binding domain consists of two 'zinc fingers', which contain cysteine residues surrounding a zinc atom. These fingers are believed to wrap around the DNA helix at hormone-responsive elements in the strand. RNA polymerase activity can be modulated and protein production is enhanced or reduced. A well-documented example of this is the increase in lipocortin production and the decrease in COX-2 production induced by glucocorticosteroid hormones (reviewed by Humphrey, 1997).

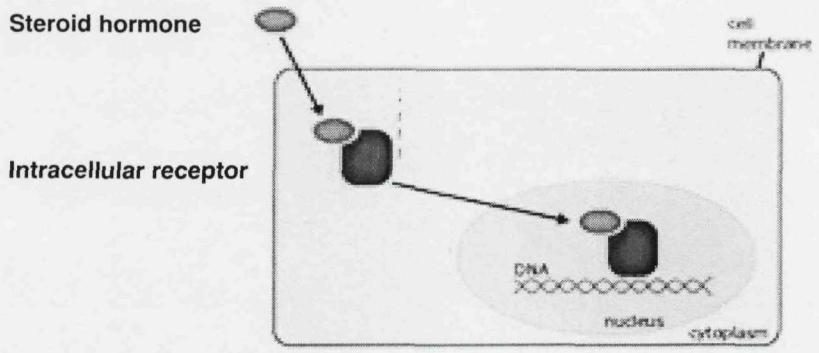


Figure 1.7 Steroid receptor. Steroid receptor diffused through the cell membrane and binds to an intracellular receptor, forming a hormone receptor complex. The complex interacts with DNA in the nucleus, affecting gene expression and cell function (reviewed by Humphrey, 1997).

1.5 Glutamate Receptors

Michaelis *et al.* (1974) and Roberts (1974) firstly described the sodium-independent binding of L-glutamate to synaptic membrane fractions derived from whole rat brain and cerebral cortex. Upon release of glutamate at the presynaptic junction, post-synaptic responses occur via pharmacologically and functionally distinct metabotropic (mGluR) and ionotropic (iGluR) receptors (Sugiyama H *et al.*, 1989). Metabotropic receptors mediate their action through GTP-binding protein dependent mechanisms that cause mobilization of Ca2+ from internal stores. The activation of ionotropic receptors [N-methyl--aspartate(NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazolepro -pionate (AMPA) and kainate (KA) receptors] on the other hand, leads to permeability to Na+, K+ and/or Ca2+. mGluRs are different from ionotropic receptors not only functionally, but also pharmacologically. They share no antagonists with the iGluRs (Sugiyama H *et al.*, 1989).

1.5.1 Ionotropic Glutamate Receptors

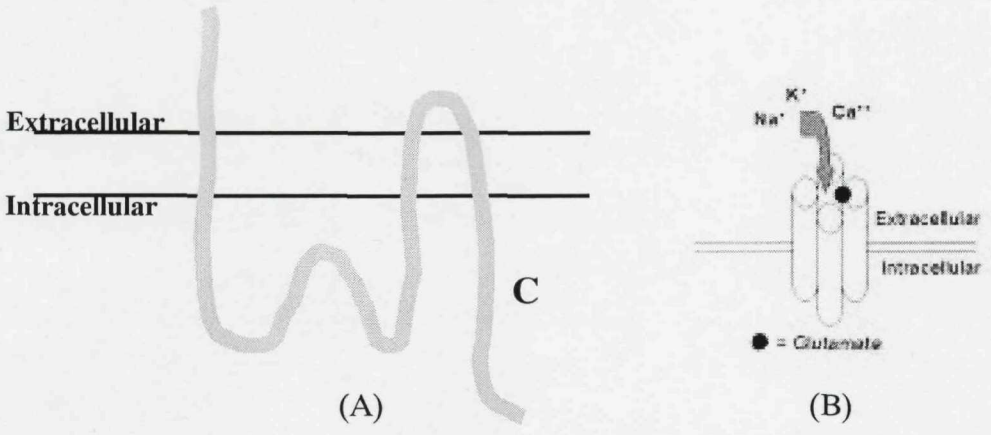


Figure 1.8. Topological organization of ionotropic glutamate receptors. iGluRs have four hydrophobic regions (A) and function as a multimer of four (B) (Laube et al. 1998).

iGluRs themselves are ligand-gated ion channels. On binding of glutamate, ions such as Na^+ and Ca^{2+} pass through a channel in the center of the receptor complex. iGluRs are divided into three groups, N-methyl-D-aspartate(NMDA),α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors, based on their pharmacological and structural properties (Barnard 1997). All iGluR subunits share a common basic structure (see figure 1.8). They possess four hydrophobic regions within the central portion of the sequence. The second transmembrane domain forms a re-entrant loop giving these receptor subunits an extracellular N-terminus and intracellular C-terminus (Dingledine, *et al* 1999).

The receptors appear to be tetrameric (Laube et al. 1998) and the subunits that comprise these are specific for each of the three families (Dingledine and Conn, 2000). Fifteen functional subunits assemble together in heteromultimeric complexes to form these receptors as follows: GluR1, 2, 3 and 4 for AMPA; GluR5, 6, 7 and KA1, KA2 for kainate; and NR1, NR2A, 2B, 2C, 2D and NR3 for

NMDA receptors. The subunit composition determines the biophysical properties of the receptor and its pharmacology.

Ionotropic receptors have functional properties beyond that of opening ion channels. The intracellular carboxy terminals interact with a variety of intracellular proteins. For example, the AMPA receptor activates a protein tyrosine kinase, Lyn, that activates the mitogen-activated protein kinase pathway (Hayashi *et al.*, 1999).

1.5.1.1 NMDA Receptors

NMDA receptors have critical roles in excitatory synaptic transmission, plasticity and excitotoxicity in the CNS. They have a voltage-sensitive block by extracellular Mg^{2+}, a high permeability to Ca^{2+} and unusually slow 'activation/deactivation' kinetics. A variety of NMDAR subunits have been identified and all NMDA receptors appear to function as heteromeric assemblies (Reviewed by Cull-Candy *et al.* 2001). A further specific feature is the need for glycine as a coagonist. Each receptor unit appears to have two glycine and two glutamate binding sites (Laube *et al.*, 1998).

1.5.1.2 AMPA Receptors

AMPA receptors mediate fast excitatory synaptic transmission in central nervous system. They are tetramers containing various combinations of subunits (GluR1–4). The most notable modification in AMPA receptor function is provided by the presence of a GluR2 subunit, which prevents the open channel from showing a Ca^{2+} conductance. Receptors expressing only GluR1 and GluR3 subunits show a significant Ca^{2+} conductance (Meldrum, 2000).

1.5.1.3 Kainate Receptors

Kainate receptors have five different subunits and kainate receptors are tetrameric assemblies of subunits that have a structure similar to those of the other ionotropic glutamate receptors. They are distributed both presynaptically and postsynaptically. Postsynaptic kainate receptors contribute to synaptic transmission, and the slow time-course of their response endows synapses with longer integration time. Presynaptically, kainate receptors modulate neurotransmitter release from excitatory and inhibitory synapses (Lerma, 2003).

1.5.2 Metabotropic Glutamate Receptors

The term 'metabotropic' means mediating its effect by activating enzymes. In 1985 glutamate was reported to stimulate phospholipase C (PLC) in cultured striatal neurones via a receptor that did not belong to the NMDA, AMPA or Kainate receptor families (Sladeczek *et al.,* 1985). Soon after, a similar effect of glutamate was described in hippocampal slices (Nicoletti *et al.,* 1986a, b), cultured cerebellar granule cells (Nicoletti et *al.,* 1986c) and cultured astrocytes (Pearce *et al.,* 1986). These results suggested that glutamate not only activated ligand-gated channel receptors but also receptors coupled to GTP-binding proteins (G proteins).

To date, eight distinct metabotropic glutamate receptor subtypes (termed mGluR1–GluR8) have been identified by molecular cloning (Conn and Pin, 1997; Pin and Duvoisin, 1995). They have little sequence homology with other metabotropic receptors, except for a modest resemblance to $GABA_B$ receptors and Ca^{2+}-sensing receptors. The hydropathy plots suggest that the membrane topology of mGluR may be similar with other G protein–coupled metabotropic receptors, i.e., they are inferred to have seven trans-membrane domains with an extracellular N-terminal and intracellular COOH

terminal. Despite these similarities, there are clear differences between mGluR and other G protein–coupled receptors with regard to the functional roles of different receptor domains. For example, the ligand-binding domain of mGluR lies within the extracellular N-terminal portion of the receptor rather than involving the transmembrane-spanning regions. Also, the second intracellular loop and the carboxy tail region appear to be critical for coupling of mGluR to G proteins, rather than the third intracellular loop (Dingledine and Conn, 2000).

Later a Ca^{2+}-sensing receptor, isolated from a bovine parathyroid cDNA library, was found to have about 30% sequence identity with mGluRs (Brown *et al.,* 1993). This receptor is also sensitive to Mg^{2+} and it is possible that there exist additional ion-sensitive receptors related to mGluRs.

1.5.2.1 Classification and Transduction Mechanisms of mGluRs

Nakanishi (1992) proposed that members of the mGluRs family could be classified into three different groups based on their degree of shared sequence homologies (about 70% homology within a group and 40% homology between groups), similar coupling mechanisms (phosphoinositide hydrolysis for group I and negative coupling to adenylate cyclase for groups II and III), and pharmacological properties (group I mGlu receptors were most potently activated by quisqualate, group II receptors by (2*S*,1*S*,2*S*)-2- carboxycyclopropyl glycine (L-CCG-I), and group III receptors by L-2-Amino-4-phosphonobutyric acid) (Schoepp *et al* 1999).

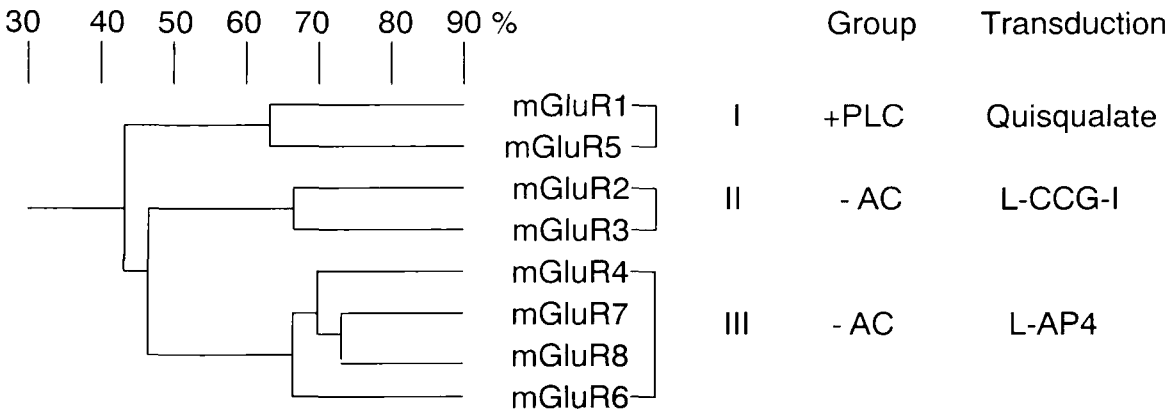


Figure 1.9 Dendrogram and pharmacological classification of the members of mGluRs. The numbers on the top indicate the % amino acid sequence identity between members of this receptor family. Eight mGluRs can be classified into three groups. Group I is coupling to phosphoinositide hydrolysis (G α o) while group II and II are negatively coupling to adenylate cyclase (G α i). Three groups are most potently activated by different agonists (Schoepp *et al* 1999).

According to the above system of classification, group I receptors consist of mGluR1, mGluR5 and their splice variants; group II mGluR include mGluR2 and mGluR3; group III consists of mGluR4, mGluR6, mGluR7 and mGluR8.

As already discussed, the classification of mGluRs is also supported by their respective transduction mechanism (figure 1.4). Group-I receptors, including their splice variants, stimulate phospholipase C as revealed by an increase in phosphoinositide turnover, and Ca^{2+} release from internal stores. The Ca^{2+} response induced by mGluRlb and c is slower but longer in duration than that induced by mGluRla. The reason for this difference is not known but may reflect a difference in the affinity of the receptors for the G-protein. The G-proteins involved in the activation of PLC by Group-I mGluRs have not been clearly identified.

In addition, mGluR have been shown to activate other effector systems through a variety of direct or indirect mechanisms. These include activation of phospholipase D (Boss et al. 1994, Holler et al.

1993), potentiation of cyclic AMP responses to activation of Gs-coupled receptors (Alexander et al. 1992, Winder and Conn 1993), increases in cyclic GMP levels (Okada 1992), activation of phospholipase A2 and release of arachidonic acid (Dumuis et al. 1993, Stella et al. 1994).

1.5.2.2 General structure of the mGluRs

Each mGluR has a signal peptide, indicating that the amino-terminal domain of the receptor is extracellular. The extracellular domains of mGluRs are composed of two globular domains with a hinge region (Kunishima *et al.*, 2000). Glutamate binds to amino acids residues in this hinge region. Seven closely located hydrophobic segments are often referred to as the seven transmembrane domains. The intracellular carboxy-terminal domain is variable in length and not as conserved between members of this receptor family. The presence of numerous phosphorylation sites and of numerous threonine and serine residues at the C-terminus suggested that it is the target of several types of kinases that could regulate receptor activity. The most conserved regions are an additional hydrophobic domain in the extracellular domain, which is postulated to form the ligand binding domain, and the first and third intracellular loops, which are involved in G-protein coupling (Ostrowski *et al.,* 1992; Savarese and Fraser, 1992; Pollak *et al.,* 1993). Several transmembrane segments, especially the sixth, are conserved between mGluRs. Twenty-one cysteine residues are conserved in all mGluRs. Nine cysteines are closely located in the carboxy-terminal portion of the extracellular domain and this region has been called the cysteine-rich region (O'Hara *et al.,* 1993).

1.5.2.2.1 Dimerization

The receptors have a large N-terminal extracellular ligand binding domain that forms a cysteine-linked dimer (Tsuji *et al.,* 2000), Roles of dimerization of mGluRs in ligand binding and

signal transmission remain to be elucidated. The significance of dimerization in signal transduction of single-span transmembrane receptors for growth factors or cytokines has been studied extensively (Weiss and Schlessinger, 1998). The main focus is on whether structural details of extracellular regions have an influence on intracellular signaling. For GPCRs, hetero- or homodimer formation has been reported in a few cases such as some subtypes of dopamine receptors (Ng *et al.,* 1996), 2-adrenergic receptor (Hebert *et al.,* 1996), and muscarinic acetylcholine receptors (Maggio *et al.,* 1993). More convincing evidence has emerged of dimer formation in GPCRs. A heterodimeric opioid receptor dimer with an affinity and ligand selectivity different from those of the homodimer has been reported (Jordan and Devi, 1999). Dimerization in the intracellular region is also reported to be essential for functional expression of the $GABA_B$ heterodimer receptor (Jones *et al.,* 1998; White *et al.,* 1998; Kaupmann, *et al.,* 1998).

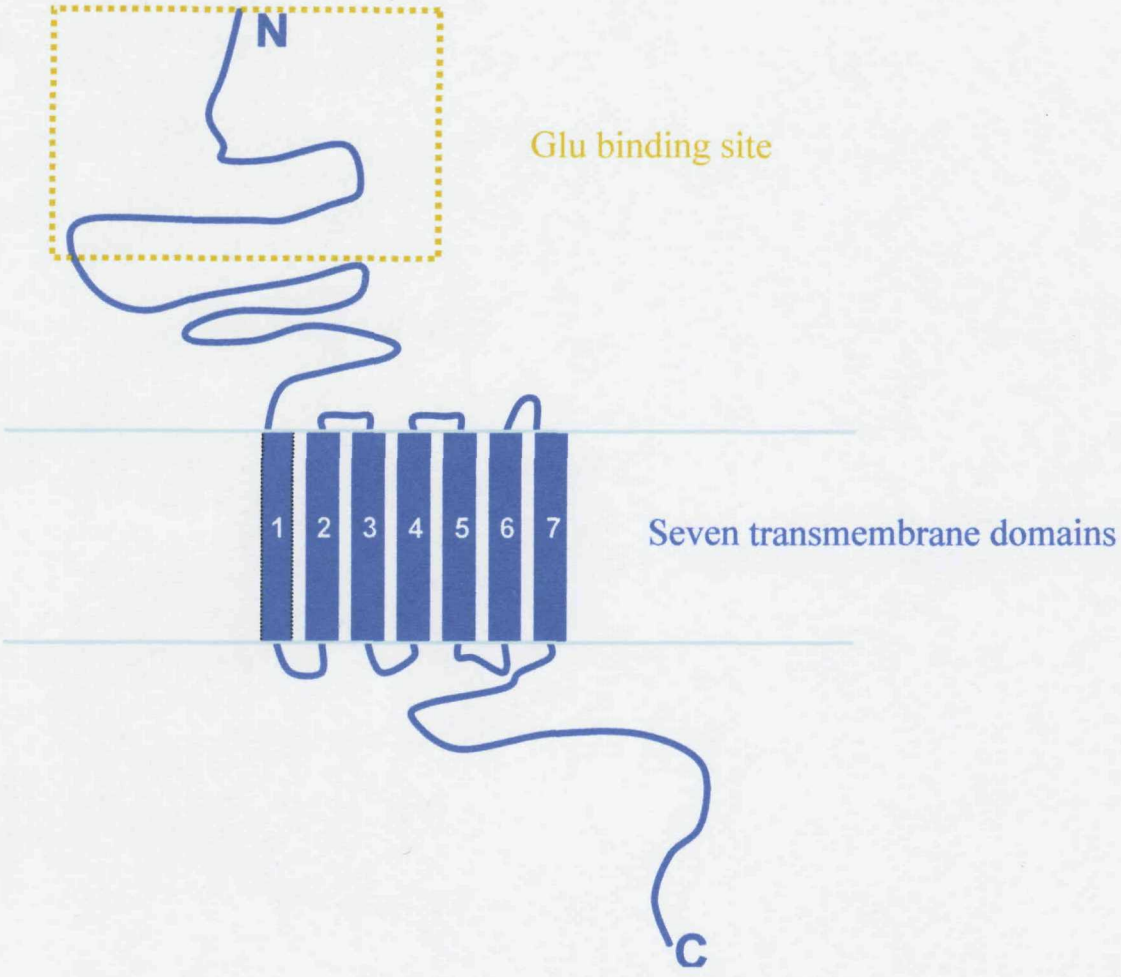


Figure 1.10 Topological organization of metabotropic glutamate receptors. The mGluRs possess the common 7-transmembrane domain. The N-terminus contains the ligand binding domain and the C-terminus generally undergoes splice variation. Each mGluR has a large N-terminal extracellular domain with 17 cysteines in conserved positions. The Co-immunoprecipitation experiments using wild type and epitope-tagged receptors demonstrated that the receptor undergoes a disulfide- dimerization. The intermolecular disulfide that mediates dimerization occurs in the extracellular domain, within about 17 kDa from the N terminus (Romano *et al.,* 1996).

1.5.2.3 Synaptic Localization of the mGluRs

Electron microscopic immunogold analyses have revealed a highly differentiated arrangement of glutamate receptors at synapses in the central nervous system (Lujan *et al.,* 1996; Kharazia and Weinberg, 1999). The differentiated distribution of metabotropic glutamate receptors reflects their functional heterogeneity.

To understand the diversified physiological roles of different mGluRs, it is important to know the precise localization in the brain of receptors relative to neurotransmitter release sites. Several studies demonstrated that group II mGlu receptors (particularly mGluR2) are located on both sides of the synaptic cleft, and group I (mGluR1 and mGluR5) and group III (mGluR4, mGluR7, and mGluR8) receptors are localized post- and presynaptically, respectively (Shigemoto *et al.*, 1997; Takumi, *et al.* 1999). In particular, mGluR7 was found to be highly enriched at active zones in terminals of hippocampal pyramidal cells (Shigemoto *et al.*, 1996). This selective localisation and its low glutamate affinity have led to the hypothesis that mGluR7 inhibits synapses which are firing above certain frequencies (Shigemoto *et al.*, 1996).

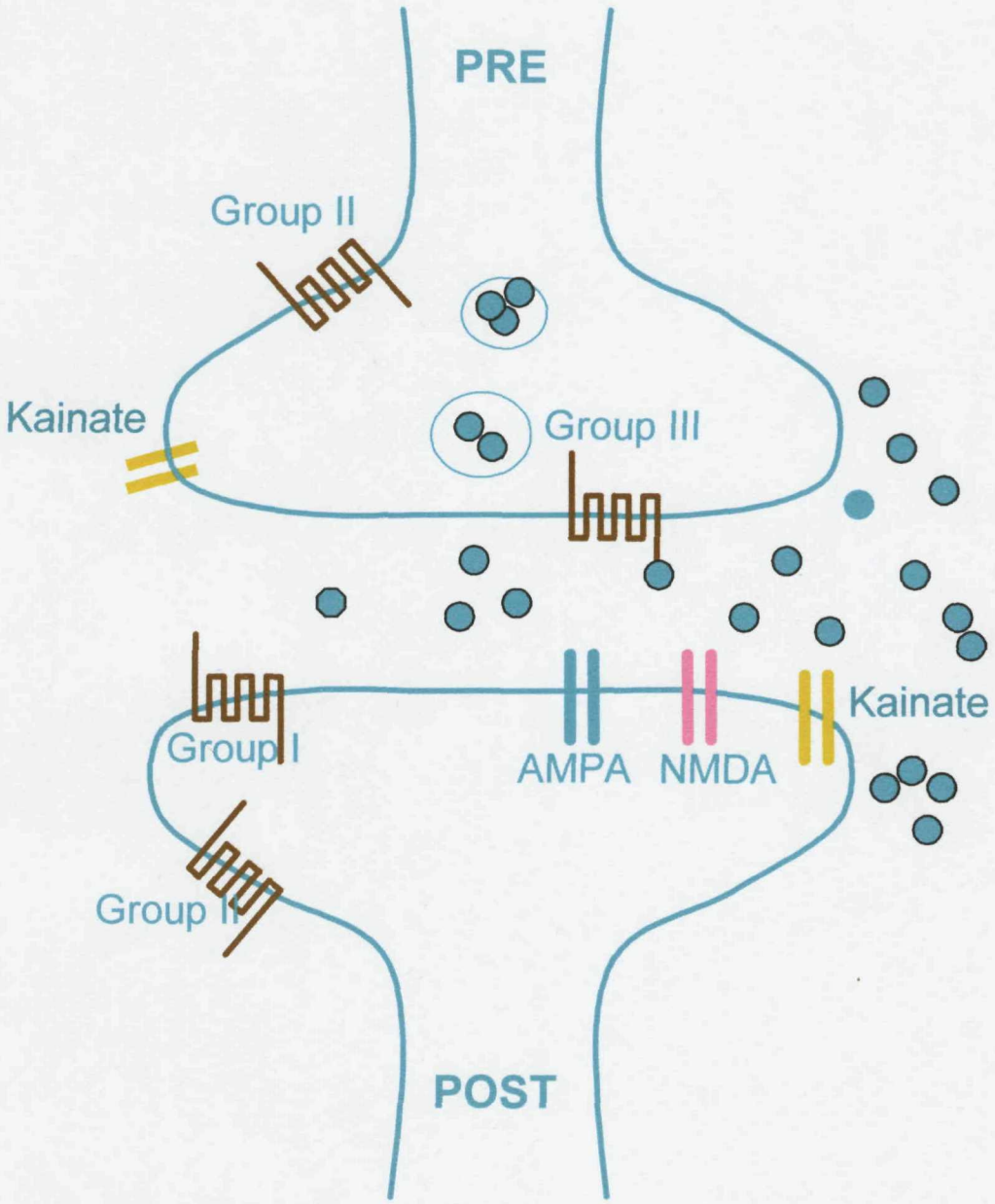


Figure 1.11 Synaptic localization of glutamate receptors. NMDA and AMPA type receptors are concentrated opposite the release site (Nusser *et al.,* 1994; Kharazia and Weinberg, 1999). Kainate receptors are localized both presynaptically and postsynaptically (Represa *et al.,* 1987; Chittajallu *et al.,* 1999). The different groups of metabotropic receptor exhibit distinct distributions at the synapse: group I receptors occur in the postsynapse ; group II receptors are located on both sides of the synaptic cleft; whereas group III receptors are found in the presynaptic release site to regulate the glutamate release (Ottersen *et al.* 1999).

