GENERATION AND ANALYSIS OF SUPPRESSORS OF *Caenorhabditis elegans unc-2* ALLELES

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ABSTRACT

The *unc-2* gene of the nematode worm *C. elegans* encodes a voltage-gated calcium channel (VGCC) that plays a central role in regulating neurotransmitter release. The objective of this project was the identification of genes whose products are important in UNC-2-mediated neurotransmission. Towards this end, twelve suppressors of the uncoordinated phenotype of *unc-2(e55)* were generated. While most of the suppressors appeared not to affect worm locomotion to a large degree, a subset resulted in uncoordinated phenotypes when the *unc-2(e55)* mutation was absent. Among these suppressors, only one was found to be allele-dependent. Based on aldicarb-sensitivity tests, the localizations of the suppressor gene-encoded products relative to the synapse structure was determined. Six of the twelve suppressors localize to the presynaptic neuron. Of the remainder, four appeared to function synaptically, while only two localized to the postsynaptic cell. The linkage of two of the suppressors was determined via SNP mapping.

DEDICATION

This thesis is dedicated to mother and father.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACh	acetylcholine
AChE	acetylcholinesterase
AID	α_1 subunit interaction domain
AP	action potential
Ba^{2+}	barium
BID	β subunit interaction domain
bp	basepair
Ca^{2+}	cadmium
CDI	calcium-dependent inactivation
CICR	calcium-induced calcium release
DHP	dihydropyridine
DHPR	dihydropyridine receptor
DNTPs	deoxyribonucleoside triphosphates
E-C	excitation-contraction
EDTA	disodium ethylenediaminetetraacetate
egl	egg laying defective
GABA	γ-aminobutyric acid
HVA	high voltage-activated
kb	kilobase
LVA	low voltage-activated

mM	millimolar
ms	millisecond
mV	millivolt
nAChR	nicotinic acetylcholine receptor
Na^+	sodium
Ni^{2+}	nickel
NMJ	neuromuscular junction
ω-Aga	ω-agatoxin
ω-CTx	ω-conotoxin
ω-CgTx	ω-conotoxin GVIA
PCR	polymerase chain reaction
PKA	protein kinase A (cAMP-dependent protein kinase)
PKC	protein kinase C
pS	picosiemen
rel. T_{50}	normalized T_{50} value
rel. TPM	normalized TPM value
RyR	ryanodine receptor
SNP	single nucleotide polymorphism
suX55	sX;*unc-2(e55)*-homozygous animal or group
suX612	sX;*unc-2(ra612)*-homozygous animal or group
sX	sX-homozygous animal, group, mutation associated with strain
T_{50}	time point of 50% paralysis
TPM	thrashes per minute
UV	ultraviolet

τ	time constant
μl	microlitre
μg	microgram
unc	uncoordinated
VGCC	voltage-gated calcium channel

CHAPTER ONE.
INTRODUCTION

Voltage-gated calcium channels are involved in many important biological processes

Voltage-gated ion channels mediate ion influx in response to changes in membrane potential, and they are of great importance to complex multicellular animals; indeed, without the ion currents that they generate in cells, processes such as muscle contraction and nervous impulse transmission would not be possible. The way in which ion channels perform their function is nothing short of remarkable. Upon sensing a change in voltage across the plasma membrane in which they are embedded, these channels switch from being impermeable structures to conduits that are able to select specifically for just one ion type from the extracellular fluid, and pass them into the cell at high rates.

It could be argued that of all the voltage-gated ion channels, voltage-gated calcium channels (VGCCs) are the most interesting, for not only do they generate electrical activity in cells, they also are responsible for controlling the intracellular concentrations of calcium, and therefore are critical participants in excitation-contraction coupling, neurotransmitter release, cell migration and regulation of gene expression (Catterall, 1991; Uchitel *et al.*, 1992; Komuro and Rakic, 1992; Bading *et al.*, 1993), to name a few. Given the multitude of processes that are regulated by the calcium current, it is perhaps not surprising that VGCC dysfunction can lead to disease states. Mutations in seven of the sixteen known human calcium channel genes have each been linked to

disorders such as familial hemiplegic migraine and epilepsy (Burgess and Noebels, 1999). Conversely, drugs that affect VGCC function may also be used to treat or moderate the effects of certain medical conditions.

It is therefore imperative to achieve a better understanding of VGCC function. Some of the most important questions yet to be answered at this point are: how do the differences in the structure of various calcium channels give rise to differences in the calcium current produced? What components are involved in the various pathways that are regulated by calcium channels? What components regulate calcium channel activity? And how can we better control the calcium current using pharmacological compounds?

For this study, I therefore sought to identify genes that are involved in modulating the function of a VGCC encoded by *unc-2* in the nematode worm *Caenorhabditis elegans*. To this end, mutations that suppress the effects of *unc-2(e55)* were generated and isolated via a genetic screen, and in certain cases, mapped to locations within the genome of the animal. It is hoped that the results of this work will form the foundation for a high throughput drug screening procedure that will be used to identify novel compounds that are capable of modulating calcium channel activity and the calcium current indirectly.

Several types of calcium currents and channels are found in biological systems

Several calcium current types, and the respective channels with which they are associated, can exist within organisms, and even within single cell types. Data generated from initial whole-cell calcium current studies indicated that, based on electrophysiological criteria, there existed two broad classes of currents, High-Voltage

Activated (HVA) and Low-Voltage Activated (LVA). The former is characterized by high single-channel conductance and slow inactivation, and requires large membrane depolarisations for activation. LVA currents, on the other hand, have relatively smaller conductances and deactivate quickly, and are responsive to small changes in membrane potential (reviewed in Jones, 1998). Because of the transient nature of its kinetics, LVA current is also known as "T-type" current. The HVA current was initially fractionated into L- and N-type currents based on differences in electrophysiological characteristics, then the N-type current was further resolved into N-, P/Q-, and R-type current primarily on the basis of pharmacological criteria. LVA current, however, consists solely of T-type current. Various aspects of the different current types are discussed in further detail in the sections that follow, with particular emphasis on their distribution, function, and electrophysiological and pharmacological properties.

L-type calcium current

First observed in chick dorsal root ganglion cells in 1985 (Nowycky *et al.*), L-type calcium current appears to be present in almost all tissue and cell types except platelets. Most abundant in cardiac, smooth and skeletal muscle cells, L-type calcium channels represent the major route of calcium entry, and are required for excitation-contraction coupling to take place. L-type calcium currents are also found in endocrine cells, where they coordinate hormone release, and in neurons, where they regulate gene expression and signal integration (reviewed in Catterall, 2000).

When Ba^{2+} is used as the charge carrier, L-type currents have single channel conductances of about 25 pS and do not open until membrane potentials from –30 to –10 mV (depending on tissue localization) are sensed. And although voltage-dependent

inactivation (VDI) of the current does occur, it is comparatively slow (reviewed in Yamakage and Namiki, 2002). Hence, this current has been designated "L-type," for "long-lasting."

However, when the charge carrier is changed to Ca^{2+}, L-type currents become smaller, and inactivation takes place more quickly. This effect, termed calcium-dependent inactivation (CDI), was first studied by Brehm and Eckert (1978) using *Paramecium*. It was found that inactivation could be slowed by intracellular application of calcium buffers, indicating that calcium ions were essential for the process. CDI is now thought to represent a negative feedback process, where calcium ions that flow through VGCCs restrict further calcium entry, that acts to supplement VDI in preventing calcium concentrations within cells from rising to toxic levels (reviewed in Budde *et al.*, 2002).

It was initially proposed that the primary mechanism for CDI involved direct binding of calcium ions to a "calcium sensor site" on the channel protein. This "site" was localized to the C-terminus of the L-type channel (Imredy and Yue, 1995), which contains a calcium-binding EF-hand motif. However, this particular element was later determined to be unnecessary for CDI (Zhou, 1997). Zuhlke and Reuter (1998) identified two sections distal to the EF-hand, one of which contains a Calmodulin-binding IQ motif, to be the key determinants for CDI. It therefore appears that Calmodulin bound to the calcium channel protein may be the "calcium sensor," instead of an element intrinsic to the channel itself. The events following calmodulin binding that lead to the attenuation of L-type calcium current have not been elucidated.

In addition to this, other secondary cellular events also appear to be capable of affecting CDI, albeit on longer time scales. Of these, the phosphorylation and dephosphorylation of calcium channels appear to be of particular importance. The current model describing this involves regulation of the phosphorylation state of the VGCC by the antagonistic actions of cyclic-AMP-dependent kinase (or PKA) and various phosphatases. More specifically, it is thought that phosphorylation of particular serine residues by PKA inhibits CDI whereas calcium-induced activity of calcineurin and/or calcineurin-regulated phosphatase PP1 dephosphorylates the channel to promote CDI (Kalman *et al.*, 1988). However, the involvement of calcineurin has since come into question, and it has been proposed that another phosphatase, PP2A, might be a better candidate (Hartzell *et al.*, 1995). Regardless, it is clear that the phosphorylation state of the channel protein is a determinant for CDI (Meuth *et al.*, 2002).

The cytoskeleton has also been suggested to play a role in CDI (Johnson and Byerly, 1993). This finding is not surprising, as calcium concentrations are important for the regulation of microtubule and microfilament stability (Bennett and Weeds, 1986). Although the specific mechanisms that effect this remain unclear, it is possible this phenomenon represents functional cross-talk between VGCCs and the cytoskeletal system, which may have serious implications in the processes of cell/neuronal development and plasticity.

It is important to note that CDI is not limited to L-type current, even though L-type current is considered the prototypical current type in which CDI occurs. CDI has also been reported for P/Q- and N-type currents, and this will be discussed further in later sections.

The initial distinction between L- and N-type currents was based on electrophysiological criteria (Nowycky *et al.*, 1985), but the contemporary definition separates these two current types on the basis of their pharmacological response to dihydropyridines (DHPs).

DHPs block L-type currents with high affinity but do not affect other current types. The action of DHP appears to be voltage-dependent, with channel inhibition becoming more severe with greater membrane depolarisation, which suggests that DHP preferentially binds to channels with an "open" conformation. While L-type current in all tissue types are blocked by DHPs, subtle tissue-to-tissue variations exist, and might be the result of the expression of different tissue-specific isoforms of L-type channels. However, DHPs, at high concentrations, also block voltage-dependent potassium and sodium channels (Nerbonne and Gurney, 1987; Jones and Jacobs, 1990), and therefore current block by DHP antagonist is no longer regarded as the most reliable diagnostic for L-type current. Rather, potentiation of current by DHP agonists, such as (S)-(-)-Bay K 8644, is now considered as a more suitable test (Nowycky *et al.*, 1985).

Other pharmacological compound classes, such as phenylakylamines and benzothiapines, also block L-type calcium current selectively. These, along with DHPs, are used as drugs in the treatment of cardiovascular disorders (Catterall 2000). L-type current in muscle cells and neurons is blocked by cadmium and nickel ions, as well as the peptide toxin ω–agatoxin IIIA of *Agelenopsis aperta* (Mintz *et al.*, 1991). However, the effects of these chemicals are not specifically restricted to this current class, as N-type current is similarly sensitive.

N-type calcium current

First described in chick dorsal root ganglion (DRG) cells in a study by Nowycky *et al.* (1985), N-type current was so named because the researchers found that its characteristics resembled "n"either of the current types that were known at the time, namely the T- and L-type currents. Coincidentally, this current type is also found primarily in "n"eurons (Yamakage and Namiki, 2002) of the peripheral nervous system.

It has been observed that, although N-type channels are initially localized to the soma and axons of immature neurons, their distribution is rearranged as the cells begin to mature and polarize, so that the channels are eventually excluded from the axon entirely, and are instead "moved" to the synaptic regions (Chambard *et al.*, 1999; Prevettoni *et al.*, 2000). Similarly, N-type VGCCs are also located mainly on the presynaptic neurons of neuromuscular junctions, where they are closely apposed to the acetylcholine (ACh) receptors on the postsynaptic muscle cells. Given their preferential association with vesicle-rich regions of neurons, it is perhaps not surprising that calcium N-type channels have been demonstrated to provide the calcium ion influx that triggers the release of neurotransmitters (Rittenhouse and Zigmond, 1999). The finding that N-type VGCCs colocalize with presynaptic soluble *N*-ethylmaleimide-sensitive fusion factor attachment protein receptor, syntaxin, synaptosome associated protein, and synaptotagmin (Dememes *et al.*, 2000), all of which are components involved in the coordination of exocytosis, further underscores the role of N-type VGCCs in neurotransmission.

Although it has been generally accepted that it represents a current with kinetic properties intermediate to T- and L-type currents (Catterall, 2000), the results of certain studies have raised the possibility that this definition may have resulted from the

inclusion of other high-voltage-activated, non-L-type currents in the initial measurements of N-type current (Elmslie *et al.*, 1994). Regardless, until evidence is produced to the contrary, the original definition is still largely "correct." The threshold of activation of N-type current, at approximately –20 mV, is "intermediate," as is its single-channel conductance of 13 pS. The values for its inactivation range, which vary from –120 to –30 mV, also fall between those for T- and L-type currents (Yamakage and Namiki, 2002). Although most sources quote the rates of inactivation of the N-type current as being between 50 to 80 msec (also "intermediate"), this is, in fact, an oversimplification of the inactivation process, which is perhaps the most complex and interesting aspect of this current type.

In the initial study performed by Nowycky *et al.* in 1985, it was observed that the calcium current of chick dorsal root ganglion cells were consisted of two distinct components, one of which inactivates more rapidly than the other when the cells were depolarised from a holding potential of –80 mV. When similar experiments were performed with a depolarised holding potential of –40 mV, it was found that the rapid inactivation had been eliminated, and that the peak current amplitude had also been reduced. The researchers therefore suggested that the sustained component of current was carried by non-inactivating L-type channels, while the inactivating component was attributed to the novel N-type channel. However, a more recent study performed by Cox and Dunlap in 1992 demonstrated that the HVA current of chick DRG cells was carried almost entirely by N-type channels. Therefore, the biphasic inactivation of current seems to result not from the presence of both L- and N-type channels. Rather, a mechanism involving only N-type channels must be responsible. One theory, proposed by Boland

and Dingledine (1990), suggests that the slow and fast phases of inactivation arise from the expression of two or more kinetically distinct types of N-type channels. Alternatively, it might also be possible that some intrinsic property of the N-type VGCC could allow a single channel type could carry both current types.

It is the latter model that has received the greatest experimental support. Results from a study by Plummer and Hess (1991), in which the single channel activities of N-type VGCCs in rat superior ganglion cells were analysed, revealed that, when stimulated by depolarisations, N-type channels will produce either a single, short burst of current lasting approximately 40 msec, or a longer, sustained pulse with a duration of over 1 sec, until the cells become repolarised. It thus seems that rapid inactivation can indeed be uncoupled.

But what is the exact mechanism underlying the rapid phase of inactivation? Although it is clear that slow inactivation is a voltage-dependent process, the manner in which fast inactivation is effected is still poorly understood at this point. Two possibilities have been proposed: rapid, but incomplete VDI, and L-type current-like CDI. To determine which of the two processes governs rapid inactivation, Cox and Dunlap in 1994 performed a study to determine if factors known to affect CDI can also influence the initial attenuation of the current conducted by N-type channels. It was seen that lowering the levels of calcium chelators in the patch pipette and /or increasing the amplitude of the calcium current by elevating extracellular calcium concentrations (both of which will promote CDI) had the effect of increasing fast inactivation. Also, when sodium was substituted as the charge carrier in lieu of calcium, rapid inactivation was eliminated. But, in the same study, it was also found that additional calcium buffering

reduced, but did not abolish rapid inactivation. Increasing extracellular calcium levels under these conditions did not relieve the suppression that was observed, and had negligible effects on channel kinetics when depolarised holding potential was used. These results appeared to indicate that CDI is not the primary mechanism of rapid inactivation. The researchers thus concluded that though CDI might serve to provide a certain degree of current inactivation, it was not the mechanistic correlate of the first phase of biphasic inactivation. More recently, results from some studies have raised the argument that CDI might not operate in all N-type channels (Patil *et al.*, 1998; Jones *et al.*, 1999), and this further supports the view that a voltage-dependent process is responsible instead. However, it is important to note that this aspect of N-type channel function is still relatively poorly understood at this stage and more work must be performed before any conclusions can be drawn regarding the unusual inactivation profile of N-type calcium channels.

N-type calcium channels, unlike their L-type counterparts, are insensitive to DHPs. They are, however, inhibited by a class of toxins present in carnivorous cone snails of the genus *Conus*. Collectively known as ω-conotoxins, these are small peptides that the animals produce and store within harpoon-like teeth, which are then used to paralyse prey species. While the effects of this family of toxins on mammalian N-type channels are virtually irreversible (Biagi and Enyeart, 1991), the calcium channels of frogs do recover over time (Boland *et al.*, 1994). At low concentrations (up to 1 μM), the effect of this toxin is specifically restricted to N-type channels, but when higher concentrations are applied, L- and T-type currents can become transiently blocked as well (Kasai *et al.*, 1987).

P- and Q-type calcium currents

First characterized in "P"urkinje neurons of the cerebellum (Llinas *et al.*, 1989), the P-type channel was categorized separately from N- and L-type channels based on the fact that blockers specific for either of the latter channel types failed to inhibit the current carried by the former (Regan *et al.*, 1991). Though unaffected by DHPs and most conotoxins, P-type channels are more sensitive to ω-Aga IVA, a peptide component of the venom of the funnel web spider *Aglenopsis aperta*, than other channel types. P-type current is potently and specifically blocked when this toxin is present at 100 nM, while T-, L- and N-type currents are inhibited only at far greater concentrations (Mintz *et al.*, 1992). P-type current is also irreversibly blocked by ω-conotoxin MVIIC. But, as this toxin affects Q-type current as well (Zhang *et al.*, 1993), ω-conotoxin MVIIC inhibition cannot be used as a diagnostic for this current type.

In addition to its pharmacological profile, the electrophysiological characteristics of P-type channels are also unique. For instance, the threshold of activation is relatively low, at –50 mV (Regan *et al.*, 1991), and is more akin to the values observed for T-type current. Also, unlike other current classes, the unitary conductance of the P-type varies greatly, even within a single tissue type, with values ranging from 9 to 20 pS (Usowicz *et al.*, 1992; Umemiya and Berger, 1995) when Ba^{2+} is used as the charge carrier. However, it is perhaps its ion selectivity that is the most intriguing aspect of this channel. As with the HVA channels, P-type channels will also preferentially conduct Ba^{2+} over Ca^{2+}. However, P-type channels are additionally able to select among monovalent ions if divalent ions are not available, with Rb^{+} being the most permeable and Cs^{+} being the least favoured (unlike HVA channels, which conduct these ions in a non-selective manner). P-

type inactivation kinetics resembles that of L-type channels, with little inactivation seen over a period of one second.

Though it is most prevalent in Purkinje neurons, P-type current is, in fact, widely distributed throughout all neurons of the PNS and CNS. However, it was observed that, even after the application of N-, L- and P-type channel-specific blockers, some HVA current still persisted in certain nervous tissues, which suggested the existence of yet another channel-type (Mintz *et al.*, 1992). Subsequently classified as "Q-type" by Randall and Tsien (1995), this channel type appears to carry most of the HVA current in neurons of the hippocampus and the sympathetic nervous system (Mintz *et al.*, 1992).

As previously mentioned, Q-type current is blocked irreversibly by ω-conotoxin MVIIC (Zhang *et al.*, 1993; McDonough *et al.*, 1996; reviewed in Jones, 1998). However, as Q-type current is 10- to-100-times less sensitive to ω-agatoxin IVA than P-type current, it can be isolated with the application of appropriate concentrations of this toxin. Doing so reveals a current with electrophysiological properties not unlike that of P-type current, and differences are only observed where inactivation is concerned. Whereas P-type current does not inactivate to any appreciable extent, Q-type current decays rapidly, most likely through a calcium-dependent mechanism (Lee *et al.*, 2000), resulting in the current becoming undetectable in some cells when the current conducted by P-type channels is not eliminated first. As a result of the similarity between these two currents, for most practical purposes, they are often considered as a single entity.

Immunohistochemical studies have determined that P/Q-type channels are mainly localized to the dendrites and cell bodies of the neurons that comprise the CNS (Westenbrock *et al.*, 1995), and may be involved in neurotransmitter release at certain

synapses. Indeed, the application of P-type channel-specific blockers appears to be effective in preventing glutamate release from neurons of certain tissues (Turner *et al.*, 1992). Also, calcium entry, and, by extension, neurotransmitter release, was blocked in neurons that innervate mouse muscle when either FTX, an inhibitor of P-type channels, or sFTX, a synthetic analogue, was present (Uchitel *et al.*, 1992).

R-type calcium current

It has been observed that most neurons have a component of HVA current that remains even in the presence of L-, N-, and P/Q-channel specific blockers (Pearson *et al.*, 1995). This R-type current, so named for its "r"esistant or "r"esidual nature, is also occasionally classified as an intermediate voltage activated, or IVA current (Yamakage *et al.*, 2002).

Certain biophysical characteristics of R-type current resemble those carried by N- and Q-type channels. For instance, the threshold of activation of this current type is –40 mV, and its single channel conductance is 14 pS. However, R-type current inactivates very rapidly by comparison, with a time constant of approximately 22 msec. In this respect, it is more reminiscent of T-type current (Yamakage *et al.*, 2002).

Due to its relative novelty, studies have not yet been performed to determine the pharmacological sensitivity of the channels that support this current. And although it is now considered as a single current class for the same reason, it is likely that R-type current will be fractionated further in the near future, since it has already been observed that at least two R-current subtypes possessing distinct electrophysiological properties can exist in rat cerebellar granule cells (Tottene *et al.*, 1996).

Low voltage gated (LVA)/T-type calcium current

LVA current was first observed in voltage-clamp recordings of starfish eggs by Hagiwara *et al.* in 1975, and was subsequently characterized in detail in the years that followed through the work of Carbone and Lux (1984), Fedulova *et al.* (1985), Nowycky *et al.* (1985), and Swandulla and Armstrong (1988). Since then, attempts to achieve a greater understanding of this current had been hampered by repeated failures to identify and clone the molecular correlate(s) of the current using standard methods that had already been successfully applied towards the HVA channels. However, the situation changed drastically once human, yeast, and *C. elegans* genome sequence data became available, providing what was, in essence, a library that was amenable to in computer-based screens. Using an "in silico" approach, not one, but three LVA-current-supporting channels were cloned recently (Cribbs *et al.*, 1998; Perez-Reyes *et al.*, 1998; Lee *et al.*, 1999; reviewed in Perez-Reyes, 2003).

LVA channels possess electrophysiological properties that are quite different from those of HVA channels. For instance, single channel conductance of LVA channels, which has been measured to be approximately 8 pS in 110 mM Ba^{2+}, is comparatively low. More importantly, activation of this channel type occurs with relatively small membrane depolarisations; currents are elicited at –70 mV and reach a maximum value at approximately –30 mV. In fact, the majority of LVA channels are inactivated at the resting membrane potential of most cell types, and therefore are not available for opening unless a hyperpolarized condition is first established (Veselovsky and Fedulova, 1983). Once elicited, the current decays rapidly, with a time constant that ranges between 11 to 69 msec (Perez-Reyes, 2003). This current was therefore also dubbed "T"-type, for its

"t"ransient nature. The rates of both activation and inactivation of current also appears to be voltage dependent, increasing with greater depolarisations away from threshold potential (Nowycky *et al.*, 1985). LVA channels differ from HVA channels with respect to ion selectivity as well. While the HVA channels preferentially conduct Ba^{2+} over Ca^{2+}, T-type channels exhibit no such bias, and are equally permeable to both ions (Perez-Reyes, 2003).

There are currently no known drugs or peptide toxins that selectively and specifically block LVA current only. However, the sensitivity of T-type channels to certain compounds is sufficiently different from that of other channel types to allow these channels to be classified separately on a pharmacological basis. One drug to which LVA channels are particularly sensitive is mibefradil. Originally produced by Roche to be used for the treatment of hypertension and stable chronic angina pectoris, but later voluntarily withdrawn from market due to reports of harmful drug-drug interactions and other problems (Krayenbuhl *et al.*, 1999), mibefradil has been shown to be 10- to 30- times more selective for T-type channels than it is for L-type channels (Martin *et al.*, 2000; Perchinet *et al.*, 2000). The degree to which mibefradil inhibits LVA current appears to be dependent on a number of factors, including the identity and concentration of the divalent cation that is used as the charge carrier. More specifically, a solution containing Ca^{2+} compared to one with Ba^{2+} promotes inhibition, as does elevated levels of either of these ions. Membrane potential can affect mibefradil function as well. It was observed that current block is more pronounced at holding potentials that cause T-type channels to become inactivated, which indicates that drug binding occurs with greater affinity to channels in that state (Martin *et al.*, 2000). This characteristic of mibefradil provides

researchers with an additional level of selectivity, as HVA channels inactivate at higher membrane potentials than LVA channels (Perez-Reyes, 2003).

Although dihydropyridine drugs are generally more selective for L-type channels, some analogues, such as flunarizine, nicaripine, and nifedipine, can also inhibit the activity of certain subsets of T-type channels at micromolar concentrations (Akaike *et al.*, 1989). The binding sites for these drugs are, however, likely different in the two channels types. This is evidenced by the fact that the application of a racemic mixture of the L-type channel agonist Bay K 8644 to T-type channels causes current inhibition, instead of potentiation (Lievano *et al.*, 1994), while a similar preparation of niguldipine blocked both L- and T-type channels with equal potency (Romanin *et al.*, 1992). However, the mechanism of inhibition appears to be common, and involves the high-affinity binding of the drugs to, and the subsequent stabilization of, the inactivated state of the channels (Bean, 1984).

Antiepileptic drugs such as ethosuximide and methyl-phenylsuccinimide can block T-type currents as well, and it is believed that this is the basis for the therapeutic effects of these compounds (Coulter *et al.*, 1989). As with mibefradil, the effect of these drugs is voltage-dependent (Gomora *et al.*, 2001), with greater blockage of current occurring at more depolarised membrane potentials. Neither drug appears to be completely selective for T-type channels only, however, as ethosuximide can affect recombinant R-type channels at high concentrations (Nakashima *et al.*, 1998), and methyl-phenylsuccinimide blocks L-type currents in thalamic neurons (Coulter *et al.*, 1990).

T-type calcium channels are widely expressed in many tissue types, and although little is known of the specific functions of these channels, their unique electrophysiological profiles and subcellular localisation may provide some clues. For instance, the low thresholds of activation of these classes of calcium channels suggest that they may participate in the generation of rhythmic electrical activity. More specifically, in neurons with highly depolarised membranes, T-type channels can act as "secondary pacemaker[s]," opening in response to excitatory postsynaptic potentials to activate sodium and high-threshold calcium channels (Perez-Reyes, 2003). Indeed, T-type current has been found to be associated with cell types exhibiting regular spontaneous firing behaviour, such as those of the thalamus (Llinas *et al.*, 2001).

The speed with which the T-type channels recover from the inactivation phase, coupled with their sensitivity to low voltages, may also enable them to facilitate the restoration of charge distribution across the membrane following an inhibitory signal, particularly in cells that have relatively depolarised resting-state potentials. Inhibitory postsynaptic potentials received at the dendrites hyperpolarize these neurons, exerting an inhibiting effect on HVA channels. However, the LVA channels that were previously inactivated quickly become available for opening again, and the resulting influx of calcium ions from the extracellular fluid rapidly re-establishes the resting state. In this way, T-type channels allow neurons to "rebound" from IPSPs (Perez-Reyes, 2003), and prime them to receive subsequent signals.

Finally, unlike L- and N-type channels, which localise primarily to the synaptic termini of neurons, T-type current appears to be most prominent at the dendritic regions

instead (Destexhe *et al.*, 1998; Markram and Sakmann, 1994). It is therefore also likely that T-type channels play a role in signal amplification.

