Dopamine mediated modulation of electrotactic swimming behaviour in *Caenorhabditis elegans*

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By

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Title: Dopamine mediated modulation of electrotactic swimming behaviour in *Caenorhabditis elegans*

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ABSTRACT

The nematode *C. elegans* is a multicellular model organism to study the neuronal-basis of behaviour. *C. elegans* demonstrates an innate response to swim towards the cathode in the presence of a DC electric field(EF), a behaviour known as "electrotaxis". We examined mutants affecting sensory and dopaminergic neurons and found that these mutants moved with reduced speed with intermittent pauses, abnormal turning, and slower body bend. A similar phenotype was observed in worms treated with neurotoxins 6-OHDA, MPTP and rotenone. Pre-exposing worms to a known neuroprotective compound acetaminophen could suppress the effects of neurotoxin on movement.

Further, this study demonstrates that dopamine and the D2-type dopamine receptor are necessary to modulate electrotactic movements in worms. A reduction in extracellular dopamine leads to a significant increase in the swimming speed as judged by the analysis of *bas-1*(dopa decarboxylase) and *cat-1*(VMAT) mutants. The dopamine transporter *dat-1* acts genetically downstream of *bas-1* and *cat-1* since *dat-1* mutants efficiently suppress *bas-1* and *cat-1* phenotypes. We also found that DOP-3(D2-type receptor) acts as the sole receptor for dopamine-mediated regulation of electrotaxis. Interestingly, we found that prolonged exposure to EF resulted in a gradual decline in the swimming speed such that animals were 40% slower at the end of ten minutes exercise period. This change is mediated by DOP-3 since *dop-3* mutants continue to swim at the initial speed and don't slow down. This conclusion is supported by the analysis of animals treated with Heloperidol(D2 antagonist) and SKF38393(D1 agonist). Overall, our work demonstrates that D2 receptor-mediated neuronal signalling is required to restrict

muscle activity not only during the initial phase of electrotaxis swimming but also for the entire duration of the assay. We suggest that such a role of dopamine signalling might serve as an important and conserved mechanism to limit muscle overuse during prolonged physical exercise.

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I dedicate my work to my work to my family, friends and colleagues, who have made this journey memorable.

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Abbreviation

AADC	Aromatic Amino Acid Decarboxylase
AC	Adenylyl Cyclase
cAMP	cyclic Adenosine Mono Phosphate
AMPA	A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CNS	Central Nervous System
DA	Dopamine
DAergic	Dopaminergic
DARP-32	Dopamine and cAMP Regulated Phosphoprotein
DAT	Dopamine Transporter
DC	Direct Current
DEG/ENaC	Degenerin/Epithelial Sodium Channel
FMRF amide	Phe-Met-Arg-Phe amide
GABA	γ-amino butyric acid
GFP	Green Florescence Protein
GPCR	G-protein couple receptor
HSN	Hermaphrodite Specific Neuron
L-DOPA	Dihydroxyphenylalanine
MSN	Medium Spiny Neurons
MPTP	1-methyl-4-phenylpyridinium
NMDA	N-methyl-D-aspartate

6-OHDA	6-hydroxy dopamine
PD	Parkinson's Disease
PDMS	Polydimethylsiloxane
PET	Positron Emission Tomography
PKA	Protein Kinase A
ROS	Reactive Oxygen Species
SNpc	Substantia nigra pars compacta
TEP	Transepithelial Potential
TH	Tyrosine Hydroxylase
VC	Ventral Chord
VMAT	Vesicular Monoamine Transporter
VTA	Ventral Tegmental Area

1. INTRODUCTION

Multicellular eukaryotes have evolved with sophisticated sensory systems in order to respond to environmental cues. These systems enable them to adapt to seasonal changes, alter their diet according to the availability of food resources, and sometimes demonstrate immediate physiological changes to acclimatize quickly to environmental fluctuations. These behavioral responses contribute to the overall fitness of the organism, with regards to their survival and reproduction. Every behaviour is mediated by a specific set of connected neurons, which work together to trigger the organism's reaction to external stimuli. Each of these neurons may be interconnected to other sets of neurons to regulate the diverse behavioral responses of the organism to the environmental cues it encounters. Since the inception of evolutionary studies, many naturalists have focused their research on animal behaviour, which has lead to our understanding of the basic principles of behavioral responses to the environment. During the mid 19th century, Charles Darwin made reference to the brain as a major site for behavioral regulation (Smulders, 2009). Subsequently, the neuronal basis of behaviour emerged when Claude Bernard demonstrated that specific drugs/toxin bind to particular site (receptors) on the cell surface and not anywhere else on the cell, which pave ways to the foundation of neuron doctrine and synapse (Conti, 2002). This study led to the discovery that nerve cells communicate via chemical means/neurotransmitters and neurotransmitter signalling allows organism to respond to environmental stimuli by eliciting a behavioral response.

1.1. THE COMPLEX HUMAN BRAIN:

The ultimate goal of neurobiology is to understand how our nervous system, particularly the brain, is formed, and how it regulates our behaviour. The human brain is the most complex organ in the body consisting of billions of neurons and trillions of synapses, which are organized in a complex temporal and spatial multiscale structure. Furthermore, structures within any given scale are organized into modules to perform distinct functions. For instance, the posterior region of the frontal lobe of the brain controls the ability to speak, neurons from the basal ganglia regulate movement, the amygdala controls fear, the lateral hypothalamus regulates sleep and so on. Each of these actions is managed by a set of neurons that form neuronal circuits. The neurons in a neuronal circuit are connected by neurotransmitters and ion channels, which transfer the neuronal signals from a presynaptic to a postsynaptic neuron. Understanding the complexity of these brain structures and neuronal connections is integral to any study of nervous system development and its control over behaviour, as well as the study of the greater than 400 disorders and diseases that are linked to the brain in humans (NIH).

In order to understand the heterogeneity of the human brain at the cellular and molecular level, efforts have been made in studies focusing on non-human model organisms. Though mammalian models such as mice and monkeys are considered closely related model systems to humans, their longer life span and expensive maintenance relative to simpler organisms hinders their progress in scientific breakthroughs. Many remarkable discoveries in neuroscience such as axonal conductance, synaptic transmission, integrative neurobiology and behaviour were made from studies utilizing

invertebrate animal models (Sattelle and Buckingham, 2006). Major discoveries in the fruit fly *Drosophila melanogaster* have lead to the identification of many genes (proneural and neurogenic classes) involved in neuronal development. Forward genetic approaches in this organism have resulted in the discovery of genes regulating circadian rhythms, which is considered a major advancement in behavioral neurogenetics (Bellen et al., 2010).

Similar to Drosophila, the nematode *Caenorhabditis elegans* is another invertebrate animal model that, with a small and well-defined nervous system (White et al., 1986), serves as an excellent model to understand the development of the nervous system and how it regulates behaviour. The advanced molecular genetic techniques initially developed in the worm system, e.g., green florescence protein (GFP) tagging of proteins (Chalfie et al., 1994) and RNAi knock down of desired genes (Fire et al., 1998), have allowed researchers to uncover conserved cellular and molecular mechanisms of neurogenesis, axonal growth, regeneration, and age-related neurodegenerative diseases (White et al., 1986) (Link, 2006).

1.2. *C. elegans* AS A MODEL SYSTEM:

1.2.1. Life style:

The nematode *C. elegans* is a small soil dwelling organism, about 1.5 mm in length (Riddle et al., 1997). It is a commonly used model organism in the study of development and behaviour. In 1965, Sydney Brenner was the first to establish *C. elegans* as a model organism, and was later in 2002 awarded a Nobel Prize in physiology or medicine along with Robert Horvitz and John Sulston for their work in *C. elegans,*

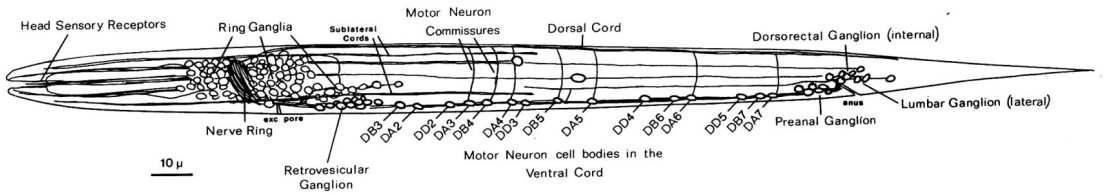
contributing to our understanding of genetic regulation of organ development and programmed cell death. Many features of *C. elegans* make it an ideal invertebrate model organism for laboratory studies. *C. elegans* has a short life cycle of 3 days consisting of four larval stages (L1, L2, L3 and L4) that molt into an adult (Riddle et al., 1997). Its rapid life cycle makes it suitable for genetic analysis and cultivation in the laboratory using *E. coli* culture. Additionally, it has a large brood size (300-350 progeny), hermaphroditic life style, short life span of approximately 18 days, transparent body, and stereotypic cell lineages (Riddle et al., 1997). A complete cell lineage map is available for every cell in the organism (Riddle et al., 1997). Furthermore, it was the first multicellular eukaryote to have its complete genome sequenced. The *C. elegans* genome is about 30 times smaller than the human, yet it encodes for more than 22,227 proteins coding genes which is only slightly fewer proteins than the human genome (Spieth, J. and Lawson, 2006). Combined, these features make *C. elegans* amenable to laboratory research on molecular and cellular studies and particularly well suited to studies of nervous system development and behaviour.

1.2.2. Genome of *C. elegans* nervous system:

The *C. elegans* nervous system consists of 302 neurons in hermaphrodites and 385 neurons in males (Riddle et al., 1997). These neurons are grouped into two distinct and independent nervous systems: the pharyngeal, comprised of 20 neurons and the somatic (voluntary control), comprised of 282 neurons (Riddle et al., 1997). A complete circuitry diagram of these neurons is available which was constructed from serial sections obtained from electron micrographs. The circuitry diagram consists of all chemical synapses,

electrical and neuromuscular junctions (White et al., 1986). A complete lineage map of each of these neurons provides a substantial tool for neuro-developmental studies. Moreover, the *C. elegans* genome has many parallel features to the vertebrate nervous system related genes as well as a few prominent differences (Bargmann, 1998). *C. elegans* normally has one identifiable gene related to known gene families whereas vertebrate genomes often have more than one similar gene. The conserved gene families include neurotransmitter synthetic pathway genes, their receptors such as the G protein coupled receptors (GPCR) and ligand gated ion channels and vesicular trafficking components (Bargmann, 1998). *C. elegans* also has voltage activated ion channels such as the voltage gated calcium and potassium ion channels, however there are none of the voltage gated sodium channels. (Bargmann,1998). The absence of voltage gated sodium channel is compensated for by the small size of *C. elegans* neurons and its high membrane resistance, thus being able to propagate signals effectively without the presence of voltage gated sodium channels (Davis and Stretton, 1989)(Goodman et al., 1998).

A.



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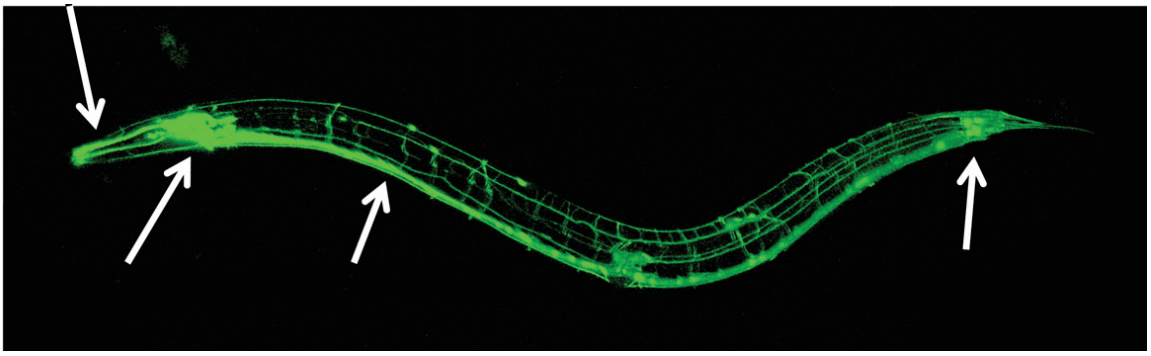


Figure 1. The *C. elegans* nervous system. A) A general view of *C. elegans* nervous system at the L1 stage. This diagram was published in Wormatlas (Durbin, 1987). B) *C. elegans* worm expressing pan neuronal green florescent protein (GFP) in its nervous system .This picture was originally from Hang Ung, Jean-Louis Bessereau's laboratory, France (Emmons, 2015).

C. elegans' neurotransmitters include excitatory acetyl choline and glutamate, inhibitory γ-amino butyric acid (GABA) and monoamines such as dopamine, serotonin and the invertebrate specific octopamine (Bargmann, 1998). Additionally, *C. elegans* uses neuropeptides for neurotransmission such as the FMRF amide (Phe-Met-Arg-Phe amide)(Bargmann, 1998). These features of the *C. elegans nervous* system have allowed the study of the neuronally regulated behaviours described in the next section.

1.3. NEURONAL REGULATION OF *C. elegans* BEHAVIORS:

1.3.1. Feeding:

In *C. elegans*, the pharynx connects the mouth to the intestine, and the rapid pumping of this organ requires neuronal inputs to the muscle. The three neurons in the pharyngeal system M4, MC and M3 control the start, termination and rate of pharyngeal pumping (Avery and You, 2012). Neurotransmitters such as acetylcholine, glutamate and serotonin regulate feeding mechanisms (Avery and You, 2012). The muscarinic acetylcholine receptor encoded by *eat-2* and a novel protein encoded by *eat-18* are required for rapid pharyngeal pumping (McKay et al., 2004), while *eat-4*, which encodes a vesicular glutamate transporter, is required for pharyngeal relaxation (Avery, 1993).