1.5.2.4 Functions of mGluR in mice

The generation of mGluR knockout mice has provided an important tool to study mGluR functions. The knock out of mGluR in mice does not lead to the death of animals. However they show deficiently in several behaviours. This suggests that glutamate transmission plays a role in regulating animals' behaviour and supports the idea that they act in a neuromodulatory fashion.

	mGluRs	Phenotypes
Group I	mGluR1	Knockout (K/O) mice exhibit locomotory abnormalities with an abnormal and unstable gait, and spatial learning deficits (Aiba *et al.*, 1994).
	mGluR5	K/O mice, after overnight food deprivation, eat significantly less than the wild type (WT) controls when refeeding (Bradbury *et al.*, 2005).
Group II	mGluR2	K/O mice have hyperlocomotion phenotype in the light/dark transition test. Total distance traveled by mGluR2 K/O mice was significantly greater than that of WT mice (Morishima *et al.*, 2005).
	mGluR3	K/O mice are not caused anxiety by the selective and potent mGlu3 receptor agonist LY354740 whereas WT exhibit anxiolytic-like activity (Linden *et al.* 2005).
Group III	mGluR4	K/O mice show lower motor response to Ethanol than WT (Blednov *et al.*, 2004).
	mGluR6	K/O mice show a very weak suppressive effect of locomotory activity to light, whereas in WT mice a suppressive effect was induced by light (Takao *et al.*, 2000).
	mGluR7	K/O mice impair short-term working memory shown by the experiment of retrieving food from several locations (Holscher *et al.*, 2004).
	mGluR8	K/O mice have a reduced locomotory activity. The hourly distance traveled by it is shorter than WT (Duvoisin *et al.*, 2005).

Table 1.2 Some Phenotypes of mGluR knockout mice. The knockout of mGluRs leads to several behaviour deficiencies.

1.6 *Caenorhabditis elegans* as a Model Organism

Many aspects of biology are similar in most or all organisms and it is easier to study particular aspects in particular organisms. We chose *Caenorhabditis elegans* as the model organism for metabotropic glutamate receptor research as it has strong advantages compared to other model organisms (table 1.3).

Arabidopsis thaliana	It has a small genome and is now the main model plant system for genetics.
Yeast	It is the most thoroughly researched eukaryotic microorganism and being used as a model organism in modern cell biology research.
Dictyostelium discoideum	It has been used as a model organism in molecular biology and genetics, and is studied as an example of cell communication, differentiation, and programmed cell death.
Drosophila melanogaster	It has only four pairs of chromosomes and its compact genome was sequenced. is the most studied organism in biological research, particularly in genetics and developmental biology.
Caenorhabditis elegans	It is easy to grow in the laboratory, it has a fast life cycle and a sequenced genome. It is one of the simplest organisms with a nervous system. It is relatively straightforward to disrupt the function of specific genes by RNA interference (RNAi).
Mouse	It is the closest model organism to humans especially used in development, genetic and immunology studies. But its neuronal system is very complicated.

Table 1.3 Popular model organisms. They have been used in research laboratories to advance our understanding of life and human diseases.

C. elegans are predominantly hermaphroditic with males making up 0.1% of wild-type populations. The advantages of this soil nematode for studies of the genetics and behaviour include its rapid (3-day) life cycle, small size (1.5-mm-long adult), ease of laboratory cultivation, a fully sequenced

and mapped genome, a complete anatomical map of the 302-cell nervous system and several well-described behaviours (Grant and Wilkinson, 2003; Segalat and Neri, 2003). Every neurone in *C. elegans* is known and has been anatomically described, making it easier to go from gene to identified neuron to behaviour than it is in an organism with a larger, more complex nervous system (Hobert, 2003).

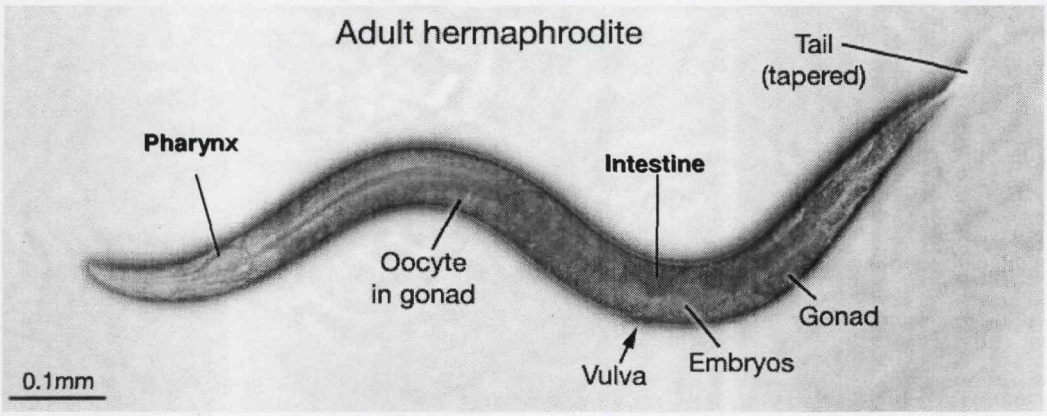


Figure 1.12 Stereomicroscopic image of an adult *C.elegans* hermaphrodite. The nematode has a simple cylindrical body plan. At the anterior end of this simple elongated tube-like body, the two-bulbed pharynx opens with six lips, while the tail ends in a tapering whip-like structure and accomodates an anal opening. Midbody is marked by vulval opening that is used to lay fertilized eggs generated by a two-armed gonad.

1.6.1 *C.elegans* Genome and Genes

C. elegans mitochondrial genome (13,794 bp) has been fully sequenced (Okimoto *et al.*, 1992). The nuclear genome contains approximately 100 million base pairs, organized into six chromosomes ranging in size from 14 Mbp to 22 Mbp (Coulson *et al.*, 1991), which is about about 1/30 the size of the human genome.

More than 8% of the 20,000 total genes estimated by the genome sequencing project (Waterston *et al.*, 1992; Wilson *et al.*, 1994) have been genetically identified. They consist of 716 visible and 787

essential genes that have been classified into 150 categories. Park and Horvitz (1986) estimated that mutants in up to 50% of *C. elegans* genes may be wild type when null. One striking feature of the *C.elegans* nervous system that eases the genetic analysis is that most of the neurones are nonessential in hermaphrodites under laboratory conditions. Only three neurones are required for normal development (CANL and CANR in the body and M4 in the pharynx (Wood, 1988). Thus, many mutations leading to defects in the nervous system need not have lethal phenotypes.

1.6.2 *C.elegans* Life Cycle

One of the advantages of working with *C. elegans* is that it has a short life cycle. The life cycle is temperature-dependent. *C. elegans* goes through a reproductive life cycle (egg to egg-laying parent) in 5.5 days at 15°C, 3.5 days at 20°C, and 2.5 days at 25°C. They live a total of about 2-3 weeks. *C. elegans* eggs are laid by the hermaphrodite. After hatching, they pass through four larval stages (L1-L4). When crowded or in the absence of food, *C. elegans* can enter an alternative third larval stage called the dauer state. Dauer larvae are stress-resistant and do not age. Hermaphrodites produce all their sperm in the L4 stage (150 sperm per gonadal arm) and then switch over to producing oocytes. The sperm are stored in the same area of the gonad as the oocytes until the first oocyte pushes the sperm into the spermatheca (a kind of chamber where the oocytes become fertilized by the sperm). When self-inseminated the wild-type worm will lay approximately 300 eggs. When inseminated by a male, the number of progeny can exceed 1,000. Hermaphrodites can mate with males or self-fertilize. A male responds to contact with a hermaphrodite by apposing his tail against her body; he moves backward until he approaches the end of the hermaphrodite, at which time he turns and continues backing until he locates the vulva. At the vulva, he inserts his copulatory spicules and forms a seal with his cloaca apposed to the vulva; he then transfers sperm

and seminal fluid, retracts his spicules, and moves away.

1.6.3 *C.elegans* Nervous System

The entire nervous system of the adult *C.elegans* hermaphrodite, which contains 302 neurons, has been reconstructed from serial section electron micrographs (White *et al.*, 1986). The 302 hermaphrodite neurons can be grouped by anatomical criteria into 118 classes. These include 39 classes of predicted sensory neurons, 27 classes of motoneurons, and the remainder classes of interneurons (White *et al.* 1986). Most neurons in C. elegans are extremely simple in structure and contain one or at most a few processes. The 302 neurons make approximately 5000 chemical synapses, 600 gap junctions, and 2000 neuromuscular junctions (White *et al.,* 1986). For comparison, the number of chemical synapses in *C. elegans* is approximately equivalent to that made by any single hippocampal pyramidal neuron. Twenty of the cells are contained within the pharynx (Albertson and Thomson 1976); the remaining neurons are found throughout the body of the animal. The major process bundles of the nematode are the nerve ring and the ventral and dorsal nerve cords. The nerve ring is the central region of neuropil in the animal. The ventral cord emanates from the nerve ring and is made up of the processes of interneurons and motor neurons. The dorsal cord is composed of axons of motor neurons that originate in the ventral cord and enter the dorsal cord via commisures (White *et al.*, 1986).

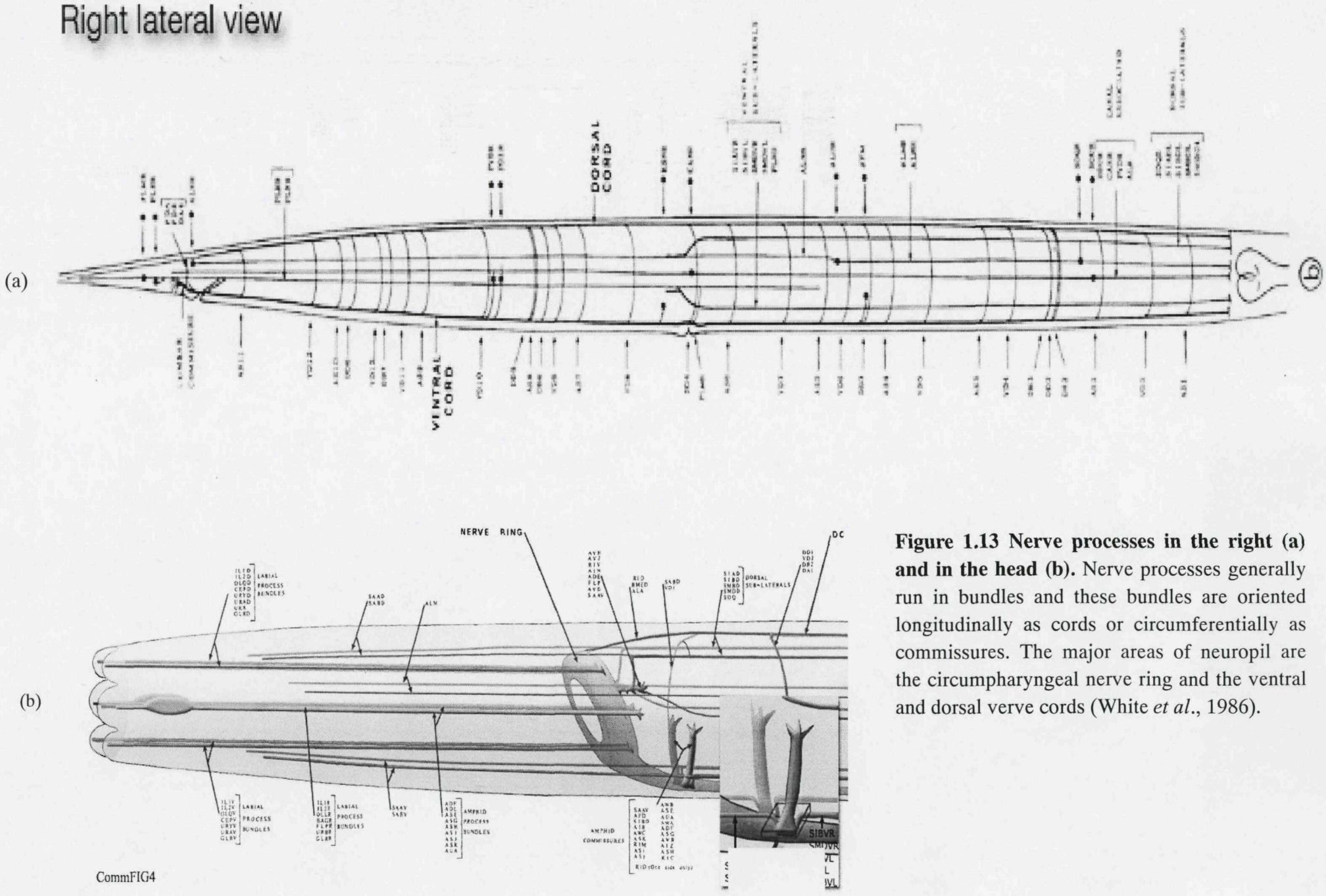


Figure 1.13 Nerve processes in the right (a) and in the head (b). Nerve processes generally run in bundles and these bundles are oriented longitudinally as cords or circumferentially as commissures. The major areas of neuropil are the circumpharyngeal nerve ring and the ventral and dorsal verve cords (White *et al*., 1986).

1.6.3.1 The Motor Nervous System

Over a surface *C.elegans* lies on its side and moves forward with periodic backward movement by propagating waves. The waves are generated by the coordinated activation of the verntral and dorsal body muscles. The muscles are innervated by motor neurons and these neurons receive the synaptic input from cord interneurons. There are six major classes of motor neuron that innervate the body musculature, three innervate dorsal muscles (DB, DD, and DA), and three innervate ventral muscles (VB, VD, and VA). Forward movement is mediated by the activation of the DB and VB motor neurons via their associated interneurons AVB and PVC. Backward movement is mediated by the activation of the DA and VA motor neurons by their associated interneurons AVA, AVD, and AVE. The DA, VA, DB, and VB motor neurons are excitatory and contain the neurotransmitter acetylcholine. The DD and VD motor neurons are inhibitory and contain the neurotransmitter GABA (Wood *et al.*, 1988). Laser ablation of motor neurons in the mid-cord regions of L1 larvae reveals that DBn neurons are required for normal forward movement, DAn neurons for normal backward movement, and DDn neurons for coordinated movements in both directions (Chalfie *et al.* 1985). Killing the interneurons (AVB and PVC) that innervate VB/DB neurons leads to defective forward movements in adults and ablating the interneurons (AVA and AVD) that innervate VA/DA neurons lead to defective backward movements (Chalfie *et al.* 1985).

1.6.3.2 The Sensory Nervous System

The bulk of the nervous system of *C. elegans* is situated in the head, which is richly endowed with sensory receptors. Information is received by sensory neurons and integrated by interneurons before being output by motor neurons. There are arranged in groups of sense organs, known as sensilla. Two large sensilla, the amphids, are located laterally and have internal channels, formed by the

sheath and socket cells, which open through the cuticle to the outside. Eight neurons have their ciliated endings in this channel; a further four are associated with the sheath cell. There are two analogous structures, the phasmids, in the tail, but they are simpler in that they only have two neurons ending in the channel. The amphids and phasmids are generally considered to be the main chemoreceptive organs in the animal, because their structure permits a group of nerve endings to be exposed to the external environment of the animal (Wicks and Rankin, 1995)..

1.7 *C.elegans* Behaviour

C. elegans shows a diversity of behaviors. It can taste, smell, and sense light and temperature. These characteristics make *C. elegans* a good experimental organism to study behavior. *C.elegans* behaviours that have been studied by mutational analysis include coordinated movement, chemotaxis, thermotaxis, osmotic avoidance, male mating, egg laying, mechanosensation, pharyngeal pumping, thrashing, nose touch and memory; all of these have led to new insights into how genes can influence behaviour.

1.7.1 Movement

The wild type displays a smooth sinuous movement on the agar surface. The motion of the body is confined to the dorsoventral plane, and on plates the animals are lying on their sides. The head can move in all directions, but surface tension restrains it to the surface as well. The animals can reverse with the same wave-like motion. Reverse motion can be stimulated either by tapping the surface of the plate in front of the animal or by touching the tip of its head. This normal movement of *C.elegans* is affected in uncoordinated (Unc) mutants. This covers a very wide range of phenotypes from paralysis to quite small aberrations of movement (Brenner, 1974).

1.7.2 Chemotaxis

Chemotaxis was one of the first sensory behaviours examined in *C.elegans*, which describes the animals are attracted by at least four classes of attractants: by cyclic nucleotides, cAMP and cGMP; by anions, Cl^-, Br^-, I^-; by cations, Na^+, Li^+, K^+, Mg^{2+}; and by alkaline pH values. The nematode's behavioral response to gradients of these attractants involves orientation and movement up the gradient, accumulation, and then habituation. It is defective in several mutants (*tax-1, 2, 3, 4, 5 and 6* [Dusenbery *et al.,* 1975], *che-1*, 2, *3*, *5, 6, 7*[Lewis and Hodgkin,1977]).

1.7.3 Thermotaxis

When grown at a temperature from 15°C to 25°C and placed on a thermal gradient, the nematode *C.elegans* migrates to its growth temperature and then moves isothermally. Behavioral adaptation to a new temperature takes several hours. Starved animals, in contrast, disperse from the growth temperature. A number of mutants have been tested for thermotactic abnormalities (*ttx*,[Hedgecock and Russell, 1975]). Several mutants selected for chemotaxis defects have thermotaxis defects as well.

1.7.4 Osmotic Avoidance

Wild-type animals have been shown to avoid high concentrations of a number of sugars and salts (Culotti and Russell 1978). All the mutants (*osm-1*, *che-3*, *osm-3*, *daf-10*, *osm-5*, and *osm-6*) failed to avoid both NaCl and fructose. The mutants move normally, exhibit normal touch sensitivity, and, like wild type, follow isotherms in a radial thermal gradient. None of the mutants is temperature sensitive, and all exhibit defective osmotic avoidance behaviour as young L1 larvae.

1.7.5 Male Mating

Male mating is one of the most complex behaviours in *C.elegans*, and it is composed of a series of sub-behaviors, which are under separate neuronal and genetic control. A male responds to contact with a hermaphrodite by apposing his tail against her body; he moves backward until he approaches the end of the hermaphrodite, at which time he turns and continues backing until he locates the vulva. At the vulva, he inserts his copulatory spicules and forms a seal with his cloaca apposed to the vulva; he then transfers sperm and seminal fluid, retracts his spicules, and moves away (Liu and Sternberg, 1995). Many of the *unc* mutants and some of the *che*, *daf*, *osm* mutants have reduced or no ability to mate.

1.7.6 Egg Laying

The anatomical components of the egg-laying system have been defined using a combination of light and electron microscopy: the egg-laying system consists of a 22-nuclei vulva, 16 sex (vulval and uterine) muscle cells and 16 neurons (White *et al.* 1976; Sulston and Horvitz 1977). The neurons providing direct synaptic inputs to the sex muscles are the two hermaphrodite-specific neurons (HSNs) and the six ventral C neurons (VCs). Other neurons provide synaptic inputs indirectly, via the HSNs: five mechanosensory neurons (the microtubule cells: AVM, ALMS and PLMs; see Chalfie and Sulston 1981), two interneurons (BDUs) and a single neuron with apparent mechanosensory endings in the region of the vulva (PVT). The *egl* mutants are defective in egg laying (Trent *et al.*, 1983).

1.7.7 Mechanosensation

Mechanosensation is the ability in which *C.elegans* response to various touch. It is mediated by six

mechanosensory neurons called touch receptor cells. Genetic analysis has resulted in the identification of over 400 mutations that disrupt the function of the touch receptors (Driscoll and Tavernarakis, 1997). Touching the animals often causes a transient cessation of pharyngeal pumping (Chalfie *et al.*, 1985). Over 400 mutations causing this touch insensitivity have been identified (Sulston *et al.*, 1975; Chalfie and Sulston 1981; Chalfie and Thomson 1982).

1.7.8 Pharyngeal Pumping

Pharyngeal pumping is an eating behaviour. Feeding is accomplished by two separately controlled muscle motions, isthmus peristalsis and pumping. The pharyngeal nervous system consists of 20 neurons. Pharyngeal muscle cells are controlled by motor neurons which are innervated by interneurons. The single neuron M4 was necessary and sufficient for isthmus peristalsis. M3 neurons are glutamatergic and control the timing of pharyngeal relaxation. The NSM neurons are serotonergic and signal the presence of food. MC motor neurons control the rate of pumping and release ACh onto pharyngeal muscle and receptors (Avery and Horvitz, 1989). Mutations in *cha-1*, *unc-13*, and *unc-18* slow pharyngeal pumping (Wood, 1988). *cha-1* encodes a choline acetyltransferase, which plays an important role in acetylcholine synthesis. *unc-13* is required to stimulate acetylcholine release. *unc-18* gene encodes a protein expressed in motor neurons.

1.7.9 Locomotion

The locomotion behaviour has been studied since 1980's. Worms normally move over a surface by going forward and backward. The neurons AVB and PVC initiate or control the forward movement, while AVA and AVD/AVE control the backward movement. Specialized sensory neurons that detect touch to the body (anterior body touch: ALM, AVM; posterior body chronitouch: PLM) provide

input to neurons in both the forward and back-ward circuits (Chalfie *et al.,* 1985). The *glr-1* gene encodes an AMPA-type glutamate receptor that is expressed in both the forward (AVB and PVC) and backing (AVA and AVD) interneurons. The *glr-1* mutant worms are deficient in their ability to withdraw backwards when mechanically stimulated (Maricq *et al.,* 1995). Thus glutamate plays an important role in worm locomotory behaviour.