Calcium channels are heterooligomeric complexes

Initial investigations into the molecular structure of voltage-gated calcium channels were performed primarily on skeletal muscle, and this was due to the fact that the transverse tubule membranes of this tissue are highly dense in these molecules. In 1984, Curtis and Catterall reported on a study in which digitonin-solubilized channel proteins were purified and isolated based on their affinity for tritiated nitrendipine, a DHP analogue. Separation by polyacrylamide gel electrophoresis revealed that the fraction comigrating with the nitrendipine activity contained three different polypeptides, which the researchers named α_1, β, and γ. This provided the first experimental evidence that VGCCs were multimeric structures. Through further purification of the calcium channel complex using photoaffinity labelling with synthetic fluorescent phenylakylamine analogues, two other groups demonstrated that there existed yet another subunit (Hosey *et al.*, 1987; Striessnig *et al.*, 1987), that was composed of two dissimilar peptides. Dubbed $\alpha_2\delta$, this dimer had not been detected previously as it migrated with α_1 as a single band in the polyacrylamide matrix due to the very similar relative masses of the two subunits. A thorough analysis of the biochemical characteristics, glycosylation patterns, and hydrophobicity of each of these components has led to the presently accepted model of the skeletal muscle voltage-sensitive calcium channel, the structure of which is illustrated in Figure 1.1. In this model, the 175-kDa α_1 subunit forms the principal transmembrane calcium ion-conducting pore, and contains the voltage-sensing mechanism as well. The membrane-bound γ (30 kDa) and δ (27 kDa) subunits, the latter

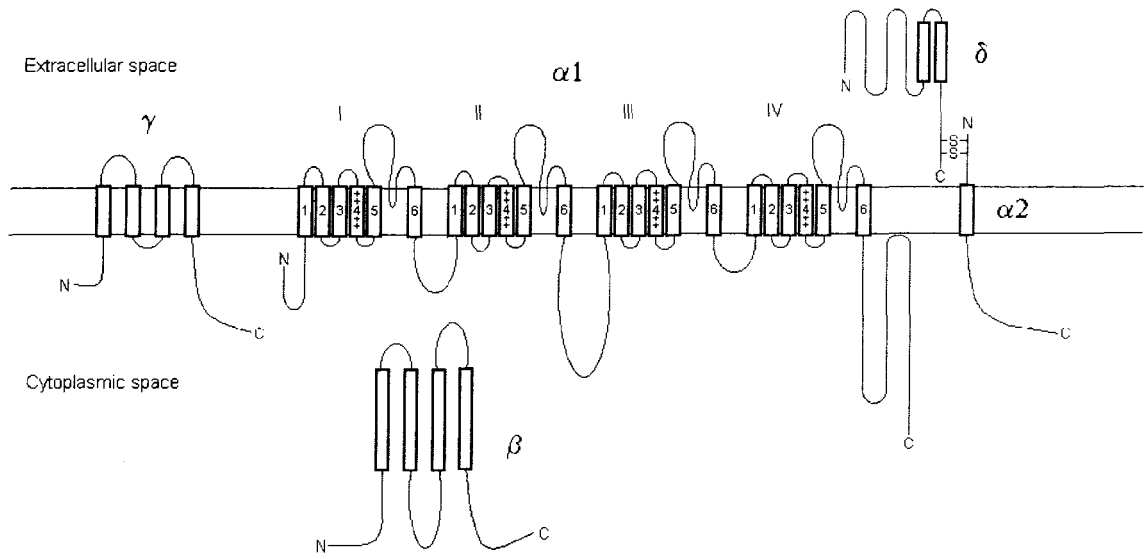


Figure 1.1 The heterooligomeric structure of VGCCs

The voltage-sensing apparatus and the ion-conducting pore are entirely contained within the α_1 subunit. The auxiliary subunits $\alpha_2\delta$, β, and γ serve to modify the characteristics of α_1. Cylinders highlighted in bold in lipid bilayer correspond to transmembrane segments, while those in the cytoplasm and extracellular space represent predicted α-helices. Features to note include the membrane-associated loops linking transmembrane segments 5 and 6 in each of the four domains of the α_1 subunit, and the dimeric structure of the $\alpha_2\delta$ subunit, which is linked by a pair of disulphide bridges. Refer to text for a more detailed discussion of the structure and properties of each of the subunits.

of which is disulphide-linked to the heavily-glycosylated extracellular α_2 (143 kDa), interact with α_1 via non-covalent interactions. The 54-kD β subunit is entirely cytoplasmic, and associates with the C-terminus of the α_1 subunit (Takahashi *et al.*, 1987).

Other HVA calcium channel complexes have also been purified using similar methods, and their structures are now fairly well understood. It is quite illuminating to compare the differences in subunit composition. The cardiac muscle-derived L-type VGCC is largely similar in this respect to the skeletal muscle form, as is to be expected (Kuniyasu *et al.*, 1992). But, the neuronal form of the L-type VGCC lacks a γ subunit, and so is composed of just four, as opposed to five peptides (Ahlijanian et al., 1990). Rat brain N-type calcium channels, likewise, do not associate with γ subunits either (Witcher et *al.*, 1993). The inclusion of the γ subunit in the calcium channel, therefore, is not absolutely critical for all HVA VGCCs; a "core" composed of the α_1, β and $\alpha_2\delta$ subunits seems to be sufficient. P/Q-type calcium channels were, until recently, thought to not include γ subunits as well (Liu *et al.*, 1996), but evidence now suggests that this might not be the case (Letts *et al.*, 1998).

However, even the β and $\alpha_2\delta$ subunits are unnecessary for the formation of a functional calcium channel. Indeed, the α_1 subunit alone, when expressed heterologously, is capable of conducting calcium currents in response to changes in membrane potential, albeit with a low expression level and with altered electrophysiological and kinetic characteristics (Perez-Reyes *et al.*, 1989). What, then, are the functions of the auxiliary subunits? In two separate studies published in 1991, Lacerda *et al.* and Singer *et al.*

demonstrated that the β and $\alpha_2\delta$ subunits, when co-expressed with α_1, lead not only to the increase of expression levels, but also the restoration of normal channel gating. The auxiliary subunits, therefore, serve to modulate the activity of α_1, and therefore are important in determining the properties of the channel. In the sections that follow, the structure, function and other aspects of each of the subunits will be discussed in greater detail.

The function and common structural features of VGCC α_1 subunits

In an attempt to elucidate the structure of the α_1 subunit, Tanabe *et al.* cloned the cDNA of the dihydropyridine receptor of rabbit skeletal muscle in 1987. Analysis of the predicted amino acid sequence of the protein revealed that considerable similarity existed between it and voltage-sensitive sodium channels. It was therefore suggested in their report that the α_1 subunit supports both the ion-conducting and excitation-contraction coupling activities of the skeletal muscle form of the VGCC, as with these other two channel types. This was subsequently demonstrated experimentally in another study, where the expression of the cloned α_1 subunit in dysgenic myotubes was sufficient to "rescue" the mutant phenotype, and re-established the ability of the cells to respond to depolarisations and support charge movement across their membranes (Tanabe *et al.*, 1988).

With the cDNA obtained from skeletal muscle now available for use as a probe, the cardiac (Mikami *et al.*, 1989) form of the α_1 subunit was also cloned. A study, performed by Tanabe *et al.* (1990), in which measurements of the electrical activity of dysgenic myotubes transformed with the new cDNA were taken showed that although the

cardiac and skeletal muscle forms of α_1 both conduct slow currents with L-type signatures, the currents differed in several key areas, the most notable of which are activation kinetics and ion selectivity. It was clear that the differences observed for the calcium currents of various tissues are mainly defined by the α_1 subunit of VGCCs. It was also seen in the same study, that cardiac α_1, when expressed heterologously in skeletal muscle-derived myotubes, transformed the excitation-contraction coupling mode of the cells into one that more closely resembled that seen for cardiac cells. In other studies, it was reported that cardiac α_1 cDNA injected into systems devoid of auxiliary VGCC subunits still produced voltage-sensitive calcium currents that were susceptible to block by L-type calcium channel antagonists (Perez-Reyes *et al.*, 1989; Bosse *et al.*, 1992). Therefore, not only are the α_1 subunits of VGCCs responsible for the characteristics of the calcium current, they also appear to contain the determinants for the mode of excitation-contraction as well as those for pharmacological sensitivity.

The VGCC α_1 subunit, as mentioned above, is similar to that of voltage-gated sodium channels. Both, however, are based on the basic structure of the voltage-sensitive potassium channels, as are most channel-forming proteins of eukaryotes. But, unlike the potassium channels, which require four separate peptides to form a functional unit, the expression of only one peptide suffices in the case of calcium channels, owing to the fact that all of the necessary domains have been merged into a single entity. This most probably happened via two separate rounds of tandem gene duplication during the course of evolution (Strong *et al.*, 1993), and has resulted in the structure of the α_1 subunit having a modular composition. Each of the four modules (named domains, or repeats I through IV) conforms to a potassium channel-like 6-transmembrane pass motif, with six

putative transmembrane helices (S1-S6, as predicted by hydropathy analysis) connected by loop segments. Of these, the S4 helices are particularly interesting, as they contain one positively-charged residue in for every three amino acids. Extrapolating from the results of site-directed mutagenesis and fluorescent labelling studies performed on sodium and potassium channels (Yang and Horn, 1995; Mannuzzu *et al.*, 1996), it has been proposed that the S4 helix in each of the modules act as voltage sensors, responding to membrane depolarisation by causing a conformational change in the channel to permit the entry of calcium through the ion-conducting pore, which is composed of the S5 and S6 helices, and the peptide loop between them. The external and internal limits of the pore are defined by the S5 and S6 helices respectively, while the extracellular membrane-associated S5-6 loops forms the lining of the pore.

Calcium selectivity is mediated solely by the pore region of the VGCC α_1 subunits

While seemingly simple, the pore is in fact a highly evolved structure that contains the elements that are the keys to the remarkable selectivity and permeation characteristics of the channels. Under physiological conditions, VGCCs are consistently able to conduct only calcium ions despite the fact that other cations are present in far greater concentrations (Tsien *et al.*, 1987). While the mechanism responsible for this was not known at this point, it was observed that, in the absence of calcium, VGCCs become non-specific conductors of monovalent cations (McCleskey and Almers, 1985). It was additionally seen that the passage of these ions are blocked by micromolar calcium, and current is not restored until calcium ions are reintroduced to millimolar levels (Almers and McCleskey, 1984; Hess and Tsien, 1984; reviewed in Jones, 1998). It was thus clear that there did not exist a physical "filter" sized to permit the entry of a specific ion type

only within VGCCs, and that some form of competition between ion types must be at work instead. It was therefore proposed that calcium channels most likely contained two separate and independent ion-binding sites possessing low affinity for monovalent ions but high-affinity for Ca^{2+}. According to this model, the block of monovalent ion flow by calcium at micromolar levels is due to calcium ions binding to, and occupying, both high-affinity sites, and denying the access of other ions to the pore. At higher concentrations of calcium, the close apposition of ions within the binding pockets leads to mutual electrostatic repulsion, and this results in the rapid influx of calcium through the channel. This model gained wide acceptance for a time as it imposed no initial restriction on permeable ion species, but still was able to account for all observed phenomena.

The molecular determinants controlling calcium selectivity were first identified in 1992, not in calcium channels, but in sodium channels. Comparing sequence data, Heinemann *et al.* saw that the well-conserved glutamates, found in the S5-S6 loop of each of the internal repeats of all HVA calcium channels, had been instead replaced with other negatively charged amino acids in equivalent positions in sodium channels. Substitutions that diminished the net negative charge at these sites of cloned rat sodium channel II led to a considerable decrease in single channel conductance. More importantly, it was also seen that when either or both of lysine 1442 of repeat III and alanine 1714 of repeat IV were replaced with the corresponding glutamate residues from calcium channels, the sodium channel took on characteristics that closely mimicked those of VGCCs instead. Indeed, all of the major features of calcium permeation mentioned previously were represented in this mutant channel, including calcium selectivity under

physiological conditions, non-specific cationic currents in the absence of calcium, and the block of this current by micromolar calcium.

Following this, additional studies were then performed to determine the exact functional roles of the conserved glutamate residues in the pore loops of calcium channels. In the first of these, glutamates 1469 and 1765 (of repeats III and IV, respectively) of the brain calcium channel B1 were targeted for single point mutations to glutamine. Upon measuring the macroscopic properties of the current, it was seen that although loss of calcium selectivity resulted in both cases, the impact of the repeat III mutation appeared to be greater (Kim *et al.*, 1993). The researchers therefore concluded that the different glutamate residues probably played different roles in controlling ion permeability, despite being equivalently situated within each of the "modules." In 1993, Yang *et al.* reported on a set of experiments in which a series of glutamate-to-lysine substitutions were introduced into each of the four pore loop regions of an L-type calcium channel. Their initial findings agreed with those of Kim *et al.*, and thus confirmed that there was indeed a certain hierarchy among the individual glutamates with respect to selectivity, with the one in repeat III being most critical. However, it was further observed that disruptions at certain sites abolished the block of non-specific current by micromolar calcium entirely, and this strongly indicated that the two separate binding sites described in the model proposed by Almers and McCleskey (1984) were in fact non-independent of each other, and requires the presence of all four glutamate residues. Based on this, it was suggested that the first model be modified so that the pore loop structures, originally described as forming two rigidly localised pockets, would instead be flexible, dynamically interacting with Ca^{2+} travelling through the pore in a single file. According

to this "car wash" model, under low calcium conditions, glutamate availability becomes comparatively high, and so any calcium ions that are present become bound tightly to the pore site, resulting in the block of the current carried by monovalent ions. If calcium is present in abundance, however, the carboxyl groups of the residues become "overwhelmed" with excess binding partners. In this case, the coordination effect weakens, and permeation occurs (Yang *et al.*, 1993), accelerated by repulsion between closely-apposed calcium ions, as with the previous model.

The necessity for ion-ion interactions within the pore stems from the need to reconcile the discrepancy between the measured affinity of pore for calcium ions and the rapid rate of calcium conduction. Specifically, the observed current conducted by VGCCs is up to a thousand times faster than that allowed by the tight association between the calcium ion and the binding site, the K_a of which has been experimentally determined to be comparatively low. It therefore becomes necessary, in the case of the two models discussed previously, to invoke a mechanism (inter-ionic repulsion, in this case) to account for the apparent increase in the rate of dissociation. Using rate theory calculations, Dang and McCleskey (1998) recently demonstrated the validity of a novel model that does not require the presence of any additional driving forces. According to this model, the pore of VGCCs contains a single high-affinity calcium-binding pocket, with two low-affinity ones on either side of it. Initial binding and ion selection (via competition) occurs at the flanking sites, and the ions are then "handed off" to the central site. Ion permeation proceeds through two energetically-favourable steps instead of a single jump, and ion flux is thus accelerated. Besides its ability to explain rapid calcium ion flow, this model successfully predicts micromolar block and other hallmarks of

VGCC function as well. Also, its general nature allows it to be applied, with only slight alterations, to voltage-gated potassium and sodium channels too. This further bolsters the validity of this model, as the basic mechanism of selectivity is likely conserved amongst these evolutionarily related channel types.

Phylogeny and structural diversity of the VGCC α_1 subunits

Ten distinct voltage-sensitive calcium channel α_1 subunit genes have been identified in mammals through molecular cloning thus far, seven of which encode for HVA channels, and three for LVA channels (Moreno, 1999). Via heterologous expression studies, the physiological correlates for each of these have also been determined. Based on the pairwise analysis of the sequences that comprise the transmembrane segments and the pore loops (which are regions that exhibit the greatest degree of homology between the individual α_1 subunits), three major families - namely, DHP-sensitive HVA channels, DHP-insensitive HVA channels, and LVA channels - may be defined, within which whole sequence identity exceeds 60%. By contrast, interfamilial identity, calculated for the consensus sequences for each of the families, averages only approximately 25% (Ertel *et al.*, 2000) across the three families. This grouping reveals much about the phylogeny of the channel proteins. It is readily apparent, for example, that the current three-family arrangement resulted from a two major rounds of gene duplication, one early on in the evolution of VGCCs, forming the HVA and LVA classes, followed by another within the HVA class to form the DHP-sensitive and DHP-insensitive channel types. Subsequent duplication and divergence events then occurred, resulting in the ten calcium channel types that are known today.

The structures of each of the VGCC α_1 subunits represent variations on a single theme. When the structures of the ten known α_1 subunits are compared, it is seen that the greatest amount of conservation occurs in the portions of the proteins that are known to be central to channel function. The transmembrane helices, for instance, are remarkably similar in all α_1 classes, even in distantly related ones, as are the pore loop regions. This is evident from the fact that if only these segments are taken into account, the degree of interfamilial similarity rises to approximately 55% between the DHP-sensitive HVA and DHP-insensitive HVA channel classes, as opposed to the 30% that is calculated when whole sequences are used (Ertel *et al.*, 2000).

Outside of these regions, however, the structure of the various α_1 subunits can vary to a greater degree. In the channels that support DHP-sensitive L-type currents, for example, the intracellular loops that connect repeats I and II are short compared to those found in channels carrying P/Q- and N-type current. In other cases, some elements may be absent from certain isoforms. The most notable of these is the β subunit-binding alpha interaction domain (AID) that is found in the I-II loop in all HVA channels, but is missing entirely from the LVA channels. As a consequence of this, the LVA channels do not interact with any β subunits. Other more subtle sequence alterations confer different pharmacological sensitivities, channel activation/inactivation characteristics, and current kinetics on the channels. The cloning and heterologous expression of each of the calcium channel α_1 genes has allowed for the identification of their physiological correlates (Figure 2). With this information, researchers can thus begin to dissect the channels in greater detail and understand how the structural differences between the various α_1 subunits result in their unique properties.

VGCC α_1 subunit nomenclature

There were initially no standard procedures for the naming of novel VGCC α_1 subunits. Birnbaumer *et al.*, in 1994, suggested that a system be put in place whereby all α_1 subunits, with the exception of the skeletal isoform that was first isolated - now to be designated as α_{1S} - would be assigned alphabetical suffixes based on the order of their discovery (α_{1A}, α_{1B}, α_{1C}, and so on, for example). It eventually became clear that this system had certain weaknesses, however. For one, it conveyed little information besides that regarding chronology, and therefore, while it was useful for the purposes of unification, it precluded the systematic organization of the various channel types on the basis of electrophysiological or pharmacological characteristics. Additionally, it revealed nothing about the phylogenetic relationships that exist between the various calcium channel types. The system was found to be lacking in practical respects as well: with the cloning of more channels, there likely will be, at some point in the future, a need to name one of them α_{1L}, and this may potentially lead to some confusion if that particular channel protein does not mediate a L-type calcium current.

Ertel *et al.* (2000) therefore proposed a replacement for the original nomenclature that was based closely on those that were already in use for voltage-gated potassium and sodium channels. According to the new system, VGCCs would now be given names composed of four parts, which are, in sequence: the chemical symbol for the target ion species (Ca, in this case), a descriptor for the activation stimulus in the subscript ($_v$, to indicate that the channels respond to voltage changes), the designation for the specific channel family (with '1' identifying DHP-sensitive HVA channels, '2,' DHP-insensitive HVA channels, and '3,' LVA channels), and finally, a number "tag" corresponding to the

order in which a particular member of a family was discovered, delimited from the previous component by a period symbol. To provide some examples, the original DHP-sensitive L-type HVA channel clone that was derived from skeletal muscle, formerly known as α_{1S}, would now be called $Ca_v1.1$, while the DHP-insensitive P/Q-type channel that was isolated subsequently from nervous tissue, nee α_{1A}, would be renamed $Ca_v2.1$. The recently cloned T-type channels were now to be known as $Ca_v3.1$, 3.2 and 3.3, instead of α_{1G}, α_{1H}, and α_{1I} (Figure 2).

With the present nomenclature, systematic organization is facilitated as the structural relationships, electrophysiological properties and pharmacological sensitivities of the various voltage-gated calcium channel α_1 subunit are now readily apparent. As an added advantage, the chronological element of its predecessor is also retained.

The structure, function and properties of the VGCC $\alpha_2\delta$ subunits

$\alpha_2\delta$ is perhaps the auxiliary subunit about which most is known. It has a widespread pattern of expression, and has been found in all manner of tissues, including the brain, heart, adrenal gland, intestine and skeletal muscle (Qin *et al.*, 2002). The presently accepted model of this protein describes it as consisting of two components, linked to each other by a disulphide bridge. The first, δ, is bound to the plasma membrane by a single hydrophobic helix which makes up a major part of its structure, and terminates with a short extracellular N-terminal extension and an even shorter C-terminal intracellular tail composed of five residues at most. The second, α_2, is entirely extracellular. A single gene encodes for each $\alpha_2\delta$, but once a transcript is translated in the

cytosol, it undergoes oxidation at specific cysteine residues followed by proteolytic cleavage, resulting in the distinct structure of the final product (Gurnett *et al.*, 1996).

α_2 is known to form direct contacts with the third, and possibly parts of the second, domains of the channel-forming α_1, though the exact amino acid residues involved have yet to be identified. δ has too been shown to be capable of binding to α_1 via its short N-terminal domain (Gurnett *et al.*, 1997). Given its structure, it is unlikely that δ forms additional protein-protein interactions with the cytosolic β subunit. There also seems to be little indication that $\alpha_2\delta$-γ binding occurs.

Currently, only four $\alpha_2\delta$ subunits (named $\alpha_2\delta$-1, -2, -3, and -4) have been identified in mammalian species (Gao *et al.*, 2000; Qin *et al.*, 2002; Gurnett *et al.*, 1996). Although the major characteristics central to the function of the subunit, such as overall structure, hydrophobicity profiles, as well as the cysteine residues that participate in the disulphide linkage, are well conserved in each of them, there remains a considerable amount of diversity at the protein level. This is contributed partly by alternative splicing of the RNA message, but post-translational glycosylation is mostly responsible. The latter has been experimentally determined to be crucial for the maintenance of $\alpha_2\delta$–α_1 interactions, and as is evident from the specific glycosylation patterns of the various subunit types, it appears to be a non-random, as well as tightly regulated process.

As was stated previously, $\alpha_2\delta$ subunits can profoundly affect the various properties of the whole channel. However, the specific effects that are produced are highly dependent upon the identity of the $\alpha_2\delta$ subunit being considered. As well, the identity of the associated α_1 subunit and the presence or absence of other auxiliary

subunits can also have a major bearing on the consequences. The recent cloning of the various $\alpha_2\delta$ genes has allowed researchers to perform heterologous expression experiments in order to determine the manner, and the molecular mechanisms by which they modulate the calcium current. The generation of mice carrying defects in these genes have also provided animal models for the study of the physiological role of $\alpha_2\delta$. The following are brief discussions on the results obtained from these studies.

$\alpha_2\delta$-1

The expression of $\alpha_2\delta$-1, and indeed, that of any $\alpha_2\delta$ subunit, greatly enhances the trafficking of α_1 to the plasma membrane (possibly by stabilising its structure once it is integrated into the membrane via α_2) and the open probability of the channel (Felix *et al.*, 1997). As a consequence, the amplitude of the calcium current at its peak, as well as the number of binding sites for calcium antagonist molecules, is increased. No additional subunits are necessary in the case of L-type (i.e. $Ca_v1.X$) channels, but the co-expression of β is required for the manifestation of these effects in non-L-type ($Ca_v2.X$) channels. The presence of $\alpha_2\delta$-1 also increases, by up to two-fold, the rate of activation of the L-type channels, but not that of the non-L-type ones. Both channel types experience faster inactivation rates, however, and co-expression of β is essential for this (Singer *et al.*, 1991; Stea *et al.*, 1993; reviewed in Walker and De Waard, 1998, and Arikkath and Campbell, 2003). The mechanism responsible for bringing about the acceleration of channel kinetics is currently unknown, but the results of a study conducted by Sipos et al. (2000) indicate that δ may play a part.

$\alpha_2\delta$-2

Unlike $\alpha_2\delta$-1, the co-expression of this isoform with any α_1 subunit increases only the amplitude of peak current flowing through the resultant channel. Other aspects of channel function are unaffected (Gao *et al.*, 2000). In the case of ducky (*du/du*) mice, the $\alpha_2\delta$-2 gene Cacna2d2 is disrupted by a rearrangement that leads to the expression of a protein that contains part of α_2. The δ membrane anchor is completely missing, and therefore, the gene product remains entirely intracellular (Brodbeck *et al.*, 2002). Ducky mice exhibit several defects, such as loss of coordination, and suffer from episodes of abnormal movement that are accompanied by all the classic behavioural and electrophysiological hallmarks of epilepsy. Reduced P-type current density, and poor development of the Purkinje neurons of the brain are also characteristic of these mutant mice (Barclay *et al.*, 2002).

$\alpha_2\delta$-3

This subunit, when expressed with $Ca_v1.3$, produces only a hyperpolarizing shift in the activation kinetics of the channel. However, if a β subunit is co-expressed, current density increases, and the inactivation curve becomes shifted in a hyperpolarized fashion as well (Klugbauer *et al.*, 1999). Co-expression of $\alpha_2\delta$-3 and β_3 with $Ca_v2.3$ causes a similar shift in the activation and inactivation curves (Gurnett *et al.*, 1996; reviewed in Arikkath and Campbell, 2003).

$\alpha_2\delta$-4

$\alpha_2\delta$-4 is the most poorly understood of all the $\alpha_2\delta$ subunits due to its relative novelty. Preliminary analysis indicates that as with the other isoforms, it too is capable of

amplifying current (at least that of $Ca_v2.1$), but it is still unclear if it can modulate other channel properties (Qin *et al.*, 2002).

The structure, function and properties of the VGCC β subunits

The β subunit was initially identified as a band of approximately 55 kDa that comigrated in a gel matrix with the channel-forming α_1 subunit of voltage-dependent calcium channels (Campbell *et al.*, 1988). Subsequently, four distinct β subunit-encoding genes were identified in mammals. Each of these may give rise to several alternatively spliced variants, thus creating an additional level of diversity. β appears to be fairly ubiquitous, and mRNA transcripts that correspond to the various isoforms have been detected in a wide variety of tissue types. The distribution of this subunit is clearly non-random, and different isoforms preferentially associate with specific tissue types. β_4, for example is expressed primarily in the brain, together with β_{1b} and β_{1c}, which are also found in the spleen. β_{1a} and β_{2a} have been detected in skeletal and cardiac muscle, while β_3 appears predominantly in smooth muscle (Ludwig *et al.*, 1997; Vance *et al.*, 1998; Ruth *et al.*, 1989; Qin *et al.*, 1998; Powers *et al.*, 1992; reviewed by Hanlon and Wallace, 2002).

The effects of the β subunits on channel function are numerous and are highly dependent on the identities of the α and β subunits that are being considered (Sokolov *et al.*, 2000). However, the coexpression of β will generally result in enhanced expression of the associated α subunit, which can be indirectly observed through increases in peak current amplitude and ligand binding sites. In virtually all cases, activation kinetics are accelerated, particularly in L-type channels, as are the rates of inactivation. In the case of

the non-L-type channels, a hyperpolarizing shift of up to –23 mV may also occur (Williams *et al.*, 1992; Wei *et al.*, 1991; Castellano *et al.*, 1993; De Waard *et al.*, 1991; De Waard and Campbell, 1995; reviewed in Walker and De Waard, 1998).

β is distinct as it is the only subunit that is fully contained within the cytosol. Certain splice variants, though capable of associating with the membrane, do so either via acidic sequences, in the case of mammalian β_{1b} (Bogdanov *et al.*, 2000), or palmitoylation as with rat β_{2a} (Chien *et al.*, 1998), instead of the specialized transmembrane domains that are found in other auxiliary VGCC subunits. A comparison of various β-encoding genes has revealed two sequences that are highly conserved within all isoforms and structural modelling studies have determined that they correspond to SH3 and guanylate kinase-like domains. They are flanked on either side by a PDZ-like N-terminus and a C-terminus region, and are separated by a short central section (Hanlon *et al.*, 1999). A thirty amino acid sequence, termed the beta interaction domain, or BID (De Waard *et al.*, 1994), is located between this portion of the protein and the conserved guanylate kinase-like domain. This element has been determined to be critical in mediating the interactions important for the modulation of channel properties by β by allowing it to bind to the complementary alpha interaction domain (AID). Comprised of eighteen residues, this motif is found on the intracellular peptide loop that connects the first and second hydrophobic repeats of the α_1 subunit (Pragnell *et al.*, 1994). The interaction between the AID and BID is crucial, as the disruption of the BID of an otherwise intact β subunit nullifies its ability to increase expression of α_1. Conversely, the coexpression of the BID alone, together with an α_1 subunit, is sufficient to produce some enhancement of current amplitude and acceleration of activation/inactivation

kinetics of the resultant channel (De Waard *et al.*, 1994). It is apparent, however, that the BID is not the sole participant involved in the β-mediated modulation, as only partial effects are observed when the rest of the subunit is absent. Also, the fact that sequence conservation occurs outside of the BID indicates that other portions of the β subunit may also form points of contact with α_1. They might alternatively be involved in the modification of channel properties by causing conformational changes in α_1, or the regulation of the various functional aspects of β itself. A short run of acidic residues located between residues 547 and 556 of β_{2a}, β_{1b} and β_4, for instance, has recently been identified as an additional α_1-binding site. The same motif of β_{1b} has been determined to be important in controlling the trafficking of that subunit as well (Brice *et al.*, 1997; Bogdanov *et al.*, 2000; Walker *et al.*, 1998; reviewed in Hanlon and Wallace, 2002). A sequence found in the N-terminus of β_{2a} serves a dual function, reducing inactivation of a co-expressed P/Q-type channel directly by interacting with its inactivation gate, and localising the protein to the membrane (Restituito *et al.*, 2000; reviewed in Arikkath and Campbell, 2003).