1.3.2. Egg-laying:

C. elegans has a hermaphroditic life style; it first produces sperm which is stored in the spermatheca, and then switches to produce oocytes at the adult stage. In the absence of mating, the oocytes are self fertilized(Schafer, 2005). At any given time there are 10-15 eggs inside the uterus and each of these eggs are expelled outside the body

when there are favorable conditions in the environment(Schafer, 2005). For instance, starvation inhibits egg laying. This behaviour is regulated by hermaphrodite specific neurons (HSNs) and ventral chord (VC) motor neurons(Trent et al., 1983)(Desai et al., 1988). The HSNs and VC motor neurons are located laterally and slightly posterior to the vulva, which is the reproductive organ in *C. elegans* (**Figure 2**). The neurotransmitters serotonin and neuropeptides are found in HSNs(Horvitz et al., 1982)(Duerr et al., 2001)(Schinkmann and Li, 1992). Ablation of the vulva muscles or mutation in the serotonergic pathway genes causes an accumulation of eggs inside the uterus, while treatment with external serotonin rescues this defect (Schafer, 2005). VC neurons are acetylcholinergic as well as serotonergic. Disruption of the VC neurons has a less striking effect than the HSN neurons on egg-laying, but calcium recordings indicate that the VC neurons regulate the contraction of vulval muscles (Shyn et al., 2003)(Schafer, 2005).

1.3.3. Response to touch:

The natural habitat of *C. elegans* is compost, detritus such as rotten fruits and vegetables, where the animal constantly responds to environmental cues. These responses are facilitated by the sensory neurons, which detect forces from the environment and other animals. There are 30 sensory neurons in hermaphrodites and depending on the type of stimulus, different sets of neurons are recruited to mount the animal's response (Goodman, 2006). Worms respond to touch by demonstrating a backward locomotion. One such response to touch at the body wall is mediated by the mechanoreceptor neurons. The mechanoreceptor neurons have either ciliated or non-ciliated dendrites, and have their endings embedded in the cuticle or exposed to the external environment. There are

six touch receptor neurons: ALMR, ALML, AVM, PLMR, PLML and PVM (**Figure 3**)(Chalfie et al., 1985)(Wicks et al., 1996), and The DEG/ENaC (degenerin/epithelial sodium channel) transduction channels are known to be involved in mediating this response(Goodman and Schwarz, 2003)(Canessa et al., 1993). Gene mutation affecting the DEG/ENaC channel in these neurons such as *mec-10*, *mec-4*, *unc-8* and *unc-105* are defective in the touch response(Goodman, 2006).

1.3.4. Chemotaxis:

C. elegans has a well developed chemosensory system that allows it to sense food, noxious conditions, mating pheromones and other chemicals. The chemosensory neurons that are exposed to the external environment sense these chemicals, allowing the worm to respond to their environment (Ward et al., 1975). *C. elegans* has three types of chemosensory neurons, the amphid, phasmid and labial neurons (**Figure 4**). These neurons detect a variety of chemical cues such as salt, water soluble and volatile attractants(Bargmann, 2006a). Chemotaxis of *C. elegans* towards dissolved salts (Na^+, Cl^-), cyclic nucleotides (cAMP) and amino acids (biotin and lysine) is mediated by the ASE gustatory neuron (Ward, 1973). The AWA, AWB and AWC neurons detect volatile chemicals (Bargmann et al., 1993)(Troemel et al., 1997). These chemicals are either transported through the sheath cells or diffused through the cuticle. Similar to the mammalian sweet and bitter taste system, *C. elegans* can recognize and respond to attractive or repulsive odors. ODR-10 is a GPCR normally expressed in AWA neurons; when ectopically expressed in AWB neurons, worms demonstrate repulsive behaviors to the compound 2-anone, however when the receptor is expressed in AWC, the compound

acts as an attractant (Troemel et al., 1997)(Wes and Bargmann, 2001). Thus, it has been determined that the AWA and AWC neurons mediate attraction, whilst the AWB mediates repulsion in response to odors. *C. elegans* can also respond to repellents or noxious chemical stimuli by using the ASH polymodal neurons. The repulsive behaviour in the worm is demonstrated when exposed to solutions of high osmolarity, heavy metals, bitter alkaloids, detergents, acids, and some organic compounds (Colbert et al., 1997)(Culotti and Russell, 1978)(Dusenbery, 1974)(Hilliard et al., 2002)(Hilliard et al., 2004). Phasmid neurons in the tail are also known to be involved in the avoidance response against the detergent SDS (Hilliard et al., 2002).

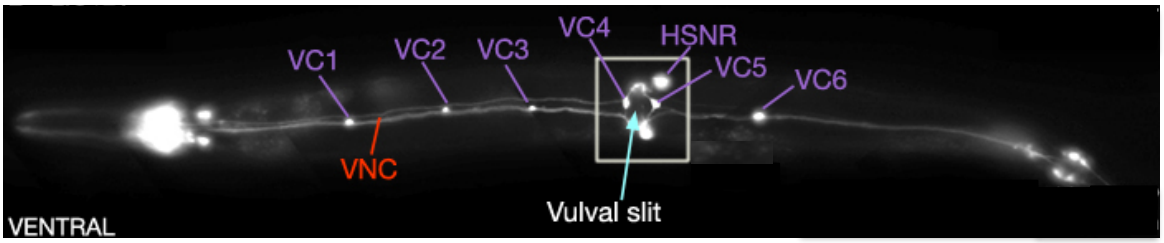


Figure 2: **Egg-laying neurons.** VC1-6 and HSN neurons expressing *ida::GFP* (Lints and Hall, 2009).

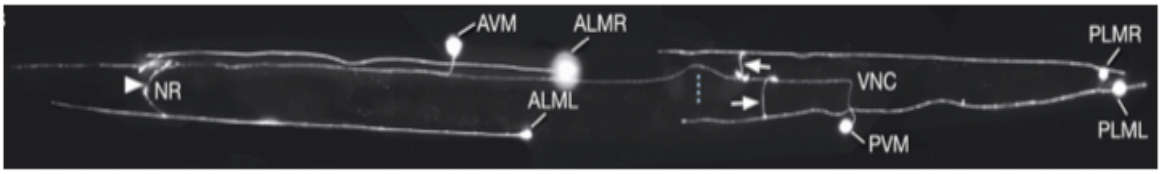


Figure 3: **Touch/Mechanosensory neurons**: The six mechanosensory ALMR, ALML, AVM, PLMR, PLML and PVM expressing *mec-4::GFP* (Altun and Hall, 2011)**.**

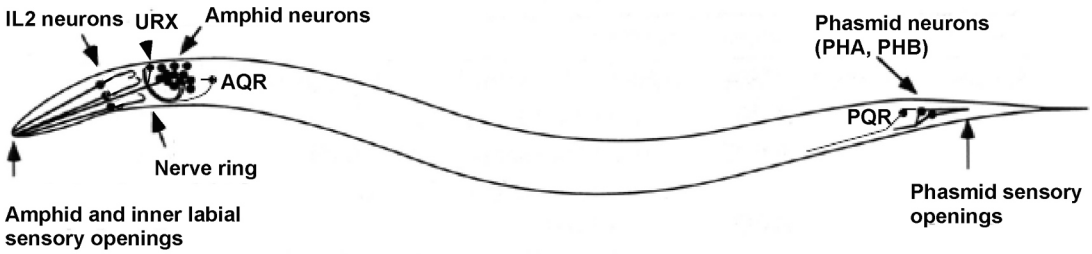


Figure 4: Chemosensory organs in *C. elegans*: each of the amphids contains 12 neurons. Each of the phasmids contains two chemosensory neurons (PHA and PHB). Each of the labial organs contain one IL2 chemosensory neuron (Bargmann, 2006b)**.**

If *C. elegans* is exposed to harsh environmental conditions during the late L1 stage (e.g., high temperatures, crowding or limited food), then the larvae utilize an alternate developmental program termed 'dauer' in which they undergo a morphological and physiological transformation (Golden and Riddle, 1982)(Golden and Riddle, 1984). Dauer animals are highly resistant to heat and desiccation, and can survive without food for several weeks. Chemosensation plays a major role in the transformation of the worm to dauer stage and the transition back to normal development when conditions are once again favorable. A pheromone called daumone, secreted by worms, is detected by the sensory neurons to determine whether or not to enter the dauer stage (Jeong et al., 2005). This mechanism is well understood and involves the ASI neuron, which is considered to be one of the daumone sensing neurons (Bargmann and Horvitz, 1991). ASI expresses DAF-7, a TGFβ family ligand, that prevents dauer formation (Ren et al., 1996). This is shown by the decreased expression of *daf-7* during starvation and in high nematode population densities (Ren et al., 1996). The ASJ neuron is also involved in the dauer transition, and ablation of the ASJ neuron prevents animals from exiting the dauer stage. ASJ expresses *daf-28*, an insulin related peptide, and is a regulator of *daf-2* which encodes for a tyrosine kinase an insulin/IGF receptor ortholog (Li et al., 2002). *daf-28* expression inhibits dauer formation and its protein acts as *daf-2* agonist (Li et al., 2002).

Another role of the chemosensory neurons in *C. elegans* is the behavioral response to oxygen levels in the environment. *C. elegans* is an aerobic organism and experiences frequent local fluctuations in oxygen levels in the soil environment (Sylvia et al., 2004). The important chemosensory neurons that mediate aerotaxis are URX, AQR

and PQR (Gray et al., 2004)(Cheung et al., 2005)(Chang et al., 2006). URX has a non-ciliated ending exposed to the external environment, suggesting that it senses external oxygen (Coates and de Bono, 2002). The AQR and PQR neurons have ciliated endings exposed to coelomic fluid, thus sensing internal oxygen levels (Coates and de Bono, 2002). Oxygen levels in the environment are known to regulate the aggregation behaviour of worms into feeding groups. Lower oxygen levels are seen to suppress the clumping of worms, while higher oxygen levels in the environment promote aggregation into feeding groups (Gray et al., 2004)(Rogers et al., 2006).

Signal transduction of chemosensation is mediated by chemoreceptors such as GPCRs. The *C. elegans* genome has more than 1000 predicted GPCRs. Sixty percent of about the 100 candidate chemoreceptor GPCR genes are expressed in the chemosensory neurons, 20% in other cells and 20% show undetectable expression (Troemel et al., 1995) (Chen et al., 2005)(Bargmann, 2006a). A single chemotactic neuron can express more than one type of chemoreceptor and chemoreceptors in the ciliated neurons are highly localized in the cilia. Two distinct pathways lie downstream of chemoreceptor GPCRs to elicit a behavioural response to external stimuli: signal transduction via cGMP as a second messenger targeting cGMP gated channels, or signal transduction via TRVP channels.

1.3.5. Electrotaxis and other behavioral responses

In addition to sensory phenomena described in previous sections, *C. elegans* also demonstrates a few other "taxis" behaviors such as electrotaxis, thermotaxis, and magnetotaxis. The electrotaxis behaviour involves the directed movement of worms

towards a cathode when placed in a direct current (DC) field (Sukul N.C., 1978). Although the reasoning behind why they exhibit such a response is unclear, it has been hypothesized that electrotaxis offers advantages in locating food sources in the wild, by sensing certain ionic gradients (**Section 1.3.6)**. In the thermotaxis response, worms that are placed in a thermal gradient demonstrate a preference for the temperature at which they were grown. The magnetotaxis response involves an upward movement with a specific angle in response to a local magnetic field (Vidal-gadea et al., 2015).

1.3.6. Electrotaxis

The electrotaxis behaviour of *C. elegans* was first reported by Sukul and Croll almost four decades ago (Sukul N.C., 1978). The authors reported that wild type animals move towards the cathode when placed in a DC field environment. More recently, the neuronal basis of electrotaxis was demonstrated by Gabel and colleagues (Gabel et al., 2007) in which the authors demonstrated that *C. elegans* move with an angle in the direction generated by the DC electric field. It was also shown that the amphid neurons mediate this electrotaxis behaviour. Ablation of specific amphid neurons, ASJ and ASH, using a laser resulted in significant disruption of the electrotaxis behaviour. In addition to *C. elegans*, a few parasitic nematode species, e.g., *S. carpocapssae* and *S. glaseri*, are also reported to exhibit this electrotaxis behaviour (Shapiro-Ilan et al., 2009a).

The electrotaxis phenomenon is not just limited to worms. Studies have shown that living cells and animals exhibit directed movement towards electric potentials. Electrotaxis is demonstrated by living systems in response to their complex microenvironment. For example, the polarized epithelial cell generates a physiological

transepithelial potential (TEP) due to the differential distribution of ion channels (Zhao et al., 2006). During wound heeling, disruption of the epithelial cell affects the physiological TEP generating ionic currents due to ionic movement, thereby acting as a signal for cell growth towards the injury site (Matsubayashi et al., 2004)(Fraser et al., 2005) (Fitzsimmons et al., 2008). Moreover, the application of electric fields activates signalling molecules required for wound healing such as epidermal growth factors, integrins and kinases (Matsubayashi et al., 2004)(Fraser et al., 2005)(Fitzsimmons et al., 2008). The cellular and molecular basis of electrotaxis behaviour is not well understood, although mounting research suggests that the molecular pathways mediating electrotaxis in cells overlaps with the pathways mediating chemotaxis behaviour (Li et al., 2011).

Whole organism electrotaxis responses have also been observed in vertebrates, fish and amphibians. Electroreception in fish is of two types: passive and active. In passive electroreception, the animals can detect low frequency, weak electric signals originating from objects or animal tissues. In active electroreception, electrogenic animals can detect distortions of a self-generated electric field using organs that can detect high frequency electric signals. Weakly electric fish species use a combination of the passive and active systems for electroreception (Kramer, 1994). The electroreceptive system of fish plays many roles in behavioral tasks such as locating and capturing prey, orientation and navigation as well as communication. These evidences demonstrate the different ways to exhibit electrotaxis behaviour and its neuronal control not only in worms but also in other organisms.