1.8 Glutamatergic Signaling Modulates *C.elegans* Learning and Plasticity

A broad definition states learning is a change of behaviour that follows experience. Worms clearly can adapt to odorants (Colbert & Bargmann 1995), habituate to mechanical stimuli (Rankin &Wicks 2000, Wicks & Rankin 1995), and change their behaviour according to their feeding state (Hills *et al.* 2004, Sawin *et al.* 2000). In vertebrates, synaptic plasticity and learning are critically dependent on experience-dependent changes at glutamatergic synapses (Bredt & Nicoll 2003, Malenka & Bear 2004, Seidenman *et al.* 2003). Olfactory associative learning and mechanosensory habituation, show that glutamatergic neurotransmission is also important for behavioural plasticity in *C. elegans* (Saeki *et al.* 2001, Rankin and Wicks 2000, Wen *et al.* 1997, Wicks & Rankin 1995).

Following pairing of diacetyl with acetic acid, wild-type worms are far less likely to track diacetyl. In contrast, *glr-1* mutants show no evidence of a learned association between diacetyl and acetic acid. They maintain their tracking to diacetyl (Morrison and van der Kooy 2001). *glr-1* mutants are deficient in an olfactory associative learning task, in which diacetyl is paired with acetic acid solution, *glr-1* mutant nematodes are also impaired in nonassociative learning (habituation) with the same diacetyl stimulus. Glutamatergic signalling modulates *C.elegans* learning and plasticity.

1.9 Glutamate Receptors in *C.elegans*

Glutamate serves as an important neurotransmitter mediating rapid excitatory synaptic transmission via a large and diverse number of postsynaptic glutamate receptors in the nervous system. There are several glutamate receptor families in *C.elegans* which have been investigated, including *glr-1* to *glr-8* (non-NMDA subtype), *nmr-1, nmr-2* (NMDA subtype), *mgl-1* to *mgl-3* (metabotropic glutamate receptor) and *glc-1* to *glc-3* (glutamate-gated chloride channel) (Bargmann, 1998). On the basis of sequence conservation, the *C. elegans* glutamate receptor subunits appear to have the same topological organization as vertebrate receptors.

1.9.1 *glr-1, glr-2, glr-3, glr-4, glr-5, glr-6, glr-7, glr-8*

glr-1, glr-2 and glr-3 encode AMPA-like ionotropic glutamate receptors, *glr-4, glr-5, glr-6 and glr-7* encode kainate type ionotropic glutamate receptors, and *glr-8* encodes a delta-like ionotropic glutamate receptors (Bargmann, 1998; Sprengel *et al.*, 2001). *glr-7* and *glr-8*, are primarily expressed in the pharyngeal nervous system (Strutz-Seebohm *et al.*, 2003). *glr-1* is expressed in motor neurones and inter-neurones, including interneurons implicated in the control of locomotion. Among these receptors, the functions of *glr-1* in regulating *C.elegans* behaviour have been extensively studied. *glr-1* was identified in a forward genetics study. Forward genetics is looking for mutant individuals with defects in a biological process and identifying gene responsible. *glr-1* modified the neuronal control of locomotion and mechanosensory signaling (Maricq *et al.*, 1995). *glr-1* mutants are defective for nose touch avoidance, which suggests that the sensory transmitter is glutamate and that *glr-1* encodes a subunit of the postsynaptic glutamate receptors at the nose touch neuron-to-interneuron synapses. GLR-1 receptors are required in both the forward and backing interneurons and that the forward interneurons also have a role in backward movement (Hart et al.

1995). Killing the PVD neurons in animals that lack touch cell function eliminates harsh touch sensitivity (Way and Chalfie 1989). The locomotion interneurons AVA and PVC are direct synaptic targets of PVD. Mutants lacking GLR-1 glutamate receptors are insensitive to harsh touch, which suggests that these synapses are functional and that glutamate is the PVD transmitter (Hart et al. 1995).

1.9.2 *nmr-1 and nmr-2*

nmr-1 and *nmr-2* are *C.elegans* NMDA-type ionotropic glutamate receptor subunits, and NMR-1 and NMR-2 are always coexpressed (Sprengel *et al.*, 2001; Strutz-Seebohm N *et al.,* 2003). *nmr-1* plays a role in the control of movement and foraging behavior. *nmr-1* mutants show a lower probability of switching from forward to backward movement and a reduced ability to navigate a complex environment. Electrical recordings from the interneuron AVA show that NMDA-dependent currents are selectively disrupted in *nmr-1* mutants (Brockie *et al.,* 2001).

1.9.3 Glutamate-gated chloride channels (GluCl)

GluCl subunits are a class of ligand-gated ion channel receptor found only in invertebrates, including nematodes. They are the targets of the avermectin and milbemycin anthelmintics, including ivermectin. Five *C. elegans* genes (*glc-1, glc-2, glc-3, avr-14* [which has also been called gbr-2] and *avr-15*) have been demonstrated to encode GluCl subunits (Horoszok *et al.,* 2001). *avr-15*, via alternative splicing, encodes two alternative spliced glutamate-gated chloride channel subunits that share ligand binding and transmembrane domains and are members of the family of glutamate-gated chloride channel subunits (Li *et al.* 1997). *avr-15* mutants lacks M3 neurotransmission, and have prolonged pharyngeal contractions (Dent *et al.*, 1997).

1.10 Three mGluR-like Genes in *C.elegans*

In the *C.elegans* genome, three metabotropic glutamate receptor (MGLs) genes have been defined—*mgl-1*(ZC506.4), *mgl-2*(F45H11.2) and *mgl-3*(Y4C6A.2a). The predicted amino acid sequences suggest *mgl-1* belongs to the Gi coupled mGluR group, while *mgl-2* belongs to the Gq coupled group. *mgl-2* is expressed in interneurons, while that of *mgl-1* is expressed in motorneurones and pharyngeal neurones as well as interneurones (Ishihara et al, 1997). Some RNAi phenotypes have been reported (see figure 1.14). *mgl-1* has a slow growth RNAi phenotype (Kamath *et al.,* 2003).

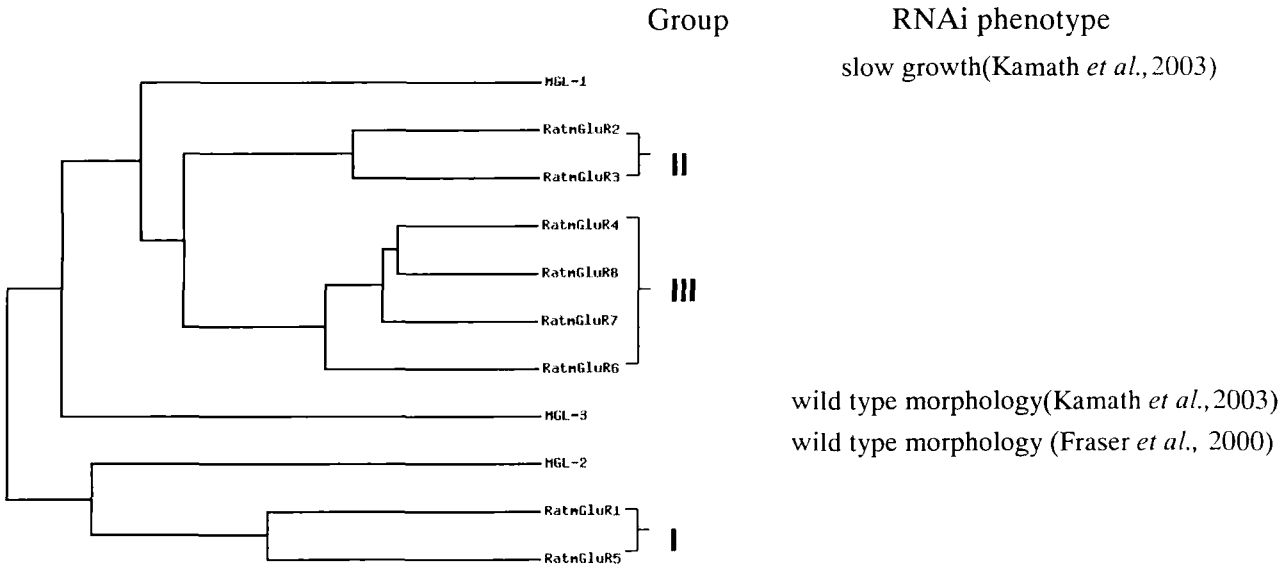


Figure 1.14 Three metabotropic glutamate receptors in *C.elegans*. The predicted amino acid sequences suggest *mgl-1* belongs to the Gi coupled mGluR group (group II and III, see figure 1.9), while *mgl-2* belongs to the Gq coupled group (group I). The use of RNA interference to inhibit the function of *mgl-1* suggests *mgl-1* mutants have got a slow growth phenotype.

1.11 Aims

Metabotropic glutamate receptors are hypothesized to modulate the transmission properties of synapses in response to the magnitude and frequency of glutamate signaling. Previous studies using *glr-1* in *C.elegans* have shown that glutamate signaling regulates behaviour. Three mGluR-like genes *(mgl-1, mgl-2* and *mgl-3)* have been identified in *C. elegans.* We expect that *mgl*s will

function at synapses known to signal using glutamate to regulate worm behaviour. The aim of this project is to investigate the roles of metabotropic glutamate receptors (mGluRs) in regulating behaviour, using the nematode *C.elegans* as a model organism.

Three investigations were proposed to study the functions of MGL receptors: DNA manipulation, identification of neurones expressing MGL receptors and behavioral characterization of *C.elegans* mutants. DNA manipulation was carried out to mutate genes and generate single, double and triple mutants for behavior study to determine the functions of MGL receptors. Behavrioual studies of the mutants were performed to learn about *C.elegans* behavior and the functions of the genes in regulating *C.elegans* behaviour. Identification of neurones expressing MGL receptors was carried out to understand in which biological process the receptor proteins are involved and through which neuronal circles the receptors regulate behaviors. All of these investigations are aimed to determine neuromodulatory roles of mGluRs.

CHAPTER 2
MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Molecular Techniques for Bacteria

Bacteria was used to feed *C.elegans* worms and for routine use.

2.1.1 Bacterial Strains

Escherichia coli (*E.coli*) OP50, a uracil-requiring mutant of *E.coli*, is used to prevent overgrowth of the bacterial lawn. The medium has limited uracil, and the bacteria cannot grow into a thick layer which obscures the worms (Brenner, 1974).

E.coli HT115 with IPTG-inducible T7 polymerase activity, lacking the dsRNA-specific RNase III, produce high levels of specific dsRNA and is used to effectively trigger strong and gene-specific interference responses when fed to *C. elegans* (Timmons *et al.*, 2001).

XL-1 Blue electrocompetent cells, which allow blue-white color screening for recombinant plasmids and are purchased from Stratagene, is for routine use.

DH5a chemical competent cells purchased from Invitrogen is for routine use.

E.coli TOP10 chemically competent cells purchased from Invitrogen were used for TA cloning.

2.1.2 Bacterial Culture

Bacterial culture was grown in sterilized LB (Luria-Bertani) media at 37ºC overnight with moderate shaking. Bacterial colonies were selected by 50μg/ml Ampicilin on LB agar plates. Bacteria for the nematode cultivation were seeded on the nematode growth media (NGM) agar plates.

2.1.3 Electroporation

The XL-1 Blue electrocompetent cells was used for transformations with 1ng plasmid DNA. The XL-1 blue was thawed on ice and plasmid DNA was added and gently mixed. The sample was

transferred to a prechilled 2mm gap eletroporation cuvette (Qbiogene). The cuvette was placed in the electroporation chamber and 2.4KV was applied. The cells were recovered with 1ml of LB and incubated at 37ºC with moderate shaking for 1hr. Antibiotic-selective LB plates were prewarmed to 37ºC and incubated for 16hrs at 37ºC. Bacteria colonies were further analyzed.

2.1.4 Ligation and Chemical Transformation

Vectors and inserts were digested with appropriate enzymes as indicated in text. Prior to ligation linearized vector DNA was dephosphorylated by alkaline phosphatase and incubated at 37ºC for 30mins. Inserts and vectors were purified and determined quantity by running a gel. 50ng vector and (vector size x 50ng / insert size) ng insert was added into a tube. 10% ligation buffer, 5% T4 DNA ligase and ddH_2O was added. After incubating at 15ºC overnight, 2μl of the ligation mixture was set aside and 1.8μl 3M sodium acetate pH5.2 was added. 40μl ice cold ethanol was added and the mixture was spun at 13,000rpm at room temperature (RT) for 10mins. Supernatant was removed and the pellet was washed with 75% ethanol. The sample was spun at 13,000rpm for 2mins at RT again. The supernatant was removed and the pelleted DNA was allowed to air dry. 2μl of ligation mixture that was set aside was added back to the pellet. 50μl of DH5a chemical competent cells were added to the tube and gently pipetted up and down to mix. After incubating on ice for 30mins, the sample was heat shocked at 37ºC for 45s. 250μl of SOC medium was added and the sample was incubated at 37ºC for 30mins. Transformation mixture was spreaded on a pre-warmed LB plate containing the appropriate antibiotic for selection of transformants. After incubating at 37ºC overnight, the colonies were analyzed further.

2.1.5 DNA Extraction From Bacteria

QIAprep Miniprep kit from QIAGEN were used to purify plasmid DNA from 1-10ml overnight bacteria cultures in LB medium. Bacterial cells were pelleted and resuspended in 250μl of Buffer P1 (50mM TrisCl, pH 8.0, 10mM EDTA 100μg/ml RNase A). 250μl of Buffer P2(200mM NaOH, 1%SDS) was added to that. The sample tube was gently inverted to avoid shearing genomic DNA. 350μl of Buffer N3 (3.0M potassium acetate, pH5.5) was added and the tube was inverted gently immediately to avoid localized precipitation. After 10min centrifugation at 14,000rpm a compact pellet was formed. The supernatants was pipetted into the QIAprep column and spun. DNA bound to the column was eluted by spinning with 50μl of Buffer EB (10mM Tris-Cl, pH 8.5).

2.2 DNA Manipulation

2.2.1 Restriction Digestion

Restriction digestion was used to characterize DNA and generate complementary DNA ends for DNA ligation. Restriction endonucleases were purchased from Roche or New England Biolabs and were used following their recommendations. Typically 1unit of enzyme was used to digest 1μg of DNA in 1hr.

2.2.2 Electrophoresis

Ethidium bromide agarose gels were used to analyze DNA under ultra violet light. Agarose gels were prepared at various percentages to achieve optimal separation of DNA fragment. A 1% gel was made by 1g of agarose and 100ml of Tris-acetate (2ml/L 0.5M EDTA pH8.0, 0.04M TrisBase, 1.14ml/L Glacial Acetic Acid, make up to 1L with ddH_2O). The mixture was heated in a microwave for 2mins. The molten gel mix was allowed to cool and 1μl of Ethidium Bromide (EtBr) (10 μg/mL)

was added. The gel was poured and allowed to set. DNA samples were loaded into the wells of the gel and the gels were run in an electrophoresis chamber containing Tris-acetate (TAE) buffer. Large gels were run at 150mV, medium gels were run at 120mV and mini gels were run at 90mV. When required, DNA was purified using the QIAGEN Gel Extraction Kit as suggested by manuals. RNA was run on EtBr agarose mini gels at 90mV for 20-30mins.

2.2.3 PCR Reactions

Enzyme used: Taq PCR System (Roche)

Expand Long Template PCR System (Roche)

The polymerase chain reaction (PCR) was used to amplify target DNA. Taq DNA polymerase PCR System was used for amplifications of up to ~5kb DNA. Expand Long Template PCR System was used for amplifications from 5kb-22kb. It is composed of an enzyme mix containing thermostable Taq DNA polymerase and Tgo DNA polymerase, a thermostable DNA polymerase with proofreading activity. Oligonucleotides used in PCR reactions were ordered from Invitrogen (Table 2.1). The melting temperatures predicted according to A/T=2°C and C/G=4°C. PCR reactions were done as recommended by the manufacturers.

2.2.4 RT-PCR Reactions

Superscript One-Step RT-PCR with Platinum Taq from Invitrogen was used for reverse transcription and cDNA synthesis. The system uses an mixture of SuperScript III Reverse Transcriptase and Platinum Taq DNA polymerase in an optimized reaction buffer, and can detect a wide range of RNA targets, from 200 bp to 4.5 kb.

2.2.5 DNA precipitation

DNA precipitation was used for obtaining a high concentration of DNA sample and sequencing DNA. To each 10µl DNA sample, 2.0µl 3M Sodium acetate (pH4.6), 50µl of 95% ethanol and 10µl ddH_2O were added in an eppendorf tube. The tube was vortexed to mix and left at room temperature for 1 hour to precipitate DNA. Samples were spun at 13,000rpm for 20mins. The supernatant was removed and the pellet was washed with 250µl of ice cold 75% ethanol. Samples were vortexed and spun at 13,000rpm for 10mins. The supernatant was removed and the pellet was air dried. Then it was sent to MWG (www.mwg-biotech.com) for sequencing.

2.2.6 TA Cloning Reactions

The TA Cloning kits from Invitrogen were used for the direct insertion of PCR products into the plasmid vector (Invitrogen). Taq polymerase (Invitrogen) has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in the kit has single 3' deoxythymidine (T) residues. That allows PCR inserts to ligate efficiently with the vector. The reactions were performed according to Invitrogen's protocol.

2.2.7 Table of Oligonucleotides Used

Oligonucleotides	Application	Sequences
mgl-1N1F	mgl-1 library screen	AATGGAGTCTTGAGCAACGC
mgl-1N1R	mgl-1 library screen	ATCTGCATTGAAGCCTTCGG
mgl-1C1F	mgl-1 library screen	GAATTTGATACTCCGCCTGG
mgl-1C1R	mgl-1 library screen	GAAAGTATCGTGAGCAGAGC
mgl-3N1F	mgl-3 library screen	AAACAACTTACTGTACCCGG
mgl-3N1R	mgl-3 library screen	TTCGATGCATTCGGATTAGG
mgl-3C1F	mgl-3 library screen	TTCCACGTGTATTCTTTGGC
mgl-3C1R	mgl-3 library screen	TACTCTTTGCAGTGTCCTGC
mgl-1N2F	mgl-1 library screen	TCTGCTCAACCAACAGTTCC
mgl-1N2R	mgl-1 library screen	CTTTACTTGCCACAACTCCC
mgl-1C2F	mgl-1 library screen	GATGCAGTCTATGCCATTGC
mgl-1C2R	mgl-1 library screen	TTGCGTGATGATCGTCTTCG
mgl-3N2F	mgl-3 library screen	AGGAGTTCAGAGAATGGTGG
mgl-3N2R	mgl-3 library screen	TTTCTCAGTCCATCTTCTCG
mgl-3C2F	mgl-3 library screen	TCGGTCCGATTTATTTTGCG
mgl-3C2R	mgl-3 library screen	TCCGACTGTATGAACACTGC
mgl-1N3F	mgl-1 library screen	GCAAATGTCAATGTGGTTGC
mgl-1N3R	mgl-1 library screen	CTTTCGAACGCTTCTGTACC
mgl-1C3F	mgl-1 library screen	GCGATATGTCCAGATGATGC
mgl-1C3R	mgl-1 library screen	GCTATTGGCACATTATCGGG
mgl-3N3F	mgl-3 library screen	CGAGATAGACAACTACTTCC
mgl-3N3R	mgl-3 library screen	GATCCCGTATCAGCAATTGC
mgl-3C3F	mgl-3 library screen	TAACATCATTGTGCATGTGC
mgl-3C3R	mgl-3 library screen	GAATCTCTTCACTGTTCTCG
mgl-1SEQ	mgl-1 sequencing	CAAAAGAAGAATGTAGCAGC
mgl-1INR	mgl-1 characterization	GGATTGTTTGATATCCTCGC
mgl-3SEQ	mgl-3 sequencing	TCAGTGTTATGGTTGCTTCG
mgl-2plus3F	MGL-2::GFP protein fusion –	TACTATAGAACAGCTGTCGG

mgl-2plus3R	MGL-2::GFP protein fusion –	TTATGGCATCTACGTCAAGG
mgl-2dnF	MGL-2::GFP protein fusion –	CAGAATTCACGGTCCAACAGTCG
mgl-2dngnmR	MGL-2::GFP protein fusion –	GTGGGCCCAGCGAGATTGGTGTC
mgl-2dnR	MGL-2::GFP protein fusion –	TCGGGCCCTCAAAAGATTTGCTTG
mgl-2up2F	MGL-2::GFP protein fusion –	TTGGTCGACGACTCATTCTTCTCT
mgl-2up5F	MGL-2::GFP protein fusion –	AAGTCGACGATCAGCATCTCTTCT
mgl-2up8F	MGL-2::GFP protein fusion –	CTGTCGACTATCCAGACGAATACG
mgl-2upR	MGL-2::GFP protein fusion –	GCGGCCGCCGTCTTTGCAACGACA
mgl-3dnF	MGL-3::GFP protein fusion –	ACAGCTAGCCAACTTACTGTACCC
mgl-3dngnmR	MGL-3::GFP protein fusion –	AAGGGCCCCAAATACCTGTTTACC
mgl-3dnR	MGL-3::GFP protein fusion –	AAGGGCCCTCAAAGAAAAGTGGA
mgl-3up2F	MGL-3::GFP protein fusion –	GGAGTCGACTAAAATCCGACTGA
mgl-3up5F	MGL-3::GFP protein fusion –	TTCGTCGACAATAGTATTCCCGAG
mgl-3up8F	MGL-3::GFP protein fusion –	TGAGTCGACGGTACTAGTCCGAAA
mgl-3upR	MGL-3::GFP protein fusion –	GCGGCCGCTTTTACTGTCGAACTG
MGL-2FPTZ1	mgl-2 characterization	CGTGGTCGATGTCGTTGC
MGL-2FPTZ2	mgl-2 characterization	GCGCACTCTTTCCAATCC
MGL-2FPTZ3	mgl-2 characterization	TCATGTTGTCAAACACCTGG
MGL-2FPOUT	mgl-2 characterization	CCATCTGATGTTTTTCAAGC
MGL-2FPIN	mgl-2 characterization	AGCACAAGCAATCAATCG
MGL-2FPTZ4	mgl-2 characterization	ATCGATTAATGACTATGG
MGL-2FPTZ5	mgl-2 characterization	CGAATAGCGAGGATATTAGC
MGL-2RPTZ1	mgl-2 characterization	ATATAGTTGAGGATGTCG
MGL-2RPIN	mgl-2 characterization	AGCAACACGCCATTGTTTCT
MGL-2RPOUT	mgl-2 characterization	TTTGGCCATGAGCCAAGAGT
MGL-2RPTZ2	mgl-2 characterization	ATTATTGACTCGTCTCGC
MGL-2RPTZ3	mgl-2 characterization	TGGTGACAGTAGCTGTCG
mgl-2dnF	MGL-2::GFP protein fusion –	CAGCTAGCACGGTCCAACAGTCG
mgl-2up2F	MGL-2::GFP protein fusion –	CTGGCGCGCCGACTCATTCTTCTC
mgl-2up5F	MGL-2::GFP protein fusion –	GAGGCGCGCCGATCAGCATCTCTT
mgl-2up8F	MGL-2::GFP protein fusion –	TGGGGCGCGCCTATCCAGACGAAT

mgl-1SEQ	mgl-1 sequencing	AATGTAGCAGCAGTAGTAGG
mgl-1HOMCHEQ	mgl-1 sequencing	TACTCGGCAGATGAATACGG
mgl-3SEQ	mgl-1 sequencing	CAAGTCAGTGTTATGGTTGC
mgl-3HOMCHEQ	mgl-3 sequencing	AGCTACAGTTCAACAGGAGC
mgl-3PLUS3F#	MGL-3::GFP protein fusion –	ACAAACTCTTCCAACGACGG
mgl-2PLUS3R#	MGL-2::GFP protein fusion –	AATGGAGAGGATGTGCAAGC
mgl-2Sequencing	mgl-2 sequencing	TCATGTCACACTTCTCATGC
mgl-3C1FU	mgl-3 library screen	TGATCGGAACATTTGTCTGG
mgl-3C1RU	mgl-3 library screen	ATATGAGCAACTGATGAGCC
mgl-3N2FU	mgl-3 library screen	ATGCATCACATCAATGTGG
mgl-3N2RU	mgl-3 library screen	TTCTCAGTCCATCTTCTCG

Table 2.1 Oligonucleotides List. Oligonucleotides were stored at -20°C as 10μM stock solutions in ddH_2O.