Various β-null mouse strains have either been generated or have arisen as the result of spontaneous mutation events, and these have allowed researchers the opportunity to observe the physiological effects of the β subunit. Presented below are brief discussions of the results pertaining to each of these mutants.

β_1

Gregg *et al.*, in 1996, reported on a strain of mice in which the *cchb1* gene encoding for the β_1 subunit of the VGCC complex had been disrupted via conventional

gene targeting techniques. Homozygous β_1-null foetuses are immobile, and also exhibit gross structural defects with respect to muscle tissue. At the cellular level, these mice show greatly reduced expression of the skeletal muscle-specific α_1 subunit, and the excitation-contraction coupling process is affected as a direct consequence of the lack of β_1 as well. Due to the fact that the mice do not survive beyond birth, it has thus far been impossible to investigate the behavioural effects of the mutation. A recently generated mouse strain that expresses functional β_1 in muscle, but not in other tissue types (including the brain), should be viable, and should allow for direct observation of the role that this subunit plays in brain development (Ball *et al.*, 2002; reviewed in Arikkath and Campbell, 2003).

β_2

β_2 deficiency results in lethality at the embryonic stage as a result of cardiac defects, thus demonstrating that this isoform is important in the regulation and modulation of $Ca_v1.2$ (Ball *et al.*, 2002). As in the case of β_1-null mice, however, the early lethal phenotype precludes the study of the physiological functions of β_2 in other systems. As a means to circumvent this problem, another mouse strain that expresses β_2 in the cardiac tissue, but not anywhere else in the animal, has been generated. These mice exhibit structural defects of the retina, and abnormal $Ca_v1.4$ distribution patterns in the vision cells as well. It therefore appears that, in addition to modulating the calcium current and regulating the expression of α_1, β_2 also participates in controlling the localization of certain VGCC complexes.

β_3

Knockout of β_3 expression in mice appears to produce no visible effects. The animals, even those homozygous for the deficiency, display no outward phenotype, and inspections of various tissues reveal no apparent morphological defects (Namkung *et al.*, 1998). It is not until the mice are examined at the electrophysiological level that any changes are seen. In the cervical ganglia, N- and L-type current density is diminished, while the kinetics of the P/Q-type current are affected slightly. On the other hand, in smooth muscle, where β_3 is predominantly expressed in normal mice, L-type currents are unaffected, as is the contractility of the tissue. Interestingly, when fed a diet high in sodium, the animals display signs of hypertension, which may indicate that certain aspects of smooth muscle cell function might still be affected by the loss of β_3, or that β_3-containing VGCCs may be involved in the regulation of blood pressure (Murakami *et al.*, 2000; reviewed in Arikkath and Campbell, 2003).

β_4

The *lethargic* mutation was mapped by Burgess *et al.* in 1999 to a locus in the mouse genome that corresponded to the *cchb4* gene. The specific lesion was a 4-base pair insertion into a splice site, which, assuming a gene product is made, would lead to the exclusion of the exon carrying the critical BID sequence, as well as a truncated protein. It was determined that homozygous animals produce no β_4 protein whatsoever, and so *lethargic* may be considered, for intents and purposes, as a null mutation.

Mutant mice are, as their name suggests, lethargic and display a general lack of movement. They also exhibit two types of seizures. The first of these causes localized

ataxia, and the other resembles absence seizure in humans. The animals show other defects, such as high mortality and deficiencies in immunological function as well. In the rare instances that a mouse is able to survive longer than two months, much of its immunity will be regained. Also, measurements of P-type current of Purkinje neurons are normal, demonstrating that there is no apparent loss of α_{1A} ($Ca_v2.1$) control even in the absence of β_4. It therefore appears that the other β subunit-types in the mutant animals are able, to a certain extent, to compensate by associating with, and modulating the activities of the calcium channels normally paired with β_4 (Burgess *et al.*, 1999). However, the fact that the lethargic phenotype is retained even in the surviving mice points to the fact that certain β_4 functions are unique to that isoform.

The structure, function and properties of the VGCC γ subunits

The first γ subunit (γ_1) was isolated by Campbell *et al.* in 1988 as part of an effort to elucidate the subunit composition of the skeletal voltage-gated calcium channel complex. It was subsequently characterized further and its DNA was cloned (Sharp and Campbell, 1989; Jay *et al.*, 1990; reviewed in Kang and Campbell, 2003), but due to the fact that it was initially thought to be specific to skeletal muscles only, was otherwise neglected. However, the recent identification of a γ subunit of neuronal origin (γ_2) in 1998 by Letts *et al.* has served to spark renewed interest in the subunit. Following this, six additional genetically distinct γ types ($\gamma_{3\text{-}8}$) were identified via in silico screens of the genome. All eight isoforms most likely arose from a single ancestral γ subunit gene via several rounds of gene duplication and, based on expression patterns and sequence similarity analysis, they are placed in one of two categories. The first of these, termed the

skeletal group, is comprised of γ_1 and γ_6. The rest of the known γ isoforms form the neuronal group (Burgess *et al.*, 2001; Chu *et al.*, 2001), which on the basis of homology can be further divided into two subclasses, the first consisting of γ_2, γ_3, γ_4, and γ_8, and the second of γ_5 and γ_7. It is important to note that, despite being classified in this fashion, γ subunits are not in fact restricted only to the particular cell type of the class to which they are assigned, nor just to skeletal or neuronal tissues. Indeed, γ_6 expression has been observed in neuronal tissue as well, while other γ isoforms may also be expressed in cardiovascular and reproductive tissues (reviewed in Arikkath and Campbell, 2003).

Certain identifying characteristics are shared between the members of the γ subunit family. The basic arrangement of the structure of the protein, for example, is well-conserved: every γ subunit is composed of four transmembrane segments that are linked by two extracellular and a single intracellular loops, and all terminate on either end with cytoplasmic extensions. Three other features have also been retained in all γ types, and these are found within the first extracellular loop alone. They are: a GLW*XX*C sequence unique to γ subunits, a N-glycosylation site, and two cysteine residues that may be linked by a disulphide group in vivo. cAMP/cGMP phosphorylation sites, as well as a PDZ binding domain in the C-terminus, are also common to all neuronal group γ subunits. In γ_2, γ_3, γ_4, and γ_8, the amino acid sequence of the latter element has been well-conserved, but the comparable motifs of γ_5 and γ_7 have undergone slight modification.

Unlike β or $\alpha_2\delta$, certain γ subunits can be trafficked to the surface membrane when expressed heterologously, independent of α_1. The γ subunits also appear to be unique in that they are relatively particular where the choice of partners is concerned. For

instance, γ_1 will bind only with calcium channels that contain $Ca_v1.1$, whereas γ_2 will not participate in the same complex (Moss *et al.*, 2002; Arikkath *et al.*, 2003; reviewed in Arikkath and Campbell, 2003). This fastidious nature of γ has been exploited by Arikkath et al., who in 2003 identified the site in this subunit that is responsible for mediating its association with α_1. For their study, they first constructed a series of γ_1-γ_2 chimeras, which they then tested for incorporation into skeletal muscle-type VGCC. It was eventually determined that the first half of the γ subunit was needed for the α_1-γ interaction, and while the specific site involved remains unknown, it is likely that the first extracellular loop is crucial, given the amount of conserved sequence found there. And, if that were the case, it is probable that N-glycosylation would be an important factor, as in the case of $\alpha_2\delta$. Besides the VGCC complex, the neuronal γ subunits also have the potential to interact with other components of the cell. The subunits of the AMPA receptor have been shown to represent one such set of targets, and γ_2 has been demonstrated to be critical for the maintenance of the expression levels of these proteins (Chen *et al.*, 2000). Also, the "post synaptic density" proteins PSD93, PSD95, SAP 97, and SAP102 may also be bound, although the physiological consequences of this association is unclear (Chen *et al.*, 2000).

The γ subunits also differ from the other auxiliary subunits in terms of their function, as they seem essential neither for the trafficking and/or expression of α_1, nor for the maintenance of the stability of the heterooligomeric channel complex. Rather, γ appears to be primarily involved in the modulation of the calcium current. When expressed in heterologous systems together with channels that contain the appropriate α_1 subunits, γ_1, γ_2, and γ_3 have inhibitory effects on the calcium current, and alter the

kinetics of activation and inactivation of the calcium channel slightly. γ_4 affects inactivation kinetics only, while γ_6 and γ_7 greatly reduce peak current amplitude (Chu *et al.*, 2001; Burgess *et al.*, 2001; Klugbauer *et al.*, 2001; Moss *et al.*, 2002; Chu *et al.*, 2002; reviewed in Arikkath and Campbell, 2003).

Ahern *et al.* and Ursu *et al.* have each independently generated strains of γ_1 null mice (2001). These animals are viable, and do not exhibit any behavioural abnormalities. At the cellular level, calcium channel expression and stability do not seem to be affected, and excitation-contraction coupling too, functions normally. However, calcium current in the muscle is measurably increased, and inactivation kinetics are altered, but not by a great extent. These observations largely appear to agree with the results of heterologous expression studies, and demonstrate that the effects of γ_1 subunits on calcium channel function is generally slight compared with that of the other VGCC subunits.

In 1998, Letts *et al.* reported on their studies of the stargazer mouse, in which the expression of the γ_2 gene had been effectively knocked out by a spontaneous retrotransposon insertion event. Unlike their γ_1 null counterparts, these animals were quite obviously affected by the mutation, and often display behavioural abnormalities consistent with absence epilepsy, such as ataxia and head tossing, as well as a lack of eyeblink coordination. These phenotypes, however, stemmed not from VGCC dysfunction (as evident from the fact that calcium current characteristics were not altered to an appreciable extent in the mutant mice), but were apparently the result of a lack of brain-derived neurotrophic factor and defective AMPA receptor trafficking (Qiao *et al.*, 1996; Chen *et al.*, 2000; reviewed in Arikkath and Campbell, 2003). It is therefore clear that, in addition to their primary function, certain γ subunits also are capable of

influencing numerous other elements of the cells in which they are expressed via pathways that have yet been elucidated.

The UNC-2 VGCC and neurotransmission in C. elegans

C. elegans is a relatively "complete" animal with respect to the cell types and organs that it possesses. Despite its small size, it contains a rudimentary nervous system, in the form of a network of neurons, that it uses to coordinate various activities. The complete structure of this organ has been elucidated (White *et al.*, 1986), as have much of the basic principles on which it operates.

Not surprisingly, the process of neurotransmission in this nematode is very similar to that is found in mammalian systems. For one, there is a considerable degree of overlap in the array of chemicals used to carry messages across neural synapses in humans and worms. Acetylcholine, dopamine, and serotonin (5-HT) have all been determined to act as neurotransmitters in *C. elegans* (del Castillo *et al.*, 1963; Sulston *et al.*, 1975; Horvitz *et al.*, 1982; reviewed in Rand *et al.*, 2000), and more recently, McIntire *et al.* (93a, 93b) demonstrated the existence of γ-aminobutyric acid (GABA) immunoreactivity and function in the worm as well. It is known that, as with mammals, neurotransmitter release in *C. elegans* is calcium-dependent, and is under the control of heteromultimeric voltage-gated calcium channels (VGCCs). To date, six VGCC genes have been identified in worms, three of which encode for α_1 subunits that form the pore of the complex, and whose structure dictates the major characteristics of the whole channel. The first of these, *egl-19*, is highly homologous to mammalian L-type channels, while a second gene, *cca-1*, corresponds to a T-type channel.

The *unc-2* gene, which is the subject of this study, was recently determined to encode for a third channel type that bears considerable structural similarity to mammalian dihydropyridine-insensitive, high voltage activated channels (Mathews *et al.*, 2003). Via a *unc-2* promoter-tagged green fluorescent protein construct, it was determined that the product of this gene is found mainly in motor and mechanosensory neurons, where it mediates the exocytosis of the excitatory neurotransmitter acetylcholine at the synapses. UNC-2 expression was also detected in the VC and HSN neurons, which are known to regulate egg-laying behaviour. In these cells, the UNC-2 calcium channel serves to control the release of GABA, which exerts an inhibitory effect on the postsynaptic neurons (Mathews *et al.*, 2003). Besides regulating the process of neurotransmission itself, the UNC-2 VGCC is also involved in determining the structure of the neural network of *C. elegans* as well as the developmental fates of the neurons contained therein, as it is involved in the control of neuronal migration and axon extension (Tam *et al.*, 2000) and gene expression (Troemel *et al.*, 1999).

The generation and isolation of the first *unc-2* mutants was reported as early as 1974 by Sydney Brenner, and since then, several additional mutant alleles have been identified. The phenotypes of different mutant strains are largely dependent upon the nature of the genetic lesion being considered, but they generally adhere to a common motif due to the shared etiology. Disruption of UNC-2 channel function in *C. elegans*, whether via null or missense mutations that lead to altered channel function, causes all physiological processes that are dependent upon the calcium current that it carries to be adversely affected. Thus, all *unc-2* mutant worms suffer from poor actuation and coordination of the body wall muscles responsible for the control of movement and

posture, and so are lethargic, kinked and appear flaccid, as opposed to wild-type worms, which move in a smooth sinusoidal fashion and whose muscles maintain a certain degree of tone even at rest. The processes of egg laying and defecation similarly operate under the influence of the channel encoded by *unc-2*, and therefore alterations in these behaviours are seen as well (Brenner, 1974). Abnormalities in the connectivity of the nervous system as a consequence of aberrant neuron placement or axonal extension during the process of development may also contribute to mutant characteristics (Tam *et al.*, 2000).

Based on the resistance of mutant worm strains to the acetylcholinesterase inhibitor aldicarb and susceptibility to the effects of nicotine, it was demonstrated that the Unc phenotype of the mutants largely arises from the failure to release acetylcholine at the neuromuscular junctions (Mathews *et al.*, 2003), and this is likely true of all other synapses at which the UNC-2 calcium channel is involved in the coordination of the exocytosis of neurotransmitter in the mutant worms as well. But, while the voltage-gated calcium channel encoded by the *unc-2* gene is clearly of great importance to proper neuronal function in *C. elegans*, it is apparently not essential, as evidenced by the fact that mutants bearing null alleles of the gene are viable and able to reproduce. This, combined with the transparency with which the nervous system of the worm operates, makes it relatively straightforward to conduct studies of this particular calcium channel. More importantly, these characteristics also facilitate the dissection of the various cellular events that lie up- and downstream of the UNC-2 calcium channel, as they allow for the generation, observation and maintenance of viable and fertile mutants with defects in these processes.

As already mentioned, the process of neurotransmission is very much alike in mammals and in *C. elegans*. Besides the array of neurotransmitters that are employed and the voltage-dependent calcium channels that are central to controlling their release, this similarity extends much further, and this is readily apparent from the fact that there is a high degree of commonality where the other neuronal components of these two seemingly disparate group of organisms are considered. For example, mammalian synaptic proteins such as syntaxin, synaptotagmin, and synaptobrevin, as well as many others, all have counterparts in *C. elegans*. Also, some of the genes that regulate the synthesis and the reception of the neurotransmitters and those that serve modulatory purposes in mammalian species have orthologs in the worm too.

C. elegans thus clearly has the potential to serve as an excellent model organism for the study of the various processes that underlie neurotransmission in humans. There are however certain gaps in our knowledge of the nervous system function that must first be addressed. The most important of these, at this juncture, concerns the fact that a considerable portion of the genes that are central to the proper operation of the nervous system of the worm remains unknown. The identification of these genes and the characterization their gene products is certainly relevant, as it not only would improve our understanding of the nematode nervous system, but would also facilitate the comparison of the various neurological phenomena that occur in humans and *C. elegans*.

Objectives of this study

The aims of the present study are: i) the identification of genes whose products that, either by interacting with the VGCC encoded by the *unc-2* gene, or by participating in the biochemical pathways in which that channel is involved, influence the process of

neurotransmission and neural function in *C. elegans*, and ii), to provide an initial description of the nature of these genes by determining the approximate position that their products occupy within the series of events that constitute nervous transmission.

Towards these goals, a genetic screen for suppressors of the *unc-2* gene was first performed. Secondary mutations were generated in worms already carrying a pre-existing null allele (*e55*) of the *unc-2* gene, and worms treated thusly were allowed to reproduce, and the progeny that exhibited some degree of alleviation of the initial *unc-2* mutant phenotype were selected by inspection. These were designated as "suppressor; *unc-2*," or "su," and ten such strains were isolated in total. Worms in which the suppressor (or "s") mutations had been segregated from the *e55* allele (otherwise known as suppressor-only, or "s" strains) generally had very mild uncoordinated phenotypes. All suppressor mutations appeared to be recessive, and none exhibited significant sex-linkage.

The suppressor mutations were then characterized further, first by crossing the "s" mutations back into the original *unc-2(e55)* background, then to a strain carrying the *ra612* missense allele of the same gene. The purposes of these experiments were, respectively, to determine if the suppression of the *unc-2* mutant phenotype was indeed produced by the putative suppressor mutations alone, and if the suppressor effects were dependent on the nature of the *unc-2* mutation. The effectiveness of the suppressor mutations in positively modifying the behaviour of worms carrying either the *e55* or the *ra612* allele of *unc-2* were then confirmed using thrashing assays, following which the various "s" and their corresponding "su" strains were tested for their resistance to the acetylcholinesterase (AchE) inhibitor aldicarb in order to determine the localisation of the products encoded by the suppressor genes with respect to the synapse.

Two of the suppressor mutations were then mapped using the Snip-SNP technique. The suppressors s10 and s6 were found to localise to chromosome I and IV respectively.

CHAPTER TWO.
MATERIALS AND METHODS

Genetics

Nematode strains and growth conditions

General

Nematodes used in this study are named according to the standard nomenclature for *Caenorhabditis elegans* established in 1979 by Horvitz *et al.* Unless stated otherwise, all worms were cultured in 10-cm Petri dishes on NGM agar streaked with *Escherichia Coli* strain OP50 (for additional information regarding maintenance of worms, refer to Brenner, 1974), and were incubated at an ambient temperature of 18 °C. Observation and manipulation of animals were carried out at 25 °C.

Nematode strains

The following nematode strains were used in this study: wild-type *C. elegans* (var. Bristol) **N2**, wild-type *C. elegans* (var. Hawaii) **CB4856**, *unc-2(e55)* **CB55**, and *unc-2(ra612)* **DM612**.

Isolation of suppressors of *unc-2*

A single suppressor screen was performed using the protocol outlined by Brenner (1974), with some modifications. Six hundred adult *unc-2(e55)* hermaphrodites were treated with 50 mM EMS for 4 hr at 25 °C. Following this, five hundred of these animals were transferred onto three 25-cm petri dish with NGM Agar and allowed to recover for 4 hr before being placed singly onto individual 10-cm plates and brooded over 3 days at

25 °C (with the transfer of the adult P_0 hermaphrodites to a new plate performed once every 24 hr). Five hundred worms, some of which exhibited alterations in behaviour, were selected from the various broods, and again allowed to self-cross for 3 days at 25 °C. The resultant progeny were inspected for individuals that displayed movement characteristics that were improved over those of the Unc P_0 worms. Twelve independent F_1 isolates were found to segregate offspring that satisfied this criterion, and were isolated for further analysis. The suppressor; *unc-2 (e55)* (suppressor *unc-2*/ suppressor *unc-2*) F_2 double mutants produced by these worms were given three-part names consisting of a "su" prefix - signifying the presence of both the suppressor mutation and a *unc-2* mutant allele - followed by a numeral that uniquely identifies each strain, and ending with a "55" suffix indicating that the mutant *unc-2* allele of interest in this case is *e55* (as an example, the first such strain would be named su155, the second, su255, and the X^{th}, suX55, the name by which these double mutant strains shall henceforth collectively be referred to).

Outcross of suppressor mutations

To isolate the suppressor effects from the influence of *unc-2(e55)*, hermaphrodites from each of the suX55 strains were crossed to N2 males. Fifteen wild-type progeny from these crosses, which were necessarily heterozygous for both the suppressor mutations and the *e55* allele of *unc-2* (i.e., suppressor *unc-2*/ + +), were picked to individual plates and allowed to self-cross, following which the progeny were inspected to select for individuals that were neither wild-type, Unc, nor suppressed-Unc. These animals were picked to individual plates and self-crossed for three generations to ensure that the mutant characteristic bred true in each of the lines, after which the suppressor-only strains,

referred to as "sX" from this point on, consistent with the nomenclature described in the preceding section, were outcrossed twice more to minimise the number of background mutations present (the term "sX" also applies to the suppressor mutations carried by these strains). These strains were then collected for more detailed analysis.

Construction of suppressor; *unc-2(e55)* and suppressor; *unc-2(ra612)* double mutant strains

Due to the high concentration of EMS (50 mM) used to generate mutants in the suppressor screen, there was a high probability that other genes in the su strains were affected as well. To ensure that the morphological and behavioural alterations observed in the suppressor strains were solely the products of the interactions between the suppressor mutations and the *unc-2* genetic background, the suppressor; *unc-2(e55)* double mutant strains were reconstructed. To do this, suppressor/ + males, produced by crossing hermaphrodites homozygous for the suppressors to N2 males, were crossed to Unc hermaphrodites (*unc-2/ unc-2*). Fifteen wild-type hermaphrodite progeny from these crosses, which must necessarily be heterozygous either for both the suppressor mutations and *unc-2(e55)* (i.e., suppressor *unc-2*/ + +) or for *unc-2(e55)* only (*unc-2*/ +), were picked to individual plates and self-crossed. The progeny of these worms were then inspected for the presence of suppressed-Unc (suppressor *unc-2*/ suppressor *unc-2*) double mutants. These were again picked to individual plates and their phenotypes were compared to those of the double mutants isolated in the suppressor screen.

To determine if the suppression effects of the suppressor mutations were specific for the *e55* allele of the *unc-2* gene only, suppressor; *unc-2(ra612)* double mutants were also constructed, following the procedure described above. Following the nomenclature

introduced in a previous section, these strains were assigned three-part names also, with a "suX612" format ("612" here signifying that *ra612* is the mutant *unc-2* allele that is involved in this case).

Phenotypic analysis of suppressor strains

Aldicarb resistance

Assay method

Plates to be used for this assay were prepared according to the method described in Miller *et al.*, 1996, with a few modifications. A 105 mM stock solution of aldicarb (in ethyl alcohol) was added to liquid NGM agar to a final concentration of 0.5 mM before the mixture was poured into 10-cm petri dishes. The plates were then streaked with *E. Coli* (OP50), and stored at 4 ^{o}C. To perform the assay, twenty adult hermaphrodite worms from each of the s, suX55, and control strains (wild-type N2 and *unc-2(e55)*) were placed on the aldicarb plates and monitored over a 130 min period. At ten-minute intervals, worms from each strain were tested for paralysis by assessing their response to harsh touch. Non-motile worms were then removed from the plates and the cumulative number of paralysed animals was recorded. This quantity was then plotted against time to generate the curves shown in the appendix.

Data analysis

Time was plotted against the cumulative number of paralysed animals at each of the various time points for the mutant strains and the relevant control strains. The resultant curves were fitted to third-order (of the format $y = ax^3 - bx^2 + cx + k$) or fourth-order ($y = ax^4 + bx^3 - cx^2 + dx + k$) polynomial equations, or to linear equations ($y = ax +$

k) in cases where the total number of paralysed animals at the end of the assay period was significantly below 50% of the start value. "T_{50}" values (defined as the time point at which 50% of the worms become paralysed) for each of the mutant strains were derived from these equations, and from this, the normalised T_{50} values (rel. T_{50}) were calculated.

Thrashing assay

Assay method

This assay was performed using the method outlined in Miller *et al.*, 1996, again with a number of changes. Fifteen hermaphrodites from the suX55, suX612 groups and the corresponding control strains, *unc-2(e55)* and *unc-2(ra612)*, as well as wild-type N2 worms, were placed into polycarbonate microtitre wells containing 100 μL of M9 buffer. After a short recovery period (~ 1 min), the number of thrashes performed by each worm over 1 min was tallied.

Data analysis

"TpM" values were calculated by averaging the total number of thrashes performed by the worms of the mutant and control strains by the total number of animals in each set. Normalised TpM values were then obtained by dividing the TpM achieved by each of the mutant strains by that of the respective controls.

Molecular biology

Snip-SNP mapping

The Snip-SNP mapping approach, developed by Wicks *et al.* (2001), exploits the presence of numerous single-nucleotide polymorphisms that exist between the wild-type Bristol isolate of *C. elegans* (N2) and its Hawaiian counterpart (CB4856), several of

which affect restriction enzyme recognition sites. This subset of SNPs confers upon the genomic DNA of these strains unique restriction patterns, which serve as identifiers of the genetic origin of a particular chromosome, and of specific regions within chromosomes, and this can be used to determine the approximate localisation of recessive mutations with observable phenotypes. CB4856 males are mated to hermaphrodite worms that were homozygous for the mutation of interest (which are of a N2 genetic background), and the resultant F_1 progeny were allowed to self-cross. Mutant and wild-type F_2 worms are collected to separate pools, and their genetic compositions are compared via restriction length polymorphism analysis. As mutant animals must necessarily be homozygous for the region of DNA bearing the visible marker, their genomes will therefore be enriched for DNA of N2 origin across these particular sites, and this is easily detected via visual inspection.

The genetic correlates of suppressors s6 and s10 were physically mapped using this method. The sections that follow detail the steps that were involved in doing this.

Bulked segregant analysis (BSA) and genomic DNA extraction

CB4856 males were mated to hermaphrodites homozygous for the suppressor mutations. Nine offspring from each mating were picked to individual plates and allowed to self-cross. F_2 progeny were inspected visually and phenotypically wild-type animals were picked to a thin-walled polycarbonate PCR tube containing 50 µl worm lysis buffer, to which 25 µl of proteinase K solution was added. Mutant animals were treated in an identical fashion. The tubes were frozen at –80 °C for 5 min before they were placed into a thermocycler (DYAD) and incubated, first at 60 °C for 90 min, then at 95 °C for 15 min in order to halt proteinase K activity.

The resultant lysates were diluted with additional worm lysis buffer to a final volume of 150 µL. 90 µL of this was drawn off for use as template material in the PCR reactions.

PCR amplification, restriction digest, and DNA fragment length analysis

To perform the PCR reactions, 5 µl lysate, 5 µl 10X PCR buffer and 1 µl 10 µM dNTP were mixed with 34 µl ddH_2O in polycarbonate PCR tubes. To this, 4 µl of the appropriate primer (10 µM) was added, followed by 1 µl Taq Polymerase. The mixture (final volume 50 µl) was subjected to 36 amplification cycles (95 °C for 40 s, 58 °C for 40s, and then 72 °C for 40 s) in a thermocycler (Dyad).

Restriction digest reactions were performed by mixing 3 µl of 10X restriction buffer and 1 µl restriction endonuclease (of the appropriate types), with 25 µl of PCR product in polycarbonate tubes (0.3 µl of bovine serum albumin was also introduced where necessary), which were then incubated at 37 °C for at least 2 h.

Products from the PCR and restriction digest steps were ran in parallel, alongside 1-kb DNA ladder (Invitrogen), on 2% agarose gels. Gels were treated with ethidium bromide solution and fragment bands were visualised under UV illumination.

CHAPTER THREE.
GENETIC AND PHENOTYPIC ANALYSIS OF SUPPRESSORS OF *unc-2*

Background

The *C. elegans unc-2* gene, first identified by Schafer and Kenyon in 1995, was recently characterized in detail in a study conducted by Mathews *et al.* (2003). The channel that is encoded by this gene was found to bear a high degree of structural homology to mammalian dihydropyridine-insensitive HVA channels. Primarily localized to motor and sensory neurons of *C. elegans*, as well as a subset of neurons involved in the control of egg-laying, it provides the calcium signal that triggers the exocytosis of acetylcholine and γ-aminobutyric acid (GABA), respectively, from these cells (Mathews *et al.*, 2003). Recent evidence indicates that the channel is also crucial in determining the structure of the nervous system of the worm by directing processes such as neuronal migration and axonal extension (Tam *et al.*, 2000) and by influencing gene expression (Troemel *et al.*, 1999). Given the numerous functions of this gene, it is perhaps not surprising that *unc-2* mutants display several defects. These animals are generally slow moving, and adopt a kinked posture that is particularly evident when they are at rest. Egg-laying and defecation behaviours are affected as well. The severity of the phenotype depends greatly on the specific allele being considered, but it has been observed that, even in the most extreme of cases, in which protein function is obliterated completely, mutant worms continue to be viable and fertile. It is therefore clear that this gene, while important, is not absolutely crucial.