Studies in the slime mold *Dictyostelium* have shown that the electrotactic movement is associated with changes in the calcium flux (Shanley et al., 2006). Gao and his group recently performed a genetic screen in *Dictyostelium* for changes in the electrotaxis behavior and recovered mutations in *PiaA*, which encodes for a subunit of kinase protein complex TORC2 (Gao et al., 2015). Other genes identified in their screen include *gefA*, *rasC*, *rip3* and *pkbR1* among 28 electrotaxis defective strains that encode components of TORC2-PKB pathway(Gao et al., 2015). While the findings are interesting, more research is needed to elucidate the molecular pathways of electrotaxis behavior in organisms.

1.4. DOPAMINE REGULATION OF BEHAVIOUR:

Dopamine as one of the neurotransmitter is critical in controlling behavioral responses through its receptor signalling. Dopamine in the central nervous system (CNS) is involved in the control of locomotion, cognition and endocrine secretion. Carlson, Flank and Hillarp were the first to identify dopamine in their study of catecholamines during the 1960s (Carlsson et al., 1962). Following this identification were the revolutionizing studies in the field identifying the pathways and insights into molecular signalling mechanisms by Paul Greenguard, giving a complete picture of the dopamine system in the human brain. Recognizing the 40 years of study, Carlson and Greenguard were awarded the Nobel Prize in physiology or medicine in the year 2000. Just two years after Carlson's discovery of dopamine, Dahlström and Fuxe reported the distribution of catecholamine (noradrenaline and dopamine) containing neurons in the rat brain. However, the mapping of dopamine neurons in the human brain was not well studied

until the advent of the positron emission tomography (PET) imaging technique and dopamine radiotracers which lead to many neuronal mapping studies in the recent past (Sedvall and Farde, 1995)(Volkow et al., 1996)(Shen et al., 2012).

1.4.1. Dopamine synthesis and signalling

In mammals, tyrosine is converted to dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme tyrosine hydroxylase (TH) (**Figure 5**). Aromatic amino acid decarboxylase (AADC) then converts L-DOPA to dopamine. The cytosolic dopamine is packaged into the synaptic vesicles by a transporter called the vesicular monoamine transporter (VMAT). In humans there are two types of VMATs, VMAT1 and VMAT2 (Duerr et al., 1999). Dopamine signalling is terminated when the extracellular dopamine is re-uptaken into the presynaptic dopaminergic neurons by the dopamine transporter (DAT).

The dopamine receptors belong to a seven transmembrane receptor family of GPCRs, whose signalling is mediated by interactions with the heterotrimeric GTP binding proteins (G proteins). Dopamine receptors are categorized into two types, the D1 and D2 like receptors. In humans there are five subtypes of dopamine receptors, of which DRD1 and DRD5 are of the D1 type and DRD2, DRD3 and DRD4 are of the D2 type receptors.

The D1 like receptors couple with a G protein, $G_{\alpha s}$ which stimulates adenylyl cyclase (AC) and increases the levels of the second messenger cAMP (Spano PF, Govoni S, 1978) (**Figure 6**). The cAMP activates protein kinase A (PKA), which phosphorylates several downstream target proteins that are involved in signal transduction and gene expression. The PKA substrates include DRAPP-32 (dopamine and cAMP regulated

phosphoprotein), ion channels (such as NMDA, AMPA and GABA) and CREB (cAMP response element binding protein)(Neve et al., 2004).

The D2 like receptors interact with the G protein $G_{\alpha i}$, which has an inhibitory effect on adenylyl cyclase (**Figure 7**). The D2 type receptor signalling has an opposing effect to the D1 type receptor signalling. For instance, inhibition of AC activity due to D2 type receptor signalling decreases cAMP levels, which in turn decreases the PKA dependent phosphorylation of DARPP-32. The $G_{\alpha\beta}$ subunit of the heterotrimeric G protein causes inhibition of ion channels including Ca^{2+}, NMDA, AMPA and GABA (Neve et al., 2004).

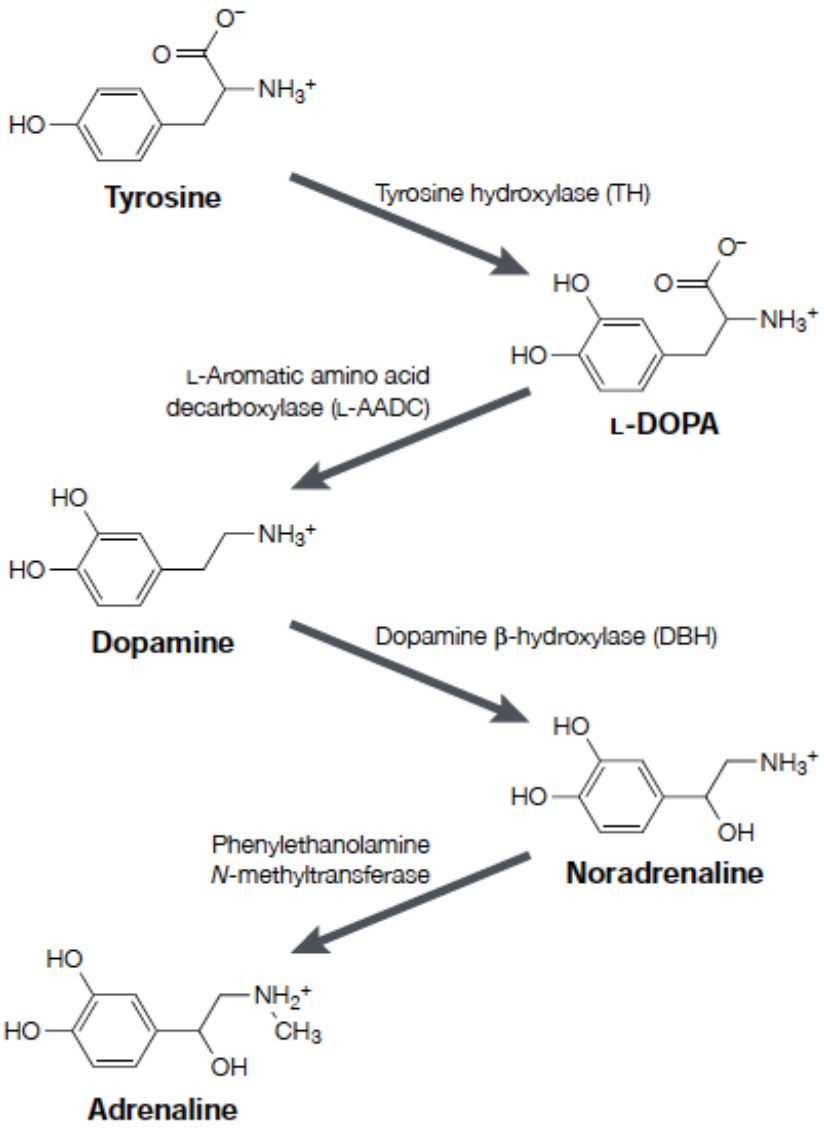


Figure 5: Enzymatic pathway of catecholamine synthesis. This pathway diagram was published by Christo Goridis and Hermann Rohrer (Goridis and Rohrer, 2002).

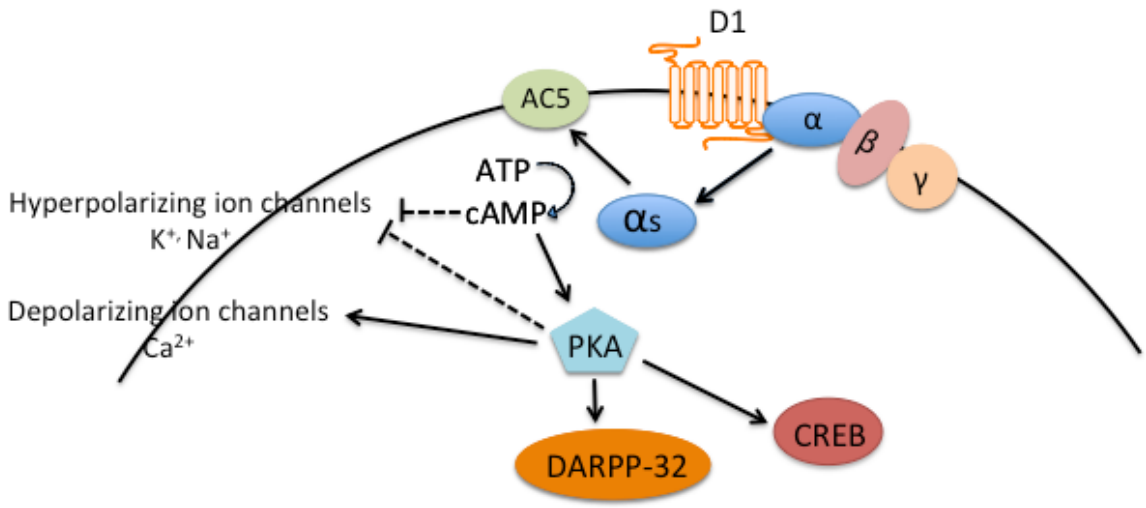


Figure 6. **D1 receptor signalling**. Solid arrowhead lines indicate stimulatory effects and the dotted lines with a bar end indicates inhibitory effects. AC5, adenylate cyclase type 5; CREB, cyclic AMP response element binding protein; PKA, protein kinase A; DARPP-32, dopamine-related phosphoprotein, 32 kDa. Adapted from (Neve et al., 2004).

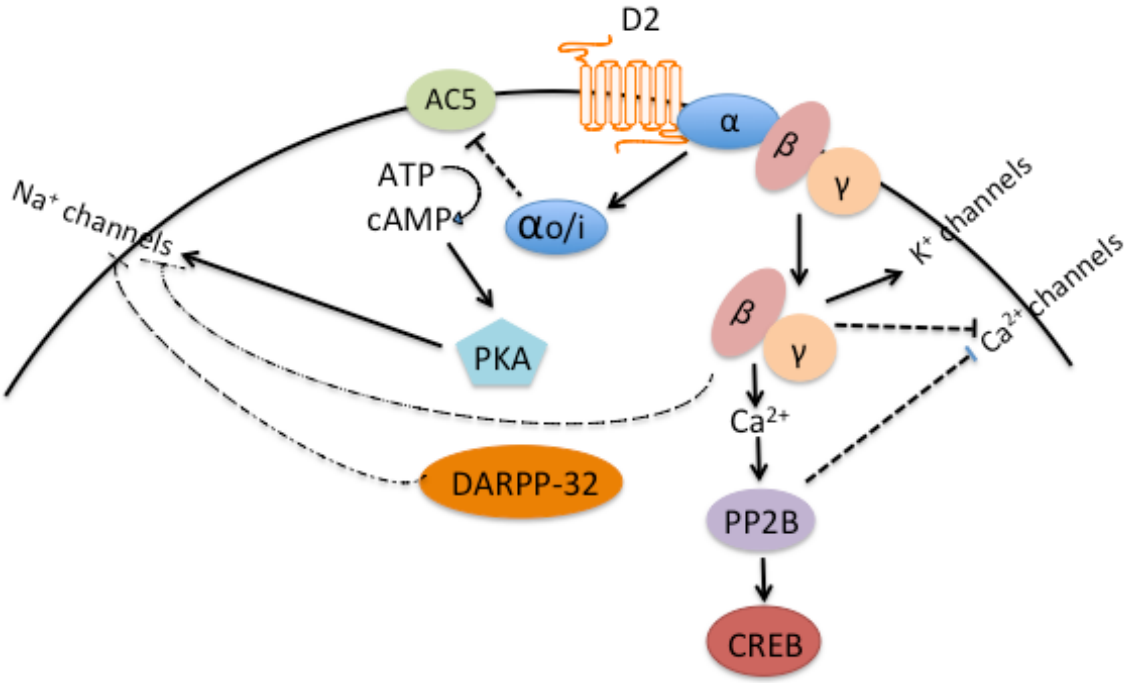


Figure 7: D2 receptor signalling . Solid arrowhead lines indicates stimulatory effects and the dotted lines with a bar end indicates inhibitory effects. AC5, adenylate cyclase type 5; CREB, cyclic AMP response element binding protein; PKA, protein kinase A; DARPP-32, dopamine-related phosphoprotein, 32 kDa; PP2B, protein phosphatase 2B Adapted from (Neve et al., 2004).

1.4.2. Dopamine pathways in the human brain:

Dopamine-producing (dopaminergic) neurons are widely distributed in the human brain and demonstrate complexity in their distribution. In the mid-brain, dopaminergic neurons have trajectories to the dorsal striatum, ventral striatum and the prefrontal cortex. Three classical pathways of these neurons are found in the brain: the nigrostriatal pathway, mesolimbic and mesocortical pathways. The nigrostriatal pathway projects from the substantia nigra pars compacta (SNpc) to the dorsal striatum and regulates motor behaviour (Ungerstedt, 1976). The mesolimbic pathway is involved in motivation and is comprised of neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens, olfactory tubercle and parts of the limbic system. The mesocortical pathway consists of neuronal projections from the VTA to the prefrontal cortex that are required for cognition (Williams and Goldman-Rakic, 1995) (Wang et al., 2004). Altered dopamine neurotransmission is involved both directly and indirectly in many human brain disorders. One key example is the degeneration of the dopaminergic neurons in the SNpc of the mid brain, which causes Parkinson's disease.