2.3 Techniques for *C.elegans*

2.3.1 *C.elegans* Strains Used

Bristol N2 wild type strain

mgl-1(tm1811), FX355 *mgl-2(tm355), mgl-3(tm1766), mgl* knockout strains, are homozygous viable, obtained from Mitani's lab. We subsequently backcrossed the strains and got HP186 *mgl-1(tm1811)*, HP188 *mgl-2(tm355)*, HP187 *mgl-3(tm1766)*. We crossed these strains and obtained HP189 *mgl-1(tm1811);mgl-3(tm1766)*, HP190 *mgl-1(tm1811);mgl-2(tm355)*, HP191 *mgl-2(tm355);mgl-3(tm1766),* HP185 *mgl-1 (tm1811);mgl-2(tm355);mgl-3(tm1766).*

pha-1(e2123) temperature sensitive embryonic lethal mutation.

NL2009 *rrf-3(PK1426)* was used in RNAi experiments. *rrf-3*, a putative RNA-directed RNA polymerase (RdRP), results in increased sensitivity to RNAi (Sijen et al. 2001; Simmer *et al.* 2002).

2.3.2 *C.elegans* cultivation media

Nematode Growth Media (NGM) were made by autoclaving the mixture of agar (10g/L), NaCl (3g/L), peptone (2.5g/L), cholesterol (5mg/L) and water at 120ºC for 90mins (Sterile). Calcium chloride (1mM), magnesium sulfate (1mM) and potassium phosphate (25mM) were added after autoclaving. Plates were poured with NGM agar in fume hoods to avoid contamination.

2.3.3 Preparation of plates

The agar plates were kept at room temperature for 3-5 days before use to allow excess moisture to evaporate. As starvation could affect the worm behaviour such as stimulating pumping (Avery and Horvitz, 1990), 50-100µl of OP50 (the optical density at 600 nm is 0.8A) was added to the plates and was grown for 3-5 days before the worms were grown on it.

2.3.4 Culturing and maintenance of worm stocks

C.elegans was maintained on NGM agar plates carrying a lawn of OP50. For general purposes, 5-cm plates were suitable; 9-cm plates were used for growing larger quantities of worms. Plates were kept at room temperature for at least 3 days before use, so that excess moisture evaporated and those contaminated with fungi and bacteria could be discarded. Plates were seeded with OP50 and OP50 was allowed to grow at least 2 days at room temperature before nematodes are added. The nematodes were transferred by means of a platinum wire pick whose end had been flattened and shaped. The standard wild-type strain N2 was grown at temperatures between 15ºC and 25ºC as indicated.

2.3.5 Trizol based RNAi Extraction from *C.elegans*

Worms were washed from plates into a microfuge tube with 1ml M9 buffer. They were left to settle

down at the bottom of the tubes at 4ºC before supernatant was removed. Worm pellets were washed with 1ml of M9 buffer to remove excess bacteria. 1ml Trizol was added to pelleted worms and vortexed to solubilize and lyse the worms. They were incubated for 1hour at RT, vortexed every 20mins and were spun at 14,000 rpm for 10mins at 4 ºC to remove insoluble material. The supernatant was removed and mixed with 200μl chloroform in a fresh tube. This mixture was vortexed for 15 seconds and incubated at RT for an additional 5mins before being spun for 15mins at 14,000 rpm. After the sample was placed at 4ºC to separate phases, the upper aqueous phase was removed into a fresh tube and 500μl isopropanol was added before the mixture was incubated for 10mins at 21-25ºC to precipitate RNA. RNA was recovered by spinning at 14,000rpm for 10mins at 4ºC. Supernatant was carefully removed and the pellet was washed with 75% EtOH in diethylpyrocarbonate (DEPC) treated H_2O. The sample was spun at 7500 rpm for 5mins at 4ºC. Supernatant was removed and pellets were allowed to air dry before being resuspended in 50ul DEPC H_2O. When resuspended, pellets were heated to 60ºC for 10mins to help redissolve the pellet.

2.3.6 DNA extraction

As required worms were grown prior to the prestarvation state and washed off with M9 buffer into tubes. Worms were allowed to settle down to the bottom at 4ºC for 5mins before removing supernatant. Pelleted worms were washed in M9 and washing procedure was repeated 2-4 times as required to remove the bacteria. 100μl worm lysis buffer containing proteinase K was added before incubation at 60ºC for 1 hour. This was followed by further incubation at 90ºC for 15mins and 2μl was used in a 50μl PCR reaction.

2.3.7 Worm Bleaching

Worm bleaching was used for decontamination of *C.elegans* cultures and synchronization of worm population. Bleaching solution was prepared by adding 1ml 1M NaOH and 6ml 4% NaOCl together. Worms are washed off with M9 into tubes and allowed to settle down at the bottom for 5 mins. Then the upper aqua is discarded and 200µl bleaching solution is added. The tubes were vortexed for 2 or 3 mins. 1ml of M9 buffer is added to dilute the bleaching solution. The tubes were centrifuged at 2,000 rpm for 1min. The upper aqua is discarded and this washing step is repeated 3 times. BSA solution (150µl 100x BSA in 150ml ddH_2O) washed tips was used to spread the eggs onto no food plates.

2.3.8 Behavioural assays

3 cm or 9 cm NGM agar plates were seeded with *E.coli* strain OP50. L4 *C.elegans* were picked up onto the NGM plates 18hours before assays and allowed to reach adulthood at 20ºC under standard well fed conditions. Right before the assays worms were removed onto no-food plates and equilibrated for 5 minutes. If selected worms contained food adhering to body wall, they were further passaged through no food plates. Assays of body bends, forward and backward movement, pharyngeal pumping, thrashing, growth rate and locomotion towards bacteria were performed at 20ºC in a temperature controlled room.

2.3.8.1 Body bends

Body bends describe the worm's general movement on plates. 50 L4 worms of each strain were grown for a further 18 hours and subjected to a body bend assay at 20ºC. Before performing the assay, worms were equilibrated on no-food plates for 5mins. Body bends were observed under a

microscope in a temperature-controlled room. One body bend is counted when the center of the worm passes through a sinusoid, regardless of whether it produces a forward or backward movement.

2.3.8.2 Forward and backward movement

Worms exhibit a characteristic movement pattern in which forward movement is interrupted by periodic brief reversal. 20 L4 worms of each strain were grown for a further 18 hours and subjected to the assay at 20ºC. Before assay, worms were equilibrated on no-food plates for 5mins and observed under a microscope in a temperature-controlled room. Subsequently the time for the worm to spend going forward and backward in a defined 5mins period was recorded.

2.3.8.3 Pumping

Pumping describes the feeding behaviour in which the number of pumps during 1 minute was monitored. Previously our pumping assays were performed after the worms were transferred to the no food plates for 5mins. The wild type worms pump at the rate of 0-20 times per minute. There is also variation on the trans-ACPD effect assays on pharyngeal pumping. Not all the drug treated wild type responded to trans-ACPD.

Then this pumping assay was redesigned. The pumping rate of the worms on the no food plates during one hour was recorded. L4 worms were picked onto new food plates and grown for 18hours further. Young adult Worms were transferred from food plates onto empty plates. After 1min, averagely the wild type pump at the rate of 10/min. As the time goes, the pumping rate increases and after 1hour it reaches up to 100/min. This assay is going to be performed on the mutants.

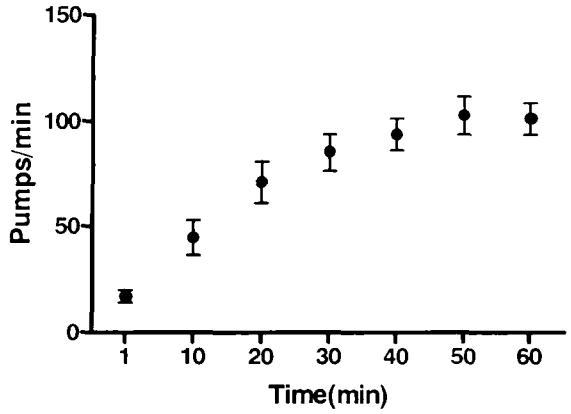


Figure 2.1 Pumping rates of the wild type along the time course of one hour. 32 worms were tested at 20ºC. Pumping rates increased as the time passed from ~10pumps/min to 100pumps/min. And after 1hour it seems to reach the maximum.

2.3.8.4 Thrashing

Thrashing describes the movement in a fluid medium. 50 L4 worms of each strain were grown for a further 18 hours and subjected to a thrashing assay at 20ºC. Before assay, worms were equilibrated in M9 buffer for 5mins and observed under a microscope in a temperature-controlled room. One thrash is counted when the tail of the worm goes from left to right and back again.

2.3.8.5 Locomotion towards bacteria

When preparing the plates, OP50 was seeded at one pole of a 9-cm agar plate. Worms were placed at the other pole of the plate and allowed to migrate towards a food source at the opposite pole for 2 hours as figure 2.2 shows. The percentage of the worms reaching the pole and the time duration were recorded.

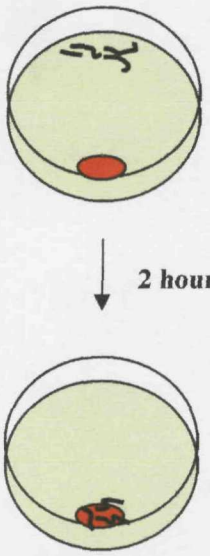


Figure 2.2 A diagram of the locomotion towards bacteria assays. Plates have OP50 seeded at one pole and worms at the other pole. After about 2 hours migration, some worms reach the OP50.

2.3.8.5.1 Optimization of the Locomotion towards Bacteria assay

9cm plates were seeded with fresh OP50 3 or 4 days before the assay. To optimize the experiment, food was seeded in three ways: on the edge; 1 cm from the edge; half radius. 5 μ l 200mM Sodium Azide was added onto the food 10 mins before the assay to avoid worms' moving away from the food after reaching it. 50 wild type young adults were transferred by picking up to no-food plates and allowed to get rid of the food on the body for 5 mins. Then they are transferred to the testing plates and the number of worms reaching the food was scored every 10mins. There is just about 60% of the total worms reaching the food when the food was put 1cm from the edge. And this assay has been repeated 9 times. We never got more than 80% of the total worms reaching the food (figure 2.3) as said in the Zheng et al paper (2004).

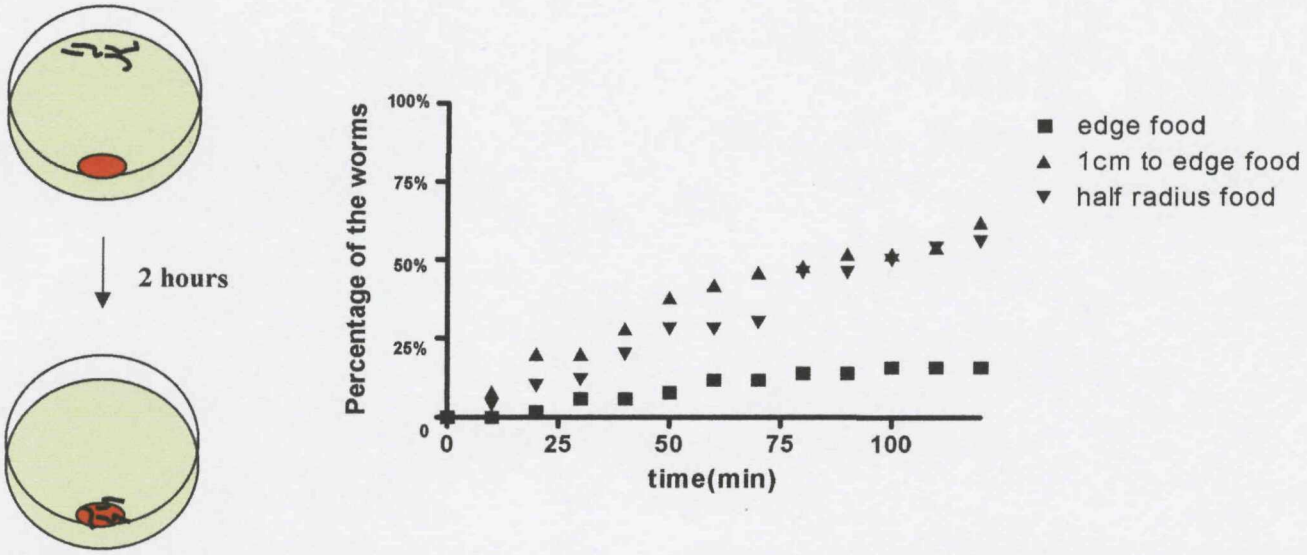


Figure 2.3 Locomotion towards bacteria assays on the wild type in three different ways. Plates have OP50 seeded at one pole and worms at the other pole. After about 2 hours migration, number of worms reach the OP50 was recorded. When food was placed on the edge, about 50% of the worms were lost on the wall of the plates or deep in the agar. Less than 5% of the worms were lost then the food was placed 1cm from the edge or at half radius.

To get higher percentage of the worms reaching the food after 2hrs, we tried Yi Zheng's method (Zheng et al., 2004): The food was about 2 cm from the edge of the plate. worms are quickly washed off with M9 for three times to remove bacteria. Approximately 150 worms were transferred to the test plate in a drop of buffer. The result shows about 90% of the worms reaching the food using Yi Zheng's method. The problem with our previous method would be behaviour disturbing when the worms are transferred or food is not removed completely by washing.

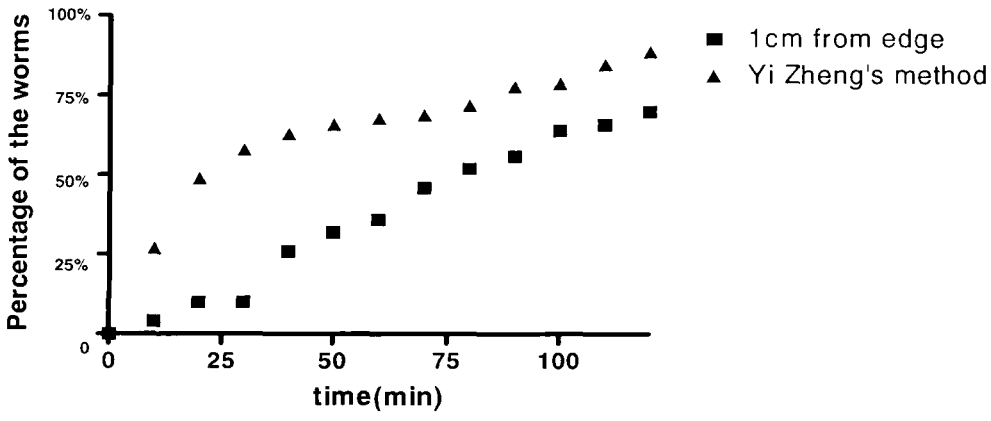


Figure 2.4 Locomotion towards bacteria assays on the wild type using two different protocols. Comparing to 70% by our method, 90% of the worms reached the food when using Yi Zheng's method.

2.3.8.6 Osmotic Avoidance

C.elegans worms were equilibrated on a no food plate before a fructose drop (2μl, 4M) was placed in the path of an advancing worm. The time taken to respond to contact with the fructose, defined by a clear backing movement, was recorded.

2.3.8.7 Growth rate

Worm strains as indicated were bleached as described in 2.3.7. Eggs are put on no-food plate overnight to hatch and arrested at L1 stage. L1 worms are washed off with M9 and transferred to food plates. Subsequently the growth stage of this synchronized population was recorded by checking worms every 12hrs.

2.3.8.8 Longevity

Worms were grown at 20° until the L4 stage of development. 200 L4 worms of each strain including wild type were picked to fresh plates seeded with OP50. The plates were incubated at 25ºC and worms on the plates were scored and moved to fresh plates everyday. Wild type worms

stopped producing offspring on days 4–5. In some mutants which are egg-laying defective, eggs are fertilized normally, but the parent lacks the structures needed for egg laying. As a consequence, eggs that are fertilized hatch inside the parent. These ultimately consume the parent from the inside called the bag-of-worms phenotype (Bagging). Worms dying as a result of bagging were censored. Death was defined as no longer being responsive to being poked repeatedly with a platinum wire. Number of live and dead worms were recorded per day.

2.3.9 Fluorescent photomicrography

Fluorescent photomicrography was used for the study of neuron identification. Nikon stereoscopic zoom microscope (SMZ800) with super high pressure mercury lamp power (C-SHG1) were used for image capture. After the microscope and the mercury lamp were switched on, the shutter was pulled out and the slider was switched to GFP filter. The fluorescent specimen was detected under this set up.

2.4 Statistical analysis

In experiments in which only two groups werer un, unpaired t tests were used to determine significance. If multiple groups were run, an ANOVA with Fisher's planned least significant difference (PLSD) planned comparison were used.

CHAPTER 3
Molecular Characterization and Backcrossing of Mutants

3 Molecular Characterization and Backcrossing of Mutants

3.1 Introduction

3.1.1 Forward and reverse genetics

Genetic analysis uses mutations to determine the function of gene products. In contrast to biochemistry which studies a protein in isolation, genetics studies the function of a protein by observing changes in the whole animal lacking the protein. There are two approaches: forward genetics and reverse genetics. Forward genetics is looking for mutant individuals with defects in a biological process and identifying gene responsible. Reverse genetics is picking an interesting gene based on the encoded protein, then mutating gene to understand in which biological process the protein is involved (Takahashi *et al.*, 1994).

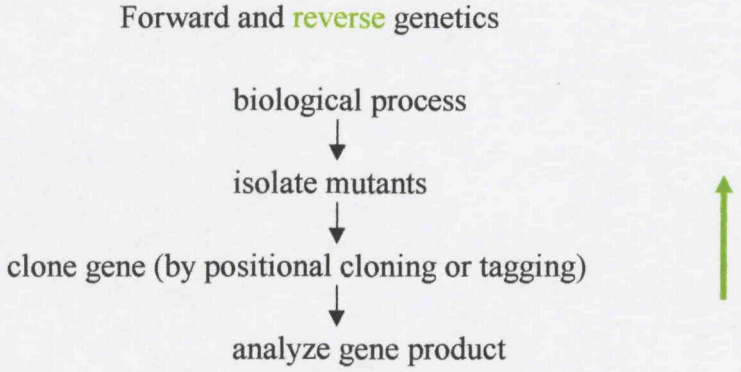


Figure 3.1 Forward and reverse genetics. Forward genetics is looking for mutant individuals with defects in a biological process and identifying gene responsible. Reverse genetics is picking an interesting gene based on the encoded protein, then mutating gene to understand in which biological process the protein is involved

Since 1974, when Sydney Brenner published his pioneering forward genetic screen, researchers have developed increasingly powerful methods for identifying genes and genetic pathways in *C. elegans*. Ion channels, transporters, and associated regulatory machinery have been identified in

forward genetic screens of various processes. Behaviors such as locomotion (Alfonso *et al.,* 1993), gonad development (West *et al.,* 2001), mechanosensory (Mano and Driscoll, 1999) and osmotic avoidance behaviour (Colbert *et al.,* 1997), chemotaxis and thermotaxis (Mori, 1999), resistance to fluoride ions (Take-Uchi *et al.,* 1998), egg laying (Elkes *et al.,* 1997), toxin and heavy metal sensitivity (Broeks *et al.,* 1996), programmed cell death (Wu and Horvitz, 1998), and neuronal degeneration (Treinin and Chalfie *et al.,* 1995) are represented by this description.

Reverse genetics programs usually begin with a well-defined class of genes. The methods include mutagenesis and RNAi technology. For years mutagenesis was the only approach available to link genotype to phenotype. Many worm genes have been knocked out using this approach. A second approach, RNAi, became available in 1990's (Fire *et al.,* 1998) and has become a genome wide approach (Kamath *et al.,* 2003).

3.1.2 Mutagenesis

Mutagenesis can be divided into three classes: spontaneous, induced, and target-selected. Spontaneous mutagenesis is the production of mutations without the deliberate introduction of a mutagenic agent. The nature and frequency of the most common type of spontaneous mutations can vary between strains. In wild-type *C. elegans* (N2 strain) most mutational events are probably due to replication error, background irradiation damage, or environmental chemical mutagenesis (Anderson, 1995).

Mutagens that can be used to induce mutations include (1) specific chemicals such as ethylmethanesulfonate (EMS), diethyl sulfate (DES), *N*-nitroso-*N*-ethylurea (ENU), or

formaldehyde; (2) irradiation with X-rays, UV light, or ionizing particles. Although the common effect is to cause G/C-A/T transitions, it does produce small deletions and other chromosomal rearrangements (Anderson, 1995). We performed EMS mutagenesis to generate mgl-1 and mgl-3 mutants as described in appendix I.

3.1.3 RNA interference

RNA interference (RNAi) is a technology used to silence the target gene. The presence of dsRNA, formed from the annealing of sense and antisense strands present in the *in vitro* RNA preps, is responsible for producing the interfering activity. Introduction of dsRNA into an adult worm results in the loss of the targeted endogenous mRNA from both the adult and its progeny (Fire *et al.,* 1998; Kamath *et al.,* 2003).

The main advantage of RNAi is that the identity of all RNAi clones is known, so that gene identification is direct and requires no mapping steps. Thus, the first genome-wide RNAi screens yielded more new genes underlying the phenotypes than the entire field of worm genetics that had generated in forty years (Zipperlen *et al.* 2001).