While much is understood about the nature and the function of the UNC-2 channel, knowledge regarding the events that occur up- and downstream, and intermediate to the calcium influx and neurotransmitter release steps remains somewhat lacking at this point in time, particularly with respect to the specific identities of the genes and gene products involved in mediating the myriad cellular pathways that constitute neurotransmission, and this poses a hindrance to the making of direct comparisons between the neurological processes observed in the worm and those occurring in mammalian species. It is therefore the objective of this project to identify the various genes and gene products that are important in these steps, as it is expected that this information would facilitate the elucidation of the various interactions that take place within these pathways, and lead to a better understanding of the neurotransmission process in *C. elegans*. Given the numerous similarities that exist between mammalian and nematode neurotransmission, it is likely that the findings from this study would be applicable to humans as well.

In this study, I isolated twelve *C. elegans* strains that bear mutations in genes whose products may participate in UNC-2-mediated neurotransmission pathways via a genetic screen for suppressors of *unc-2* mutants. The effects of each of these suppressor mutations on animals with various genetic backgrounds were characterized, and evaluated using standardized behavioural assays. The localisation of the products of the affected genes, in relation to the nervous synapse, in each of these strains were determined by testing single mutants with the suppressor mutations only, and double mutants homozygous for both the suppressor mutations, as well as a *unc-2(e55)*, for resistance to the acetylcholinesterase inhibitor aldicarb. Finally, a subset of the mutations

was physically mapped to locations within the worm genome with the Snip-SNP technique.

Results

Suppressors of *unc-2* were isolated via a suppressor screen

To isolate suppressors of *unc-2*, a population of approximately 1800 adult hermaphrodite worms homozygous for the *e55* null allele of the *unc-2* gene were mutagenized by exposing them to 50 mM EMS over a period of 4 hours. 500 of the animals that were treated in this manner were selected and allowed to self-cross on separate plates. The F_1 animals produced were then inspected, and 500 of these were selected (with particular bias towards individuals that displayed any alteration in movement characteristics) and picked to individual plates and self-crossed. The F_2 worms produced were then inspected for mutants in which the Unc phenotype observed in the P_0 generation had been suppressed (Figure 3.1). Animals that met this criterion were immediately sequestered for detailed observation, and were also self-crossed thrice more to ensure that the various suppressor mutations and their effects were stably inherited by subsequent generations.

Different suppressors provide varying degrees of suppression

Twelve independent F_1 isolates from the suppressor screen segregated offspring that, to varying degrees, exhibited some alleviation of the Unc phenotype. Qualitative evaluations of the relative efficacies of the various suppressors carried by these suX55 mutants are shown in Table 3-1A (together with the mutant phenotypes of the mutant strains, which are discussed in detail in a subsequent section). Mutant animals were listed

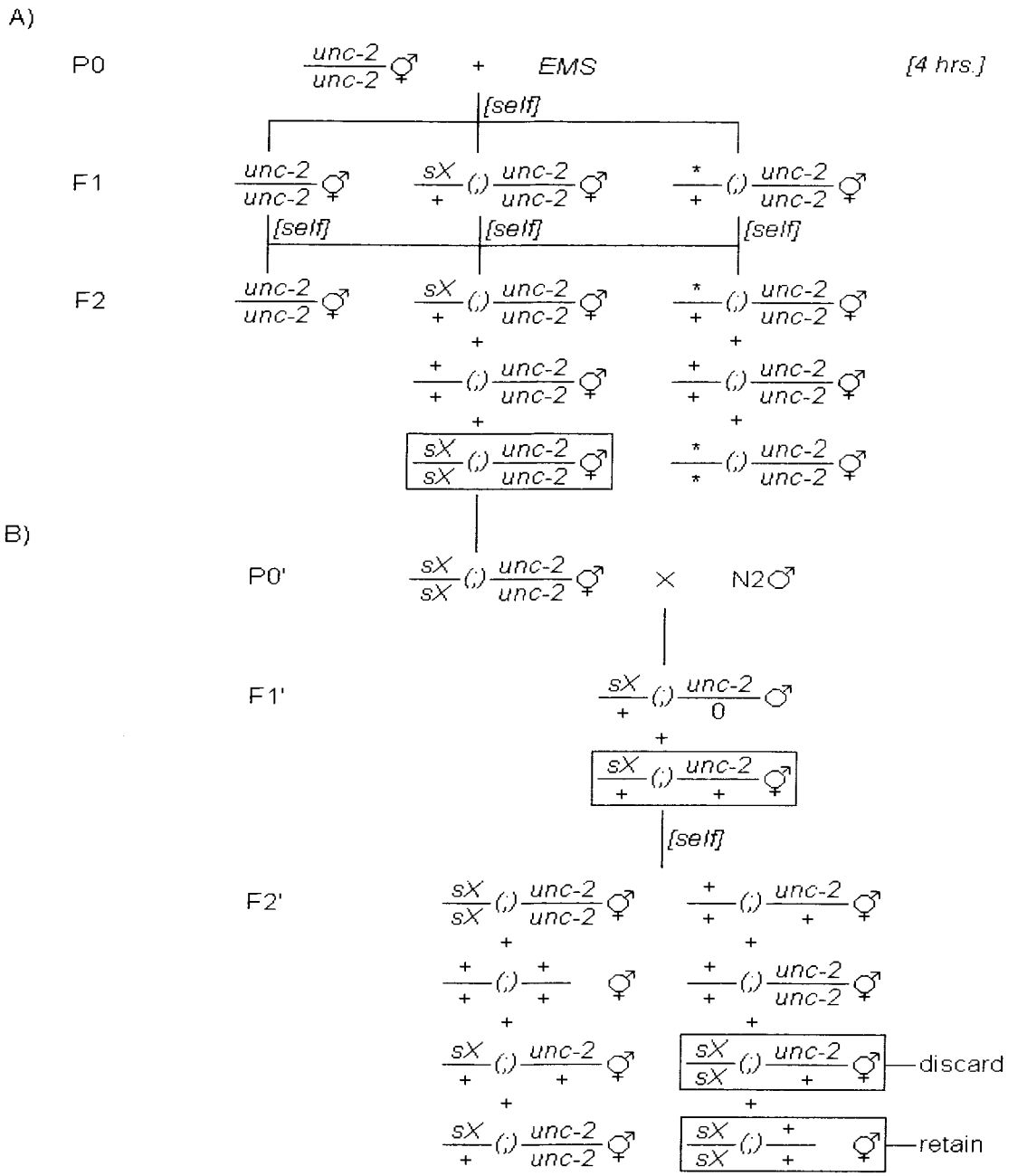


Figure 3.1 Flowchart illustrating suppressor screen

A) *unc-2(e55)*-homozygous hermaphrodites were mutagenized with 50 mM EMS and allowed to self. F1 progeny were also self-crossed, and the F2 worms were screened for individuals in which the Unc movement phenotype of the P0 worms had been suppressed. B) Suppressor-homozygous, *unc-2*-homozygous worms were crossed to wild-type males, and hermaphrodite progeny were picked to individual plates and self-crossed. F2' animals with mutant phenotypes that were unlike those of *unc-2* or N2 animals were selected, and observed over several generations. Lines that bred true and did not segregate Unc offspring were retained for analysis.

Table 3-1A Phenotypes of suX55 animals

unc-2 (e55)	severe unc., sluggish movement, kinks at rest
su155	mild unc., exaggerated sinusoidal mvmt.
su255	mild unc., depth of body bends increased
su355	mild unc., depth of body bends increased
su455	moderate unc., depth of body bends increased
su555	moderate unc., numerous deep body bends at rest
su655	mild unc., mottled appearance
su755	moderate unc., L2 and L3 larvae thin and long
su855	mild unc., appears over-extended at rest
su955	moderate/mild unc., thin, appears over-extended at rest
su1055	mild unc., uncoordinated tail movement
su1155	mild unc., thin, appears over-extended at rest
su1255	very mild unc., wt-like

Table 3-1B Phenotypes of suX612 animals

unc-2 (ra612)	moderate unc., slow-moving, numerous deep body bends
su1612	very mild unc., reduced number of body bends
su2612	mild/moderate unc., reduced number of body bends
su3612	mild unc., depth of body bends reduced
su4612	mild unc., otherwise similar to *unc-2 (ra612)* worms
su5612	mild/moderate unc., shallower body bends
su6612	mild unc., mottled appearance, depth of body bends reduced
su7612	mild unc., thin, depth of body bends reduced
su8612	moderate unc., slow-moving, numerous deep body bends
su9612	mild unc., reduced number of body bends
su10612	mild unc., depth of body bends reduced
su11612	very mild unc., depth of body bends reduced
su12612	mild unc., otherwise similar to *unc-2 (ra612)* worms

Table 3-1C Phenotypes of sX animals

Bristol N2	wild-type, smooth sinusoidal mvmt.
s1	exaggerated sinusoidal mvmt.
s2	mild/moderate unc., slightly sluggish
s3	slightly exaggerated sinusoidal mvmt., arrhythmic forage motion
s4	less responsive to harsh touch, rapid and arrhythmic forage motion
s5	arrhythmic, exaggerated forage motion
s6	mottled appearance, otherwise wild-type-like
s7	animals long and thin
s8	mild/moderate unc., slightly sluggish
s9	slightly exaggerated sinusoidal mvmt.
s10	mild unc., uncoordinated tail mvmt., over-extends on initial turn-in
s11	adults less responsive to harsh touch
s12	mild unc.

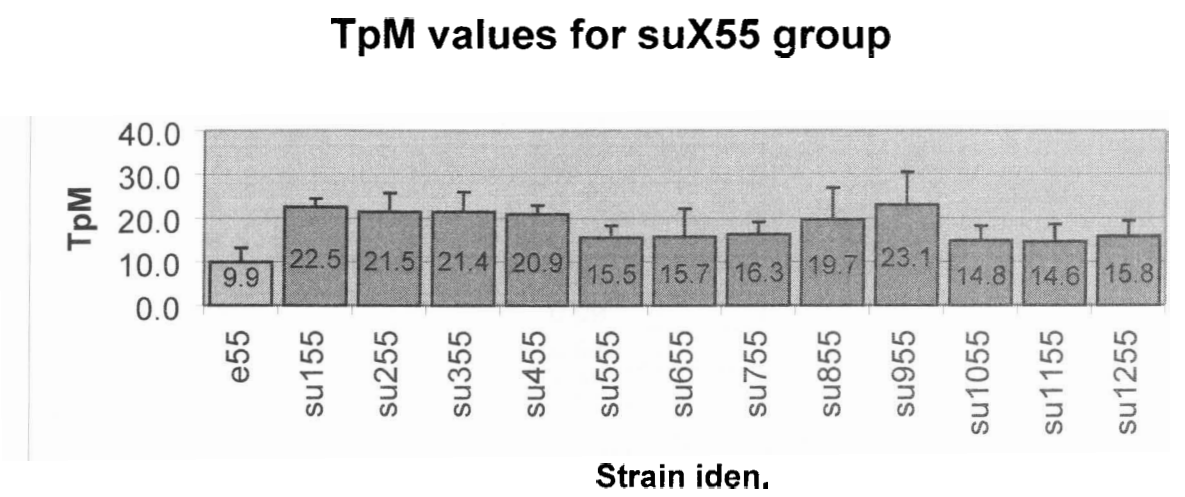
as being severe, moderate, mild, or very mild Uncs according to the effectiveness with which they improve the movement characteristics of severely uncoordinated *unc-2(e55)*-homozygous animals on solid agar medium. This was assessed via three subjective criteria: spontaneous movement, response to harsh touch, and rate and range of movement. *unc-2(e55)* mutant animals performed poorly in these three areas, while all twelve suX55 strains display movement behaviour that were comparatively more wild-type-like. Of the double mutants, su455, su555, su755 and su955 displayed detectable increases with respect to movement rates and distances, but were listed as moderate Uncs as they retained several *unc-2(e55)*-like characteristics. Mutants su155, su255, su355, su655, su855, su1055 and su1155, on the other hand, displayed substantial improvements over *unc-2(e55)* worms and were therefore listed as mild Uncs. su1255 showed a marked increase in all areas of performance that were considered, with the phenotype of this strain virtually mirroring that of the wild-type N2 animals. In all cases, resting muscle tone was also visibly improved.

The above analysis, due to its subjective nature, is ultimately limited in its usefulness. To provide additional objective evidence for the effectiveness of the various suppressors, thrashing assays were also performed with animals from the suX55 group. This assay was carried out by placing the adult worms from the various mutant strains in 100 µL of M9 buffer and recording the number of times each worm thrashes over a period of one minute (the TpM value). The "TpM" values for each of the strains were then normalized against that of the control (worms homozygous for the *e55* allele of the *unc-2* gene) to derive the relative TpM value, "rel. TpM." The rel. TpM value serves as

an objective measure of the degree of suppression provided by the suppressor mutations present in each of the suX55 strains.

It was seen that all the suppressors that were isolated in the genetic screen appreciably improved the movement rates of the worms (Figure 3.3A, Table 3-2A), with the rel. TpM values achieved by the mutants ranging between 1.5 (for su1055 and su1155) to 2.3 (su155 and su955), and averaging at 1.8 for the twelve strains. For seven of the twelve suX55 mutants, su155, su255, su355, su555, su755, su855, and su955, the rel. TpM values appeared to correlate well with subjective evaluations of the movement characteristics of the worms on solid media (i.e. NGM agar), that is, within this group, animals that were judged to be "mild" Uncs generally achieved scores that were significantly higher than those that were determined to be "moderate" Uncs. However, mutants su655, su1055, su1155, and su1255, which were judged to be as "mild" or "very mild" Uncs when observed on solid medium achieved rel. TpM scores of 1.6, 1.5, 1.5, and 1.6, respectively, which were on the extreme low end of the scale, while su455, which qualified as a "moderate" Unc based on subjective criteria scored a comparatively high rel. TpM value of 2.1. This apparent discrepancy in the results may be attributed to the nature of the thrashing assay itself. As the worms were suspended in liquid medium, they were deprived of any mechanosensory feedback that they might have otherwise received if placed on a solid surface. Consequently, the animals lacked a frame of reference, and this may have interfered with the proprioception that might have been crucial in maintaining coordinated movement, thus leading to a reduction in movement rates. While this would have applied to all the mutant strains that were tested using this method, it is important to note that, since the various suppressor strains differed in their

A)



B)

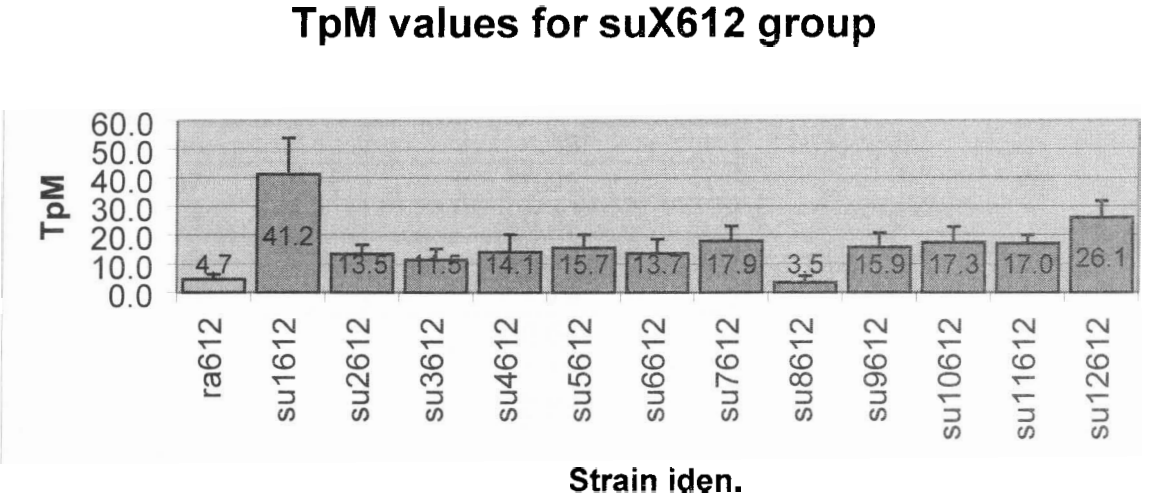


Figure 3.2 Results of thrashing assay

A) Results for the suX55 group. Every mutant strain in this group displayed suppression of the Unc phenotype. TpM values in figure are the raw values, and have not been normalized against the *unc-2(e55)*-homozygous control. Refer to text for discussion. B) Results for the suX612 group. All strains, except for su8612, achieved TpM scores that were significantly higher than that obtained by the *unc-2(ra612)*-homozygous control.

Table 3-2A Normalized TpM scores for suX55 animals

Strain	rel. T50
e55	1.40
s1	0.96
s2	1.04
s3	0.97
s4	1.04
s5	4.36
s6	3.84
s7	0.87
s8	0.72
s9	0.77
s10	0.94
s11	0.99
s12	0.98
N2	1.00

Table 3-2B Normalized TpM scores for suX612 animals

Strain	rel. T50
e55	1.00
su1	0.58
su2	0.60
su3	0.59
su4	2.11
su5	5.00
su6	4.48
su7	0.95
su8	0.37
su9	0.50
su10	1.79
su11	0.64
su12	1.10
N2	0.63

genetics (particularly where the genes that are concerned with regulating movement are concerned), they might not have been affected to equal extents. Therefore, while the data from the thrashing assay was useful for substantiating subjective observations of the effects of suppression, it was not suitable for analytical purposes.

Most suppressor mutations function in an allele-independent manner

As the suppressor screen was performed against the *e55* allele of *unc-2*, which was probably a null allele given the specific genetic lesion that is involved (see Figure 3.2) it was expected that the majority of the suppressors that were isolated would be allele-independent, that is, suppression of the Unc phenotype should occur regardless of the mutant *unc-2* allele with which these suppressors are combined. To ascertain if this was indeed true, various sX mutations were crossed into a strain of worms homozygous for the *ra612* allele of *unc-2* (see Figure 3.2 for the molecular alteration involved in this allele). Unlike worms that are *unc-2(e55)*-homozygous, a voltage-gated calcium channel is likely expressed in *unc-2(ra612)*-homozygous worms, albeit one with altered eletrophysiological properties resulting from a missense mutation in the C-terminus region (Mathews, 2000). In terms of movement rate, these worms are less uncoordinated than *unc-2(e55)*-homozygous worms, and they can also be further distinguished by their numerous mid-body bends.

When on solid media, all suX612 worms, with the exception of su8612, exhibited movement rates that were considerably improved over those of *unc-2(ra612)* worms (Table 3-1B). As with the suX55 group, these animals were also subjected to thrashing assays, the results of which supported the subjective observations. Relative to *unc-2(ra612)* worms, all the suX612 animals (excluding su8612) thrashed much more

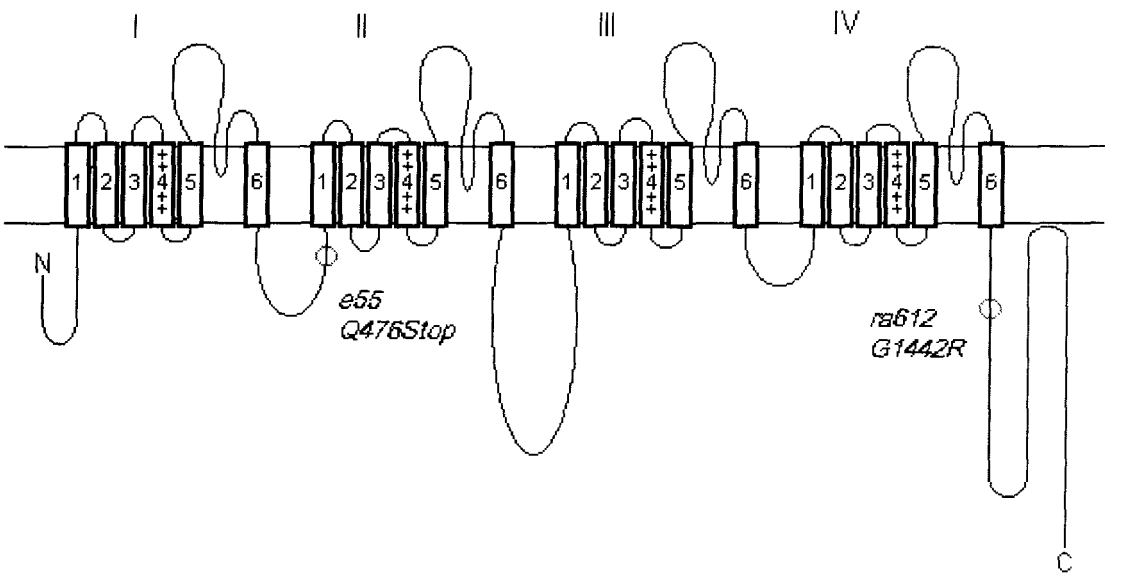


Figure 3.3 Sequence alterations involved in mutant alleles of *unc-2*

The *e55* allele, against which the suppressor screen was performed, contains a mutation that causes premature truncation of the channel protein at position 476 of the amino acid sequence, and therefore likely represents a null allele of the *unc-2* gene. The *ra612* allele contains a missense mutation that causes glycine 1442 to be substituted by an arginine residue.

frequently, achieving rel. TpM values ranging from 2.4 to 8.8 (see Figure 3.3B and Table 3-2B). su8612, which did not exhibit improved movement over *unc-2(ra612)* worms on solid medium, scored a rel. TpM value of 0.7. It is therefore likely that the s8 suppressor mutation is specific for the *e55* allele of *unc-2*.

When the thrashing assay results for the suX612 and suX55 sets were compared, it was seen that not only were the rel. TpM values were much higher for the former in general, there also appears to be little correlation between the scores achieved by each of the corresponding suX612/suX55 pairs, even after the exclusion of the expected outlier, su8612/su855 (data not shown). While this was initially surprising given that the non-allele-dependent suppressors were expected to affect cellular components that functioned distally and independently from the VGCC, this phenomenon can probably, again, be attributed to the fact that the thrashing assay was conducted with the worms in a liquid medium. The confounding effects of this became apparent when the absolute TpM values for the *unc-2(ra612)* and *unc-2(e55)* worms (Figures 3.3A and 3.3B) were compared. While the former strain out-performed the latter based on subjective evaluations of movement on NGM agar, animals from that group thrashed less than half as frequently on average (4.7 times versus 9.9 per minute) in M9 buffer. The change in the *unc-2* genetic background, combined with the differences in the nature of the suppressor mutations carried by each of the strains, could certainly account for the disproportionate manner in which suppression occured in the two groups of worms.

Suppressors can be presynaptic, synaptic, or postsynaptic

The voltage-gated calcium channel encoded by the *unc-2* gene responds to depolarisation of the membrane of the neurons in which they are embedded by becoming

transiently permeable to the calcium ions in the extracellular fluid. It is this calcium ion influx that triggers a series of cellular events (which are outlined in the next chapter) that ultimately result in the release of neurotransmitter stores from the neuron onto the effector cells with which they are associated. In the case of the cholinergic neuromuscular junction, the primary neurotransmitter that is involved is acetylcholine, which functions in a stimulatory capacity to bring about contraction of postsynaptic muscle cells. Acetylcholinesterase enzymes quickly remove acetylcholine from the synapse by catalysing the decomposition of the neurotransmitter into acetate and choline, and this ensures the specificity of the signal while ensuring that the postsynaptic muscle cells do not become over-stimulated.

Worms that are homozygous for loss-of-function alleles of *unc-2* (such as *e55*, for example) exhibit several behavioural defects, the most notable of which affect locomotion. This is due in large part to the fact that the activity of the voltage-gated calcium channels has been disrupted in these mutants, and as a consequence, the presynaptic neurons of the NMJ do not release sufficient amounts of neurotransmitter onto the body wall muscles that they innervate. Coordinated actuation of these muscles therefore does not occur, and so these animals move in a lethargic fashion. For the same reason, these mutant animals are also less sensitive to the effects of the acetylcholinesterase inhibitor aldicarb. While wild-type worms quickly hypercontract and become paralysed when exposed to this drug, *unc-2*-mutant worms can maintain motility over much longer periods of time, as the initial acetylcholine concentrations in the synapses of these animals is comparatively low.

Given the nature of the defect, suppressors can function in one of three general ways to rectify this deficiency. They can either increase the amount of neurotransmitter released by the presynaptic cell onto the muscle cell for every depolarisation event, amplify the response of the postsynaptic muscle cell to each unit of neurotransmitter that it receives, or increase the effective concentration of acetylcholine by increasing the period over which the neurotransmitter remains in the NMJ. These modes of suppression are referred to as "presynaptic," "postsynaptic," and "synaptic," respectively (a more thorough discussion on this subject is provided in the following chapter). As these three possible suppression methods involves direct modification of neurotransmission characteristics, the presence of a suppressor mutation should also alter the way in which a worm reacts to aldicarb. Mutants bearing suppressors of the presynaptic class, owing to the elevated levels of acetylcholine in their NMJ, should become more sensitive to aldicarb compared to a wild-type worm. "Postsynaptic" mutants, on the other hand, should at most exhibit a slight increase in sensitivity. Worms that are homozygous for a particular class of "synaptic" suppressors, which involve mutations that result in the reduction of acetylcholinesterase function by decreasing its affinity for its substrate, will be resistant to the effects of aldicarb, as the same mutations that negatively affect acetylcholine-acetylcholinesterase binding in such a manner will likely disrupt binding of aldicarb to the enzyme as well. The sensitivity of any suppressor strain to aldicarb can therefore provide much information regarding the localisation of the affected cellular component with respect to the synapse and aid in its identification.

Twenty animals from each of the sX strains, and from a wild-type N2 strain (used here as a control), were exposed to aldicarb, and the number of paralysed worms for each

of these strains were totalled at ten-minute intervals for each set over a period of 130 minutes. In order to perform a direct comparison of the data, "time" was plotted against "total number of paralysed worms," and third- or fourth-order polynomial or linear equations (whichever resulted in a best fit) were matched to the resultant curves. The respective T_{50} values, the time point at which 50% of the worms become paralysed, were then derived from these equations via substitution. The "rel. T_{50}" quantity was then calculated by normalizing the absolute T50 values of the mutant strains against that of the wild-type control (Figure 3.4A).

s5 and s6 appeared to be much less sensitive to aldicarb, and achieved rel. T_{50} values that were significantly higher than 1.00, indicating that the suppressor mutations in these strains most probably occured in genes that encode for synaptic components, such as the acetylcholinesterase enzymes. s8 and s9, were significantly more sensitive to aldicarb, as evidenced by their relatively low rel. T_{50} values, which indicated the suppressors in these strains were probably presynaptic. The rel. T_{50} achieved by the remainder of the mutants did not deviate significantly from a value of 1.00. While a subset of these undoubtedly corresponded to bona fide postsynaptic mutants, it is unlikely that they are all of this type. This "clustering" may have resulted from the wild-type *unc-2* genetic background on which they were based, which raised the overall level of sensitivity of these animals to aldicarb.