The nigrostriatal dopaminergic neurons synapse with the medium spiny neurons (MSNs) to activate complex downstream signalling cascades (Tsui and Isacson, 2011). The SNpc dopaminergic neurons act as autonomous pacemakers and have the ability to generate potentials without synaptic inputs. Progressive degeneration of these neurons causes bradykinesia and rigidity in Parkinson's disease. Dopamine neurotransmission in the striatum is tightly regulated by pre-synaptic mechanisms such as DAT function, which acts as a key regulator of dopamine action. It reuptakes and recycles the synaptic

dopamine, with varying densities of DAT correlating with dopamine functions. The nigrostriatal dopaminergic neurons modulate glutamatergic neuron transmission as well when inputting at the same synapse. Dopamine binding to the post-synaptic D1 receptor increases cAMP levels, which activates cAMP regulated phosphorylation of DARPP-32. DARPP-32 acts as a switch that determines the modulatory effects of dopamine on AMPA and NMDA receptor gating and trafficking. Simultaneous depolarization of MSNs by dopamine and glutamate is required for corticostriatal transmission. In contrast, there is no effect of dopamine when there is no glutamatergic activity. Differential binding of dopamine on the D1 and D2 receptors regulates the surface expression of glutamatergic AMPA and NMDA receptors (Tsui and Isacson, 2011). In an indirect pathway, dopamine binding to the D2 receptors of interneurons lowers the interneuron firing which in turn regulates cholinergic interneurons.

1.5. DOPAMINE SYSTEM IN *C. elegans*

C. elegans has eight dopamine neurons that share similar dopamine synthesis and signalling pathways to that of mammalian systems. Six of these dopaminergic neurons, two pairs of CEPs and a pair of ADEs, are anatomically located in the head region and a fourth pair, the PDEs, are located in the posterior body (**Figure 8)** (Sulston et al., 1975). The *C. elegans* genome also contains homologues for the genes involved in dopamine synthesis, transport and signalling in mammalian systems **(Figure 9).**

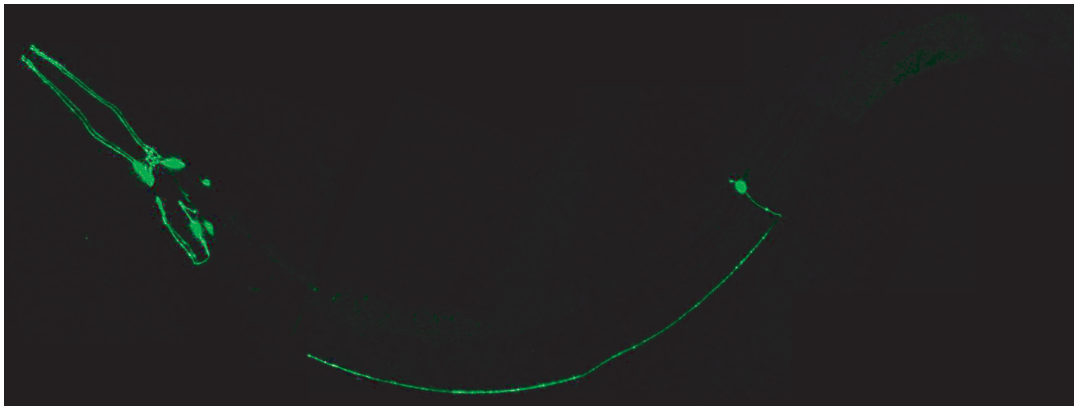


Figure 8.: **Dopaminergic neurons in *C. elegans*.** *dat-1::yfp* expression in dopaminergic neurons. In the head region we see one pair of ADEs and two pairs of CEPs extend their nerve processes towards the anterior part of the head. A pair of PDEs is found in the posterior part of the body.

In *C. elegans, cat-2* encodes a tyrosine hydroxylase. This enzyme catalyzes the conversion of tyrosine to dihydroxyphenylalanine (L-DOPA). CAT-2 shares 50% identity with the mammalian tyrosine hydroxylase (Lints and Emmons, 1999). *bas-1* encodes a homolog of AADC and it has 41% homology to the human dopa decarboxylase (AADC) (Lints and Emmons, 1999). Cytosolic dopamine is packaged into synaptic vesicles by CAT-1, the *C. elegans* homologue of VMAT. CAT-1 shares 47% and 49% identity to the human VMAT1 and VMAT2 respectively (Duerr et al., 1999). *dat-1,* expressed in all of the dopaminergic neurons, encodes for the dopamine transporter DAT-1, which functions in the re-uptake of extracellular dopamine into the presynaptic neuron (dopaminergic neurons) and terminating dopamine signalling. *C. elegans* DAT-1 has 43% shared identity with the mammalian dopamine transporter (Jayanthi et al., 1998).

In *C. elegans*, there are four known dopamine receptors DOP-1, DOP-2, DOP-3 and DOP-4. DOP-1 and DOP-4 are D1 like receptors and DOP-2 and DOP-3 are D2 like receptors (Chase et al., 2004). *C. elegans dop-1* is expressed in the support cells of the head region as well as in the neurons of the head, ventral cord and tail. It is also expressed specifically in the cholinergic motor neurons and PVD neurons (Chase et al., 2004). Additionally, *dop-1* is expressed in the mechanosensory neurons (PLM, PHC and ALM), interneurons (AUA, RIM and RIB)(Sanyal et al., 2004b), touch sensory neurons (AVM and ALM), ALN, PLN and interneuron PVQ which extends axons along the ventral nerve cord (Tsalik et al., 2003). *dop-2* is expressed in the head, ventral cord and tail neurons. Specific neurons include RIA, SIA, SIB, PDA (Tsalik et al., 2003) (Suo et al., 2009), and PVD (Suo et al., 2003).

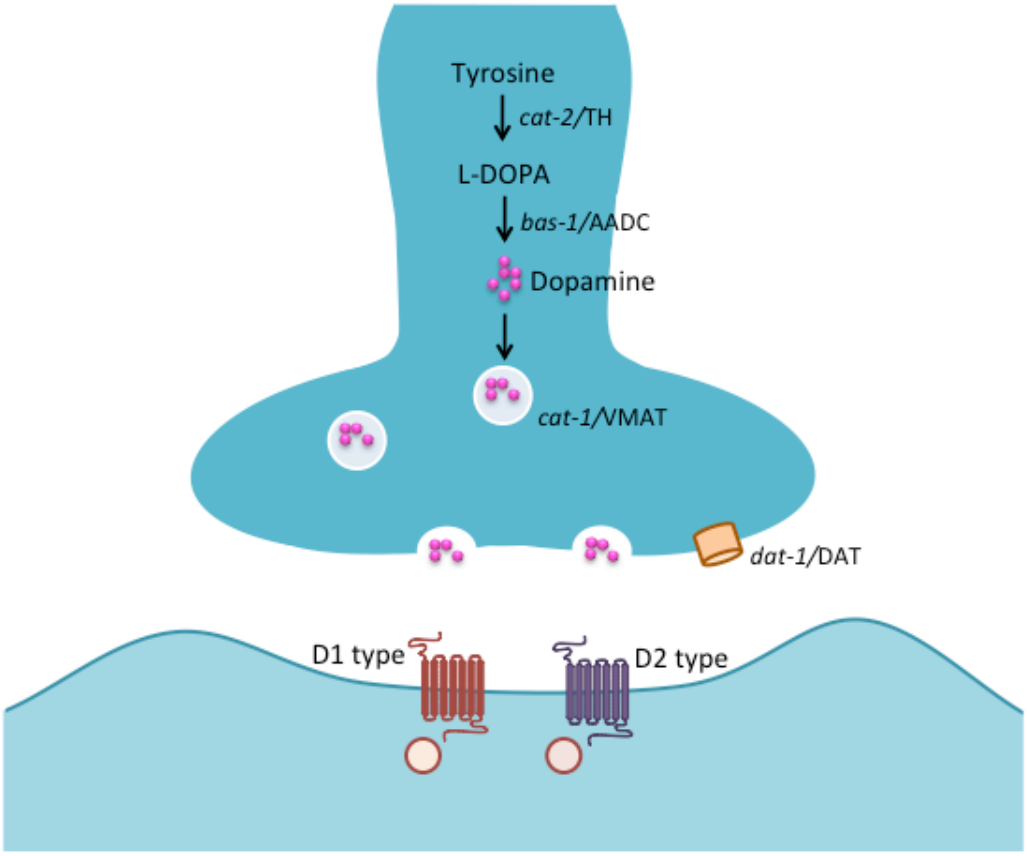


Figure 9: **Dopamine signalling in *C. elegans***. TH- tyrosine hydroxylase, AADC- aromatic amino acid decarboxylase, VMAT- vesicular monoamine transporter, DAT- dopamine transporter.

The *dop-3* receptor is expressed strongly in the GABAaergic neurons and weakly in the cholinergic neurons (Chase et al., 2004). The SIA and RIC neurons also express *dop-3* (Suo et al., 2009) as well as the ASE neurons (Etchberger et al., 2007). *dop-4* is expressed in I1 and I2 (pharyngeal), ASG, AVL, CAN and PQR neurons (Sugiura et al., 2005a). It is also expressed in other non-neuronal cells such as the vulva, intestine, rectal glands and epithelial cells. DOP-4 is reported to be an invertebrate specific D1 like receptor, sharing 42% sequence similarity in the transmembrane region to that of *Drosophila* (Sugiura et al., 2005a).

In *C. elegans,* there are 21 G_{α}, 2 G_{β} and 2 G_{γ} encoding genes (Jansen et al., 1999)(Cuppen et al., 2003). The D2 like receptor DOP-3 couples with GOA-1, a $G\alpha_{i/o}$ protein (Chase et al., 2004) and DOP-2 interacts with GPA-14, a $G\alpha_{i/o}$ subunit (Pandey and Harbinder, 2012). Further study is required to determine the G-protein that interacts with the D1 like receptors.

1.5.1. Dopamine modulation of behaviors:

i) Egg laying: Besides serotonin, dopamine also plays an important role in modulating the *C. elegans* egg laying behaviour. Schaffer and Kenyon reported that excess dopamine inhibits egg laying behaviour (Schafer and Kenyon, 1995a). Animals exposed to a high concentration of exogenous dopamine (3mM) demonstrate a decrease in egg laying and immediate paralysis, but recover after several hours, even with the continuous presence of the neurotransmitter in its environment. Once the animal has recovered, further increases in dopamine concentration do not cause inhibition of egg-laying, demonstrating an adaptation of behaviour in the presence of dopamine. Further

study has shown that dopamine is required for the activation of voltage gated Ca^{2+} channels, which are required for modulating this behaviour. Moreover, dopamine inhibits serotonin mediated egg-laying via MOD-1, an ionotropic serotonin receptor (Dempsey et al., 2005).

ii) Defecation: Defecation in *C. elegans* is a motor program, which starts with the constriction of posterior body wall muscles that builds internal pressure and squeezes the intestinal content anteriorly. Dopamine is also known to be involved in the regulation of defecation by enhancing *egl-2* encoded K^+ channel activity (Weinshenker et al., 1999).

iii) Locomotion: In a mechanism similar to egg laying, treatment of wild type worms with external dopamine can cause reversible paralysis. *unc-2* encodes for a voltage gated Ca^{2+} channel and is involved in mediating this behaviour. *unc-2* is abundantly expressed in the motor neurons, resonating its function in locomotion (Schafer and Kenyon, 1995a). Dopamine allows changes in *C. elegans* behaviour in response to the changing environment. Most of these changes in many behaviors are accomplished by the modulation of locomotion. One such behaviour is in encountering and searching for food. Well fed *C. elegans* reduce their locomotion rate when encountering a bacterial lawn, and this behaviour is called the 'basal slowing response'. This behaviour is mediated by the dopamine neurons and is considered to be due to the mechanosensory function of dopamine neurons (Sawin et al., 2000). When finding a new food source after exhausting current supplies, *C. elegans* looks at the immediate area before going to distant sources, and this behaviour is know as 'restricted area search'. This behaviour is dopamine

dependent and characterized by short angled turns during its search in the immediate area and wide angled turns during distant searches. Furthermore, dopamine modulates habituation. For instance, wild type worms respond to non-localized mechanical stimuli such as tapping the plate by exhibiting reversal turns. However, worms reduce the reversal turn response during continuous tapping, demonstrating habituation to the stimuli. Dopamine deficient mutant *cat-2* and receptor mutant *dop-1* exhibit faster habituation, highlighting the role of dopamine in this response (Sanyal et al., 2004b).

iv) Electrotaxis: My work, described in Chapter 3.1. , has provided evidence for the role of dopamine signalling in modulating electrotaxis behaviour (Salam et al., 2013). In this study, a combination of neurotoxins and dopamine pathway mutants were used to demonstrate that dopamine neurons and dopamine signalling are involved in regulating the directed movement of worms towards a cathode (when exposed to a DC electric field). In Chapter 3.2 and 3.3, I have further characterized this behaviour and demonstrated the role of the dopamine receptors in this behaviour.

1.6. DOPAMINE NEURODEGENERATIVE DISEASE:

The important role of dopamine in humans and other eukaryotes is highlighted by the association of several diseases with disruption of the dopamine system. In humans, dysfunction of the dopamine system in the CNS causes mental disorders such as schizophrenia and psychosis. Additionally, degeneration of dopamine neurons in the SNca causes Parkinson's disease (PD), which is the second most prevalent neurodegenerative disorder after Alzheimer's disease.

Parkinson's disease is prevalent in the elderly (aged 65 years and above) although there are rare occurrences in the younger population under 40. Nearly 100,000 people in Canada are living with Parkinson's disease (Canada, 2007), one million Americans suffer from the disorder and an estimate of 7 to 10 million people are considered affected with Parkinson's disease worldwide (Foundation, n.d.). The potent risk factors associated with Parkinson's disease are age, genetics and environmental toxin exposure. The hereditary presence of Parkinson's disease causing genes results in the familial form of the disease. The genetic cause of familial Parkinson's disease was first reported in 1997 with the discovery of PARK1/α-syn (Spillantini et al., 1997). Since then, several additional genes have been discovered which are linked to Parkinson's disease. Exposure to pesticides and heavy metals (eg. copper, manganese and lead) also causes Parkinson's disease-like symptoms due to damage caused to the dopaminergic neurons (Jankovic, 2008). Parkinson's disease patients suffer from motor symptoms and non-motor symptoms including cognitive dysfunction, dementia and mood disorders.