However this approach has disadvantages. Although RNAi is systemic in *C. elegans* when fed or injected to whole animals, worm neurones seemed resistant to RNAi. Partial or complete resistance of certain genes expressed in the mature nervous system was observed and all tissues are not equally sensitive to dsRNA delivered by feeding. The lack of effectiveness of dsRNA in the nervous system may be due to limiting components of the RNAi mechanism or to a factor destabilizing dsRNA in the tissue (Timmons et al., 2001).

As the level of knock down gene achieved by RNAi is variable in the neuronal system and methods for verifying the efficiency of RNAi are lacking, the behavioural studies were decided to be complimented by generating mutants of *mgl*.

3.2 Determination of the mutants: *mgl-1(tm1811), mgl-2(tm355), mgl-3(tm1766)*

mgl-1(tm1811), mgl-2 (tm355) and *mgl-3(tm1766)* strains were obtained from Mitani lab, Tokyo Women's Medical College, Tokyo, Japan. To confirm the mutations, the genomic DNA and cDNA derived from mutant lines were sequenced.

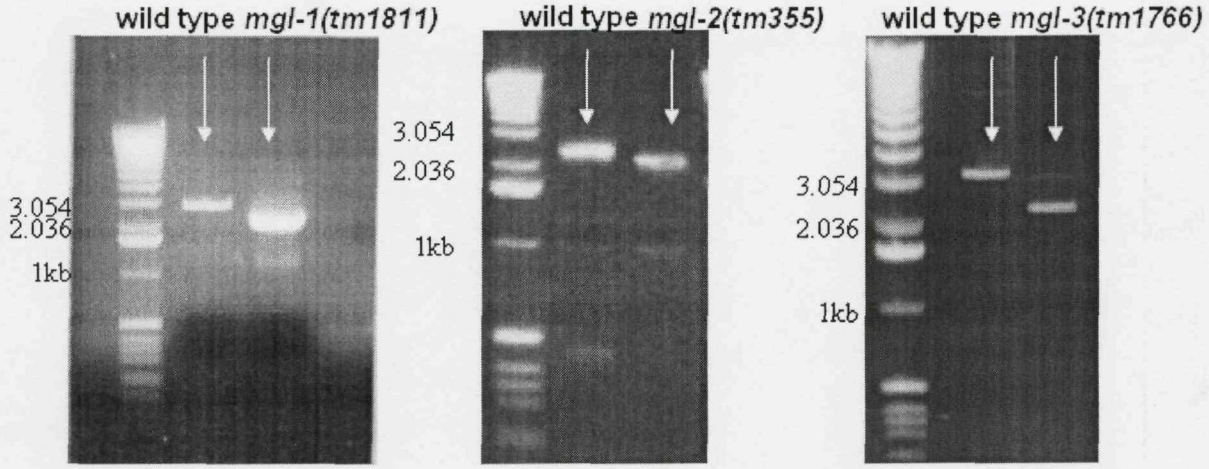


Figure 3.2 PCR products show the deletions in *mgl-1(tm1811), mgl-2 (tm355), mgl-3(tm1766).* Analysis of the genomic DNA demonstrated the deletions in the three mutant strains. Primers used for *mgl-1(tm1811)* are mgl-1N1F and mgl-1N1R, for *mgl-2(tm355)* are MGL-2FPZT3 and MGL-2RPIN, for *mgl-3(tm1766)* are mgl-3N1F and mgl-3N1R (refer to 2.2.8 Table of Oligonucleotides Used).

The sequences of genomic DNA confirm that there are 828bp genomic DNA deletions in *mgl-1(tm1811),* 271bp deletions in *mgl-2 (tm355),* 777bp deletions in *mgl-3(tm1766).* In *mgl-1(tm1811)*, the deletion crosses from the seventh exon to the tenth exon. In *mgl-2(tm355)*, the

deletion crosses part of the fifth exon and the fifth intron. In *mgl-3(tm1766)*, the deletion crosses part of the second intron, the third exon and the third intron (figure 3.3-3.5).

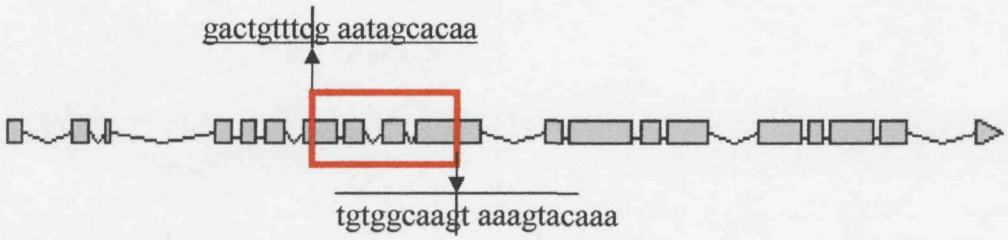


Figure 3.3 Deletion site in *mgl-1(tm1811)*. 828bp deletion crosses 7th exon to 10th intron. Sequences restricted in red-line boxes represent the deletion parts.

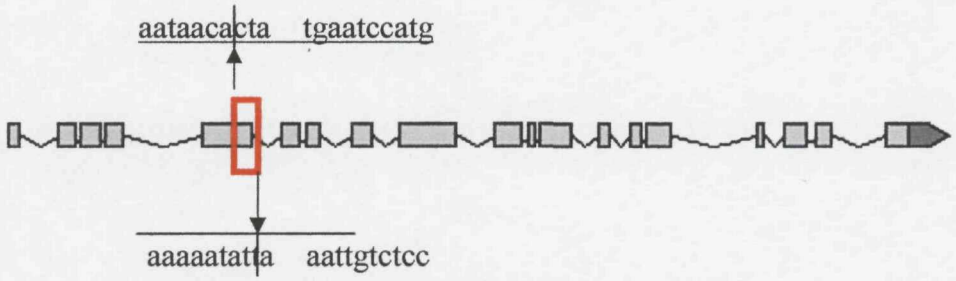


Figure 3.4 Deletion site in *mgl-2(tm355)*. 271bp deletion crosses 5th exon to 5th intron. Sequences restricted in red-line boxes represent the deletion parts.

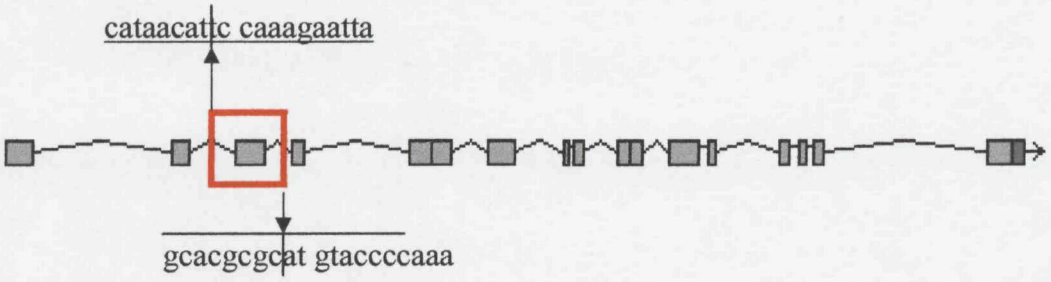


Figure 3.5 Deletion site in *mgl-3(tm1766)*. 777bp deletion crosses 2th intron to 3th intron. Sequences restricted in red-line boxes represent the deletion parts.

To find out how the mutant RNAs are spliced, RNA were extracted from mutant worms and RT-PCR was performed as described in Chapter 2. Figure 3.6 shows the RT-PCR results. The cDNA of *mgl-1(tm1811)* and *mgl-3(tm1766)* has a smaller size than the wild type. But the cDNA of *mgl-2(tm355)* has a similar size with the wild type.

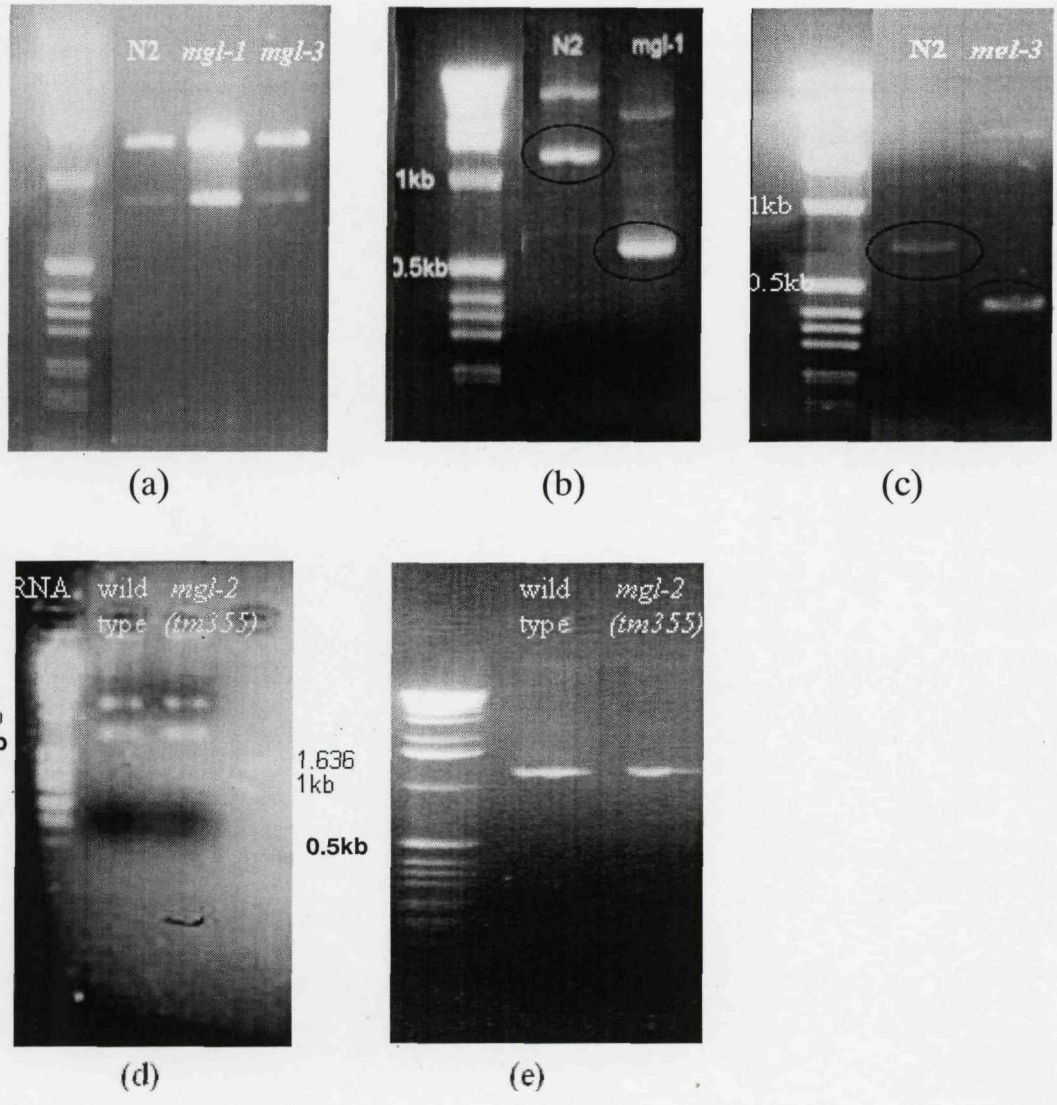


Figure 3.6 RT-PCR analysis. (a)(d) Equal amounts of RNAs from the RNA extraction before RT-PCR. (b)(c)(e) cDNA of the mutants. (The circled bands show the RT-PCR products. Others are considered as the PCR products of the genomic DNA.)

The sequencing reaction was carried out to fully elucidate the mutants as described in Chapter 2. The sequences of the cDNA suggest that the mRNA encoded by mutated *mgl-1* gene generate a protein that have a 207aa deletion at the N terminus in *mgl-1(tm1811)*. In the case of *mgl-2(tm355)*, part of the fifth intron was translated till reaching the stop codon in the fifth intron. Thus six amino acids together with a stop codon were added. In *mgl-3(tm1766)*, the third exon was deleted out and

there generate a stop codon in the fourth exon. The predicted receptor proteins generated in the mutants are showed in figure 3.7.

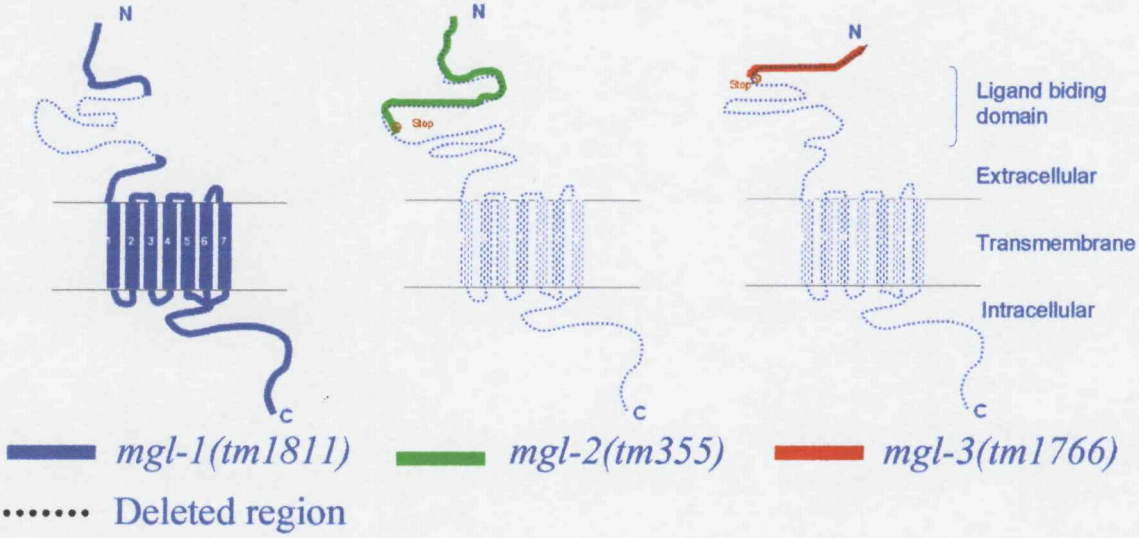


Figure 3.7 Predicted receptor proteins generated in the mutants. Part of the N terminus is deleted in *mgl-1* protein. Only part of the N terminus in *mgl-2* and *mgl-3* proteins are produced.

As figure 3.7 shows, only part of N terminus of *mgl-2(tm355)* and *mgl-3(tm1766)* are produced. Therefore *mgl-2(tm355)* and *mgl-3(tm1766)* are null mutants. In *mgl-1(tm1811)*, an in-frame deletion occurs at the extracellular N terminus. 207 amino acids are deleted from the ligand binding domain. This deletion part includes conserved residues which play the important role in ligand binding. Indeed agonists, that inhibit worm pharyngeal pumping, failed to inhibit pumping on *mgl-1(tm1811)* (James Dillon, PhD thesis). Thus *mgl-1(tm1811)* is a functional null mutant strain.

3.3 Backcrossing of the mutants: *mgl-1(tm1811), mgl-2 (tm355), mgl-3(tm1766)*

Backcrossing was carried out to get rid of other background mutations from the mutagenesis. During the process of mutagenesis, other mutations might have been induced as well. By backcrossing a number of times, and selecting for the mutations, we diluted out the other background mutations. This establishes that phenotypes we have selected for is the result of a single

gene mutation and not multiple genes that contribute to a phenotype.

mgl-1 is on Chromosome X, *mgl-2* is on Chromosome I and *mgl-3* is on Chromosome IV. The mutants and the wild type were crossed as figure 3.11-3.13 show. The worms were cultivated and mated on NGM plates seeded with OP50 at 20ºC during the process. To backcross *mgl-1(tm1811)*, we crossed N2 males to *mgl-1(tm1811)* hermaphrodites with a ratio of 5 males to 2 hermaphrodites. Male crossed progenies were *mgl-1(tm1811)*/O which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-1(tm1811)*. Hermaphrodite worms from the eggs were crossed with male N2. Male crossed progenies are *mgl-1(tm1811)*/O which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-1(tm1811)*. Hermaphrodite worms from the eggs were performed single worm PCR to determine whether it is *mgl-1(tm1811)/mgl-1(tm1811)* (Figure 3.8).

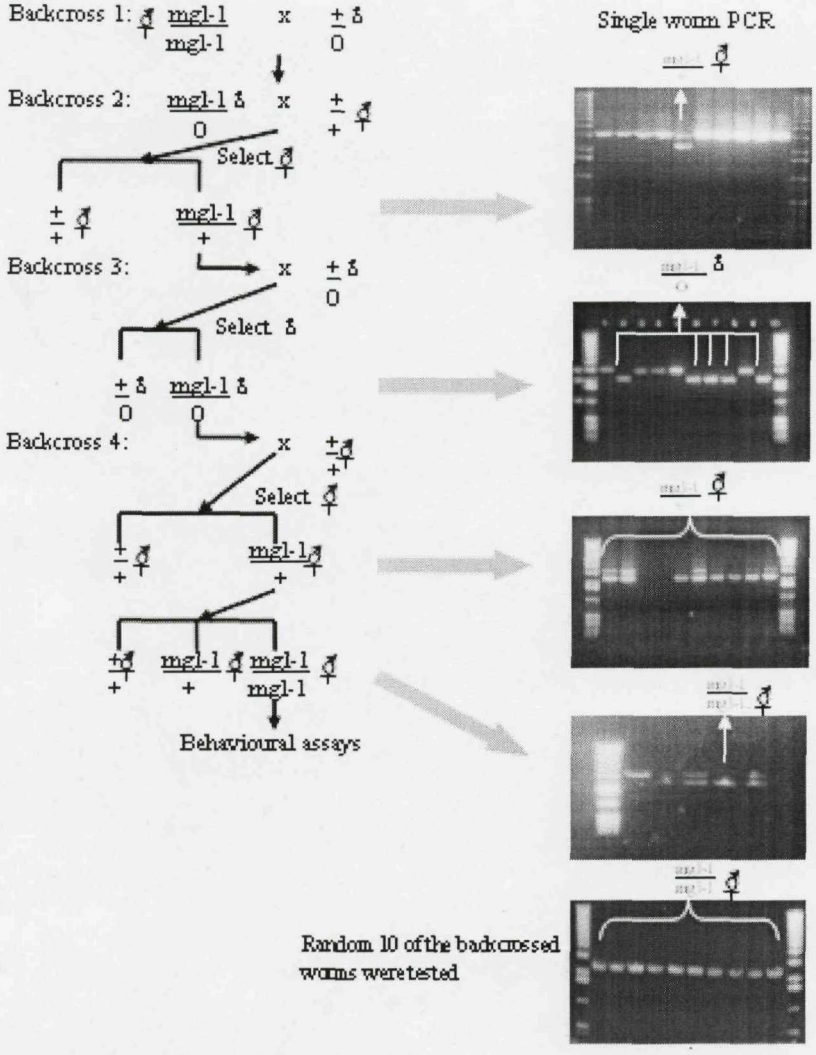


Figure 3.8 Backcrossing of *mgl-1(tm1811)*. Single-worm PCR was performed to select the required worms. We crossed N2 males to *mgl-1(tm1811)* hermaphrodites with a ratio of 5 males to 2 hermaphrodites. Male crossed progenies were *mgl-1(tm1811)*/O which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-1(tm1811)*. Hermaphrodite worms from the eggs were crossed with male N2. Male crossed progenies are *mgl-1(tm1811)*/O which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-1(tm1811)*. Hermaphrodite worms from the eggs were performed single worm PCR to determine whether it is *mgl-1(tm1811)/mgl-1(tm1811)*.

To backcross *mgl-2(tm355)*, we crossed N2 hermaphrodites to *mgl-2(tm355)* males with a ratio of 5 males to 2 hermaphrodites. Male crossed progenies were *mgl-2(tm355)*/+ which were selected and crosses with N2 hermaphrodites. Male crossed progenies are *mgl-2(tm355)*/+ which were again

selected and crosses with N2 hermaphrodites. Then again male crossed progenies are *mgl-2(tm355)*/+ which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-2(tm355)*. Single worm PCR were performed on hermaphrodite worms from the eggs to determine whether it is *mgl-2(tm355)/mgl-2(355)* (Figure 3.9).

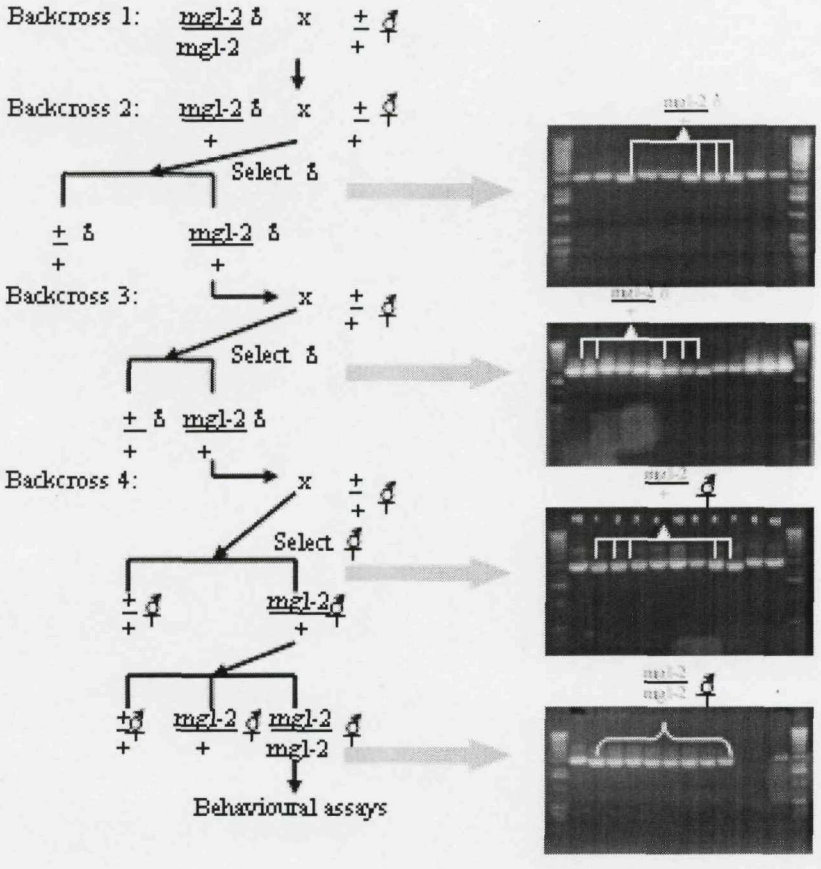


Figure 3.9 Backcrossing of *mgl-2(tm355)*. Single-worm PCR was performed to select the required worms. We crossed N2 hermaphrodites to *mgl-2(tm355)* males with a ratio of 5 males to 2 hermaphrodites. Male crossed progenies were *mgl-2(tm355)*/+ which were selected and crosses with N2 hermaphrodites. Male crossed progenies are *mgl-2(tm355)*/+ which were again selected and crosses with N2 hermaphrodites. Then again male crossed progenies are *mgl-2(tm355)*/+ which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-2(tm355)*. Single worm PCR were performed on hermaphrodite worms from the eggs to determine whether it is *mgl-2(tm355)/mgl-2(355)*.