To resolve the effects of the genetic background, aldicarb assays were also performed on the suX55 mutants (with *unc-2(e55)* worms as a control) that, due to the fact that they were all homozygous for the mutant allele of *unc-2*, were as a whole significantly less sensitive to aldicarb. T_{50} values were also calculated for this group

A)

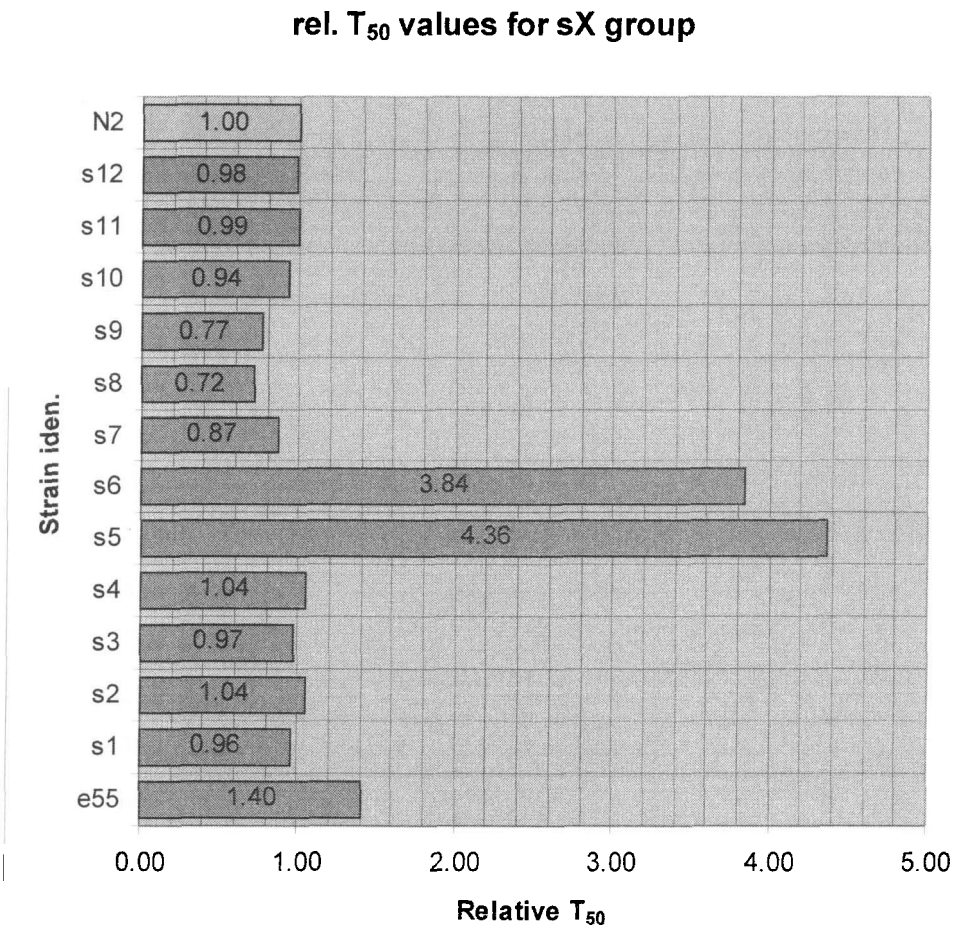


Figure 3.4A Results of aldicarb assay for sX group

Normalized T50 values of the sX group, with wild-type N2 as the control (refer to appendix for graphs from which these values were derived). With the exception of s5, s6, s8, and s9, most sX mutants achieved scores of about 1.00. The significance of these results is discussed in the text.

B)

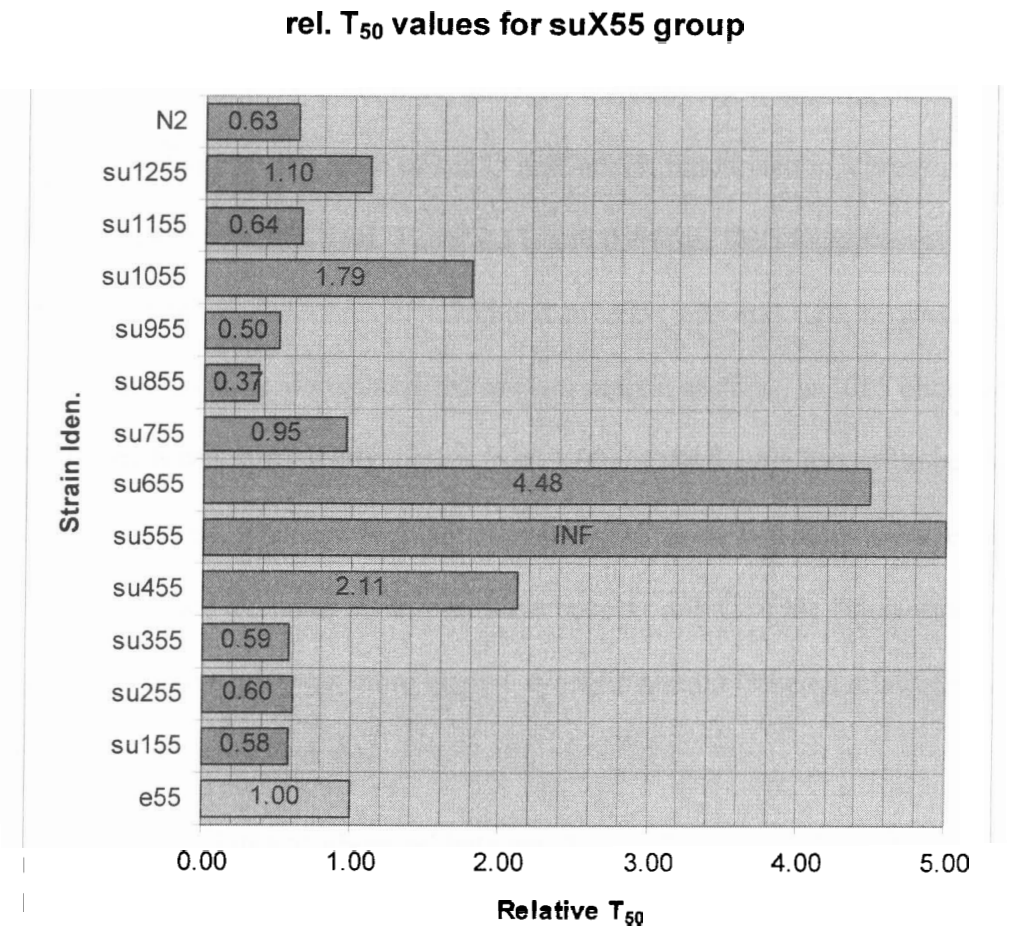


Figure 3.4B results of aldicarb assay for suX55 group

Normalized T50 values of the suX55 group, with *unc-2(e55)*-homozygous worms as a control. The presence of the *e55* allele extends the time course of paralysis for most strains, and allows the mechanism of suppression to be determined for s1, s2, s3, s6, s7, s10, s11, and s12. See text for further details.

using a method identical to that used for the sX group, and the rel. T_{50} values were derived by normalising the T_{50} scores for the mutants against that of the control. The results are shown in Figure 3.4B. su555 and su655 had rel. T_{50} scores that were above 1.00, while su855 and su955 scored below 1.00 by a significant margin, as was predicted by the results of their corresponding sX mutants. su155, su255, su355, and su1155 scored rel. T_{50} values of 0.58, 0.60, 0.59, and 0.64 respectively, indicating that the suppressors in these three strains, like those of su855 and su955, functioned in a presynaptic manner. Mutant su455 returned a rel. T_{50} of 2.11, and therefore the s4 suppressor was likely synaptic. The rel. T_{50} scores of su755 and su1255 (0.95 and 1.10, respectively) were close to 1.00 and so these were classified as postsynaptic mutants. su1055 obtained a score of 1.79, which was sufficiently distant from 1.00 for the double mutant to be classified as a synaptic mutant, whereas the suppressor-only s10 strain had achieved a rel. T_{50} of 0.94. However, since the latter result may have been a product of the "clustering" effect discussed above, s10 was more likely a synaptic mutant (albeit a relatively weak one, compared to s4, s5, and s6).

Correlation was observed between the rel. T_{50} scores of the sX mutants and those of the corresponding suX55 strains. This indicated that, at least in the case of the suppressors that were isolated in this study, the alleviation of the mutant Unc phenotype probably occurred via direct mechanisms. The fact that the rel. T_{50} values of s8 and su855 displayed such a striking level of association was quite surprising though, as it did not agree with the thrashing assay, the results of which showed that the s8 suppressor functioned in an allele-dependent manner. It is unlikely that the latter was a result of the peculiarities inherent to the assay, as this observation had been substantiated previously

by observations of the movement characteristics of strains su8612 on solid medium. Additionally, the s8 mutant also appeared to be moderately Unc (Table 3-1C), indicating that unfavourable interactions may have existed between the suppressor and the wild-type channel. Unfortunately, as further information regarding the nature of the s8 suppressor is currently unavailable, this apparent conflict cannot yet be resolved or explained with any degree of certainty.

There did not appear to be any correlation between aldicarb sensitivity and effectiveness of a particular suppressor within suppressors of the presynaptic kind. This was surprising, as the former quantity is a measure of the amount of neurotransmitter released, while the latter is under the influence of the same factor (a similar comparison was not performed for the other suppressor classes would not have yielded any useful information due to the comparatively small sample sizes that would have been involved). The results of this analysis may indicate that neither the thrashing assays nor the subjective observation of worm behaviour can provide adequate descriptions of the actual amount of suppression provided by the suppressors, and that another standard must be used if one wishes to perform more detailed investigations regarding the relationships between data of different origins.

Suppressor mutations can cause additional modifications to animal morphology and behaviour

Beyond the alleviation of the Unc phenotype, certain animals from the suX55 group also exhibited associated phenotypes in the form of modifications to their morphology and/or movement characteristics (the phenotypes of suX55 strains are described in Table 3-1A) that allowed most of the various mutant strains to be

distinguished from the others on the basis of visual inspection alone. Among the suppressor strains, su655 and su755 provided the most striking examples. The former had a mottled appearance (most probably resulting from disorganization of body wall muscle), while the latter was slightly longer and thinner than *unc-2(e55)*-homozygous animals, particularly when larvae in the L2 to L3 phase of development were compared. The presence of these additional characteristics was not surprising, given that the suppressors generated in the screen were predicted from the outset to represent genes whose products were integral to the UNC-2-dependent cellular processes, most of which are instrumental in the regulation of larval development and numerous aspects of worm behaviour. As such, disruptions to any parts of these pathways were expected to result in observable changes. In the case of certain suX55 double-mutants, specifically, su955 and su1055, multiple phenotypic changes were observed. This, again, was expected. Most defects in the *unc-2* gene itself frequently result in pleiotropic effects, due to the fact that its gene product participates in several biological pathways and processes. As it is probable that some suppressors represented cellular factors that are closely associated with, and directly facilitate the function of the UNC-2 channel in a wide variety of contexts, it followed that the mutations with which this subset of suppressors were associated should have pleiotropic effects also.

It was also seen that a third of the suppressor-only sX animals, represented by s2, s8, s10, and s12, possessed certain Unc behaviours. In the case of s10, the tail region suffered from mild uncoordination or partial paralysis, and much of the propulsive force for movement appeared to have been generated by muscle contractions in the first half to two-thirds of the worm body. These worms tended to over-extend on initial turn-in during

forward motion, and deep bends were observed to occur at the midsection of the body when the animals were backing up in response to harsh touch to the head region. However, these behavioural changes did not appear to detrimentally affect movement rates to a large extent. Furthermore, the same phenotype was observed in su1055 worms, and therefore this phenotype may have been a property inherent to the suppressor mutation carried by the s10 strain. Mutants s2, s8, and s12 were more extensively affected. These strains exhibited greatly reduced overall movement rates, whether under resting conditions or in response to harsh touch, when compared to wild-type N2 worms. The severity of the phenotypes qualified these strains as mild-to-moderate Uncs when evaluated using the subjective standards discussed earlier in this chapter. This was especially surprising, considering that the uncoordinated nature of these strains was not in any way predicted by the phenotypes observed for their corresponding suX55 equivalents, which were not noticeably less coordinated compared to other animals of the same group (see Tables 3-1A, 3-1C and 3-2A). As was previously mentioned, because the suppressors in this study were isolated against an UNC-2-null genetic background, the majority of these were expected to involve cellular components that acted distally to the UNC-2 channel. These suppressors, when present together with the wild-type form of the *unc-2* gene, were therefore not expected to negatively influence movement rates to a great extent. Only if a suppressor mutation occured in a gene that encodes for a factor that directly interacts with, or is involved in the expression of, the UNC-2 voltage-gated calcium channel would it be possible for an Unc phenotype to arise when the wild-type form of the *unc-2* gene was substituted for the mutant form, as in the case of the sX mutants in this study. These were not expected to be generated with great frequency, and

indeed, the thrashing assay data, as well as subjective observations, indicated that s8 was the sole example of suppressors that fell into this category. The Unc phenotype of mutants s2 and s12, on the other hand, cannot be explained with the same reason, as the s2 and s12 suppressors were capable of suppressing the mutant phenotype of worms homozygous for the *ra612* allele of the *unc-2* gene (refer to Table 3-1A and 3-1B). While it might have been possible that the alterations associated with the suppressor mutations were incompatible with the wild-type channel, but not with the mutant forms, it is also highly improbable that this was the case, given that the suppressors in question were generated against an UNC-2-null background in the first place.

Genetic linkage of suppressors

It was observed during outcross that the F_1 animals, which were heterozygous for both the suppressors and the mutant *e55 unc-2* allele, segregated offspring in the ratio appropriate for a dihybrid cross. This ratio was again seen during construction of the suX55 and suX612 double mutants (data not shown, see previous chapter for a detailed description of these processes). Additionally, males heterozygous for the suppressor mutations exhibited no detectable mutant phenotypes. It therefore appeared that there was no significant genetic linkage between any of the twelve suppressor mutations and the X-chromosome on which the *unc-2* gene is found. This negated the possibility that any of these suppressors of *unc-2* were intragenic.

The linkage of two of the suppressors, s6 and s10, were determined to a higher degree of resolution via the Snip-SNP method (see Materials and Methods). This method was developed specifically for the purpose of mapping recessive mutations within a N2 genetic background, and exploits the numerous single-nucleotide polymorphisms (SNPs)

that exist between the N2 strain and another wild-type strain, CB4856. A subset of these SNPs affects restriction sites, and therefore, differences in restriction patterns may be used to identify (Wicks *et al.*, 2001). Hermaphrodite animals homozygous for a particular mutation of interest (and which also have a N2 genetic background) are first crossed with CB4856 males to produce offspring heterozygous for both the mutation as well as overall genetic composition. These animals are then allowed to self-cross. The resultant F2 worms are then separated into two pools, one of worms displaying the phenotype that is associated with the mutation being mapped, and one of phenotypically wild-type worms. The first group must necessarily be composed of individuals enriched for the genetic region containing the mutation of interest, which is of N2 origin, while being deficient for DNA of a CB4856 background across that same region. The second group, on the other hand, is likely not enriched for any particular region of DNA. Restriction digestion is then performed on PCR products of various chromosomal regions, and the differences in the restriction pattern of the two pools are used to determine the specific region of DNA that the "mutant" worms are enriched for.

Due to the fact that the success of this mapping method depends on the differentiation of the "mutant" worms from the "wild-type" ones, Snip-SNP mapping functions best if the mutation of interest has a high degree of expressivity and penetrance, coupled with a phenotype that is easily detected. As s6 and s10 best satisfied these criteria, they were selected as candidates for snip-SNP mapping in this study. Animals homozygous for the s10 suppressor were subjected to snip-SNP mapping using the method described, and the resultant restriction fragments were visualised and analysed via agarose gel electrophoresis. As differences in band intensities between the wild-type

A)

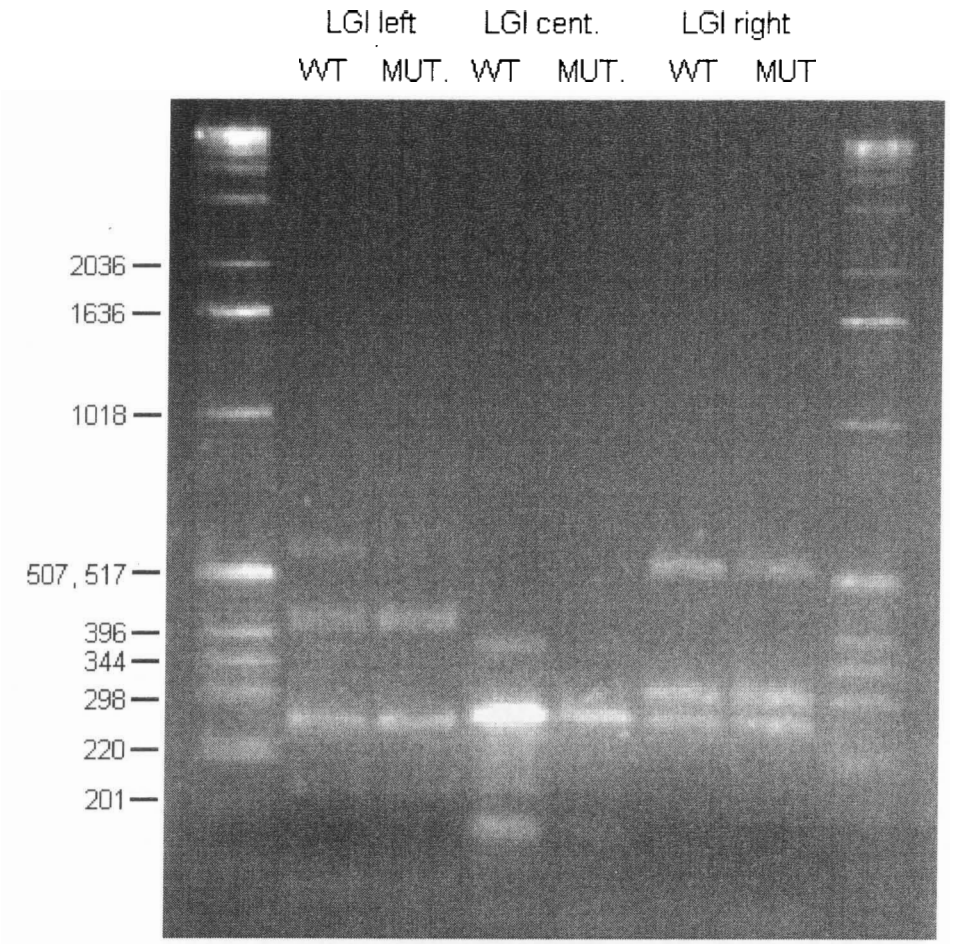


Figure 3.5A Linkage of suppressor s10 as determined by snip-SNP mapping

Restriction fragments generated from digestion of wild-type (WT) and mutant (MUT.) pool DNA from three regions across chromosome I (left, center, and right). 1-kb DNA ladders are shown alongside, in the left- and right-most lanes for comparison purposes (fragment masses, in kb, are shown on left side of figure). Band intensity bias is strongest in the lanes that correspond to the center of chromosome I, indicating that the mutation associated with the s10 suppressor has significant linkage with that region of the chromosome.

B)

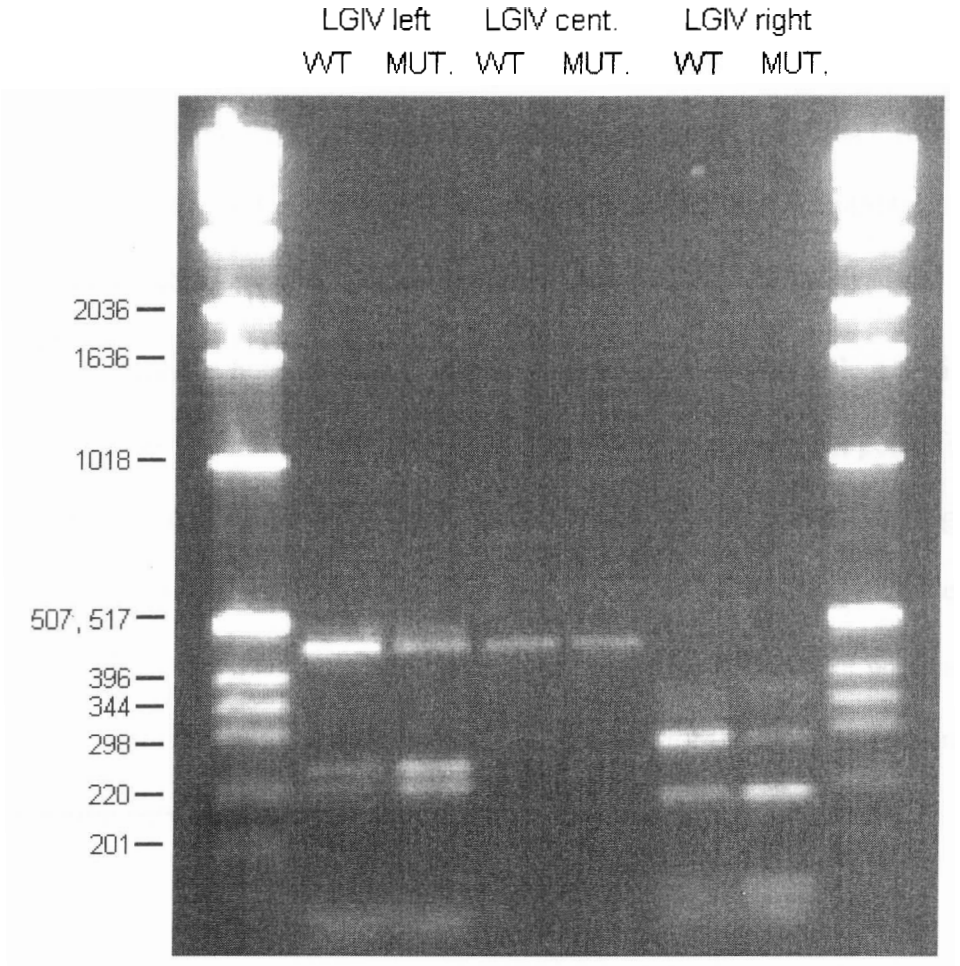


Figure 3.5B Linkage of suppressor s10 as determined by snip-SNP mapping

Restriction fragments generated from digestion of wild-type (WT) and mutant (MUT.) pool DNA from the left, centre, and right regions across *LGIV*. 1-kb DNA ladders are loaded alongside for comparison (fragment masses, in kb, are shown on left side of figure). Band intensity bias is strongest in the lanes that correspond to the left and center regions of chromosome IV, indicating that the mutation associated with the s6 suppressor is located between these two regions.

and mutant pools were observed only for the set of bands corresponding to chromosome I, the mutation associated with this suppressor was most probably linked to *LGI*. Figure 3.6A depicts the restriction patterns for chromosome I of the wild-type and mutant pools isolated in the bulked segregant analysis step for s10. Differences in band intensities were seen across all three chromosomal locations, but the bias was most apparent in the lanes corresponding to the centre region of that linkage group. It is therefore very likely that the mutation is located in this region, in a position roughly between 4500 kbp from the left end of the chromosome.

Animals homozygous for the s6 suppressor were treated in a similar fashion. Differences in band intensities between the wild-type and mutant pools for this strain were seen only for the set of bands corresponding to chromosome IV. Figure 3.6B shows the results of the restriction digest for that chromosome. While differences in band intensities were again observed between the wild-type and mutant pools across all three chromosomal locations, the greatest bias was seen for the lanes corresponding to a position between 1600 and 4500 kb from the left end of *LGIV*.

CHAPTER FOUR.
DISCUSSION

Neuronal VGCCs play a central role in the process of neurotransmitter release

The controlled release of neurotransmitter from neurons, or neurosecretion, represents the key pathway through which signals are relayed in the nervous systems of animals (Bajjalieh and Scheller, 1995). Current models describe neurosecretion as a three-step process, consisting of docking, priming, and fusion (Rand and Nonet, 1997). In the first two phases, neurotransmitter-filled vesicles synthesized in the cell bodies of the neurons are targeted to the synaptic regions of the cell, and become stably associated with the plasma membrane. It was hypothesized in 1993, by Sollner *et al.*, that docking is mediated via the interactions between complementary SNARE proteins that are present on the surfaces of both structures, that are termed "v-" (for vesicle), and "t-" (for target) SNAREs respectively. The identities of the various proteins that are involved, as well as the interactions between these proteins, have since been determined through cross-linking and immunoprecipitation studies. It is believed that, prior to docking, the primary v-SNARE synaptobrevin and the primary t-SNARE syntaxin are bound to synaptophysin and n-Sec 1, respectively. The latter proteins then disassociate, and this permits the formation of a 7S SNARE complex, composed of synaptobrevin, syntaxin, SNAP-25 (25 kDa synapse-associated protein) and the vesicle protein synaptotagmin. This structure then recruits α-SNAP (soluble NSF attachment protein), which in turn allows NSF (N-ethylmalemide-sensitive fusion protein) to bind, forming a 20S complex (reviewed in Bajjalieh and Scheller, 1995). The attached NSF protein then effects an ATP-dependent

conformational change in the complex, which causes the vesicles and the plasma membrane of the neurons to come into close apposition, and this primes the synapse for neurotransmitter release.

Upon sensing depolarisation of the membrane, VGCCs localized at the synaptic regions of the neurons open, and allow Ca^{2+} to enter. It is this Ca^{2+} influx that provides the trigger for vesicle fusion and the concomitant release of neurotransmitter, which can occur within a period of microseconds. Due to this comparatively short time scale, it is thought that the Ca^{2+} influx brings about vesicle fusion only, and that the vesicles are already docked and primed even before the depolarisation is first received by the synaptic terminus. It therefore appears that instead of initialising the process of neurosecretion from the start, intracellular Ca^{2+} may serve solely as an "off" switch for inhibitory mechanisms that prevent the neurotransmission process from progressing past the priming stage without the appropriate signal. While the nature and identities of the synaptic components controlling this release currently remain unknown, it is highly probable that the synaptic proteins are involved. Of the four already mentioned, synaptobrevin, syntaxin, SNAP-25, and synaptotagmin, synaptotagmin is potentially the most important, as it is known to be capable of binding with both Ca^{2+} and membrane phospholipids, and could therefore act as both the calcium sensor as well as the fusogenic element. In 1993, DiAntonio *et al.* provided a clear demonstration of the importance of this SNARE protein, by showing that neurotransmission is greatly reduced in synaptotagmin mutants of *Drosophila*. However, the fact that a fraction of synaptic activity remains in the mutants indicates that secondary release mechanisms must exist.

The rab3 GTPases, as well as the product of the *C. elegans unc-13* gene rab3 GTPases, are possible candidates (Rand and Nonet, 1997).

There has also been evidence to suggest that the synaptic VGCCs may also be involved. N and P/Q type neuronal channels have been co-purified with SNARE complexes, and colocalization of the channels with syntaxin has been observed. It was determined that this interaction is mediated by the "synprint" (synaptic protein interaction) site found in the intracellular II-III loop of $Ca_v2.1$ and $Ca_v2.2$, and that this region exhibits variable affinities for the SNARE complex and for synaptotagmin depending on intracellular Ca^{2+} concentrations. In experiments conducted at physiological Ca^{2+} levels, the SNARE proteins syntaxin and SNAP-25 were observed to bind strongly to synprint peptides, and this situation is thought to correspond to the initial state, in which neurotransmitter release is inhibited. However, as Ca^{2+} concentrations begin to rise following a depolarisation event, the synprint region preferentially binds to synaptotagmin instead, and this interaction may promote, or result in, vesicle fusion. It therefore appears that, in addition to providing the Ca^{2+} influx that triggers neurosecretion, the synaptic VGCCs may form part of the regulatory machinery as well.

UNC-2 *mutants provide ideal systems on which to conduct studies of mammalian neurotransmission*

The *unc-2* gene of *C. elegans* has recently been found, by Mathews *et al.* (2003), to encode for a voltage-gated calcium channel that bears structural homology to P/Q and N-type mammalian VGCCs. In the same study, it was additionally determined that the channel was expressed primarily in motor and mechanosensory neurons, and a subset of neurons involved in regulating egg-laying behaviour in worms. As *unc-2* mutants are less

sensitive to the effects of the acetylcholinesterase inhibitor aldicarb, it was proposed that the channels encoded by the gene acted in a presynaptic capacity to regulate neurosecretion. It was therefore concluded that UNC-2 functioned in a capacity similar to that of the neuronal VGCCs of mammals.

Neurotransmission is a highly conserved process, that is, its general form differs very little between one group of organisms and the next. The specific components involved in mediating the process, too, appear to be similar, even in animals as different as humans and nematode worms. In fact, several *C. elegans* SNARE protein genes, *snb-1*, *unc-64*, *ric-4*, and *snt-1*, (corresponding to synaptobrevin, syntaxin, SNAP-25, and synaptotagmin, respectively) have been identified via the analysis of neurotransmission-defective mutants, and further, genes encoding for products that bear structural homology to other mammalian synaptic proteins have also been found in the worm genome (Rand and Nonet, 1997). This similarity, combined with the genetic tractability of *C. elegans*, makes this animal highly suitable for use as a model organism on which studies of mammalian neurotransmission may be conducted, particularly where the discovery of novel genes is concerned. Such an approach has already resulted in some success. The products of the *C. elegans unc-13* and *unc-18* genes, which have been demonstrated to be central to neurotransmission in worms, have no known counterparts in mammalian species. UNC-13 has been determined to be a diacylglycerol-binding protein that interacts with the G-protein EGL-30 and phospholipase C-β to facilitate neurotransmitter release at the neuromuscular junctions (Lackner *et al.*, 1999), while UNC-18 is believed to effect vesicle docking by mediating the binding of the t- and v-SNAREs (Weimer *et al.*, 2003). These findings are likely applicable to mammalian neurotransmission as well.