Parkinson's disease is pathologically defined as the loss or degeneration of dopaminergic neurons in the SNca and a hallmark of the disease is the development of Lewy bodies in dopaminergic neurons. Lewy Bodies are the abnormal intracellular aggregates of various proteins including alpha-synuclein and ubiquitin that impairs the normal functioning of neurons. Mutations in the genes Parkin, PINK1, DJ1, ATP13A2, LRRK2 and SYNUCLEIN are linked to Parkinson's disease (Nass et al., 2008). Exposure to environmental toxins such as pesticides, (eg. methamphetamine) and stress increases inflammation in the brain which causes cellular senescence of the dopaminergic neurons

over time. In addition, impaired protein degradation system like the ubiquitin-proteasome and mitochondrial dysfunction also results in neuronal degeneration (McNaught et al., 2001).

Parkinson's disease represents a major clinical challenge, as there is no cure or effective treatment for the disease that works in the majority of Parkinson's disease patients. Knowledge of the disease pathogenesis is required to uncover some of the many "unknowns" associated with Parkinson's disease, and much further study is necessary. Due to the complexity of the human brain and ethical issues associated with experimenting on human subjects, several animal models have been developed to study Parkinson's disease more effectively (**Table 1 and 2**).

Table 1: Mammalian Parkinson's Disease Model

Toxin Models		**Animals**	**Phenotypes**
MPTP		Primates, mice and rats (resistant in rats)	Selective lesion of SNca neurons
Paraquat		Mice	Loss of striatal dopaminergic neuron nerve fiber
Rotenone		Rats	Nigrostriatal neurodegeneration
6-OHDA		Widely use	90-100% damage of SNca neuron
Lipopolysaccharide		Rats	Nigrostriatal neurodegeneration
Genetics Models			
α-synuclein	A30P A53T E46K	Mice	Non-motor symptoms
LRRK2	R1441G	Mice	Progressive motor deficit
	G2019S	Mice	Reduction in striatal dopamine content, uptake and release
PINK1	Knock out	Mice	Increased susceptibility to ROS
	G309D	Mice	Reduction of mitochondrial pathogenesis
PARKIN	Q311X	Mice	Progressive motor deficits, Nigrostriatal neurodegeneration
DJ1	Knock out	Mice	Sensitive to toxins and oxidative stress
α-synuclein	A53T A30P	Rats	No motor impairment, olfactory loss
LRRK2	G21019S	Rats	Impair dopamine reuptake by dopamine transporter

Table 2: Invertebrate Parkinson's Disease Model (Harrington et al., 2010)(Muñoz-Soriano and Paricio, 2011)

Toxin Models	**Animals**	**Phenotypes**
MPTP	*C. elegans*	Dopaminergic neuron degeneration, mobility defects
Paraquat	*C. elegans*	Oxidative stress
	D. melanogaster	Dopaminergic neuron degeneration, movement defects
Rotenone	*C. elegans*	Mitochondrial stress
	D. melanogaster	Dopaminergic neuron degeneration, motor deficits
6-OHDA	*C. elegans*	Dopaminergic neuron degeneration
Manganese	*C. elegans*	Oxidative stress
Genetics Models		
α-synuclein	*C. elegans*, *D. melanogaster*	Dopaminergic neuron degeneration, reduced movement
LRRK2	*C. elegans*	Mitochondrial stress
PINK1 and PARKIN	*C. elegans*	Hypersensitivity towards proteotoxic stress
	D. melanogaster	Dopaminergic neuron loss, motor deficits

1.7. *C. elegans* AS A PARKINSON'S DISEASE MODEL TO STUDY NEURONAL DEGENERATION AND DOPAMINE MEDIATED MOVEMENT BEHAVIOUR

Mammalian Parkinson's disease models are invaluable in understanding the basis of Parkinson's disease, however they do not fully reiterate the disease's pathological impairments. Additionally, these models are expensive to maintain, difficult to manipulate in the laboratory, and time consuming due to their slow growth (long life cycle). In this regard, the invertebrate models such as *Drosophila melanogaster* and *C. elegans* are used for genetic and drug screens to study Parkinson's disease.

C. elegans presents a particularly powerful Parkinson's disease model as it shares conserved cellular and molecular mechanisms in addition to fewer dopaminergic neurons and conserved dopamine pathways with mammals. Furthermore, the *C. elegans* genome has almost all of the conserved Parkinson's disease-associated genes (**TABLE 3**). This has led to the development of genetic and toxin induced *C. elegans* Parkinson's disease models to be used to gain a molecular understanding of Parkinson's disease pathogenesis (Nass et al., 2001)(Nass et al., 2008).

Dysfunction of protein degradation machinery such as the ubiquitin-proteasome system is a major culprit that underlies the neurodegenerative mechanisms leading to Parkinson's disease symptoms. *C. elegans* PDR-1 interacts with worm E2 and E3 enzymes (UBC-2, UBC-18, UBC-15 and CHN-1) which is consistent with findings in mammalian systems (Springer et al., 2005). Additionally, the *pdr-1* knock-out increases susceptibility to mitochondrial complex I inhibitors including rotenone (Ved et al., 2005). TOR-2 is a worm ortholog of torsinA, a chaperone like activity protein and *catp-6* is

orthologous to ATP13A2. Co-expression of *α-syn* and TOR-2 ameliorated *α-syn* misfolding. RNAi knock-down of *catp-6* in the *α-syn* + TOR-2 background enhanced *α-syn* misfolding (Hamamichi et al., 2008). Interestingly, this interaction was found in *in* vitro in mouse dopaminergic neuron cell culture as well. Overexpression of wildtype and mutant (G2019S) LRRK2 in worms increases dopaminergic neuron neurodegeneration and reduces dopamine levels, as well, G2019S enhances vulnerability to rotenone (Saha et al., 2009). The rotenone toxicity result was consistent with RNAi knock-down of *lrk-1* (Saha et al., 2009). This result shows the similarity in function of LRRK2 to *lrk-1* of *C. elegans* and adds to the using of *C. elegans lrk-1* model for the study of neurodegenerative disease.

Table 3: *C. elegans* orthologs of Parkinson's disease (PD) genes (Harrington et al., 2010**)**.

Parkinson's disease gene	Parkinson's disease protein	*C. elegans* orthologs	E value
PARK1	α-syn/SNCA	No ortholog	n/a
PARK2	PRKN/parkin	*pdr-1*	3.4e-38
PARK5	UCHL-1	*ubh-1*	1.2e-33
PARK6	PINL1	*pink-1*	7.8e-53
PARK7	DJ-1	*djr-1.1* *djr-1.2*	1.6e-45 8.9e-36
PARK8	LRRK2	*lrk-1*	5.5e-180
PARK9	ATP13A2	*catp-6*	2.5e-180
PARK11	GIGYF2	n/a	n/a
PARK13	HTRA2	n/a	n/a

Environmental factors are major contributors to non-familial Parkinson's disease. Therefore, some of the known environmental toxins that causes Parkinson's disease can be used to induce parkinsonian symptoms. MPTP (1-methyl-4-phenylpyridinium) metabolizes to MPP^+ which is a neurotoxin causing dopaminergic neuron specific degeneration. MPP^+ is transported to the dopaminergic neurons via the monoamine transporters and acts as an inhibitor of the mitochondrial enzyme complex I of the respiratory chain. MPTP induced dopaminergic neuron degeneration models have also been shown to increase lethality and reduce mobility in worms and the MPP^+ Parkinson's disease model has been used for drug screening looking for anti-Parkinson's disease drugs (Braungart et al., 2004a). 6-hydroxy dopamine (6-OHDA) is an endogenous compound and analogue of dopamine found in the brain and urine samples of Parkinson's disease patients (Andrew et al., 1993). This compound is transported across the membrane through DAT-1 and noradrenergic transporters and causes monoaminergic neuron cell death. Exposing *C. elegans* to 6-OHDA induces dopaminergic neuron specific degeneration (Nass et al., 2002), and *dat-1* is required for this toxicity (Nass et al., 2005). The 6-OHDA toxicity model was used to confirm the protective effect of TOR-2 (human torsinA-related protein) and to identify dopamine, GABA and NMDA receptor agonists which are neuroprotective in *C. elegans* (Cao et al., 2005)(Marvanova and Nichols, 2007a). Rotenone and paraquat are pesticides shown to be linked to Parkinson's disease (Tanner et al., 2011). They are known to affect the mitochondrial enzyme complex. These compounds cause phenotypes such as degeneration of dopamine neurons, increase oxidative stress and reduced mobility in worms, thus they add to using *C. elegans* as a

Parkinson's disease model(Ved et al., 2005)(Marvanova and Nichols, 2007b) (Nass et al., 2002).

1.8. INVESTIGATION OF DOPAMINE SIGNALLING IN THE ELECTROTAXIS BEHAVIOUR OF *C. elegans*:

Our findings demonstrate the role of dopamine modulation on the electrotactic swimming response in *C. elegans*. An appropriate dopamine level is required for normal electrotactic swimming and longer exposures of *C. elegans* to the electric field induces slow electrotactic swimming speed, regulated by dopamine. In all these studies, we employed a novel microfluidic platform that facilitates the on-demand control of worm movement using an electric field (**Figure 10**) (See Chapter 2 for the device set up).

Microfluidic technology has emerged significantly with applications in biomedical research during the last decade. It's uses have been demonstrated in drug screening, diagnostics and behavioral studies. Microfluidic applications involve the fabrication of microstructure devices that can manipulate a minute amount of fluid in a fast and accurate manner. Microfluidic devices are usually made of PDMS (polydimethylsiloxane), a polymer that is optically transparent, non-toxic and insoluble in aqueous solutions. These properties of PDMS make the microfluidics device suitable for biomedical research. Additionally, fabrication of these devices is easy, quick and inexpensive. Because of these advantages offered by microfluidics, the technology has been embraced as a platform to study cells and small organisms such as zebrafish (Wielhouwer et al., 2011), *Drosophila* (Lucchetta Elena M, Lee Ji Hwan, Fu Lydia A, Patel Nipam H, 2005) and *C. elegans* (Ben-Yakar et al., 2009)(Chung et al., 2008) for whole animal studies.

Microfluidic technology has significantly contributed to *C. elegans* research in the development of a platform for easy worm handling and manipulation for behavioral studies, nerve regeneration, imaging and screening. It also allows for precise control over flow and chemical stimuli delivery in chemotaxis studies (Chokshi et al., 2011)(Chronis et al., 2007). Furthermore, this technology allows for the immobilization of worms without anesthesia or glue, thus making it possible to observe and recover worm samples without any effects from these chemicals.

The novel microfluidic electrotaxis system used in this thesis, developed by our lab in collaboration with Dr. Selvaganapathy (McMaster Mechanical Engineering) (Rezai et al., 2010), offers many advantages in studying the role of dopamine signalling in electrotaxis behaviour. By keeping the electric field lines in the micro-environment parallel, the system ensures that worms travel in a straight line. This makes it possible to calculate the speed and other movement parameters, thereby enabling a comparison of mutant and wildtype phenotypes. The following sections summarize the objectives, rationale and key findings of this thesis.

1.9. OBJECTIVES:

The overall objective of this thesis is to investigate the role of dopamine in electrotaxis behaviour in *C. elegans* using neurotoxins, mutants and pharmacological approaches involving receptor agonists and antagonists.

1. To examine the electrotaxis behaviour of neurotoxin treated animals using the electrotaxis assay.

2. To investigate the electrotaxis behaviour of dopamine synthesis and signalling pathway mutants.

3. To characterize the dopamine modulation of electrotaxis behaviour by performing prolonged electric field exposures on dopamine mutants as well as pharmacologically treated wild type animals.

1.11. RATIONALE:

As described above, electrotaxis behaviour is generated when the precise sensory input in response to a DC electric field is converted into a defined movement output. Although amphid neurons are known to be involved in sensing the electric field, many of the fundamental questions regarding electrotaxis behaviour are unanswered. How is the undulating movement during electrotaxis being driven from the sensory neurons to the motor neurons? Is there a time varying pattern to the sensory system? How does dopamine signalling modulate electrotaxis? To answer these questions, the sensory motor map needs to be understood in detail. Dopamine is a well-established neuromodulator, which is involved in locomotion. *C. elegans* dopamine neurons also have mechanosensory functions. While this neurotransmitter is known to modulate gustatory plasticity in chemotaxis behaviour, the role of dopamine in mediating electrotaxis behaviour has not been studied. Thus one hypothesis that I addressed initially was, "**dopamine signalling modulates the electrotaxis behaviour of *C. elegans***". Subsequently, I addressed another hypothesis in this thesis "**dopamine synthesis and transport genes are required to maintain normal electrotaxis movement of *C. elegans***". To this end, a custom designed microfluidic device has been used in order to

facilitate the movement of the worm inside the channel and allow for the investigation of dopaminergic neuron function as well as the genes involved in dopamine signalling that modulate electrotaxis behaviour.

1.12. KEY FINDINGS:

Work in the Gupta lab prior to starting my thesis had shown that *C. elegans* swim with a sinusoidal pattern inside the channel in response to an applied external electric field (Rezai et al., 2010). I have made major contributions to characterizing the electrotaxis behaviour and its regulation by the dopaminergic neurons.

My work has demonstrated that the movement of worms in the microchannel device can be reliably analyzed in young adults in terms of speed, frequency, amplitude of turns and the angle of body bends.

By analyzing the electrotaxis responses of animals, I have conclusively shown that the microfluidic setup can be used to detect the differences between normal (healthy) and neuron defective worms.