To backcross *mgl-3(tm1766)*, we crossed N2 hermaphrodites to *mgl-3(tm1766)* males with a ratio of 5 males to 2 hermaphrodites. Male crossed progenies are *mgl-3(tm1766)*/+ which were selected and crosses with N2 hermaphrodites. Male crossed progenies are *mgl-3(tm1766)*/+ which were again selected and crosses with N2 hermaphrodites. Then again male crossed progenies are *mgl-3(tm1766)*/+ which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-3(tm1766)*. Hermaphrodite worms from the eggs were performed single worm PCR to determine whether it is *mgl-3(tm1766)/mgl-3(tm1766)* (Figure 3.10).

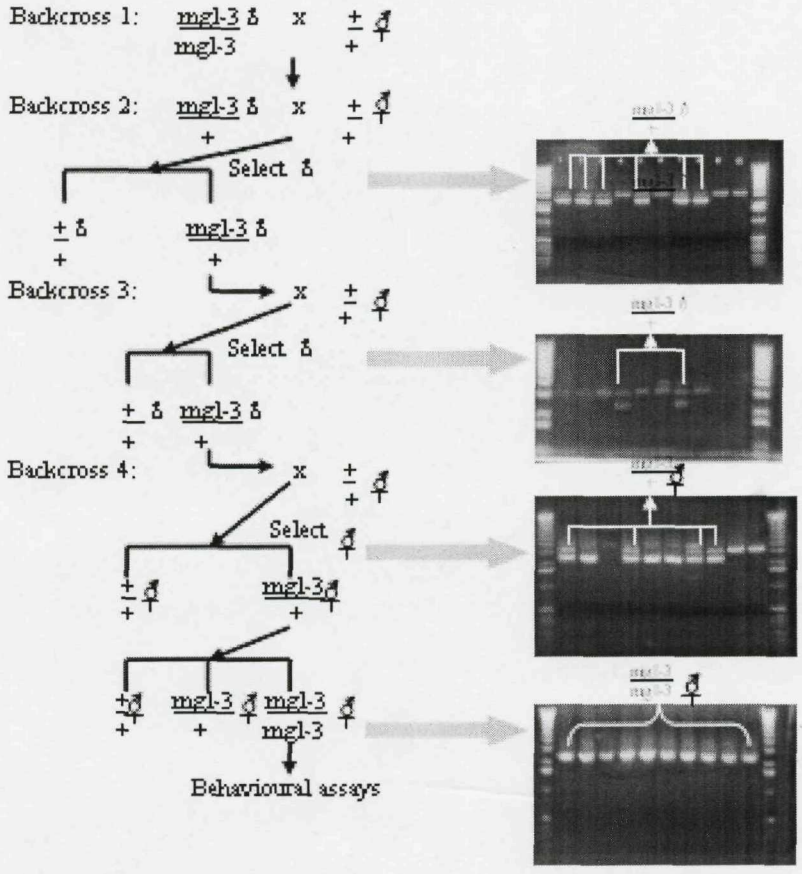


Figure 3.10 Backcrossing of *mgl-3(tm1766)*. Single-worm PCR was performed to select the required worms. We crossed N2 hermaphrodites to *mgl-3(tm1766)* males with a ratio of 5 males to 2 hermaphrodites. Male crossed progenies are *mgl-3(tm1766)*/+ which were selected and crosses with N2 hermaphrodites. Male crossed progenies are *mgl-3(tm1766)*/+ which were again selected and crosses with N2 hermaphrodites. Then again male crossed progenies are *mgl-3(tm1766)*/+ which were selected and crosses with N2 hermaphrodites. Hermaphrodite crossed progenies were selected,

laid eggs and then single worm PCR performed to determine the presence of *mgl-3(tm1766)*. Hermaphrodite worms from the eggs were performed single worm PCR to determine whether it is *mgl-3(tm1766)/mgl-3(tm1766)*

We backcrossed these 3 stains for 4 times. Then strains of HP186 *mgl-1(tm1811)*, HP188 *mgl-2(tm355)*, HP187 *mgl-3(tm1766)* were created.

3.4 Generation of double and triple mutants

It is interesting to know whether *mgl-1*, *mgl-2* and *mgl-3* are functionally dependant. By studying *mgl* double mutants and triple mutants, we may be able to figure out the relationships among this class of genes. As backcrossed *mgl* single mutant strains are available, we generated double mutants and triple mutants by crossing these single mutant strains with each others as shown in figure 3.14 Male *mgl-1(tm1811)* were crossed to hermaphrodite *mgl-3(tm1766)* with a ratio of 5 males to 2 hermaphrodites. Hermaphrodite crossed progenies were selected, laid eggs and then single worm PCR performed to determine the presence of *mgl-1(tm1811)* and *mgl-3(tm1766)*. Hermaphrodite worms from the eggs were performed single worm PCR to determine whether it is *mgl-1(tm1811);mgl-3(tm1766)* (figure 3.11).

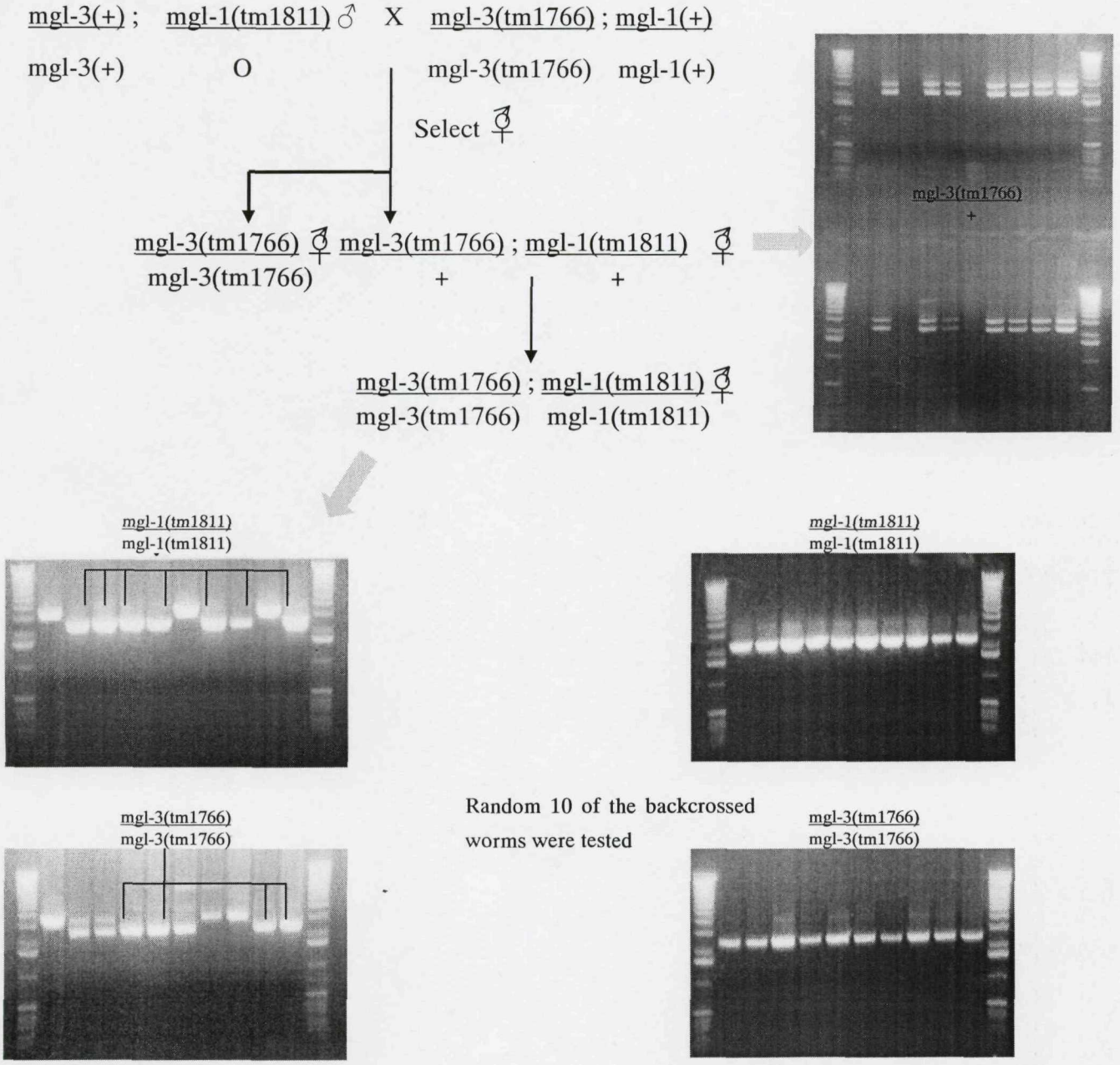


Figure 3.11 Crosses of *mgl-1(tm1811)* and *mgl-3(tm1766)* for double mutant generation. Single worm PCR was performed to select mutants.

Similarly, *mgl-1(tm1811);mgl-2(tm355)* and *mgl-2(tm355);mgl-3 (tm1766)* double mutants were generated by crossing single mutant strains with each others and verified by PCR as figure 3.12 shows.

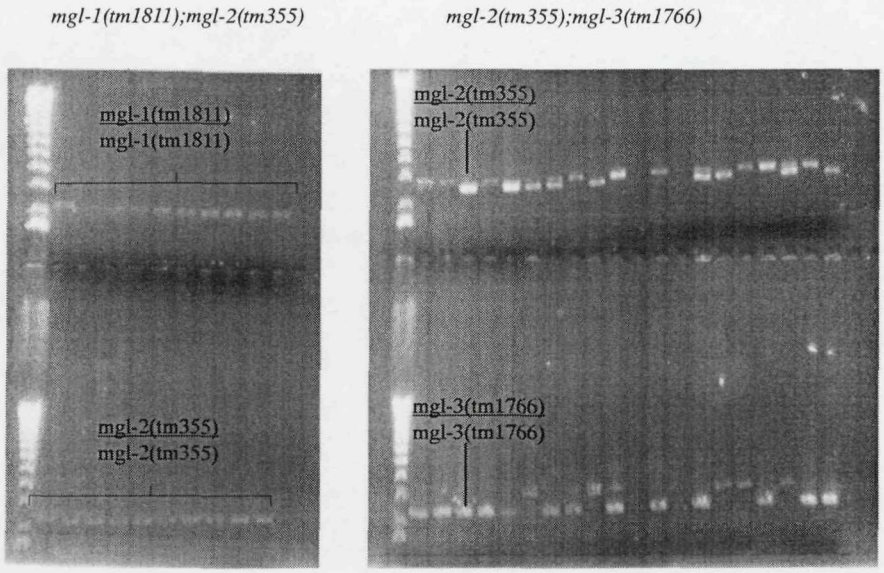


Figure 3.12 *mgl-1(tm1811);mgl-2(tm355)* and *mgl-2(tm355);mgl-3 (tm1766)* double mutants were generated. PCR was performed on the genomic DNA of the double mutant worms.

After we generated double mutants, we crossed *mgl-1(tm1811);mgl-2(tm355)* with *mgl-1(tm1811);mgl-3(tm1766)*. The *mgl-1(tm1811);mgl-2(tm355);mgl-3(tm1766)* triple mutant was obtained and it is viable on NGM plates. This triple mutant was verified by PCR on its genomic DNA as figure 3.13 shows. No obvious behavioural phenotypes were observed.

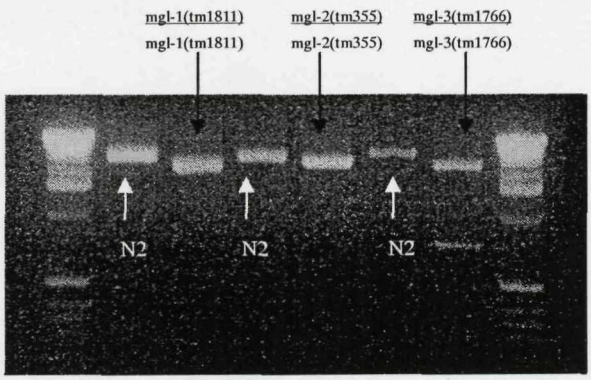


Figure 3.13 *mgl-1(tm1811);mgl-2(tm355);mgl-3(tm1766)* triple mutants were generated. PCR was performed on the genomic DNA of the triple mutant worms. The second band of *mgl-3(tm1766)* amplification is thought to be a result of an unspecific amplification.

3.5 General well being of *mgl-1(tm1811), mgl-2 (tm355), mgl-3(tm1766)*

A published RNAi experiment suggests that *mgl-1* has a slow growth phenotype (Kamath, 2003). To further analyze the roles of *mgl* in *C.elegans*, we started with a lifespan experiment to elucidate these genes' general effect on worm's well being. Worms were grown at 20° until the L4 stage of development. 200 L4 worms of each strain including wild type were picked to fresh plates seeded with OP50. The plates were incubated at 25ºC and worms on the plates were scored and moved to fresh plates every one or two days. Wild type worms stopped producing offspring on days 4–5. Worms dying as a result of bagging were censored. Death was defined as no longer being responsive to being poked repeatedly with a platinum wire. Before day 8, 30% of wild type worms died as a result of bagging. 50% wild type worms died from aging. *mgl* mutants showed similar phenotypes with the wild type, no significant differences. *mgl* genes have no effects on aging (table 3.1).

Genotype	Number of worms	Deaths % from bagging	Deaths%after21days	Alive%after21days
N2	59	30.5%	47.5%	22%
mgl-1(tm1811)	74	33.8%	56.8%	19.4%
mgl-2(tm355)	63	22.2%	55.6%	22.2%
mgl-3(tm1766)	61	27.9%	57.4%	14.7%

Table 3.1 *mgl* genes have no effects on aging. Life span was measure at 25ºC. The number of worms presented is the total number minus the number of missing worms. This experiment was carried out along the time course of 21days.

3.6 Discussion

We characterized *mgl-1(tm1811), mgl-2(tm355), mgl-3(tm1766)* mutant strains and showed that they are functional null mutants. We backcrossed these mutant strains for 4 times, and generated

double, triple mutants by crossing the strains with each other. No obvious phenotypes were observed.

As suggested by Kamath, *mgl-1* shows a slow growth phenotype by RNAi experiments. We would expect *mgl-1(tm1811)* has a slow growth phenotype and a longer life span. Longevity experiments were performed and showed the mutants have a normal life span. *mgl* genes have no effects on aging.

CHAPTER 4
mgl Expression Patterns

4 *mgl* Expression Patterns

4.1 Introduction

With its sequenced genome and simple, well-defined neuroanatomy, the nematode *C. elegans* is a useful model system in which to correlate gene expression with neuron identity. The *C. elegans* hermaphrodite nervous system is composed of exactly 302 neurons. The morphology and connectivity of each one of these neurons has been defined at high resolution (White *et al.,* 1986).

By adding the presumed promoter region of a gene up to a reporter system, we can study the developmental regulation and tissue specific expression of a gene. GFP is a naturally fluorescent protein which can be used to mark the cells in which a promoter is active (Fuhrmann et al. 1999). GFP has the benefit that the animals can be observed live. Studies of gene expression pattern could help with the behavior study if we know which neuron the *mgl* genes are expressed in. For example, LEV-8 is expressed in all six DD motor neurons which innervate the dorsal muscles and is likely to regulate their relaxation (Tower *et al.,* 2005).

To elucidate the expression pattern of the *C. elegans* mGluR genes, Ishihara T *et al.* generated transgenic worms carrying a mGluR-GFP fusion gene. From their experiments, the fusion gene of *mgl-2* is reported to be expressed in some interneurons (AIBL, AIBR, AVE, AVER, RIBL, RIBR, RMDDL, RMDDR, RMDL, RMDR, RMDVL, RMDVR, SMDDL, SMDDR, SMDVL, SMDVR), while that of *mgl-1* is reported to be widely expressed in motorneurons and pharyngeal neurons as well as interneurons (AIAL, AIAR, AIYL, AIYR, RIBL, RIBR, RMDDL, RMDDR, RMDL, RMDR , MDVL , RMDVR). Thus a further study of *mgl-2* expression pattern is necessary to verify this restricted expression. The expression pattern of *mgl-3* has not been elucidated yet. In our lab J

Dillion has characterized the *mgl-1* expression pattern which shows *mgl-1* is expressed in pharynx and tail. This chapter describes my efforts to define the expression patterns *mgl-3*.

4.2 GFP Tagging

GFP is a protein of 238 amino acids which spontaneously emits green light at 508 nm when excited with blue light at 395 nm in the presence of O_2. Its major advantage results from its intrinsic property of fluorescing in the absence of any added cofactor or substrate, thus allowing nondestructive detection of recombinant cells expressing this reporter gene. GFP is very stable and photobleaches very slowly even after repeated observations under the epifluorescence microscope.

We used PCR based GFP tagging to study the *mgl* expression patterns. DNA encoding GFP can be fused to other genes. In transfected cells the GFP-fusion protein can be observed. 5' region of the gene of interest can be inserted into GFP vector and transfect cells. GFP vector L3691 pPD117.01 obtained from Fire's lab (Stanford University School of Medicine) was used to make the constructs. Upstream from the position +2 after the cleavage site of the signal peptide, 5kb *mgl-3* promoter region was amplified by PCR. After *Sal*I and *Not*I enzyme digestion, 5kb *mgl-3* promoter regions were inserted into the GFP vector. Sequence analysis was carried out to determine the insertions.

Transgenic animals in *C. elegans* were generated by microinjection. The DNA construct with *mgl-3* was mixed with a co-injection marker *pha-1* using the vector pBX and injected into the distal gonad

(syncytium) of the temperature sensitive embryonic lethal mutation *pha-1(e2123).* The mutation grows normally at 15°C, but it is 100% embryonic lethal at 25°C. The injected DNA is taken up into the mature oocyte's nucleus. The DNA exists as an extrachromosomal array which segregates randomly and can be lost, that is why we need a marker to follow which animals have the array. At 25°C the plasmid pBX complements the embryonic lethality of the *pha-1* mutation. Thus transgenic worms can be selected by shifting F1 larvae of injected hermaphrodites from 15°C to 25°C. Only transformed progeny of these worms will survive this selection. To maintain the strain it is grown at 25°C.

4.3 Results

We generated an *mgl-3* transgenic line. 5kb *mgl-3* promoter region was amplified as shown in figure 4.1 and inserted into GFP vector. This construct was analyzed by sequencing. It confirms the fusion of *mgl-3* promoter with GFP.

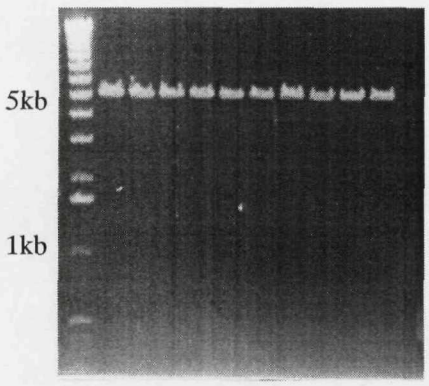
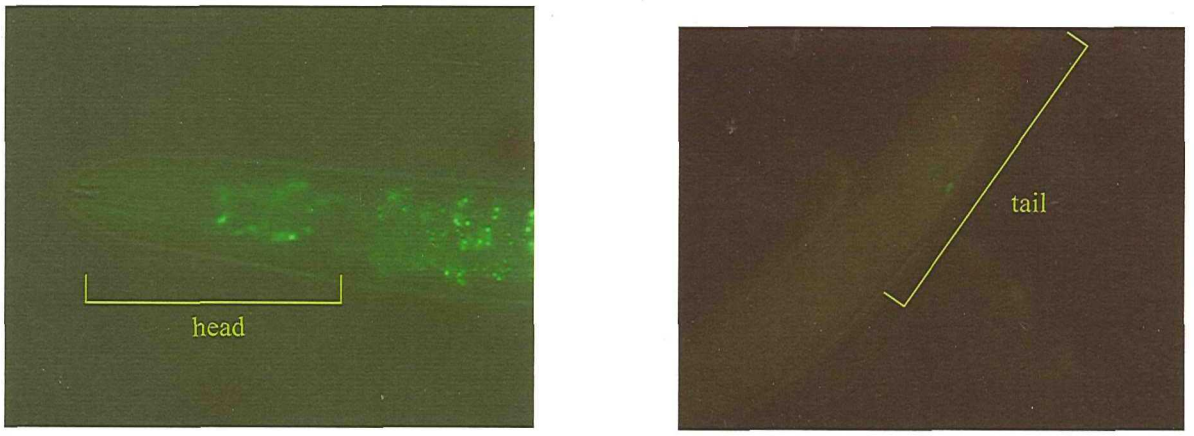


Figure 4.1 PCR of 5kb *mgl-3* promoter region. 10 PCR reactions were performed to generate a large amount of 5kb *mgl-3* promoter region. It was then digested by enzyme and inserted into GFP vector.

The construct was micro-injected into young adult animal. The gene expression pattern is shown in figure 4.2. This study suggests that *mgl-3* is expressed in some head neurons and one tail neuron.



(A) (B)

Figure 4.2 Expression pattern of *mgl-3*. *mgl-3* is expressed in the head neurons of the worm (A) and one tail neuron (B).

There are 302 neurons in *C.elegans* including sensory neurons, interneurons and motor neurons. The cell bodies of most sensory and interneurons are found in ganglia that reside just anterior and posterior of the nerve ring and a set of ganglia in the tail. Most motor neuron cell bodies reside in the ventral nerve cord. Most of the sensory neurons have dendrites that extend into the nose, a cell body in a nerve ring ganglia and then extend a process into the nerve ring where they receive and make synaptic connections with other neurons. Most interneurons have a cell body in the nerve ring ganglia and extend a process into the nerve ring where they make contacts with sensory and command interneurons.

To identify the neurons where *mgl-3* is expressed, we divided the neurons into 3 groups: pharyngeal neurons, head neurons and tail neurons. When the pharynx was cut off the worms, a pair of GFP expressed neurons could be seen as figure 4.3 shows.