The dissection of the various pathways that facilitate neurotransmission in *C. elegans* requires the identification of genes important for neural function. The UNC-2 channel may yet prove to be invaluable in this search, for two primary reasons. Firstly, as this channel represents a central regulating mechanism in the neurosecretory process, it interacts, either directly or indirectly, with several components that must also perform essential functions. UNC-2-null, or UNC-2-mutant strains should therefore provide a suitable platform against which additional synaptic mutations may be generated. And, due to the fact that UNC-2 is involved in the regulation of the nervous circuits that control several observable and predictable behaviours in the worm, such as locomotion, egg laying and defecation, the effects of mutations in these related components can easily be assessed and or quantified.

Several suppressors of **unc-2** *were generated and isolated via a suppressor screen*

Previous studies that have focussed on cataloguing the genes required for proper neurotransmission in *C. elegans* have mostly relied on approaches in which the normal function of a protein is inferred from the consequences of its dysfunction or absence. The majority of these employed some variation of the genetic screen technique, where random mutations are induced in the germline cells of a population of worms whose offspring are inspected to select for individuals exhibiting alterations in nervous function, which are then sequestered for behavioural and genetic analysis. Although such a strategy has proven itself to be quite successful, the nature of the methodology introduces an element of uncertainty to the results, and therefore its usefulness is limited if one wishes to investigate a specific portion of a particular pathway or system, as is the case here.

The use of a yeast-two-hybrid system would also have been impractical for a number of reasons. The UNC-2 channel, a membrane-bound protein, would make for a poor "bait" molecule, as it is highly probable that it would be improperly folded in the aqueous environment of the yeast cell, which will result in a high number false negatives due to the disruption or occlusion of relevant epitopes, or false positives, from the exposure of sites that are normally hidden. Using only the cytoplasmic domains of the protein instead would aid in circumventing this obstacle somewhat, but would in turn have led to a laborious experimental procedure, as the large number of intracellular loops that are found in the UNC-2 channel protein would have required several separate screens in order to achieve sufficient coverage of the sites in the protein that could potentially support interactions with other cellular components. Even if successful, such a screen would ultimately yield relatively little information, as only cellular elements that form direct contacts with the channel would be identified via this approach, and several of these are already known. Furthermore, molecules that exhibit physical interactions with the channel may not necessarily affect its function to any appreciable extent, and therefore may not be significant.

Given the requirements of this study, it was decided that a screen for suppressors of *unc-2* would be the ideal method for the identification of genes and gene products that work in concert with the VGCC to facilitate the process of neurotransmission, as this approach combines the strengths of the aforementioned methods while avoiding their inherent pitfalls. For such a screen, random secondary mutations would be generated in the germline cells of a population of worms homozygous for a mutation in the *unc-2* gene. These animals, as was discussed in previous chapters, exhibit several observable

defects, the most notable of which is uncoordinated and sluggish locomotion. The offspring of these worms (F_1), as well as the successive generation (F_2), would then be screened for individuals that exhibit visible alleviation of this Unc phenotype of the P_0 generation, and these could then be isolated for further analysis.

As with the yeast-two-hybrid system, this approach is highly selective. However, it possesses several additional unique advantages. Firstly, the approach does not require exogenous expression of any *C. elegans* proteins. Further, for a suppressor to be identified as such, direct physical interactions need not exist between the UNC-2 channel protein and the product of the suppressor gene, and so the spectrum of the potential targets that may be identified is broadened considerably. Also, as the sole selection criterion is the visible suppression of the Unc phenotype, the mutations isolated in the screen must necessarily occur in genes that are significantly involved in UNC-2-mediated neurotransmission pathways.

The selection of the initial mutant background against which the suppressor screen is conducted, as well as changes to the conditions under which mutagenesis is preformed or to the screening procedure itself can, to a certain degree, influence the identities of the suppressors that are isolated. Conversely, these aspects of the screen may also be modified to accommodate the specific natures of the potential suppressors. For the screen that was performed in this study, a relatively high concentration of mutagen was used (50 mM EMS), as a similar screen that had been conducted previously with lower concentrations had been unsuccessful (Mathews, 2000). As it was expected that the majority of suppressors of *unc-2* would involve recessive, null or loss-of-function mutations (due to the nature of the mutagen employed), F_2, as well as F_1 animals were

screened in order to ensure that these were identified (details of the procedure are described in the preceding chapter, and again in the following section). Finally, in order to restrict the set of suppressor genes found in this screen to only those whose products act distally to the UNC-2 channel (and so exclude the genes that encode for auxiliary subunits of the channel), the *e55* allele of the *unc-2* gene was used as the initial background mutation against which the screen was performed (see Chapters 2 and 3 for detailed descriptions of the screening process).

The unc-2(e55) *mutant allele and possible mechanisms of suppression*

Animals homozygous for the mutant alleles of the *unc-2* gene exhibit several behavioural defects, with the most readily observable of these relate to movement. While the bodies of wild-type worms adopt a sinusoidal posture during movement, and at rest, these worms appear kinked. Also, mutant worms are comparatively sluggish, and suffer from moderate flaccid paralysis, unlike wild-type worms, which move quickly, and maintain a certain degree of muscle tone even when at rest. In addition, worms homozygous for the *e55* allele of *unc-2* often appear to be less responsive to harsh touch, and exhibit particularly poor backing behaviour when such stimulus is applied to the head region of the animal. Given the nature of the mutation that is involved in this particular allele, the mutant phenotype may be the product of a number of contributing factors.

It is known that calcium signalling is required for the guidance of neurons during postembryonic development. The VGCC encoded by *unc-2* has been demonstrated to be central to this process, and mutants carrying defective alleles of the gene exhibit improper placement of the mechanosensory neurons AVM and SDQR (Tam *et al.*, 2000), and it is probable that the same might apply to other cells of this type as well. While the exact

physiological consequences of this defect are unclear at the present, it is possible that the proprioceptive signals that are required for the maintenance of coordination, or the sensory inputs that allow for the response to touch stimuli, might be compromised or misdirected in *unc-2*-mutant animals, and this could partly account for the mutant phenotype observed in these animals. In addition to these afferent processes, efferent processes might be affected as well if the UNC-2 channel is also involved in the regulation of the migration of motor neurons during development. It is probable that the mutant characteristics of *unc-2*-mutant animals may be the result of the misplacement of the cell bodies within the animal, or of the synapses linking the presynaptic neurons and the muscle cells involved in locomotion.

Disruption to the proper regulation of the expression of genes that are critical for the determination of the fates of neurons (or other cells involved in locomotion) may also partially contribute to the mutant phenotype of *unc-2(e55)* worms. In 1999, Troemel *et al.* reported on their finding that the correct asymmetric expression of a putative odorant receptor gene *str-2* in a pair of symmetric olfactory neurons found in the head region of the animal (AWCL and AWCR) required signalling between these cells. Besides CaM kinase II (UNC-43), the UNC-2 calcium channel also appeared to be an important participant in this process. While the exact mechanisms through which this "lateral" signalling is effected have yet to be completely identified, it is clear that UNC-2-mediated calcium signalling can have a major impact on the identity, or at least the function, of certain cell types. It is conceivable that identical, or similar UNC-2-dependent cellular pathways also operate in the various neuron-neuron interactions that occur during, and following development that are important in determining the ultimate

structure and/or function of the nervous system of the nematode worm. If any of these pathways are disrupted, as might be the case in *unc-2*-mutant animals, the connectivity of the network of neurons involved in coordinating movement may therefore be adversely affected, resulting in the mutant phenotype.

The single overriding factor that may be most important in causing the behavioural alterations that are observed might be the lowered levels of acetylcholine secretion at the neuromuscular junctions, a result of the fact that UNC-2 VGCC function has been abolished or greatly reduced in the presynaptic neurons of the mutant animals (Mathews *et al.*, 2003). Since the neurotransmitter acetylcholine is the sole trigger for the contraction of the body wall muscles, this seriously impacts upon the ability of the *unc-2(e55)*-homozygous worm to perform sustained and fluid locomotion. However, the severity of this particular defect also made it the most likely conduit through which alleviation of the mutant phenotype was effected; as the NMJ defects lead to the most easily observable changes to animal behaviour, so should positive modifications to the same system result in the most overt improvements to it. In addition, the primary criterion employed in the genetic screen used to isolate the suppressors in this study was based primarily on movement characteristics. These two factors, when taken together, heavily biased the identities of the isolated suppressors towards genes that serve important roles in regulating the functional characteristics of the NMJ.

As the NMJ is composed of three discrete parts - namely, the presynaptic neuron from which neurotransmitter secretion occurs, the postsynaptic effector muscle cell, and the synaptic cleft, the nature and composition of which exert a considerable influence over neurotransmission – there existed three possible distinct routes through which the

neurotransmitter deficiency may have been compensated for by the mutations associated with the various suppressors that were isolated in this study. First, a suppressor could amplify the sensitivity of the postsynaptic muscle cell to the neurotransmitter, and as a result, boost the responses of that cell to every unit of acetylcholine that it receives. Second, a suppressor could influence the function of elements within the synaptic cleft, to allow the acetylcholine signal to persist over a longer period of time, thereby increasing the effective concentration of that neurotransmitter. Finally, a suppressor might modify the activities of the presynaptic neuron in such a way that the amount of neurotransmitter that is released by the cell is increased.

The postsynaptic suppressor class was expected to be composed of relatively few members. This was primarily due to the severe constraints governing this system, as well as the expected nature of the suppressor mutations (i.e., reduction-of-function or null) involved, both of which ensured that the mechanisms underlying suppression remain comparatively direct. For instance, it is unlikely that suppression could occur via the mutation of the contractile elements of the muscle cells, such as myosin, actin, tropomyosin, or the troponin complex, as drastic changes to, or elimination of, these essential molecules would have result in gross structural defects of the muscle, and in the animals being non-viable. The elements that serve to limit Ca^{2+} concentration within the muscle cells – the sarcoplasmic Ca^{2+} pumps, calsequestrin, and the ryanodine receptor, for example – are more probable targets. It is not inconceivable that a certain subset of the suppressor-associated mutations might have modified the structure of these elements in such a way as to cause a reduction in their functional efficiencies or capacities. As these components are involved in the sequestration of Ca^{2+}, such changes might result in

a situation in which an elevated sarcoplasmic Ca^{2+} concentration exists prior to the initiation of any stimulatory action potentials in the muscle. If the Ca^{2+} concentration remains at sub-threshold levels, muscular contraction will not occur. However, the contractile elements of the muscle cells will become "primed," and therefore be more sensitive and responsive to any stimuli (in the form of action potentials) that it might subsequently receive, even if these are weaker than what is normally required to elicit strong contractions.

Other mechanisms of suppression might involve molecules that lie further upstream in the process. The α_1 subunit of the DHPR voltage-gated calcium channel (EGL-19) that is involved in the control of body wall muscle contraction (Jospin *et al.*, 2002) is one such possibility. While it may be difficult to imagine how the mutation of this channel could have a positive influence on E-C coupling, it is important to note that, even in the case of EMS-induced mutations, reduction or elimination of the function of the channel need not occur. It is conceivable that a mutation could have occurred in a region of the channel protein involved in the regulation of its deactivation or inactivation kinetics, which in turn led to a situation where a greater amount of Ca^{2+} is allowed to permeate into the muscle cells for every depolarisation event. Alternatively, a mutation might have altered the nature of the interaction between DHPR and RyR so that Ca^{2+} is released more readily from the SR (the existence of calcium-induced calcium release in nematodes has not been demonstrated however). Mutation of the genes that encode for the auxiliary β, $\alpha_2\delta$, and γ subunits that support the function of the α_1 subunit may also lead to suppression, particularly if the expression of subunit isoforms that serve an inhibitory function is affected.

Suppression of the mutant phenotype may have also arisen through changes to the nicotinic acetylcholine receptor (nAChR) molecules. These are pentameric receptor channels that are embedded within the plasma membrane of the muscle cells and they respond to the binding of the neurotransmitter acetylcholine by changing from a closed state to one that rapidly fluctuates between closed and open conformations. In this latter state, the channel becomes capable of conducting a transient cationic current, and this influx of ions through these channels contribute to the action potential that ultimately results in muscular contraction (reviewed in Itier and Bertrand, 2001). Changes to any of the subunits of the molecule that caused a prolongation the duration over which the channel remains open or that disrupted the closing of the channel, as may be the case, would have led to a stronger AP and thus cause the postsynaptic muscle contract more strongly in response to a given amount of neurotransmitter.

As expected, only two examples of suppressors of the postsynaptic class were isolated in the genetic screen conducted in this study, as indicated by the results of the aldicarb assays. Ostensibly, mutants s7 and s12 appeared to share no traits in common, but the mutant phenotypes of these two strains were consistent with the mechanisms of suppression proposed above. The phenotype of s7, which appeared to be longer and thinner than wild-type worms, could have been the result of developmental defects of the muscle, while the uncoordinated nature of s12 may point to the fact that E-C coupling had somehow been affected in this strain.

The second class of suppressors achieves suppression of the UNC phenotype of *unc-2(e55)* animals by modifying the nature of the synaptic cleft. This "synaptic" group of suppressors was expected to contain the least amount of diversity, for the reason that

there are comparatively few synaptic components that play significant roles at the neuromuscular junctions. In fact, only one class of molecules, the acetylcholinesterase (AChE) enzymes, have been identified as key participants in the process of neurotransmission at the cholinergic synapses.

While vertebrate animals possess just a single AChE type (Massoulie *et al.*, 1993), several kinetically distinct forms are expressed in both *C. elegans* and *C. briggsae*. The first two classes, A and B, are encoded by the structural genes *ace-1* and *ace-2* (located on chromosomes X and I respectively, Johnson *et al.*, 1981; Culotti *et al.*, 1981), represent the major AChE forms in the nematodes. Classes C AChE, on the other hand, account for only one-twentieth of the total AChE activity (Kolson and Russell, 1985). The genetic region on chromosome II containing the coding sequence for this enzyme, originally named *ace-3*, was recently discovered to contain two genes in close proximity (Grauso *et al.*, 1998). As the data that is available at present does not yet allow for the positive identification of the specific gene governing type-C AChE expression, these have been tentatively named *ace-x* and *ace-y*. The existence of a fourth class of acetylcholinesterase has also been inferred from observations conducted on worms deficient in type-C AChE expression. As no mutant alleles have been isolated, the localization of the gene encoding for this enzyme remains undetermined.

There are therefore, at least five AChE types in nematodes, with each representing a possible route through which suppression of the Unc phenotype of *unc-2(e55)*-homozygous worms might occur. Due to the fact that the mutant phenotype of these worms arises due to reduction in the amount of acetylcholine released by the presynaptic neuron, suppression could have occurred simply via the elimination of the expression of

any of these AChE types, as this would have effectively caused an increase in the concentration of the neurotransmitter in the synaptic cleft. However, worms that carried such a defect would likely display increased sensitivity to the AChE inhibitor aldicarb, due to the fact that these animals would possess a smaller pool of functional enzymes to begin with, and this may have led to the suppressor with which the strain is associated being classified as "presynaptic" (there is, in fact, a high probability that a subset of the suppressors that have been identified as "presynaptic" in this study actually have a synaptic mode of function). For a synaptic suppressor to have been correctly identified as such by the aldicarb assay, the suppressor mutation that is involved must instead have caused a reduction the efficiency with which a particular AChE catalyses the breakdown of acetylcholinesterase, specifically by reducing its affinity for the neurotransmitter. The affected enzyme would also become less susceptible to inhibition by aldicarb, as the same mutation would necessarily reduce aldicarb-acetylcholinesterase binding as well.

Four of the suppressors that were isolated in this study, s4, s5, s6, and s10 conferred upon the worms that carried them some degree of insensitivity to aldicarb and were therefore classified as being "synaptic." These suppressors probably correspond to a subset of the AChE enzymes in *C. elegans*. s6 is particularly interesting as it has been found to map to chromosome IV, and so may represent the genetic correlate of class D AChE, which, as mentioned above, has not been identified so far. Alternatively, this suppressor, as well as other in this group, may represent other novel synaptic components that are important in mediating the process of cholinergic neurotransmission.

The movement defects of the animals homozygous for the *e55* allele of *unc-2* is due, in large part, to the reduced levels of acetylcholine released by the presynaptic

neurons that innervate the body wall muscles, and thus, the suppressors that modify the nature of those cells should result in the most effective alleviation of the mutant phenotype. In addition, since most of the cellular components whose activities directly regulate and/or influence neurotransmission are associated with the presynaptic neuron, the group of suppressors that have a presynaptic mode of function is also likely the largest. Finally, as the excitation-release mechanisms that operate in the presynaptic neurons are likely largely common to all neuronal types as well, these suppressors (and, by extension, the genes with which they are associated) should also be functional at other synapses involved in fast neurotransmission. For these reasons, the presynaptic suppressor class may be the most interesting and significant.

As already discussed earlier in this chapter, excitation-release coupling may be considered as being comprised of three major steps, which are, in the order in which they occur, docking, priming, and fusion of the synaptic vesicles and the concomitant release of the neurotransmitters (reviewed in Catterall, 1999). As the voltage-gated calcium channels embedded in the presynaptic membrane do not appear to be directly involved in docking and priming, it is probable that these processes remain unaffected in an UNC-2-null setting (as might be found in the mutant worms), and that it is the final step in the sequence that is defective. It is therefore also highly likely that the presynaptic suppressors involve the molecules that participate in, or that regulate, the events of this final phase, such as the t- and v-SNARES, NSF, synaptotagmin, and synaptobrevin among others. Unfortunately, without further information regarding the specific interactions that occur between these synaptic proteins or knowing the identity of the genes that were affected by the suppressors that were isolated in this study, it is

impossible to make any speculations regarding the possible mechanisms through which suppression of the mutant phenotype might occur in this highly complex system. However, as the majority of the synaptic elements generally serve in an inhibitory role with respect to the release of the neurotransmitter, it is conceivable that a reduction their function might result in suppression of the Unc characteristics of *unc-2*-mutant worms.

Instead of directly increasing the rate of neurosecretion from the presynaptic neuron, "presynaptic-type" suppression of the Unc phenotype may alternatively result due to modifications to the structure of the synapse. It is possible that certain suppressor mutations may cause multiple synapses to form between neurons and muscle cells, and this would, in effect, increase the volume of neurotransmitter released onto the muscle cell for every depolarisation event. Genes such as *rpm-1* and *syd-2* (Schaefer *et al.*, 2000; Zhen and Jin, 1999), the products of which have been demonstrated to be important in the regulation of synapse differentiation and morphology, would likely be involved in these cases, although it is also possible that a subset of suppressors that functioned in this manner may also be linked with previously unidentified genes that perform similar functions.

It has so far been assumed that no functional UNC-2 channel is produced by the *unc-2(e55)*-homozygous mutants, and that neurotransmitter secretion is completely absent in these animals. While this provides an ideal conceptual framework in which to consider the various proposed suppression mechanisms, this assumption cannot entirely be true, due to the fact that such a severe defect would most likely result in the mutant worms being inviable. Therefore, either the elimination of UNC-2 function is incomplete in the *unc-2(e55)*-homozygous mutants, or else there must be alternative pathways

through which neurotransmission takes place. If either of these possibilities is indeed the case, then presynaptic suppression of the Unc mutant phenotype may occur via the direct enhancement of UNC-2 function, or that of the components that are involved in the parallel pathways.

Six suppressors, s1, s2, s3, s8, s9, and s11, with presynaptic modes of suppression were isolated in the genetic screen. Although, for reasons already discussed, a subset of these may correspond to AChE-null mutations, most of these probably involved genes encoding for the various cellular factors that are important in mediating the neurotransmitter release from the presynaptic neurons. Worms that were determined to be homozygous for this set of suppressors all displayed movement-related phenotypes that clearly deviated from those of typical wild-type animals, as was to be expected for animals in which the neurotransmission process had been significantly altered. s2- and s8-homozygous animals, in particular, were affected to the greatest degree, as these were observed to exhibit mild-to-moderate Unc characteristics. While the phenotype of the former group can perhaps be attributed simply to the severity of the neurotransmission defects present in these worms, the latter case appears to be more complex, as the s8 suppressor had also been demonstrated to be specific for the *e55* allele of *unc-2*, that is, it was able to suppress the mutant phenotype of *unc-2(e55)*-homozygous worms only. It therefore seems that the gene in which the s8-associated mutation occurs must have encoded for some cellular element that experiences direct interactions with UNC-2, which, in turn, implies that the expression of a functional channel protein may not have been abolished completely in *unc-2(e55)*-homozygous worms.

Limitations of current study and possible directions for future research

In this study, a suppressor screen was conducted to identify genes that are important to proper neurotransmission. Animals homozygous for the *unc-2(e55)* allele were exposed to a chemical mutagen, and their offspring were screened for individuals in which the severity of the mutant phenotypes of the P_0 generation had been reduced. While the methodology employed was largely successful, as evidenced by the fact that twelve suppressors were indeed isolated in this fashion, there were certain deficiencies in the specifics of the approach that was used which may have potentially caused a reduction of the overall yield.

While the P_0 animals exhibit several behavioural and developmental defects, those that relate to movement are the most easily observed via direct means, and it was for this reason that this particular facet of worm behaviour was used as the screening criterion for individuals carrying the suppressor mutations in the genetic screen. However, as coordination of the of the body wall muscles is primarily mediated via cholinergic neurotransmission in *C. elegans*, this selection of the suppressors solely on the basis of their ability to alleviate of the Unc characteristics will allow for the isolation and identification of elements important to this pathway only. Consequently, genes specific to other neurotransmission types will not have been isolated in the mutant screen, despite the fact that most of these processes are most probably UNC-2-mediated as well. Additional potential suppressors may also have been missed due to the fact that their effects on the behaviour of the Unc phenotype of the mutant animals may have been too subtle to be detected by visual inspection alone.

While the nature of other neurotransmission types does not permit for straightforward or convenient genetic screening procedures (it is indeed difficult to imagine how one would select for suppressors specific to the GABAergic or dopamergic circuits, for instance), the latter problem might have been resolved by performing a parallel screen against a different initial mutant background. As already mentioned, a functional UNC-2 channel is necessary for mediating the lateral calcium signalling processes that is required for maintaining asymmetry in the expression patterns of the *str-2* odororant receptor gene, as assessed by a *str-2::GFP* fusion construct, in the symmetric and equivalent AWCL and AWCR amphid neurons (Troemel *et al.*, 1999). Double mutant animals of an *unc-2(e55) str-2::GFP(kyls140) / unc-2(e55) str-2::GFP(kyls140)* genotype should therefore be an ideal system on which to conduct a screen for suppressors of *unc-2. str-2* expression would be symmetric in the majority of these worms, and therefore, suppression could be detected via the restoration of asymmetry in the progeny of these worms. While the suppressor selection procedure would undoubtedly be more involved, due to the fact that several successive generations of each of the potential suppressor strains would have to be inspected to confirm that suppression had indeed occurred, a screen conducted in this manner would aid in the isolation of a wider spectrum of suppressor types. In addition, the nature of the defect that is involved provides a convenient, and readily scorable indicator of the degree of suppression effected by each of the suppressors that are isolated.

The suppressor screen that was conducted in this study was performed against a null allele of the *unc-2* gene, with EMS as the mutagen. These stringent conditions may also have restricted the number of potential suppressors that could have been isolated.

Using a reduction-of-function allele instead would have allowed for the generation of suppressors associated with factors that interact in vivo with UNC-2, which was not possible in this case, and substituting an alternate mutagen with a different mechanism of function might also have been useful for the identification of elements that participate in the portions of the neurotransmission pathways that lie further up- and down-stream of the channel. These two changes could perhaps have increased the number of suppressor types that were isolated.

Also, as has already been discussed in the previous chapter, due to the multitude of factors that can potentially influence the movement rates of the animals in a liquid medium, the performance of the various suX55 and suX612 worms in the thrashing assay might not linearly correspond with the specific physiological changes in these animals The data collected from this should therefore be considered only as a qualitative, or semi-quantitative estimations of the efficacies of the various suppressors, and is therefore not suitable for analytical purposes. Due to the fact that most of the potentially confounding variables (which may be largely attributed to the worms' lack of spatial references in a liquid environment) could be eliminated when the worms are placed on a solid medium, it might have been more ideal to conduct evaluations of the movement phenotypes of the suppressor strains on NGM agar instead, using video-based methods that allow for the analysis of the distances travelled by the animals and frequency of movement. Alternatively, the effectiveness of the suppressors could have been assessed via other UNC-2-mediated processes such as egg laying and defecation. The use of the *unc-2(e55) str-2::GFP(kyIs140) / unc-2(e55) str-2::GFP(kyIs140)* system mentioned above may also

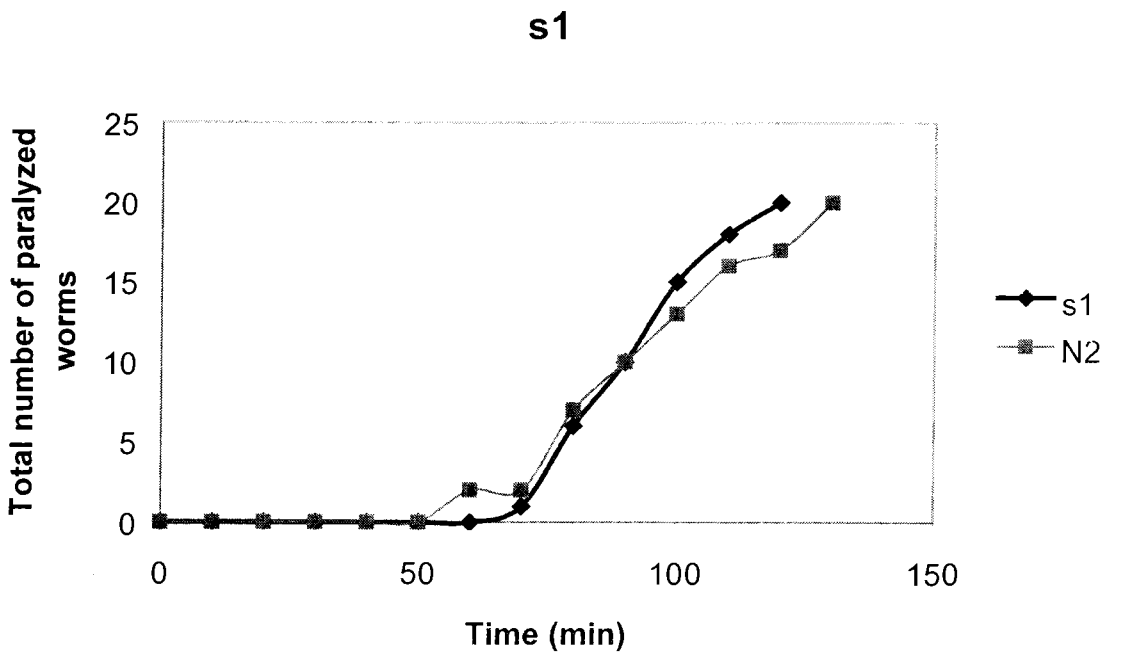
useful in quantifying the degree to which a suppressor is able to correct for the UNC-2-null defect in mutant worms.