By analyzing phenotypes of dopamine pathway mutants and animals treated with Parkinson's disease-causing neurotoxins, I have provided the first evidence of dopamine's involvement in mediating the electrotaxis behaviour of worms.

Subsequently to demonstrate the role of dopamine signalling, I have characterized the electrotaxis behaviour of dopamine synthesis and signalling pathway mutants using the microfluidics channel assay.

Finally, using a combination of exogenous dopamine, receptor mutants, and pharmacological agents, I have shown that D2 receptors play an important role in the electrotaxis behaviour of *C. elegans*.

2. METHODS AND MATERIALS

2.1. *C. elegans* CULTURES

Worms were grown at 20^0C on standard NGM-agar plates containing *E. coli* OP50 culture as previously described (Brenner, 1974). Most of the mutants were obtained from *Caenorhabditis elegans* Genetic centre (CGC), University of Minnesota. N2 (Wild type), CB1112 *cat-2(e1112),* PR813 *osm-5(p813),* PS2821 *lin-11(n389),* CB1489 *him-8(e1489)*, DY328 *dat-1$_p$::YFP (bhEx120)*, MT7988 *bas-1(ad446),* DY442 cat-1(ok411), CB1141 *cat-4(e1141),* RM2702 *dat-1(ok157),* LX645 *dop-1(vs100),* LX702 *dop-2(vs105),* LX703 *dop-3(vs106),* FG58 *dop-4(tm1392),* LX706 *dop-2(vs105); dop-3(vs106)*. Strain generated as part of this study are DY433 *dat-1(ok157); dop-3(vs106)*, DY443 *dat-1(ok157); dop-4(tm1392)* and DY434 *dop-1(vs100) dop-4(tm1392)*. Worm were grown at 20 °C on NGM plates seeded with OP50. Synchronized worms were prepared by bleaching gravid hermaphrodites and allowing L1 larvae to hatch in M9 buffer. VM6365 *dat-1::ICE* was a gift from Dr. Andres Villu Maricq's lab (Hills et al., 2004a). In *dat-1::ICE* animals, dopamine neurons undergo cell death at an early stage due to the expression of interleukin-1 beta converting enzyme.

Synchronized worms were used for all the assays and were prepared by hypochlorite treatment (Rezai et al., 2010). Briefly, gravid hermaphrodites were treated with a solution containing commercial bleach and 4N NaOH (3:2 ratio). The dead worms were washed with M9 buffer and incubated at room temperature for 24 hours to allow fertilized embryos to hatch into L1 larvae.

Except for *lin-11(n389)* animals all other plate-based and electrotaxis assays were done with 69 hours young adults. This stage was chosen based on our finding that almost all synchronized wildtype L1s, when placed on NG-agar plates, reach adulthood by 69 hours at 20^0C (97% adult and remaining younger stages, n = 1002). The *lin-11* mutants were tested at 40 hours post L1 stage because of their egg laying-defective (Egl) phenotype (Trent et al., 1983).

2.2. MOLECULAR BIOLOGY AND TRANSGENICS

The *dat-1*$_p$*::YFP* plasmid pGLC72 was made by amplifying a 710 bp fragment of *dat-1* 5' genomic region using primers GL563 (5'-AGGAAGCTTCCAGTTTTCACTAAAACGA CCTCATACACTTCTC-3') and GL564 (5'-ATGGGTACCGGCACCAACTGCATGG CTAAAAATTGTTGAG-3'). The resulting PCR product was digested with *Hind*III and *Kpn*I and subcloned into pPD136.64 (Fire lab vector, www.addgene.com). pGLC72 was injected into *unc-119(ed4)* animals to generate stable transgenic lines.

2.3. ELECTROTAXIS ASSAY

The electrotaxis assay is performed using a microfluidics device set up (Rezai et al., 2010). The set up consist of a microfluidics channel with electrodes which can be connected to the external electric field (**Figure 10**). Movement of worms inside the channel is directed by the applied electric field. The microfluidic channel provides an environment in which the electric field streamlines are confined in the axial direction of the channel. This results in a uniform stable field that stimulates worms to move along

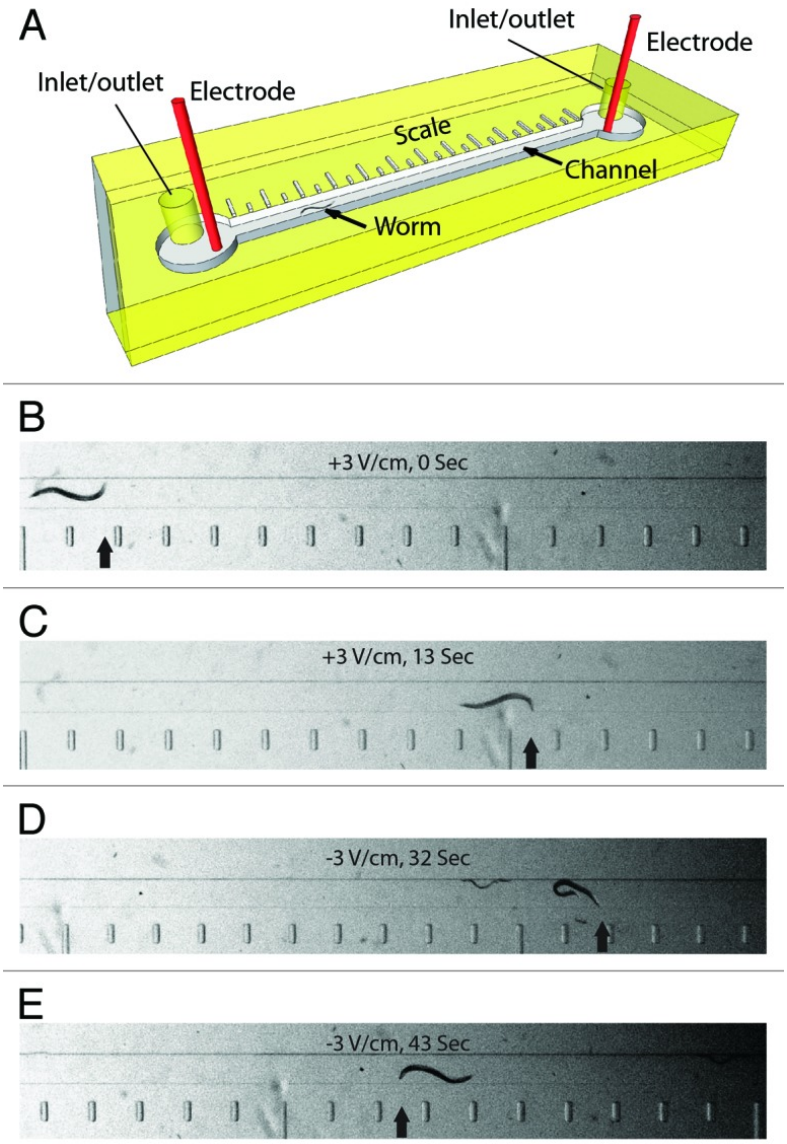


Figure 10. Microfluidic electrotaxis assay setup. (A) A detailed view of the microfluidic device. Worms are loaded and removed through inlet/outlet tubes. Electrotaxis is performed in the channel (a worm is shown). The scale along the length of the channel is used to determine the speed. (B-E) Snapshots of a worm in the channel during electrotaxis. Scale bar is visible on the bottom. The electric field voltage and time are shown in each panel. The head of the worm is marked by an arrow. Reversal of the electric field polarity (in D and E) causes the worm to switch its direction of motion.

the channel length. Furthermore, the narrow diameter (300 μm) ensures motion in a near straight line fashion. Changing the direction of the electric field causes the worm to stop transiently, turn towards the cathode, and resume motion (**Figure 1D, E**). Therefore, any deviation from these characteristics can be considered as an abnormality. As described in Materials and Methods we manually examined videos and characterized the electrotaxis behaviour using four parameters, electrotactic swimming speed, turn time, body bend frequency and Electrotaxis Time Index (ETI).

2.4. ELECTROTAXIS BEHAVIOUR DATA ANALYSIS

Four swimming parameters, electrotactic swimming speed, turn time, body bend frequency and electrotaxis time index (ETI), were quantified. The electrotactic swimming speed is an average response of animals that was obtained by dividing the total swimming distance by elapsed time. In this analysis only movement towards cathode was used to calculate the distance. Any motion towards the anode was ignored. The elapsed time is the duration of the assay. The turn time is the period to complete a U-turn following a switch in the electric field polarity. Up to 3 turning events were used to determine the average response of each animal. The body bend frequency is the average number of sine waves per second. This was obtained by dividing the total number of sine waves produced by an animal in an experiment by the duration of the assay (in seconds). Only those sine waves were counted that spanned at least half of the channel diameter. Finally, ETI is the percentage of time spent in swimming towards cathode. Thus, for a given genotype a lower ETI indicates less cathode-directed motion of animals whereas higher ETI indicates greater amount of time spent in moving towards cathode.

2.5. TOXIN TREATMENTS AND OPTIMIZATIONS

All toxin treatments were done with synchronized L1 populations. Worms were exposed to toxins for different time periods with mild shaking on a rocking platform. Following exposure, tubes were briefly centrifuged and worm pellets were washed once with M9 buffer. Worms were transferred to NG-agar culture plates. Desired concentrations of 6-OHDA (100 μM) (Sigma Aldrich, 162957), MPTP (700 μM) (Toronto Research Chemicals, M325913), and rotenone (25 μM) (Sigma Aldrich, R8875) were prepared in M9 one day before the assay and stored at -20^{0}C. The 6-OHDA solution is sensitive to light therefore it was kept in the dark.

We modified toxin exposure protocols by lowering the concentration and exposure time. This was necessary because plate-based assays in the past used high doses of chemicals resulting in pleotropic defects such as delayed growth and lethality(Nass et al., 2002)(Braungart et al., 2004)(Marvanova and Nichols, 2007a). This precluded us from carrying out electrotaxis experiments. Additionally, we were concerned about non-specific effects due to prolonged exposures to high doses of chemicals that could affect neurons other than those involved in dopamine signalling. The cuticle thickness varies between young larvae and adults (Cox et al., 1981), which may also be a factor affecting the sensitivity of animals to neurotoxins. Therefore, exposure conditions needed to be optimized.

For example, L1 animals treated with 5 mM 6-OHDA for 30 minutes (one of the lowest concentrations reported) showed extreme sluggishness, uncoordinated (Unc) movement, protruding vulva, growth arrest and early larval lethality (Marvanova and

Nichols, 2007a). We were unable to examine such worms in the channel because they were practically immobile and unresponsive to the electric field stimulus. Lowering the 6-OHDA concentration by 50-fold (100 μM) improved the overall health of worms thereby allowing us to carry out electrotaxis assays.

We also optimized the MPTP and rotenone treatment protocols. It was earlier reported that worms grown in the presence of 1.4 mM MPTP for 3 days (starting L1 larval stage) were uncoordinated and extremely slow growing (Braungart et al., 2004b). In the case of rotenone a dose of 25 μM for 4 days caused lethality (Ved et al., 2005). We found that reducing the rotenone exposure (25 μM rotenone for 12 hours) allowed animals to survive but affected their growth and movement. However, none of the above toxin conditions could be used in the channel assay since worms were too sick to move and did not respond to the electric field stimulus. We further modified the toxin treatment conditions and found that animals exposed to 700 μM MPTP or 25 μM rotenone up to 8 hours were generally healthy on plates and could be used to perform electrotaxis experiments.

2.6. NEUROPROTECTION ASSAY

The 10 mM stock solution of acetaminophen (Sigma Aldrich, A7085) was prepared fresh and diluted to a final concentration of 100 μM at the time of the assay. Synchronized L1 stage animals were incubated for 24 hours in 100 μM drug containing M9 buffer. They were wash once with M9 and placed in toxin-containing solution for 1 hr (100 μM 6-OHDA) or 8 hours (700 μM MPTP and 25 μM rotenone). After an additional

wash the animals were transferred to NG-agar plates. The electrotaxis assays were performed on 69 hours adults.

2.7. MICROSCOPY

Worms were mounted on glass slides containing agar pads and observed using Zeiss AxioImager D1 and Nikon Eclipse Nomarski fluorescence microscopes. Epifluorescence was visualized using a GFP filter (HQ485LP, Chroma Technology USA). Degeneration of dopamine neurons was examined in 6-OHDA-treated *dat-1*$_p$*::YFP* transgenic worms. For this, L1 worms were exposed to 6-OHDA for 4 hours, washed once with M9 and then plated on NG agar plates. Adults were examined for YFP fluorescence in dopaminergic neurons and their processes.

2.8. PLATE-BASED ASSAYS

Well-fed synchronized worms (described above) were used to quantify their movement responses on agar plates. The stages were 69 hours (post-L1) young adult for *osm-5, dat-1* and *him-8* and 40 hours (post-L1) larvae for *lin-11*. The corresponding stages of N2 worms were used as controls. Worms were placed, one at a time, on a fresh one-day old thin bacterial lawn. Following acclimatization for 5 min. the animal's movement was observed for 30 sec. The beginning and end positions of tracks were marked and the image was captured using a digital camera (Point Grey, FL3-GE-13S2C) attached to Leica S8APO microscope. The movement speed was calculated by NIH ImageJ software (http://rsbweb.nih.gov/ij/).

Chemotaxis assay was performed as described earlier(Rezai et al., 2012)(Hart, 2006). NaCl 100 mM solution was used as a chemo-attractant. 6-OHDA-treated animals

were washed thoroughly and placed at the center of the agar plate. The plate contained a drop of NaCl at one end and water control at the other end. The assay was run for an hour. After this, animals towards water and NaCl spots were counted and chemotaxis index (CI) was calculated.

2.9. TEN MINUTES ELECTROTAXIS ASSAY

For 10 minute long electrotaxis assays, individual worms were exposed to continuous electric field for 10 minutes. Animals were kept in the channel by reversing the direction of the electric field. The first and 10th minute of the captured videos were analyzed for comparison.