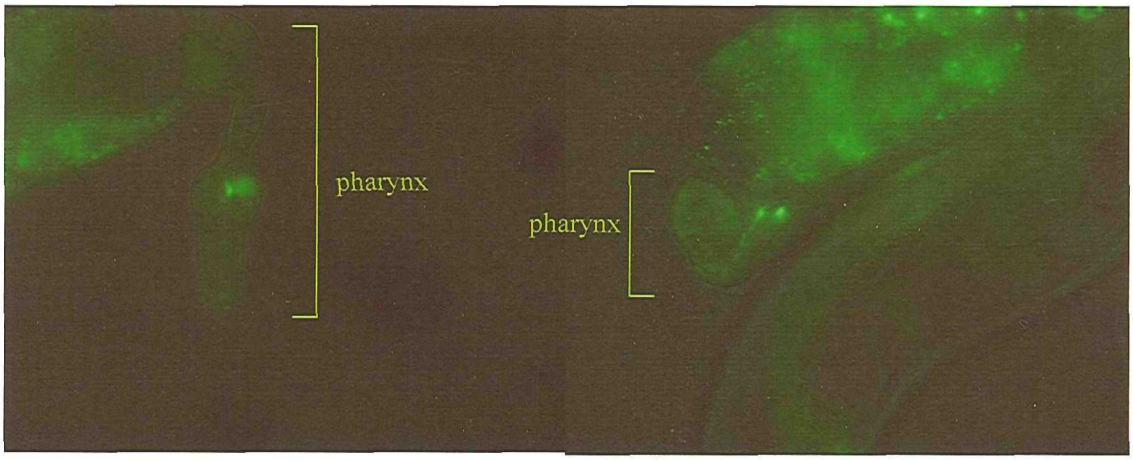
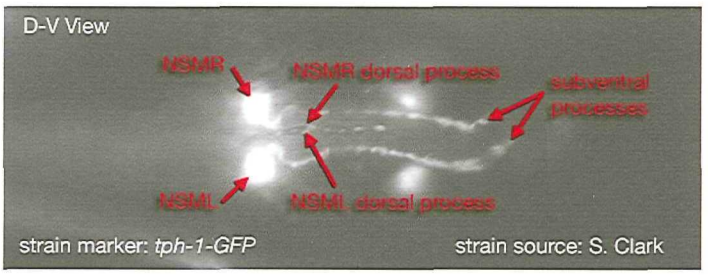


Figure 4.3 One pair of GFP expressed neurons exists in the pharynx. Here are two examples showing a pair of GFP expressed neurons are located in the pharynx which was cut off.

There are 20 neurons in the pharynx. By looking at the position of cell bodies, the length, direction and length of process, this pair of pharyngeal neurons is most likely NSM neurons (figure 4.4). Only six pharynx neurons are paired (figure 4.4C). Among these six pairs, only NSM neurons are located at the metacarpus and their processes go along to the terminal bulb.

A



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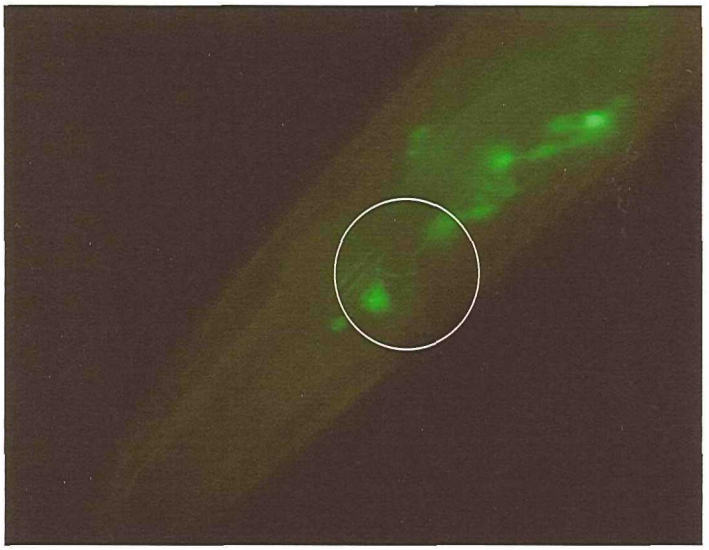


C

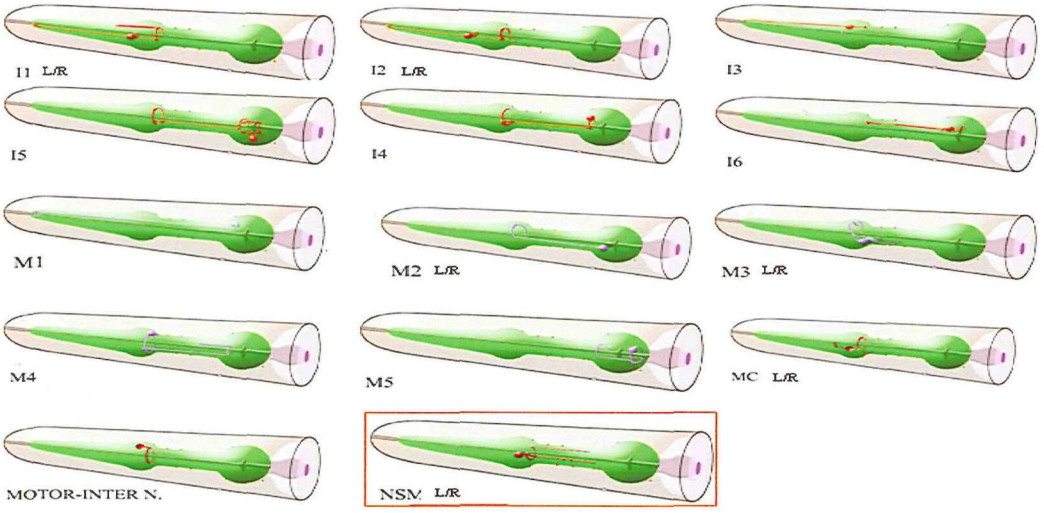
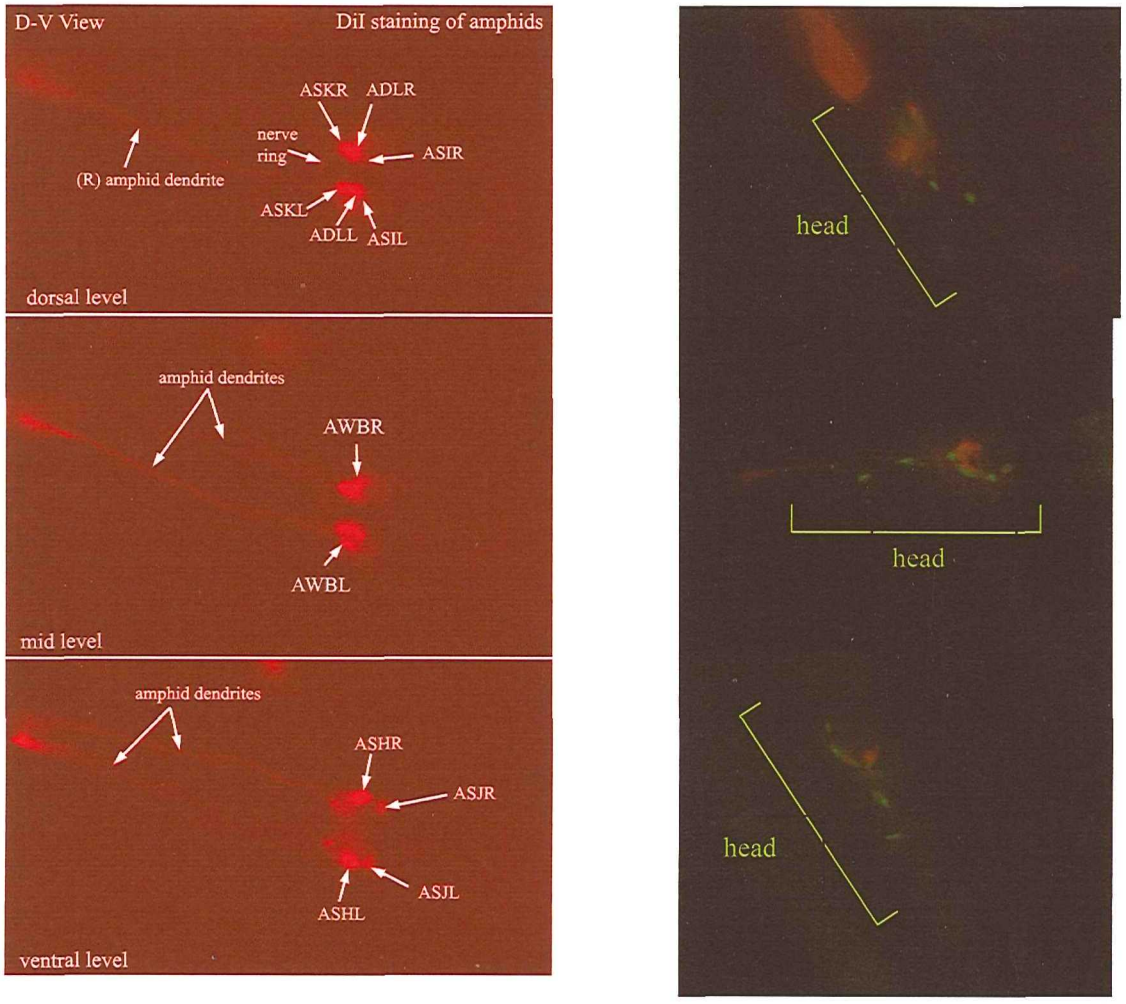


Figure 4.4 (A) NSM neurons (www.wormatlas.org). NSM has a special shape shown in the figure and it is easy to be identified. **(B) NSM neurons in transgenic worms.** From the shape of the neurons in transgenic worms, we assume they are NSM neurons. **(C) 20 pharyngeal neurons** (copied from www.wormatlas.org). It shows the shape and localization of pharyngeal neurons.

To identify whether any of the tagged neurons are amphid sensory neurons, DiI was used to stain the neurons. DiI labeling does not appreciably affect cell viability, development, or basic

physiological properties. The stained neurons did not overlay with the GFP neurons. Thus those head neurons where *mgl-3* was expressed are not amphid sensory neurons (figure 4.5).



(A) **(B)**

Figure 4.5 (A) Stained amphid neurons (molecular probes, product information 2005). It shows the localization of amphid neurons. **(B) The GFP neurons in stained transgenic worms.** It shows amphid neurons did not overlay with GFP neurons.

In summary, our candidate neurons for *mgl-3* expressed neurons are shown in figure 4.6. There are 9 pairs of neurons in the head. Based on the shape and localization of them, we assumed candidate 1 is NSM neurons. Candidate 2, 3 might be OLL, OLQ, URB or URYV neurons. Candidate 4 might be OLL, URB, or URYV neurons. Candidate 5, 6, 7, 8, 9 might be RID, ALA, URX, SMD, RIA,

AVA, AVE, RMD, AVH, AVJ, AIN, AVB, RIM, RIB, SIA, SIB, SMD, RIM, SMB, or RIC.

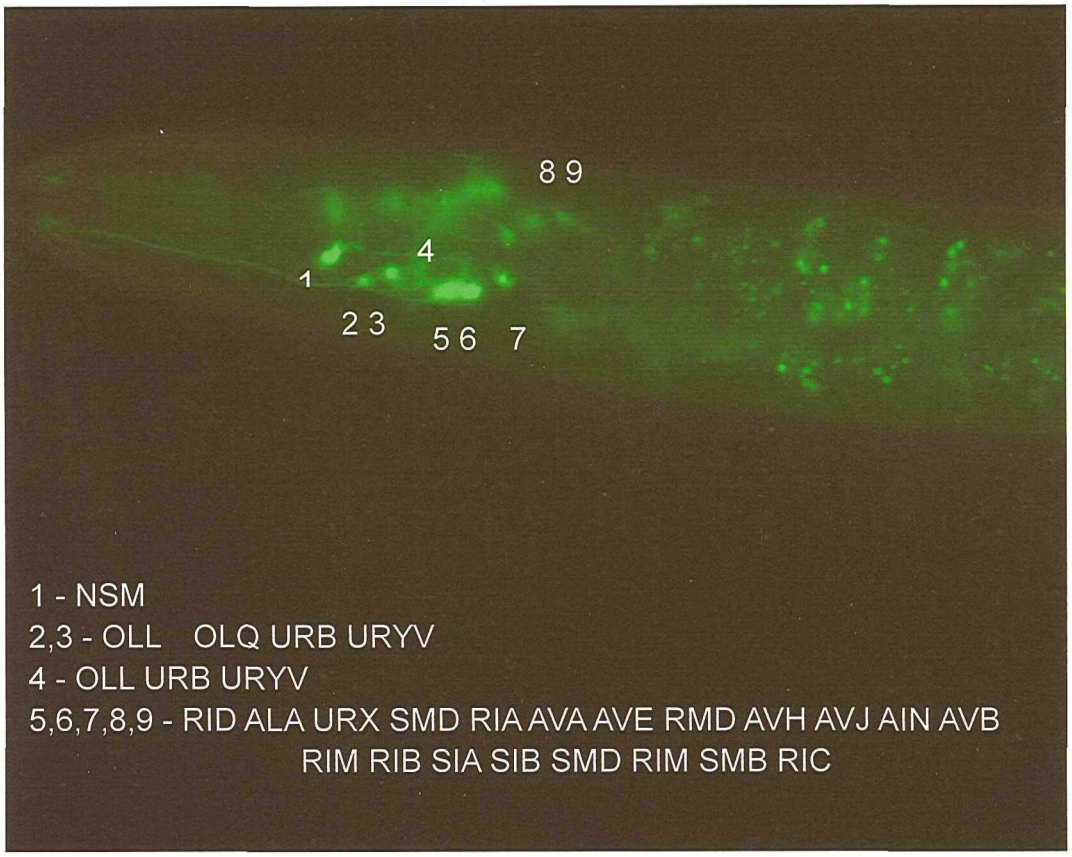


Figure 4.6 Candidate neurons. By looking at the shape and localization of these nine pairs of neurons, we assumed the candidate neurons are those listed in the figure.

4.4 Discussion

We generated an MGL-3::GFP transgenic strain to study *mgl-3* expression patterns. In the meanwhile we also made an *mgl-2*::gfp gene construct and tried to generate transgenic strain although it has not come to a success.

This study suggests that *mgl-3* is expressed in NSM neurons. The NSM neurons are serotonergic (Horvitz et al., 1982; Avery and Horvitz, 1990) neurosecretory motor neurons (Albertson and Thomson, 1976) that can stimulate pharyngeal pumping (Avery and Horvitz, 1990). It indicates that *mgl-3* might be involved in the regulation of pharyngeal pumping.

CHAPTER 5
Behavioural Studies

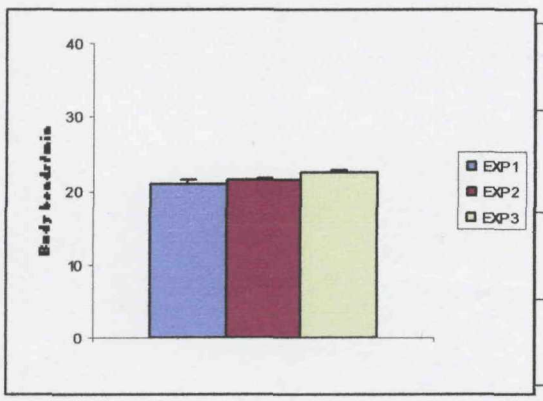
5 Behavioural Studies

5.1 Introduction

C.elegans behaviours have been studied by mutational analysis (see Chapter1 Introduction 1.10) and the functions of a number of genes have been analyzed. In this study we are analyzing *mgl* functions in regulating worm behaviour with mutant strains. The behaviours that we are looking at includes body bends, thrashing, forward and backward movement, pharyngeal pumping, foraging and habituation.

5.2 Body Bends

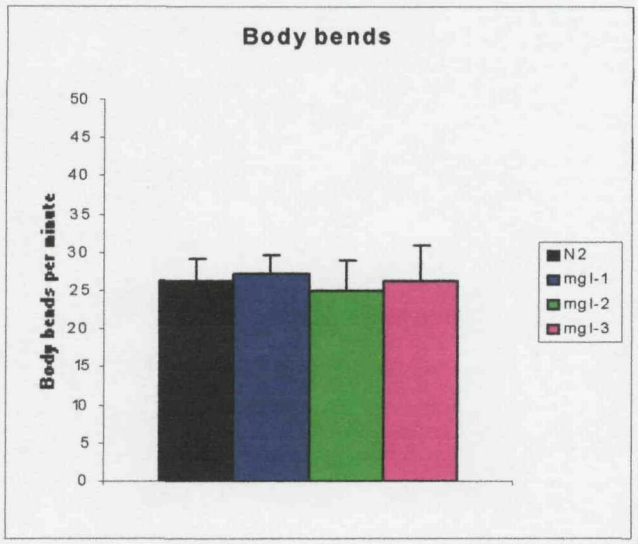
Well fed young adult worms were placed on no food plates at 20°C 5mins prior to the assays. Body bends during one minute were recorded. Studies on the wild type show that at 20°C on the no food plate their body bends during one minute is 20-30.



	Experiment 1	Experiment 2	Experiment 3
Number of worms	50	50	50
Average	20.84	21.08	22.3
STDEV	3.79	4.20	4.84

Figure 5.1 Three experiments were carried out separately to study the wild type body bends movement. In each experiment, 50 worms were tested. It shows that at 20°C on the no food plate the number of the wild type body bends during one minute is 20-30.

Studies on the mutants show that *mgl-1(tm1811), mgl-2(tm355)* and *mgl-3(tm1766)* have the similar body bends on the no food plate with the wild type (N2).

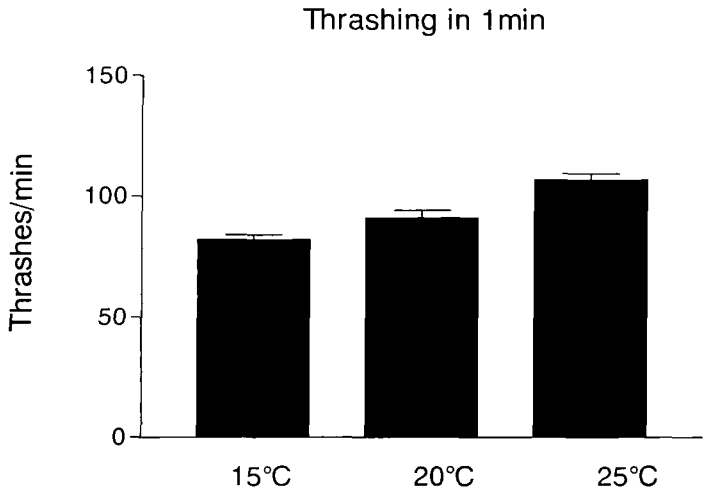


	N2	mgl-1	mgl-2	mgl-3
Number of worms	50	50	50	50
Average	26.14	27.18	24.92	26.12
STDEV	4.13	3.49	5.09	6.09

Figure 5.2 Body bends assays on *mgl* mutants. It shows that the *mgl* mutants have the similar body bends movement as the wild type. 50 worms of each strain were tested. They are not significantly different from control (ANOVA p=0.14).

5.3 Thrashing

Thrashing behavioural assays at different temperatures were performed to check the temperature effect. Worms were grown on the OP50-seeded plates placed separately in three incubators at different temperatures, 15°C, 20°C and 25°C. Three tubes containing M9 buffer were prepared and stored separately in those three incubators. Worms and M9 buffer were taken out from the incubators just before doing the assays and were kept in the incubators when not being used. 20 wild type worms from different-temperature incubators were tested in correspondent-temperature M9 buffer. As figure 5.1 shows, at 20°C wild type worms thrashed about 20% more than at 15°C, 20% less than at 25°C.

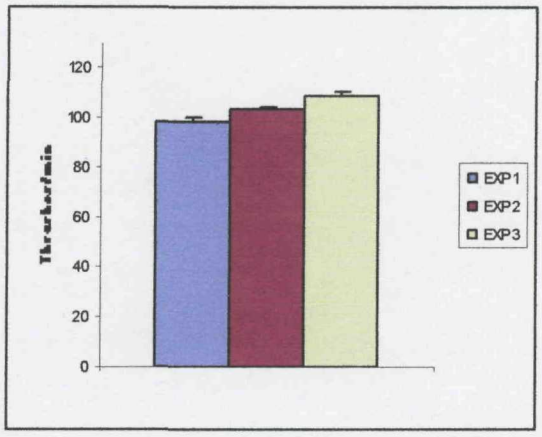


N2 Thrashes/min	15°C	20°C	25°C
Number of worms	20	20	20
Average	82	91.05	106.9
STDEV	9.35	14.78	12.09

Figure 5.3 Thrashing assays at different temperatures. Wild type at 25 ºC thrashed 40% more than at 15 ºC. N(number of worms tested for each)=20.

Temperature affects the thrashing behaviour and higher temperature stimulates a higher thrashing rate. In the following all assays, the temperature was controlled at 20ºC to avoid the variance caused by the temperature.

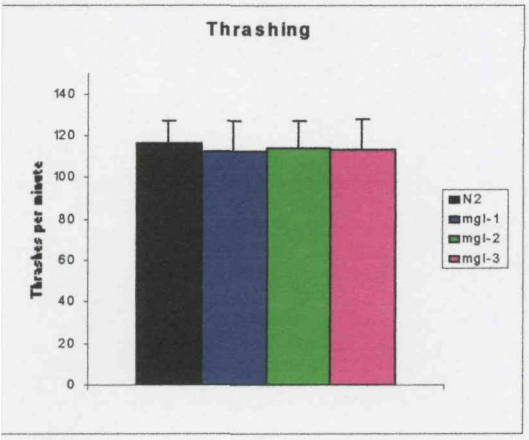
Studies on the wild type show that worms thrash 100-110 times during one minute in the M9 buffer at 20ºC. Well fed young adult worms were placed in M9 buffer at 20°C 5mins prior to the assays. Thrashes during one minute were recorded.



N2 Thrashes/min	Experiment 1	Experiment 2	Experiment 3
Number of worms	50	50	50
Average	99.3	104.58	109.18
STDEV	11.42	9.29	9.55

Figure 5.4 Three experiments were carried out separately to study the wild type thrashing in M9. In each experiment, 50 worms were tested. It shows that at 20°C the worms thrash 100-110 times during one minute in M9.

Studies on the *mgl* mutants show that they behave similarly with the wild type in M9 buffer at 20°C.



Thrashes/min	N2	*mgl-1*	*mgl-2*	*mgl-3*
Number of worms	50	50	50	50
Average	116.14	113.02	113.98	113.3
STDEV	14.88	17.50	16.34	18.09

Figure 5.5 Thrashing assays on *mgl* mutants. It shows that the *mgl* mutants have the similar thrashing movement as the wild type. 50 worms of each strain were tested. And no significant difference was seen (ANOVA p=0.79).

5.4 Forward and Backward Movement

Movement of *mgl* mutants was measured to elucidate their functions in regulating locomotion.

5mins after transfer to a no food plate a backing motion of the worms was initiated by touch.

Subsequent forward, backward and stop periods were recorded for 5mins. Worms that moved continuously forward after the initial backing were scored until they stopped or backed. On average the wild type worms go forward for about 50s then go backward once for about 4s.