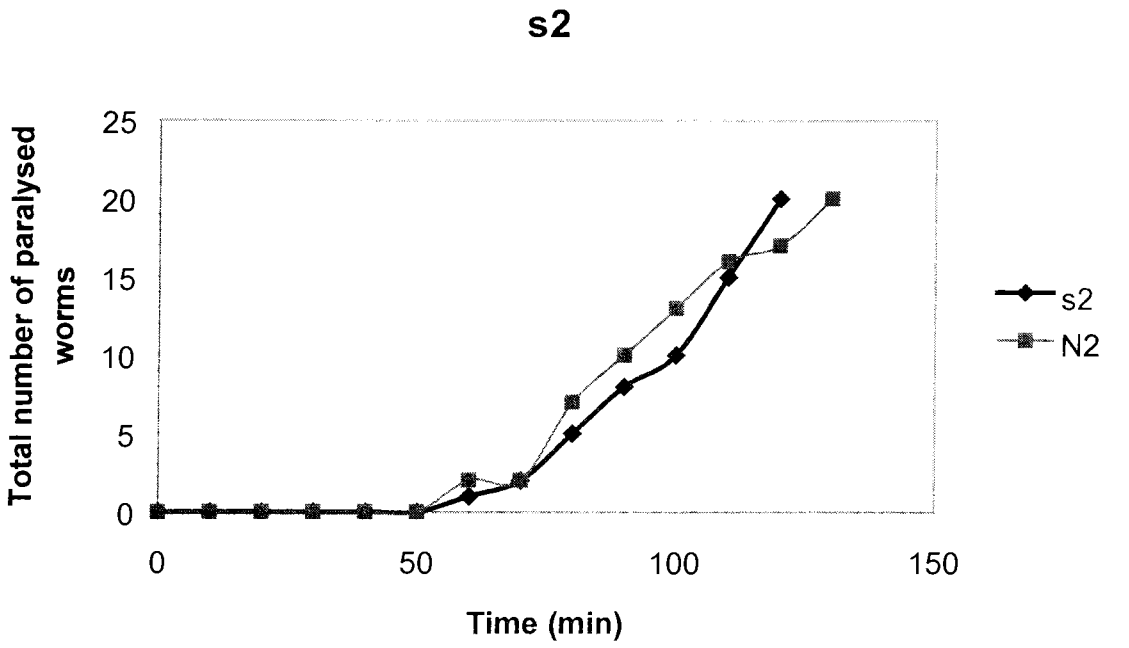
In order to be able to extract more information regarding the suppressors isolated in these study, the localisations of the associated mutations will need to be determined to a higher resolution so that the genes with which they are linked can be identified. While a large proportion of the suppressors would most probably encode for factors with known orthologs in mammals, a small fraction could possibly represent novel cellular components that are integral to the neurotransmission process, and these may prove valuable for the development of new drugs used in the treatment of cardiovascular diseases, migraine, or stroke. In the meantime however, several fundamental questions about the nature of neural function in *C. elegans* have to be answered. For instance, is the UNC-2 channel the only VGCC involved in the regulation of neurotransmitter release from presynaptic cells? Do other VGCC types operate in parallel with UNC-2 to mediate the same set of cellular processes? These, as well as other questions, once addressed, will permit for a more detailed understanding of the processes governing neurotransmission in worms and mammals.

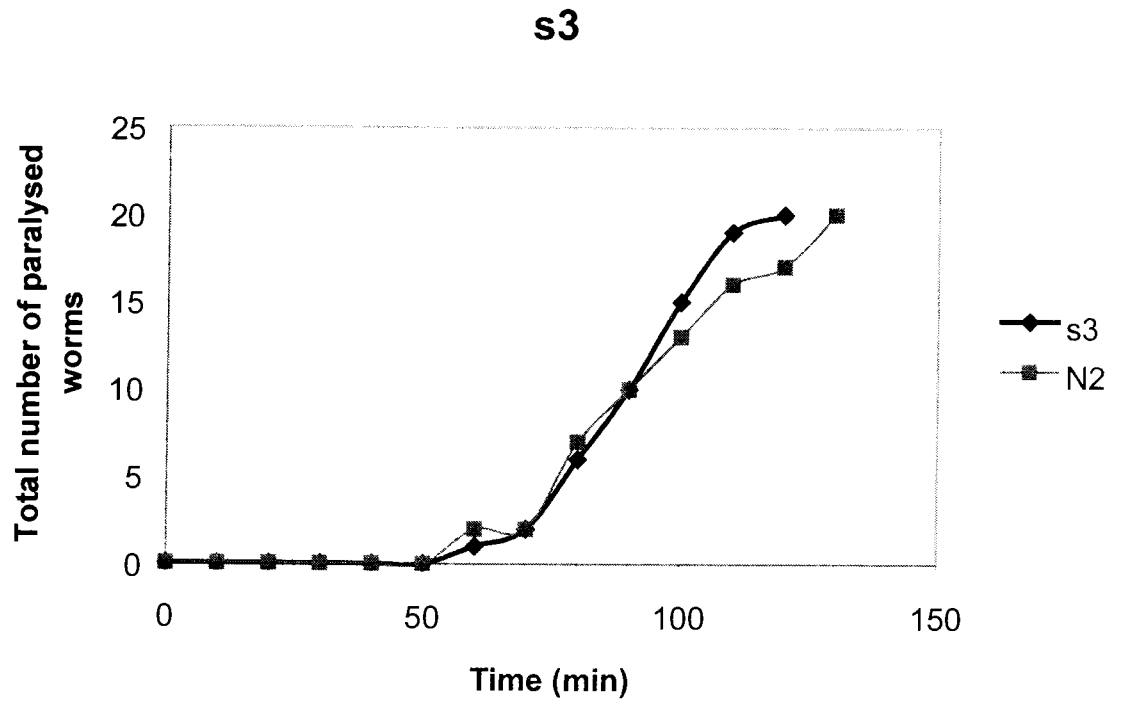
Conclusion

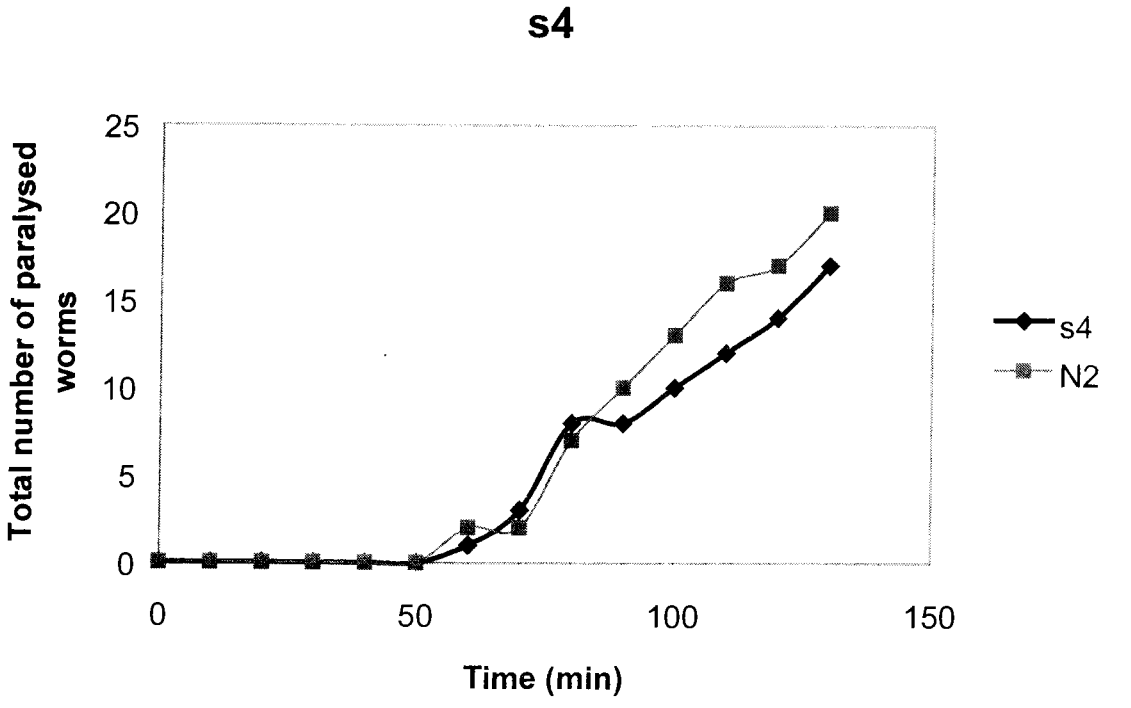
The identification of genes and gene products involved in neurotransmission can greatly facilitate the elucidation of the pathways that are involved, or provide information regarding the nature of the various interactions that take place within the process. Twelve mutants with defects in neurotransmission were generated via a suppressor screen in this study, some of which may represent previously unknown factors that are important for either the secretion, or reception of neurotransmitters at the nervous synapses in *C. elegans*. The results obtained here may form the basis for additional investigations into the mode of function, or the nature of these factors.

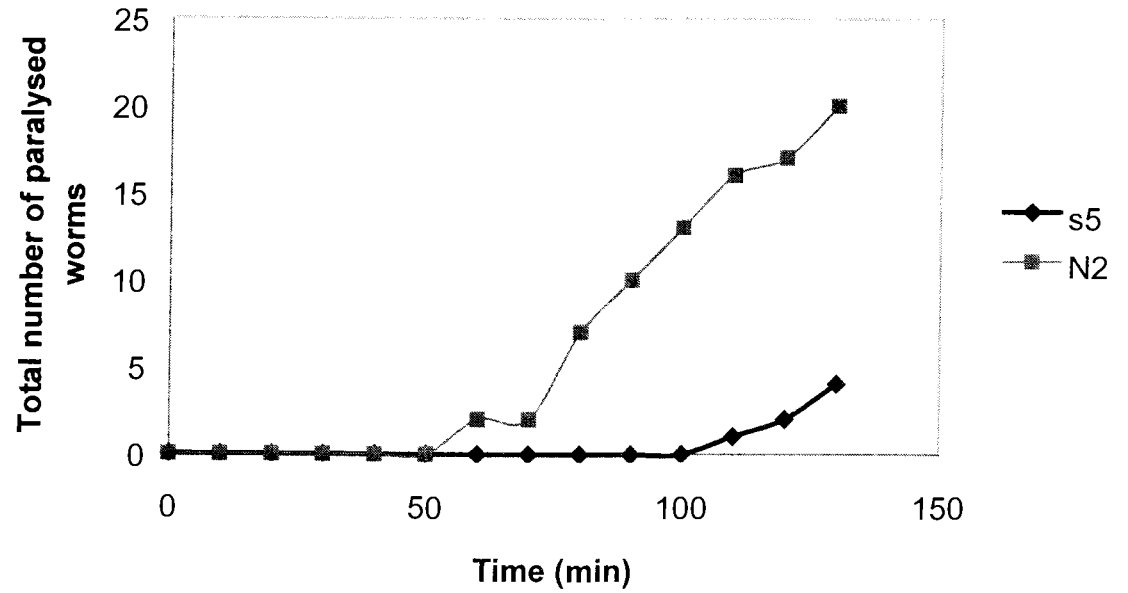
APPENDIX - ALDICARB ASSAY RESULTS FOR sX AND suX55 GROUPS

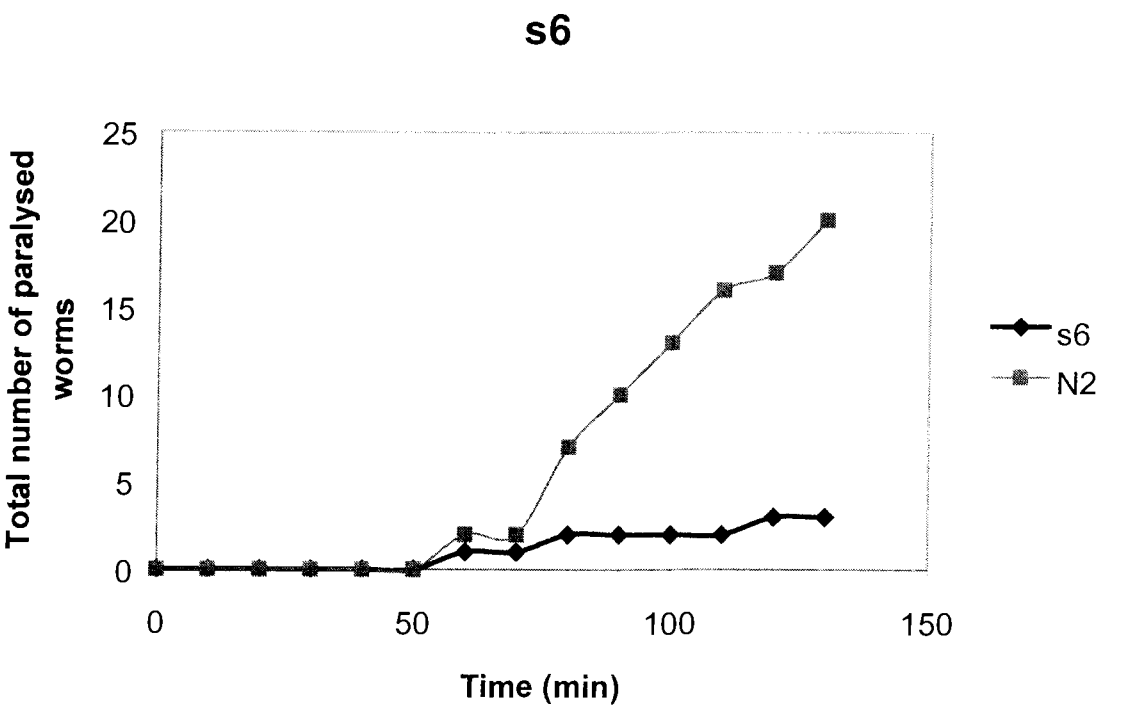




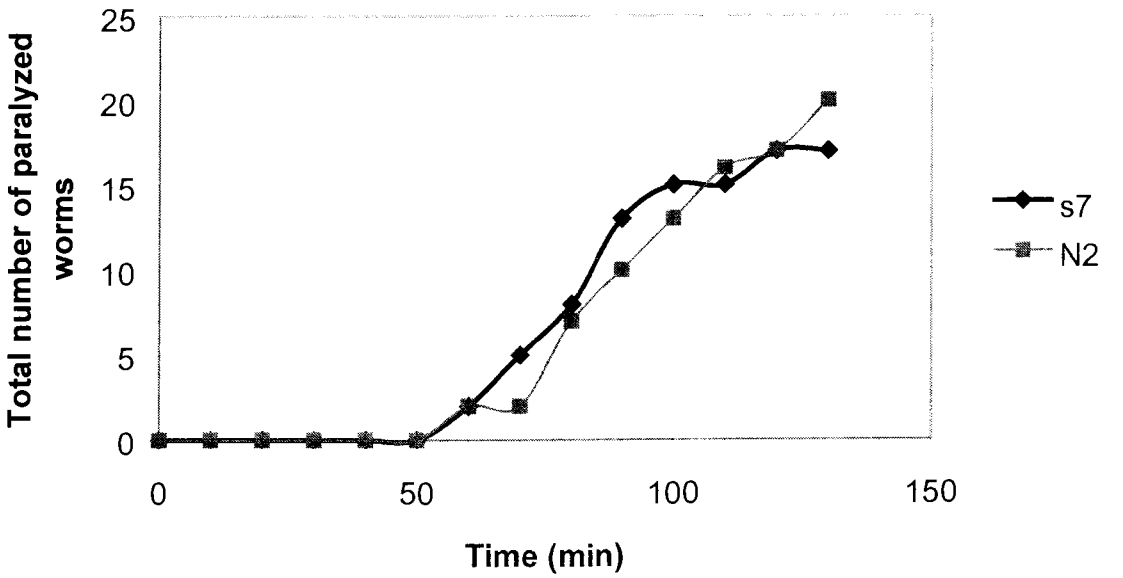

s3
Total number of paralysed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s3
N2


s4
Total number of paralysed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s4
N2

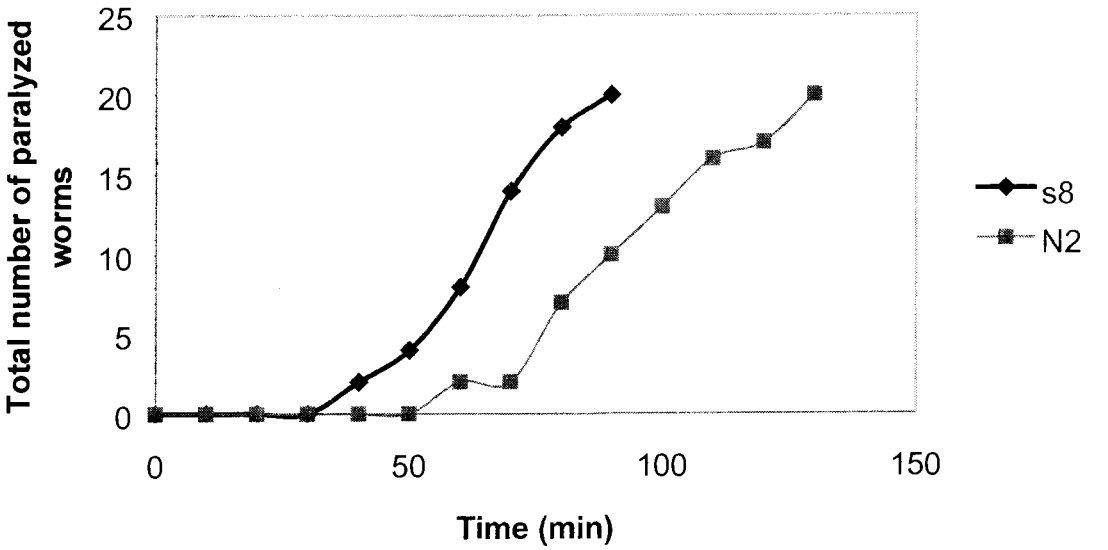

s5
Total number of paralysed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s5
N2

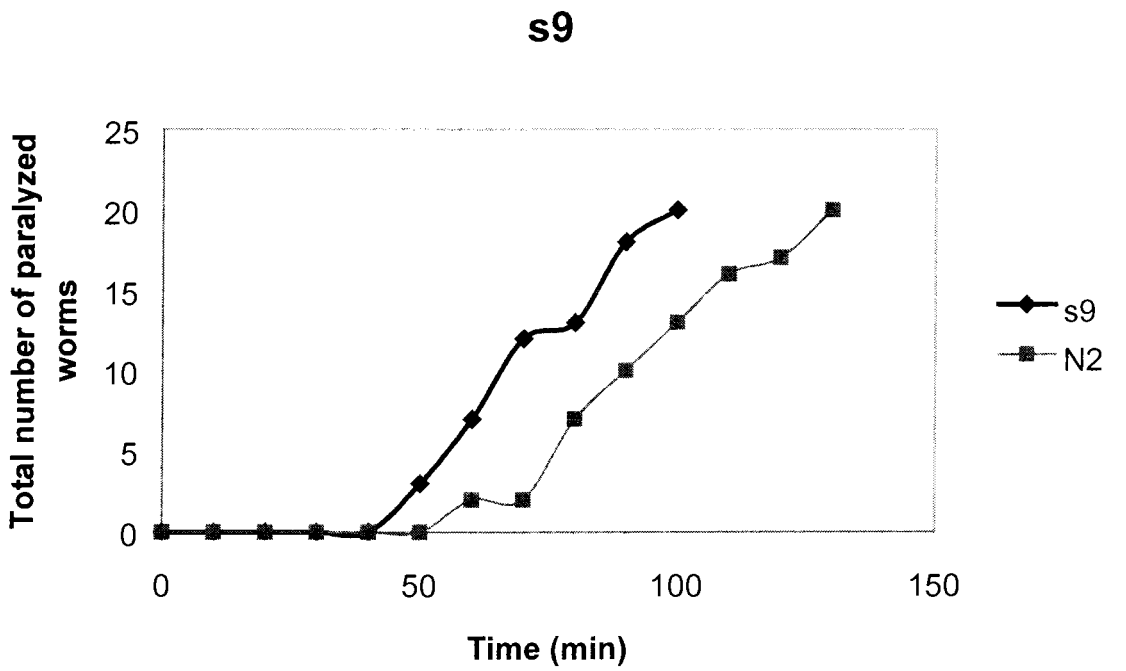

s6
Total number of paralysed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s6
N2