2.10. STATISTICAL ANALYSIS

The raw data/speed of the control and mutants animals were normalized by dividing by the median speed of the respective control. The normalized data was used to perform Mann-Whitney U test. 10 minute electrotaxis were analyzed using t-test. HPLC data was statistically analyzed using a one way ANOVA.

2.11. TEMPERATURE MEASUREMENT INSIDE MICROCHANNEL

The micro channel was filled with M9 solution. Thermocouple is embedded in PDMS device in such a way that tip of thermocouple is in contact with M9 solution filled in micro channel. 15 volts applied across the electrodes inserted at the ends of microchannel, Current observed during the process ~ 14 μAmps. Voltage applied for the duration of 12 minutes for all readings. In order to observe consistency in increase in temperature, process started at three different temperatures 21.4, 22.1 and 22.2 °C.

2.12. DOPAMINE TREATMENT

Worms were treated with 1mM dopamine six hour before the electrotaxis assay and during the assay as described earlier (Sanyal et al., 2004b). Fresh dopamine solutions were made in M9 added onto seeded NGM agar plates and allowed to dry. Worms samples were added after the agar completely absorbed the dopamine solution and kept it in the 20 degree.

2.13. AGONIST AND ANTAGONIST TREATMENT

(±)-SKF38393 hydrochloride and haloperidol were obtained from Sigma-Aldrich. Haloperidol solution was prepared by dissolving in 1% ascorbic acid (Kimura et al., 2010) and SKF38393 in water. Drug solutions were added on the seeded NGM plates and allow them to dry. Worm samples were transferred to the drug plates and fed for 5 hours before the electrotactic swimming assay.

3. RESULTS

3.1. ROLE OF DOPAMINERGIC NEURONS IN ELECTROTACTIC SWIMMING BEHAVIOUR:

Preface:

As described in **Chapter 1,** *C. elegans* demonstrates a robust electrosensory response when placed in a DC field environment (Sukul N.C., 1978). Although the significance of this behaviour is currently unknown, it was proposed earlier that in the case of plant and insect parasitic nematodes, electrosensory response may facilitate host finding (Bird, 1959)(Shapiro-Ilan et al., 2009a). Recent work on the ecology of *Caenorhbaditis* nematodes has shown that *C. elegans* is associated with plants, insects and many other animals (Kiontke and Sudhaus, 2006). Whether *C. elegans* relies on electrotaxis to find such diverse hosts in the wild remains to be determined.

Previous work showed that amphid neurons play an important role in mediating the electrosensory response in *C. elegans* (Gabel et al., 2007). In this study we examined the electrotactic swimming behaviour of worms using mutants, neurotoxins as well as genetic ablation approaches affecting different neuronal subtypes. These different but complementary methods uncovered the roles of amphid sensory neurons as well as dopaminergic neurons in sensing the electric field stimulus. These experiments were performed using a novel custom-made microfluidic channel device (**Figure 10**) that precisely controls the orientation and strength of the electric field. The small diameter of

the channel (300μm) ensures that the worm always travels in a straight line, making it possible to quantify movement parameters.

The neurotoxins used in this study include 6-OHDA, MPTP, and rotenone (a pesticide). These toxins cause the degeneration of dopaminergic neurons similar to that shown in mammalian models (Nass et al., 2002)(Ved et al., 2005)(Braungart et al., 2004). We found that toxin-treated worms displayed abnormal swimming behaviour. A comparison with plate-based behavioral assays was also carried out, which demonstrated the sensitivity of the microfluidic-based electrotaxis assay in detecting movement defects. Lastly, we investigated the effect of an analgesic, acetaminophen, on dopaminergic neurons and found that it suppresses toxin-induced damage.

3.1.1. Direct current (DC) electric fields induce the directed movement of worms mediated by sensory neurons:

Our group (Rezai et al., 2010) has shown earlier that the presence of a DC electric field inside a microfluidic channel device induces directed movement in wildtype *C. elegans*. The channel provides a uniform streamline electric field that directs the movement of the worm along the channel. Changing the polarity of the applied electric field causes the worm to stop transiently and reorient itself towards the cathode. Any alteration in the movement pattern, such as speed, body bend frequency, turn time and electrotaxis time index (ETI) can be characterized. Initially, we investigated the behaviour of wildtype N2 worms in the microfluidics channel in the absence of an electric field. The animals showed random swimming and turning activities with a mean body bend frequency of 1.3 Hz (n=6). Due to multiple reversals they failed to cover long

distances in any one direction. Thus, the electrical stimulus is required to propel worms in a directed manner.

The electrotactic swimming of *C. elegans* in the channel depends on its intact neuronal and muscular systems (Rezai et al., 2010). Consequently, defects in any of these components could alter the swimming behaviour. While the electrosensory defect may result from the inability of sensory neurons to receive and process electrical signals, general locomotion defects could arise due to problems in the neuromuscular system that controls motor responses such as speed and the amplitude of motion. Hence, electrosensory mutants will have difficulty in sensing the direction of the electric field. Such animals are expected to swim towards either pole while reversing directions frequently. However, locomotory mutants should recognize the electric field polarity and move specifically towards the cathode, albeit at an altered pace. These arguments are supported by our analysis of neuronal mutants.

To this end we have developed a new quantitative method termed electrotaxis time index (ETI). ETI allows us to distinguished between electrosensory and electrotactic swimming (locomotion) defects. For example, an animal demonstrating a normal ETI and a reduced electrotactic speed suggest that the animal is electrotactic locomotion defective but senses the electric filed normally. Contrarily, an animal demonstrating reduced ETI and normal electrotactic swimming speed suggest that the animal is electrosensory defective and has normal locomotion. If the animal demonstrates lower value in both the measures than the animal has defects in both electrosensory and locomotion.

Although the biological basis and the mechanism of electrotaxis is poorly understood, a subset of neurons in the anterior ganglion were found to respond to the electric field stimulus in an open gel surface Petri dish setup (Gabel et al., 2007). We used *osm-5* and *trp-4* mutants to examine the role of sensory neurons in our channel assay. The *osm-5* gene encodes an intraflagellar transport protein that is homologous to human IFT88 and is required for cilia formation in sensory neurons including the amphids (Haycraft et al., 2001). The *osm-5* animals appeared very active and in the absence of the electric field, exhibited swimming behaviour similar to wildtype N2 (body bend frequency of 1.8 Hz, n = 6; see above for N2 data). However, in the presence of the electric field, the animals showed severely defective electrotactic responses. The average speed of *osm-5* was nearly 60% lower than that of wildtype N2 (n = 11) (**Figure 11A**). Whereas N2 worms swam straight towards the cathode without pausing, the *osm-5* worms stopped and reversed direction many times (number of reversals for each animal ranged between 3 and 20, n = 11). Few of them became immobile after slight initial swimming and did not recover (18%, n = 11). In addition to spontaneous turning and lack of motion, *osm-5* worms also exhibited intermittent pauses, abnormal body postures, and swimming in the wrong direction (i.e., towards the anode) indicating that they lacked a sense of direction. Consequently, the turn time of animals was highly variable (**Figure 11B**). To further demonstrate the electrotaxis defect in *osm-5* animals, we computed the time of all cathode-directed swimming events and determined the ETI (**see Materials and Methods**). As expected, the ETI of *osm-5* was greatly reduced compared to N2 ($p<0.0001$) (**Figure 11C**). As a control we also tested a non-neuronal mutant, *him-*

8(e1479), that produces an increased frequency of males due to defects in X-chromosome segregation(Hodgkin et al., 1979). The speed, turn time and ETI of *him-8* were comparable to N2 (**Figures 11C**).

Similar to *osm-5, trp-4* animals also showed increased spontaneous reversals. *trp-4* belongs to the invertebrate specific TRPN/NOMP (no mechanoreceptor potentials) subfamily of TRP channels (Li et al., 2006). TRP channels are non-specific cation channels and mediate sensations such as pain, temperature, taste and pressure. TRP-4 has 40% sequence homology to the zebra-fish TRPN1 and *Drosophila* NOMPC (Sidi et al., 2003). *trp-4(sy695)* mutants show faster and exaggerated body bends (deeper angles of sine wave produced during movement) (Li et al., 2006). *trp-4* is expressed in the cilia of CEPs, ADEs and PDEs and also in the interneurons DVA and DVC (Li et al., 2006). We found that 84% of *trp-4(sy695)* animals had premature turns, leading to a highly variable speed (**Figure 12A**). The ETI was determined to be 31.69%, which is significantly lower when compared to wildtype ($p<0.0005$) (**Figure 12B).** Such a phenotype of *trp-4* is observed specifically in the electric field environment, as in absence of an electric field, animals are indistinguishable from wildtype (**Figure 12C**).

We examined another neuronal mutant, *lin-11(null),* that is weakly uncoordinated and exhibits chemosensory and thermosensory defects (Sarafi-Reinach et al., 2001)(Hobert et al., 1998)(Ferguson and Horvitz, 1985) *lin-11* is a member of the LIM Homeobox family of transcription factors(Freyd et al., 1990). It is expressed in a subset of neurons in the head ganglion (including the sensory neurons ADF and ADL, interneurons AIZ, RIC, AVG, AVA and AVE, and chemosensory neuron ASG) and is

necessary for their differentiation(Sarafi-Reinach et al., 2001)(Hobert et al., 1998). We found that *lin-11(n389)* null mutants(Freyd et al., 1990) had severely defective electrotaxis responses (experiment performed by Siavash Amon, Biology). Some animals showed no reaction to the stimulus (17%, n = 23) whereas others moved in the channel, but with multiple pauses and significantly reduced speed (**Figure 13A**). Additionally, *lin-11* worms were also defective in sensing the direction of the electric field. Upon switching the field polarity, the animals either took a very long time to turn (**Figure 13B**) or failed to turn at all (16%, n = 19). In agreement with these findings, the ETI of *lin-11* was greatly reduced ($p<0.0001$) (**Figure 13C**).

Overall, results involving neuronal mutants demonstrate that amphid sensory and dopaminergic neurons are involved in mediating the electrotaxis behaviour of *C. elegans*.

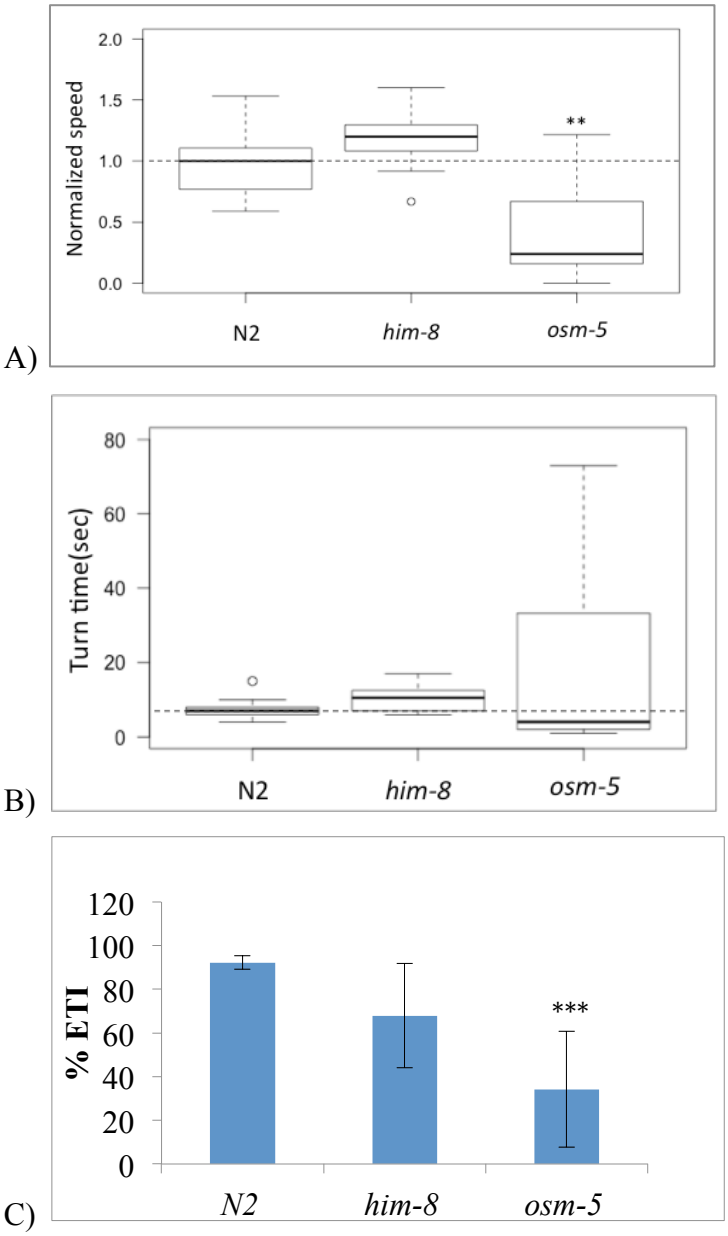


Figure 11. A and B) Electrotaxis speed and turn time of wildtype N2 and mutant animals: The lower and upper lines of each box represent the 25th and 75th quartile of data samples, respectively. The middle line inside the box marks the median. The end points of the vertical line (both top and bottom) are the maximum and minimum data points of the sample, respectively. The *him-8* (n=11) worms are comparable to wildtype whereas *osm-5*(n=11) move slower than N2(n=11). Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). C) Electrotaxis time index (ETI) of N2 control and mutant animals. The *him-8* mutant shows a normal ETI whereas *osm-5* shows a defective response.