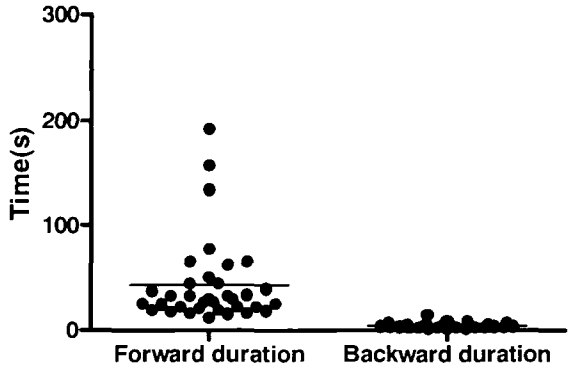
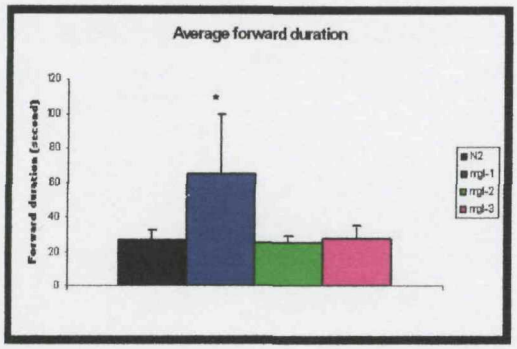


Figure 5.6 Forward and backward duration of the wild type. 22 worms were tested and it shows that averagely every 50s the worm initiates backward movement. The bar shows the mean value, each point is the value obtained from one animal.

The data from mutant strains represent averaged forward duration for individual worms meaned for 50 animals from each strain. * $P<0.0001$ (ANOVA). The switch between forward and backward locomotion is selectively disrupted in the *mgl-1* mutants.

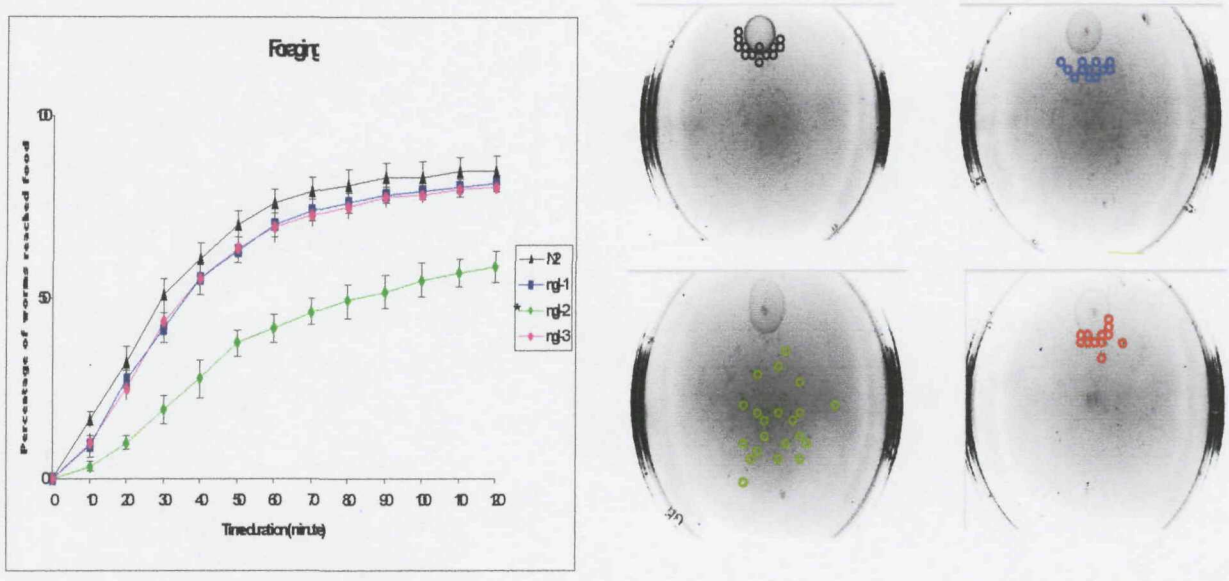


Average forward duration (s)	N2	*mgl-1*	*mgl-2*	*mgl-3*
Number of worms	50	50	50	50
Average	26.74	65.45	25.39	27.76
STDEV	7.82	69.08	5.11	15.93

Figure 5.7 Forward duration of *mgl* mutants. This measurement was carried out at 20°C on no food plates. '*' represents P <.0001 (ANOVA). It shows *mgl-1* mutants have a longer forward duration than others. The switch between forward and backward locomotion is selectively disrupted in the *mgl-1* mutants.

5.5 Locomotion towards Bacteria

9cm plates were seeded with 50 ml of fresh OP50 (OD600 0.8A) at 2cm from the edge and incubated at 20-21°C. Well washed worms (70-120) were placed at the opposite pole (2 cm from the edge) and scored for their ability to re-locate to the food source with NaN3 (200mM. 5 µl). Left panel in Fig 5.4 shows mean data from 3 independent experiments plotting % of plated worms that reach the food over time and highlights that *mgl-2* mutant is selectively retarded (P<0.001; ANOVA). Right panel shows qualitative representation of typical plates seeded with the different strains (N2 and *mgl-1(tm1811)*, *mgl-2(tm355)* and *mgl-3(tm1766)*). The *mgl-2(tm355)* mutants are deficient in sensory induced locomotory behaviour (figure 5.8).

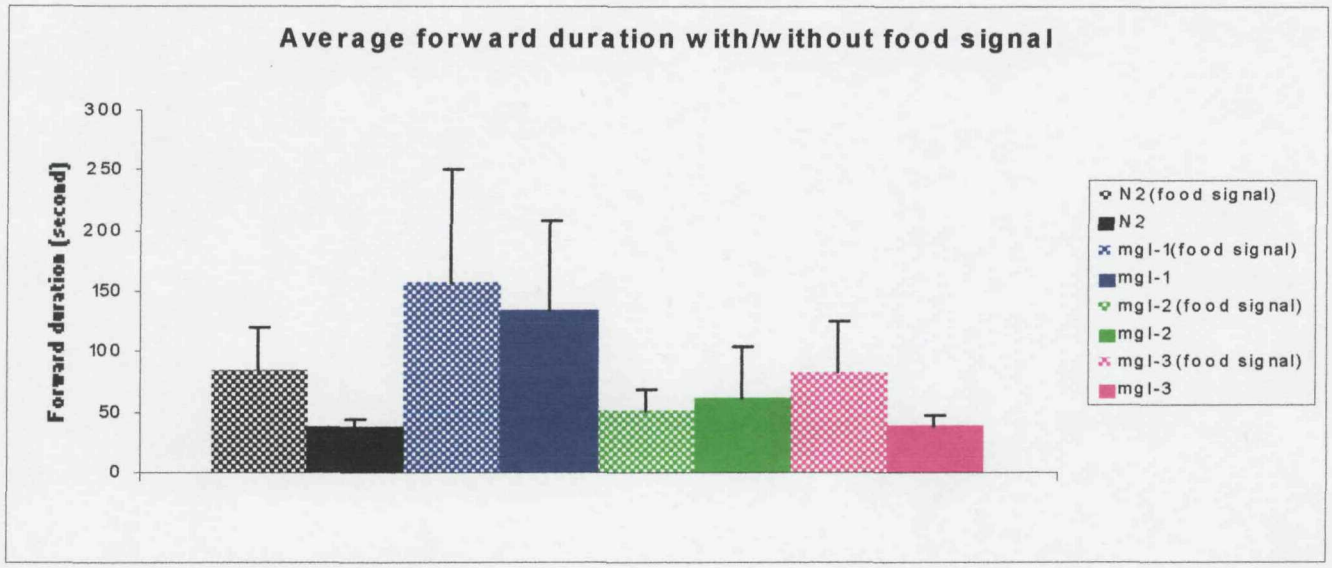


(a) (b)

Figure 5.8 Locomotion towards bacteria is disrupted in *mgl-2* mutants. (b) Black circles represent N2 worms, blue circles represent *mgl-1(tm1811)*, yellow circles represent *mgl-2(tm155)*, and red circles represent *mgl-3(tm1766)*.

5.6 Forward and Backward Movement with food stimuli

To further analyze the locomotion behaviour of mutants towards bacteria, single worm locomotion experiments were performed. Plates were seeded with bacteria or no food prior to placing individual worms at the pole opposite the food source. After 5mins equilibration worm movements were scored as described in figure 5.5. Food increased the forward duration for all strains except *mgl-2*. Note the increase forward duration *mgl-1* mutants are not significant but these animals already have an increased forward duration relative to wild-type (see figure 5.9).



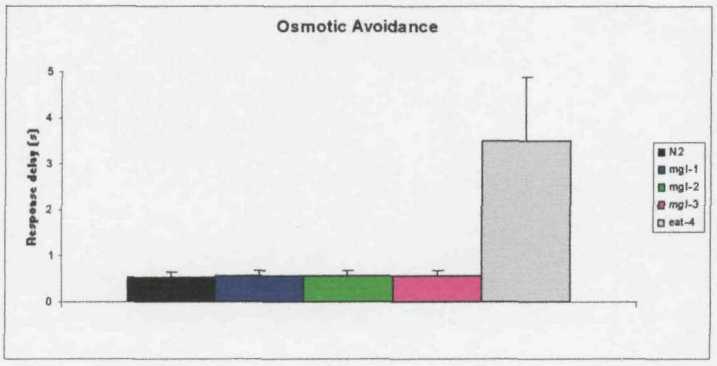
Average forward duration (s)	N2 (food)	N2	*mgl-1*(food)	*mgl-1*	*mgl-2*(food)	*mgl-2*	*mgl-3*(food)	*mgl-3*
Number of worms	30	30	30	30	30	30	30	30
Average	84.39	36.45	156.27	134.45	51.05	62.60	83.42	38.43
STDEV	54	10	109.17	92.64	21.98	67	67.52	14.32

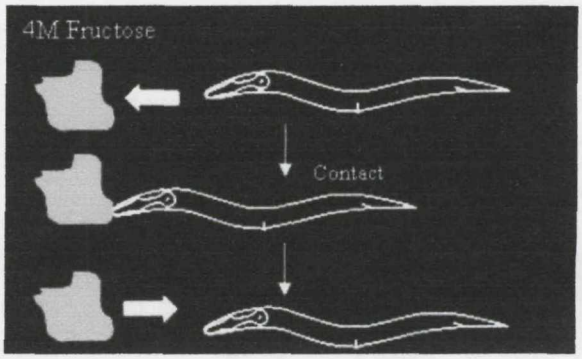
Figure 5.9 Bacteria triggers an increase in forward locomotion that is selectively disrupted in *mgl-2* mutants. It represents the average forward duration for individual worms and shows the mean value for 30 worms for each condition.

5.7 Osmotic Avoidance

To check the effects described in 5.3, 5.4, 5.5 do not reflect general disruption in sensory induced locomotion we tested whether *mgl-1*, *mgl-2* and *mgl-3* mutants reversed normally when exposed to a hyperosmotic solution (4M fructose). *C.elegans* were equilibrated on a no food plate before a fructose drop (2µl) was placed in the path of an advancing worm. The time taken to respond to contact with the fructose, defined by a clear backing movement, was recorded. *mgl* mutants performed like N2 and rapidly reversed in response to fructose (ANOVA $P>0.1$). In contrast *eat-4(ky5)* mutants which lack vesicular stores of glutamate showed a marked delay (ANOVA $P<0.01$) in their response times as described previously (de Bono and Maricq, 2005). These data

support the notion that the disrupted locomotion of the *mgl-1* mutants off food and the disruption of locomotion towards bacteria in *mgl-2* mutants reflect selective deficits in the glutamatergic pathways that regulate locomotory behaviour.





Response Delay (s)	N2	*mgl-1*	*mgl-2*	*mgl-3*	*eat-4*
Number of worms	50	50	50	50	50
Average	0.53	0.55	0.57	0.56	3.5
STDEV	0.15	0.13	0.13	0.14	1.57

Figure 5.10 Deficent glutamatergic transmission (*eat-4*) disrupts sensory (fructose) dependent changes in locomotion but is unaffected in the *mgl* mutants. This experiment was carried out at 20°C.

5.8 Discussion

We have noticed the variations of behaviour caused by environment, such as plate condition, temperature. We have defined several behavioural assays on the wild type and subsequently tested on the mutant strains. *mgl-1(tm1811)* mutants have a selectively disrupted switch between forward and backward locomotion. Backward movement is regulated during foraging. When a food source is detected by worms, backward movement dramatically decreases. Locomotion towards bacteria is

disrupted in *mgl-2(tm355)* mutants, they could not get to the food source as fast as the wild type. *mgl-3 (tm1766)* behaves normally in all the tested assays.

CHAPTER 6
DISCUSSION

6 Discussion

6.1 Limitations of the Study

In my study, there are some experimental limitations. To analyze gene effect on live worms' behavior, other factors must be eliminated such as enviroment factors. Enviroment factors which include temperature and air could affect live worms' behavior and it can not be completely avoided. Although all the behavioral assays were performed in temperature controled room, the temperature can still be 0.1° C up and down.

The age of the worms is another limitation of the study. Worms at different ages behave differently. Although we snychronized the worm population by bleaching, worms were not exactly at the same age because of the enviroment factors. And durting the experiments, worms were tested one by one, not at the same time. It makes the age of the tested worms slightly different. Thus the results of the experiments are not exactly precise.

6.2 Discussion of the Results

Glutamatergic transmission plays an essential role in the control of *C.elegans* nervous system as manifest by changes in basic and integrated behaviour. Our data provide additional evidence that G-protein coupled receptors activated by glutamate play an important neuromodulatory role for glutamatergic transmission.

In 1997 Ishihara T *et al* have reported that *mgl-1* is expressed in pharyngeal neurons. Our study of expression patterns showed that *mgl-1* and *mgl-3* both are expressed in NSM pharyngeal neurons (figure 6.1). The NSM neurons are serotonergic (Horvitz et al., 1982; Avery and Horvitz, 1990)

neurosecretory motor neurons (Albertson and Thomson, 1976) that can stimulate pharyngeal pumping (Avery and Horvitz, 1990). This indicates that *mgl-1* and *mgl-3* might be included in the same circuit and function together in pharyngeal pumping. The full understanding of *mgl* expression patterns could provide more evidences about their functions.

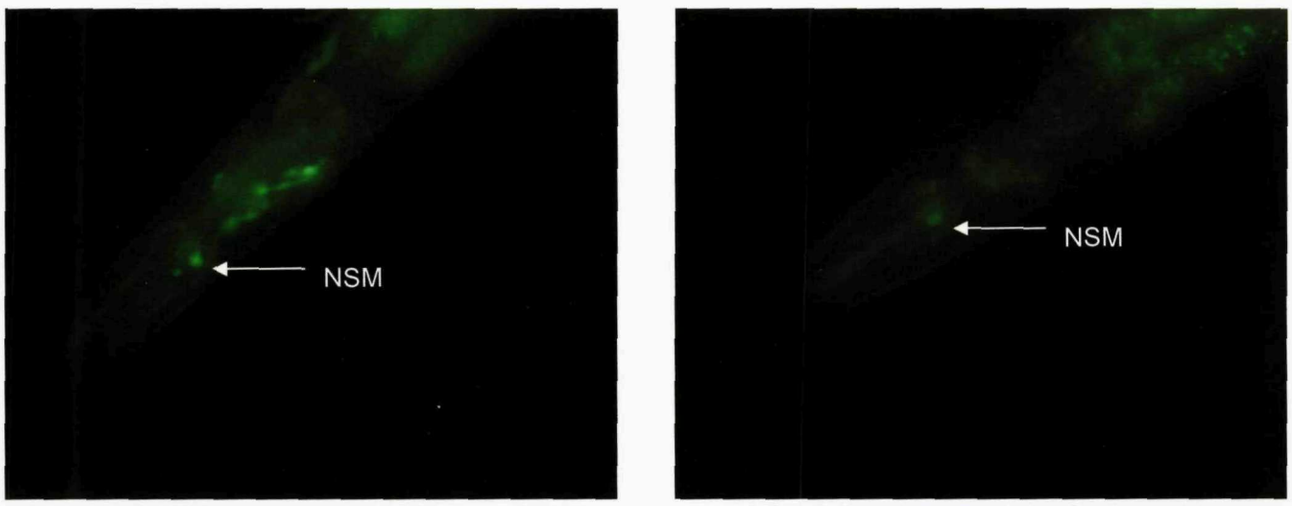


Figure 6.1 NSM neurons express *mgl-3* (left) and *mgl-1* (right). It shows that both *mgl-1* and *mgl-3* are expressed in NSM neurons (refer to Fig 4.4).

mgl genes are not essential in terms of living and have no effects in growth and aging. After knocking out *mgl* genes from *C.elegans*, mutant worms are still viable on NGM plates. This provides the availability to investigate the gene functions by behavioural studies on the mutants. Some subtle behavioural phenotypes were observed on the mutant worms. The switch between forward and backward locomotion is selectively disrupted in the *mgl-1* mutants. The *mgl-2* mutants are deficient in sensory induced locomotory behaviour. *mgl-1* and *mgl-2* regulate integrated locomotion behaviour. Investigations of locomotion and its integration by sensory inputs (e.g. food/no food) have defined a high order circuit of sensory inputs that are integrated via primary and secondary interneurons leading to modified motor outputs (figure 6.2).

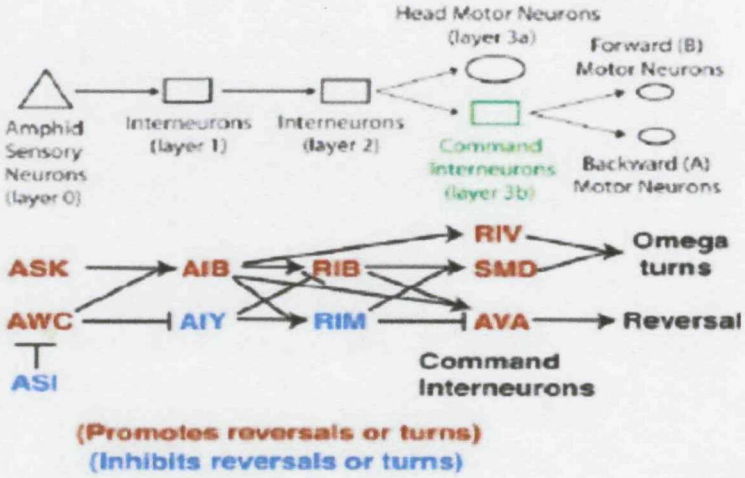


Figure 6.2 Locomotion circuit (Gray et al., 2005). Neuronal functions in the navigation circuit from sensory input to motor output. ASI may act partly by inhibiting AWC. ASK and AWC may act by inhibiting AIY and stimulating AIB. Omega bends are generated by head motor neurons, and reversals are generated by the command interneurons.

Consistent with our proposed neuromodulatory role of *mgl*s in the integration of complex behaviours preliminary expression studies (www.wormbase.org) identify that *mgl-1* and *mgl-2* are expressed in the interneurons (AIB, RIB, SMD, AIY) that integrate the sensory inputs that modify patterns of motor outputs. Although it is hard to predict what loss of function mutation will do to the circuit, our analysis provides a platform to further investigate the receptor functions.

6.3 Future Study

As we have found that *mgl-1* and *mgl-2* are modulating the locomotion behaviour, it is interesting to know how the *mgl* double mutants and triple mutants perform in the forward and backward locomotion with/without bacteria experiments. This can further analyze *mgl* roles in regulating locomotion and also the functional relationships among *mgl*s. More complex behaviour experiments (eg. Locomotion towards bacteria with barrier) are worth to be carried out for further elucidating how *mgl-1*, *mgl-2* and *mgl-3* modulate *C.elegans* behaviour. This behavioural analysis of *mgl* mutants and expression pattern study of MGL provides an insight of neuromodulatory roles

for these G-protein coupled receptors and suggest they play a broad role in defined circuits that integrate complex behaviour.

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Appendix I Isolating Deletions in PCR Screens of Mutagenized Populations

Isolating Deletions in PCR Screens of Mutagenized Populations was used for generating *mgl-1* and *mgl-3* mutants for further study.

i. Growth and Synchronization for Mutagenesis

Worms were grown on 5-10 15cm NGM plates (about 30 L4s per plate) to generate a large population of adults. The adults were washed off of the plates and treated with basic hypochlorite to harvest the eggs. Worms were collected by centrifugation in M9 Buffer (22mM KH2PO4, 22mM Na2HPO4, 85mM NaCl, 1mM MgSO4), and bleached. Eggs are collected by centrifugation (2000g, 2 minutes). Wash the eggs 4x with 10 volumes M9 and collect by centrifugation. The eggs were distributed to one unseeded plate. The plate was set at 20°C overnight. All the eggs hatched and the animals arrested as starved L1's. This produced synchrony of the population. The animals were washed off the plate with M9, spun down to a small volume (<200 μl), and distributed on 15 cm plates seeded with OP50. Six 15 cm plates generally provided an adequate amount of food to allow the animals to grow to the L4 stage without starving. The plates were set at 20°C.

ii. EMS Mutagenesis

To an 8ml suspension of worms in M9 Buffer, 8 ml of M9 containing 100 mM methane sulfonic acid ethyl ester [final EMS concentration 50mM] (Sigma #M-0880) was added. The worm suspension was incubated in a horizontal, capped 50ml centrifuge tube at room temperature for four hours with moderate shaking. Then the worms were washed 5x with 10 volumes of M9 Buffer. After each wash the worms are collected by centrifugation at 2000g for 5 minutes.

The mutagenized worms were plated on a total of 10-20 15cm NGM plates seeded with OP50 and allowed to grow for 24 hours. F1 eggs were collected by treating the worms with basic hypochlorite as described in the method 2.3.6. The eggs were allowed to hatch on the surface of a 15cm NGM plate without OP50. All of the worms were washed off with 5 mls of M9 per plate, and transferred to a 15 ml centrifuge tube. The worms were spun down, the supernatant was removed, and washed twice. The worms were bleached as stated in the method 2.3.6. The pellet of surviving eggs were transferred on two unseeded plates, and add 10 mls of S medium to the remaining empty petri dish overnight. This will produce yields of about 80-100 thousand L1 animals.

iii. Library Plating

The F1/L1 worms were collected in M9 Buffer. The worms were distributed in groups of 500 onto 1152 6cm OP50 seeded RNGM plates using a repeating pipettor. The plates were stored in groups of 96, (labeled A-L = 12 groups), in lidded plastic containers. The worms were allowed to grow for five days at 20°C.

iv. Library Harvest

0.75ml of sterile distilled water containing streptomycin (100μg/ml) and Mycostatin (= Nystatin) (12.5μg/ml) was added to each plate. 150μl of the worm suspension from each plate was transferred to the wells of a deep, 96-well microtitre plate. The spent micropipette tip was temporarily ejected into the well.

v. DNA preparation and PCR

150μl of Proteinase K solution was added to each well (50 mM KCl, 10 mM Tris- HCl pH 8.3, 2.5

mM MgCl2, 0.45% NP-40, 0.45% Tween-20, 0.01% Gelatin, 200μg/ml Proteinase K). The blocks were sealed with a AB gene Easy-Peel Polypropylene foil. The worms were frozen at –80°C for 20-30 minutes and then incubated with intermittent mixing, for six hours to overnight at 65°C. Three rounds PCR was performed to screen the mutations.