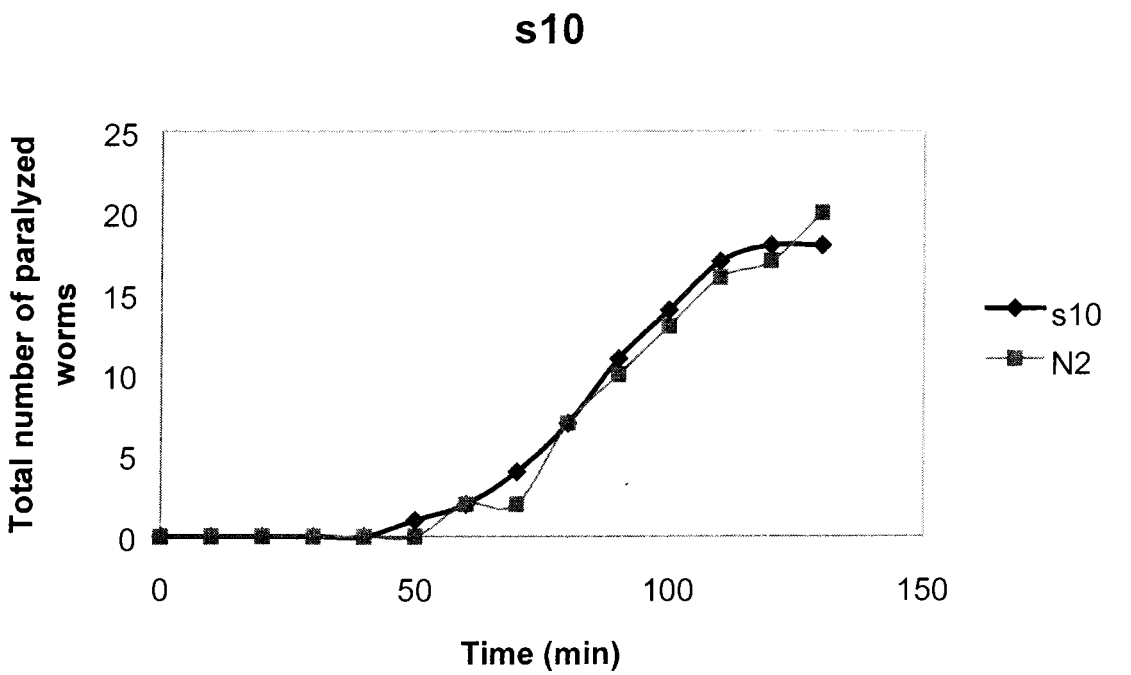
s7

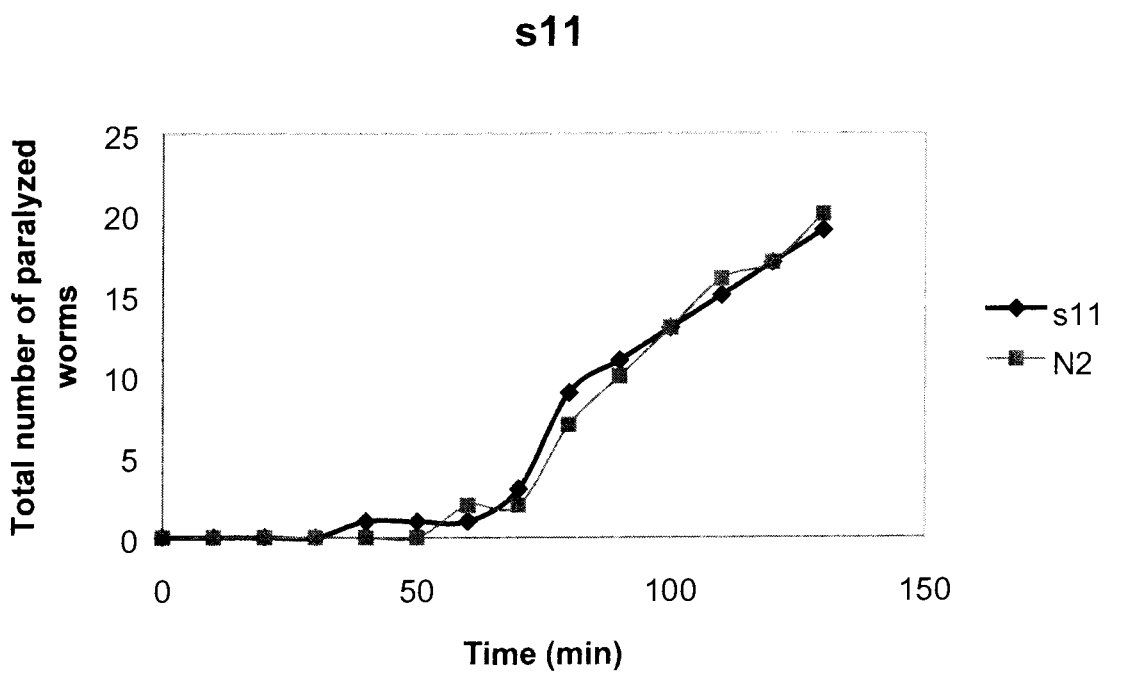

Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s7
N2

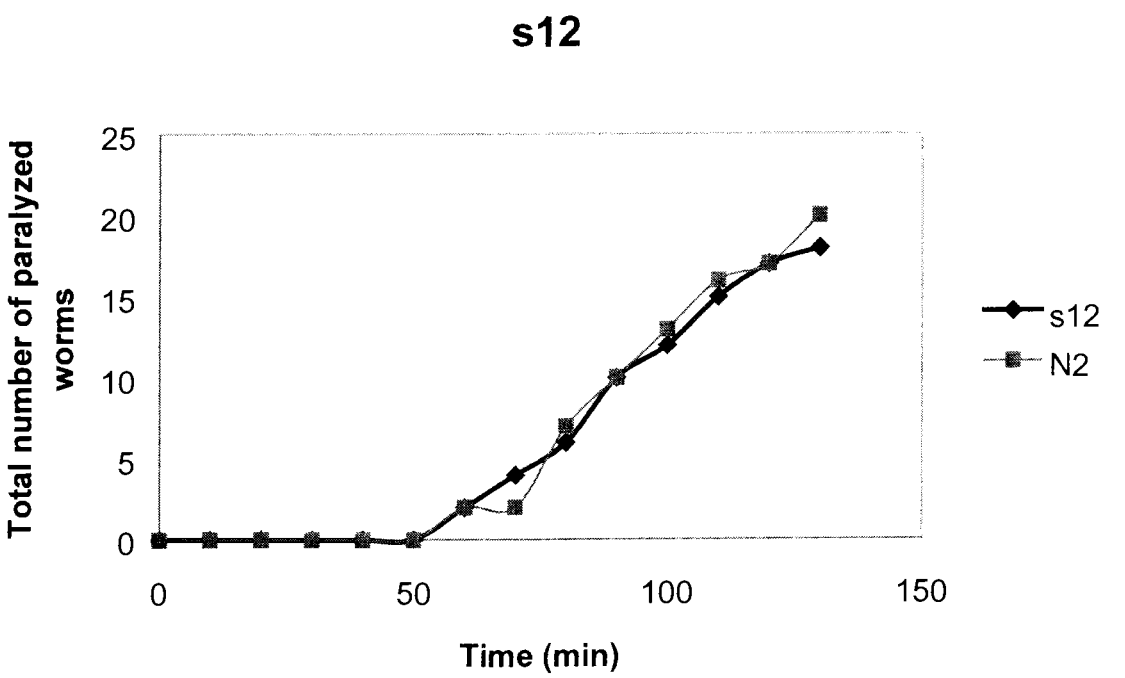
s8

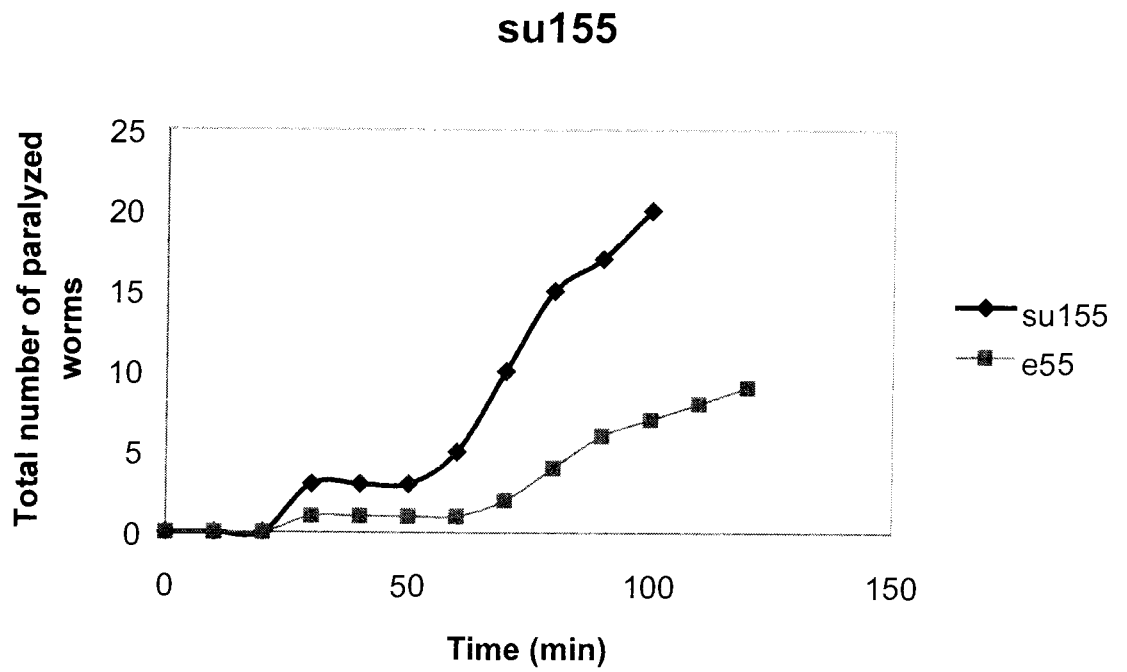

Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s8
N2

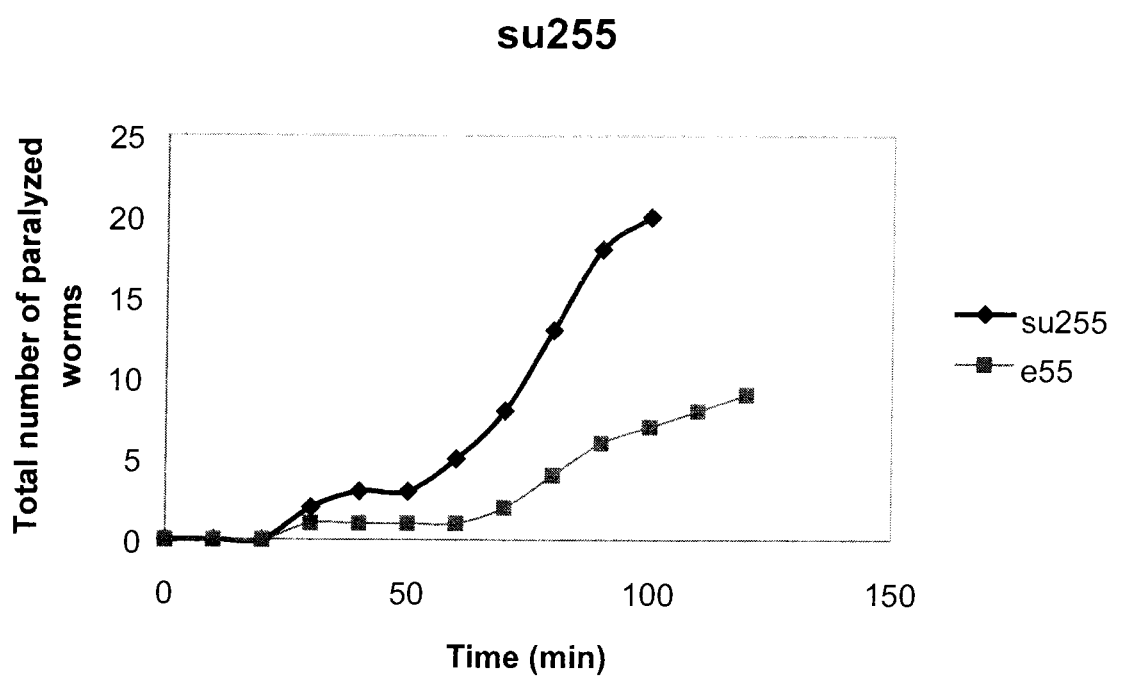

s9
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s9
N2

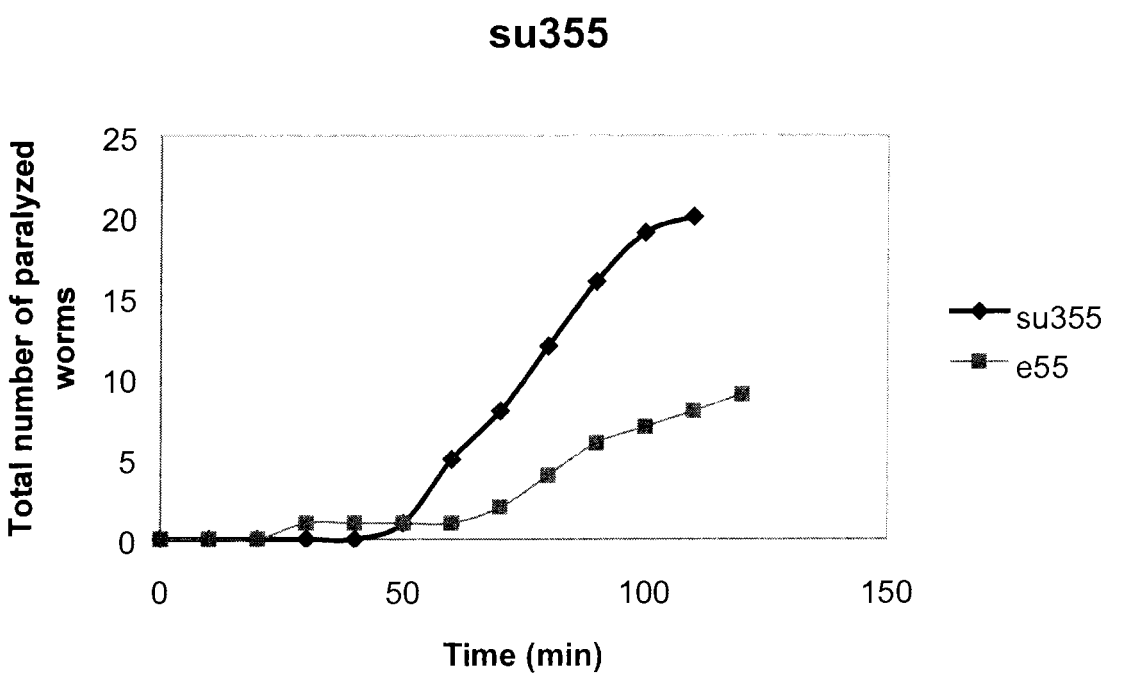

s10
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s10
N2

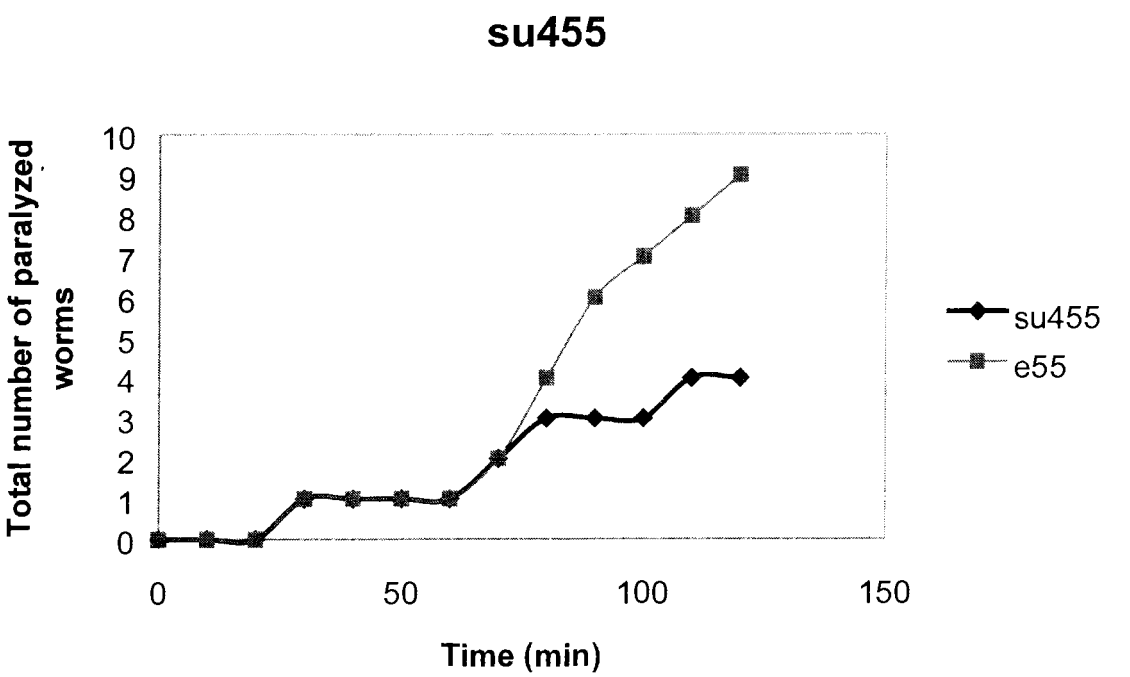

s11
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s11
N2

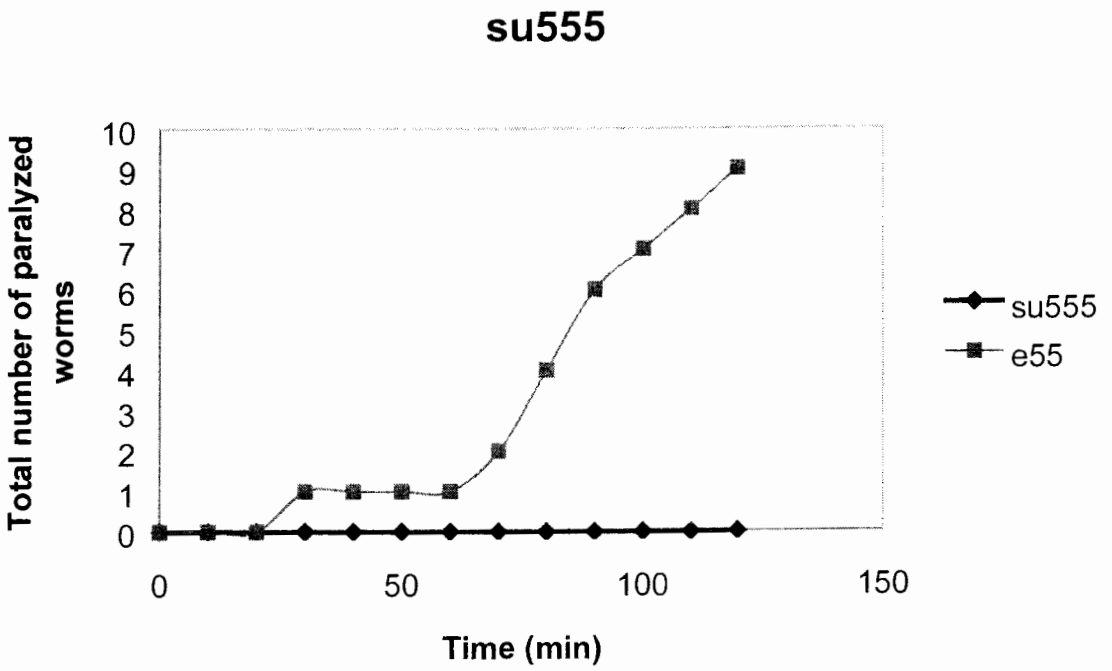

s12
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
s12
N2

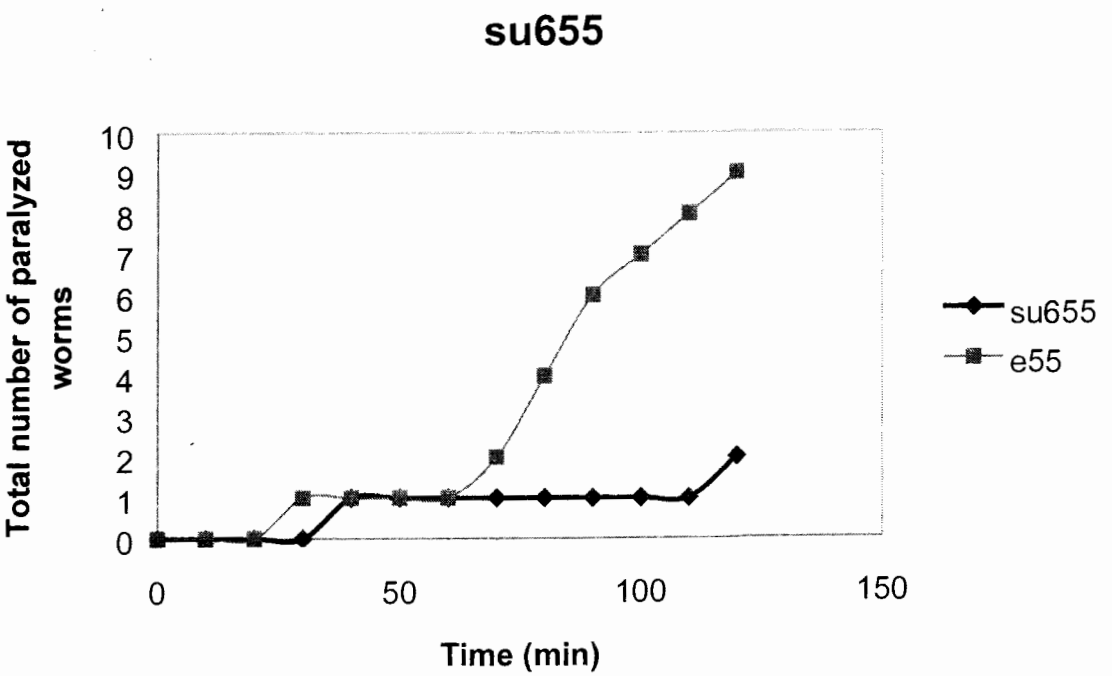

su155
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
su155
e55

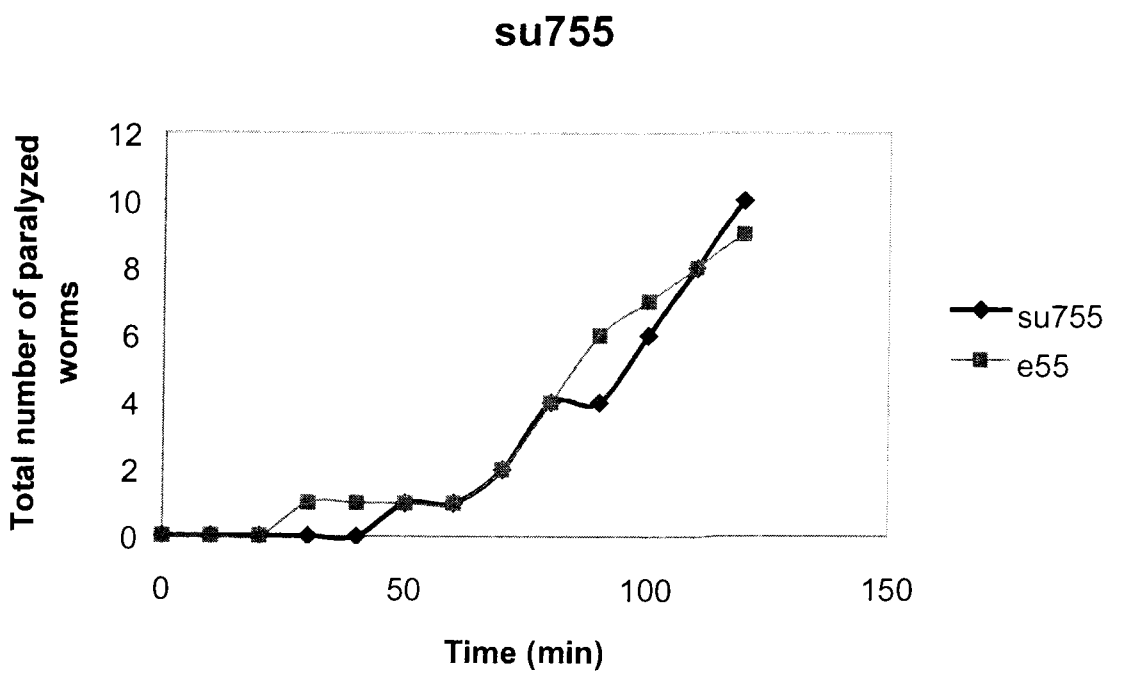

su255
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
su255
e55

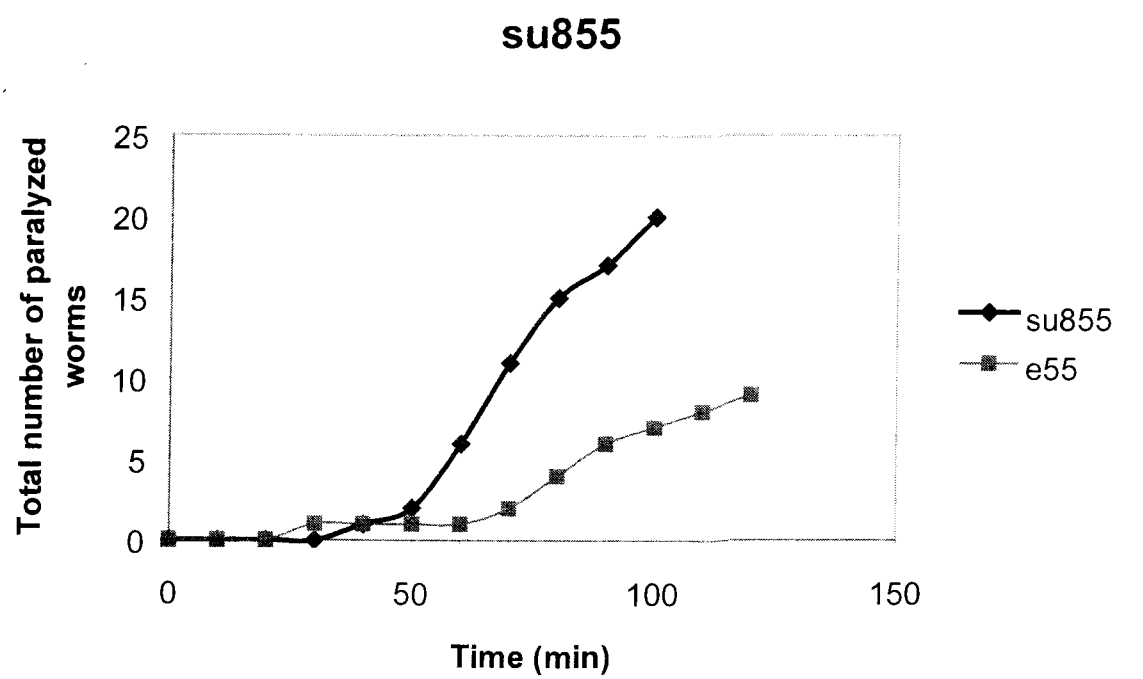

su355
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
su355
e55

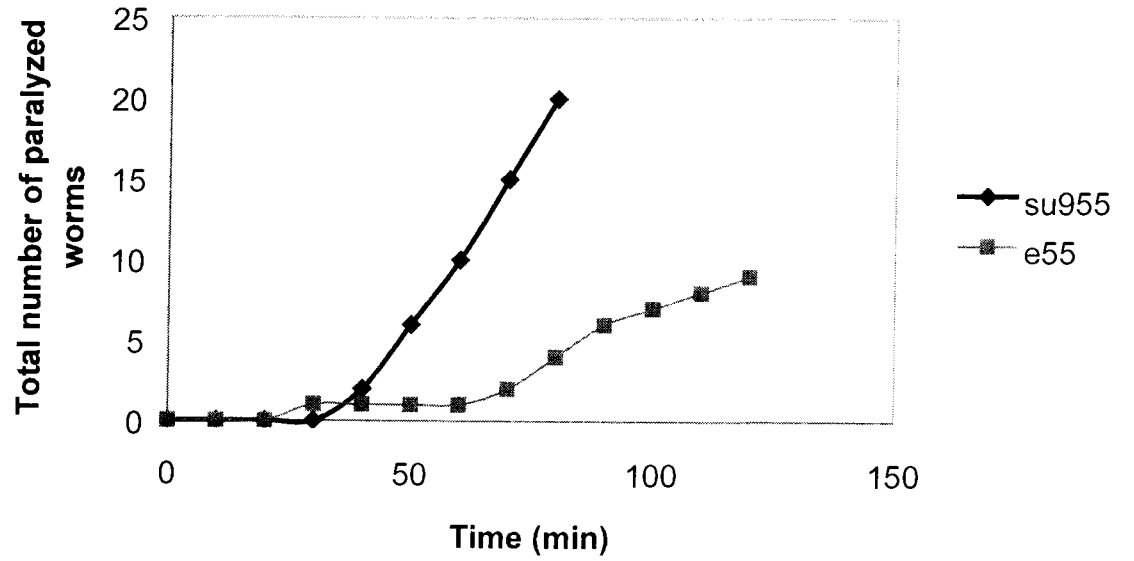

su455
Total number of paralyzed worms
10
9
8
7
6
5
4
3
2
1
0
0
50
100
150
Time (min)
su455
e55

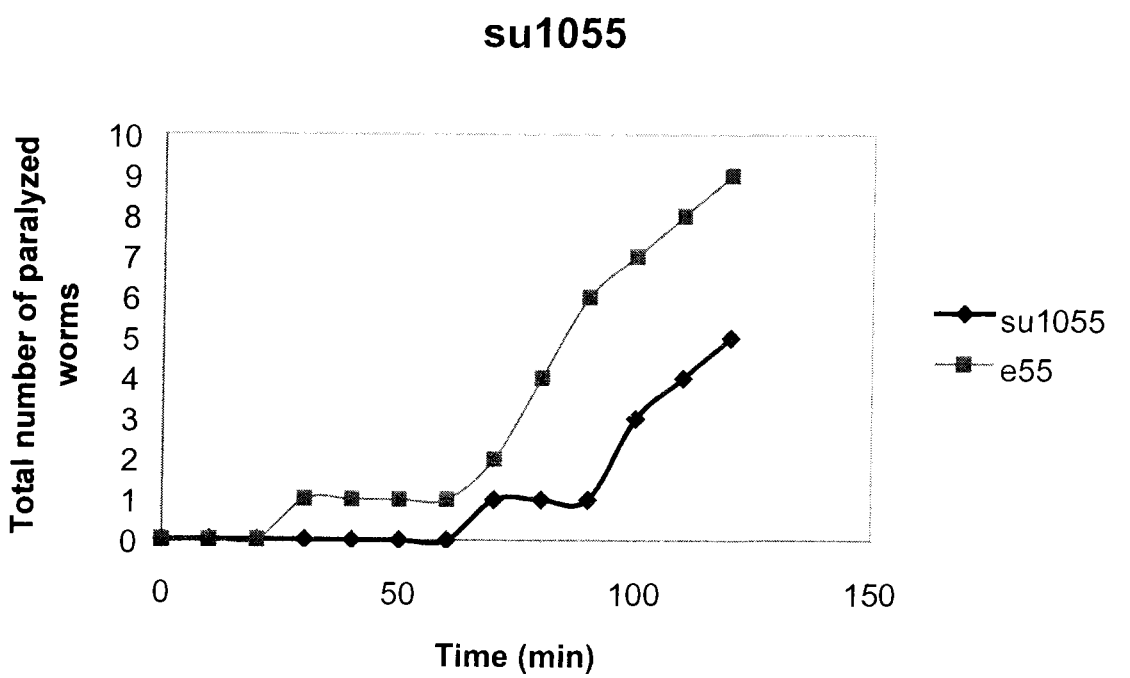

su555
Total number of paralyzed worms
10
9
8
7
6
5
4
3
2
1
0
0
50
100
150
Time (min)
su555
e55

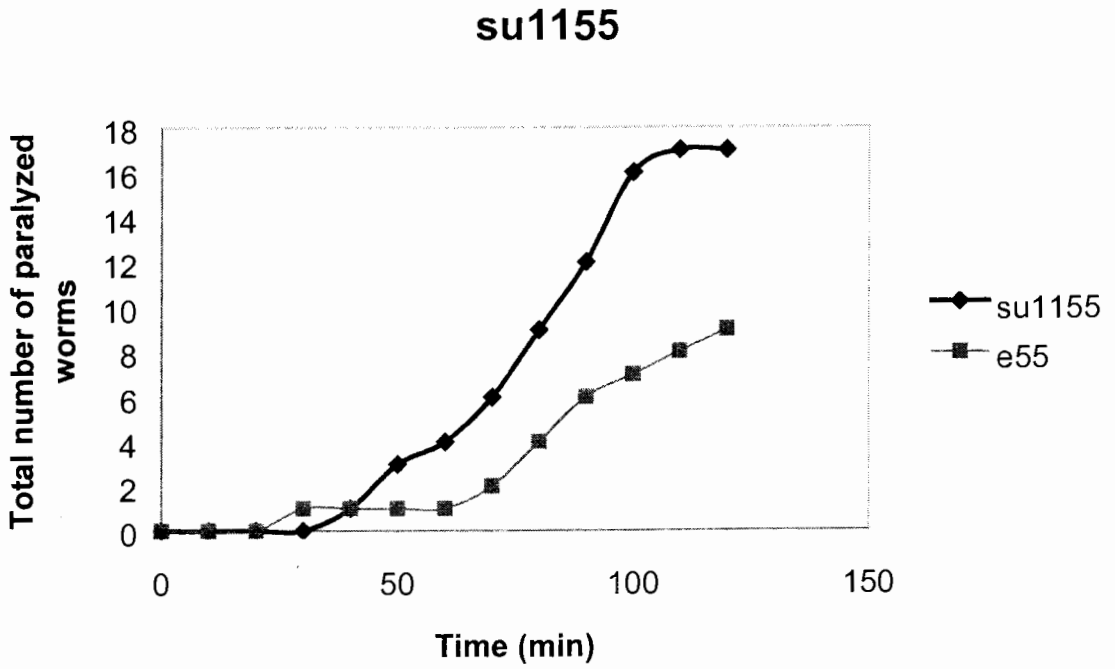

su655
Total number of paralyzed worms
10
9
8
7
6
5
4
3
2
1
0
0
50
100
150
Time (min)
su655
e55

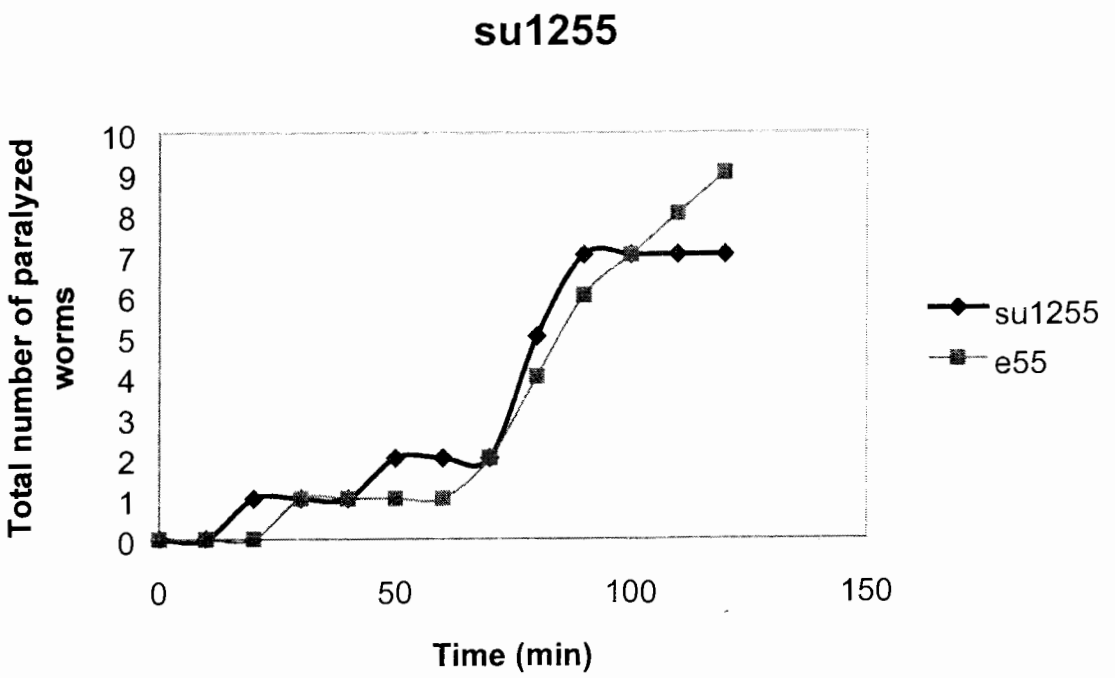

su755
Total number of paralyzed worms
12
10
8
6
4
2
0
0
50
100
150
Time (min)
su755
e55


su855
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
su855
e55


su955
Total number of paralyzed worms
25
20
15
10
5
0
0
50
100
150
Time (min)
su955
e55


su1055
Total number of paralyzed worms
10
9
8
7
6
5
4
3
2
1
0
0
50
100
150
Time (min)
su1055
e55


su1155
Total number of paralyzed worms
18
16
14
12
10
8
6
4
2
0
0
50
100
150
Time (min)
su1155
e55


su1255
Total number of paralyzed worms
10
9
8
7
6
5
4
3
2
1
0
0
50
100
150
Time (min)
su1255
e55

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