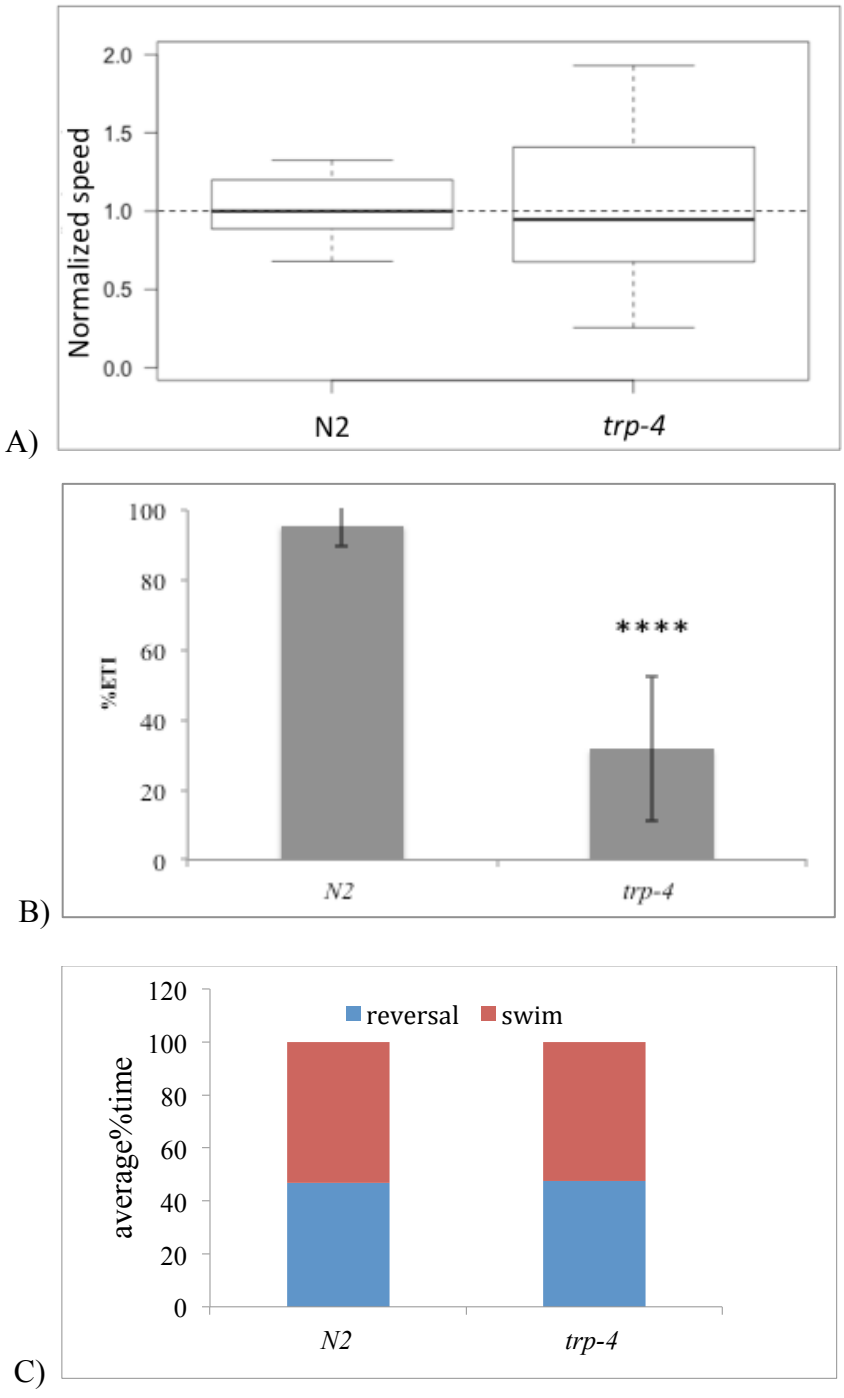


Figure 12. A) Electrotaxis assay for *trp-4(sy695)* mutants: N2(n=19), *trp-4* (n=16). B) ETI of *trp-4* during electrotaxis. N=18 for *trp-4* and n=14 N2. C) Swimming in the absence of an electric field: N2(n=5) and *trp-4*(n=5). Statistically significant responses are marked with stars (*: p<0.05, **: p<0.01, ***: p<0.001).

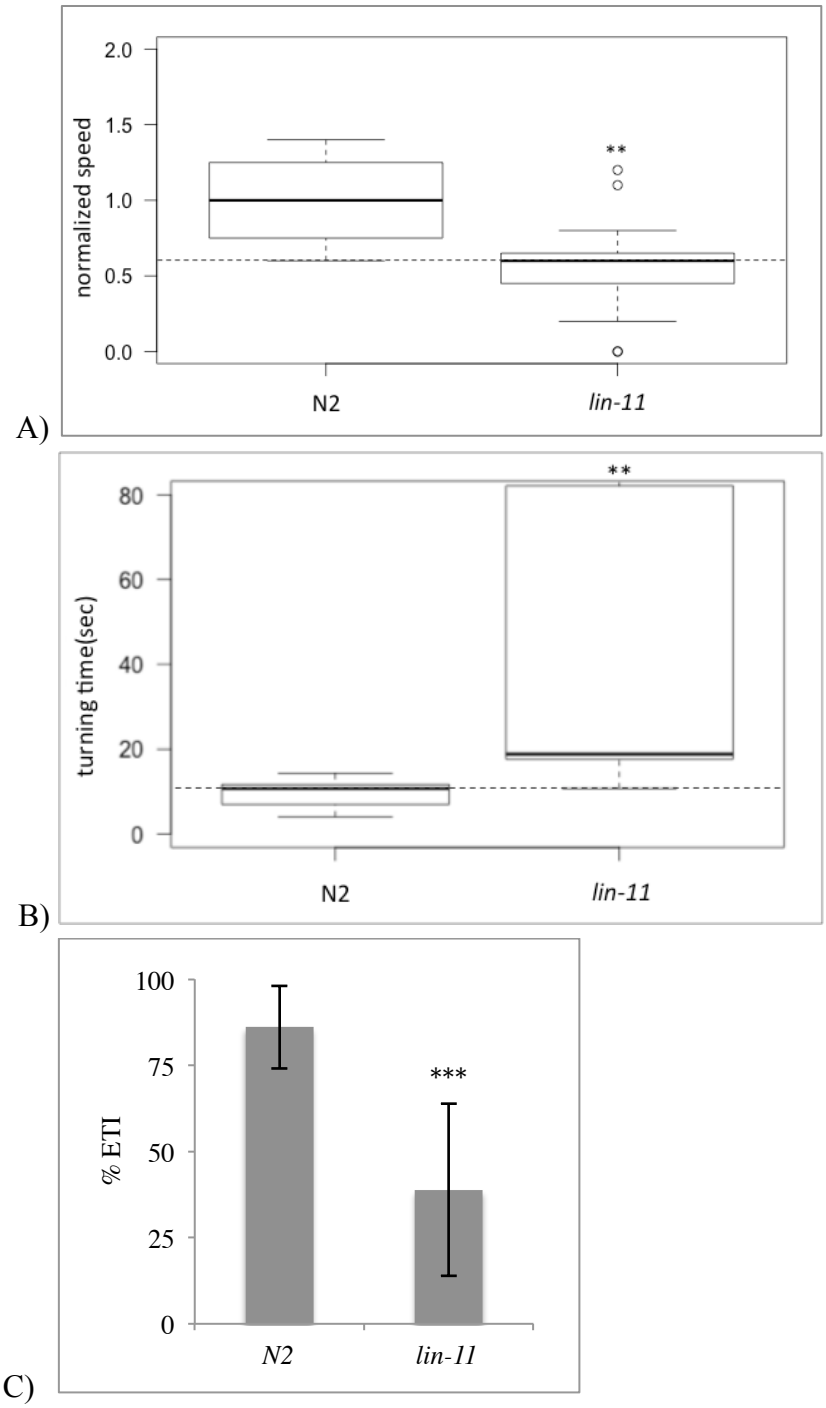


Figure 13. A and B) Electrotaxis speed and turning time of wildtype N2 and *lin-11(n389)* mutant animals: *lin-11* (n=19) move slower than N2 and take a much longer time to turn. C) ETI: *lin-11* (n=23) and N2 (n=12). Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

3.1.2. Worms exposed to an electric field have normal life span and chemotaxis behaviour:

Rezai et al., have demonstrated that electric field exposure has no adverse effect on *C. elegans* reproduction and movement (Rezai et al., 2010). However it was unclear if the electric field could still affect other aspects of behaviour and survival. Therefore, lifespan assays and chemotaxis assays were performed on worms exposed to 8V/cm for two minutes (**Materials and Methods**). It was observed that the worms had normal responses resembling that of the control. In the case of life span assays, the electric field exposed worms had 100% survival for 7 days and dropped down to 80% by the 9^{th} day (**Figure 14A**). Similar patterns were observed in the case of control animals as well.

In the chemotaxis assay, the electric field exposed worms had a chemotaxis index of 0.3 and the controls had 0.32, showing that electric field exposure does not affect chemotaxis behaviour **(Figure 14B)**.

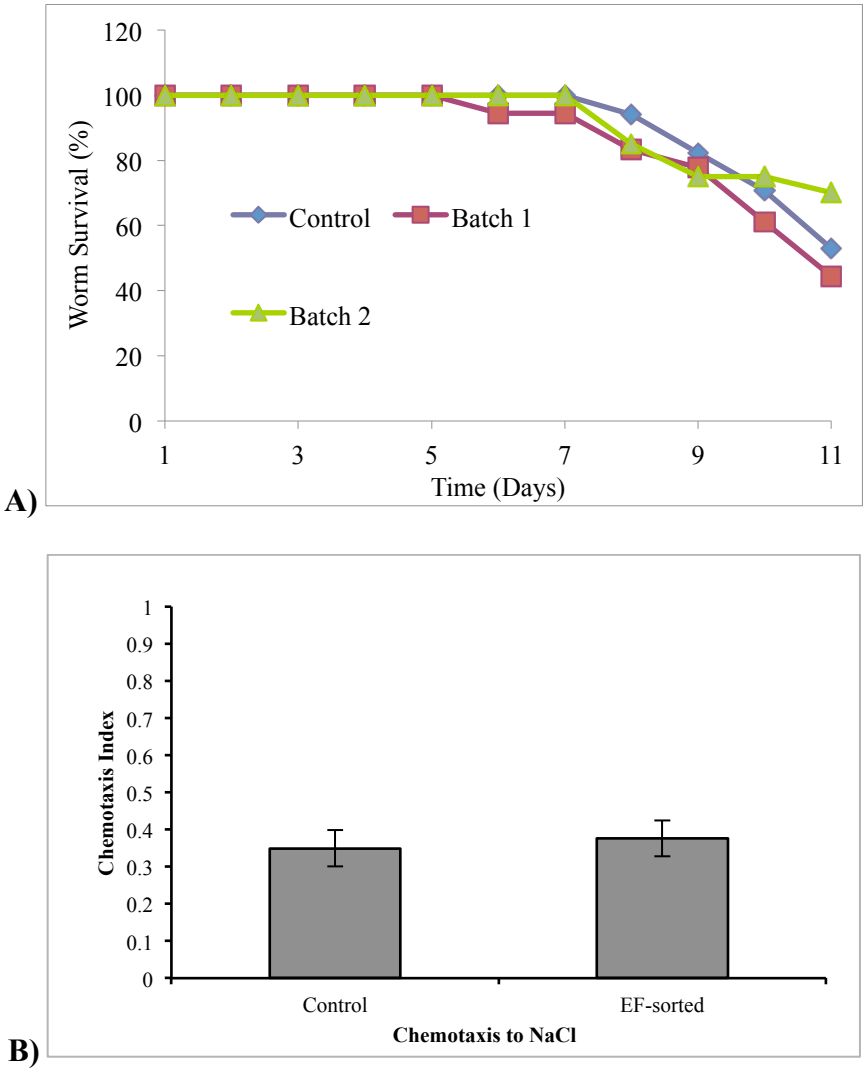


Figure 14. **A) Life span assay of worms exposed to an electric field:** Adult worms (62 hours) electric field unexposed (control, N=17) and electric field exposed worms (batch 1, N =18 and batch 2, N=20) were observed for their survival for 11 days after exposure. **B) Chemotaxis assay of worms exposed to an electric field.** Control is the electric field non-exposed worms. Electric field-exposed worms were those sorted by electrotaxis. The chemotaxis index was calculated using the formula $(N_{NaCl}-N_{H2O})$/total worms, where N_{NaCl} is the number of worms within 2.5 cm of a NaCl spot and N_{H2O} for the water spot.

3.1.3. Defects in dopaminergic neurons following toxin treatment affect electrotactic swimming:

We used three different chemical compounds: 6-OHDA, MPTP, and rotenone that are toxic to neurons. Previous work in vertebrates has shown that these chemicals cause the degeneration of dopaminergic neurons in the substantia nigra region of the brain (Betarbet et al., 2000)(Bové et al., 2005). 6-OHDA is preferentially taken up by dopaminergic neurons via the DAT transporter. Once inside the dopaminergic neuron, it causes multiple reactions including inactivation of the mitochondrial respiratory chain leading to an increase in ROS level(Glinka et al., 1997)(Jonsson and Sachs, 1975)(Glinka and Youdim, 1995). In the case of MPTP, it is metabolized into an active toxic product ($MPP^{+)}$ that enters dopaminergic neurons through the DAT-1 transporter. MPP^{+} has multiple targets inside the dopaminergic neuron including the inhibition of the mitochondrial respiratory enzyme complex I and increasing endogenous levels of ROS production (Ali et al., 1994)(Chiba et al., 1984). Exposure to rotenone in rat and *Drosophila* models has shown to cause apoptosis and oxidative damage of the dopaminergic neurons (Betarbet et al., 2000)(Coulom and Birman, 2004). *C. elegans* dopaminergic neurons are equally sensitive to the above three neurotoxins and undergo degeneration upon exposure(Nass et al., 2002)(Braungart et al., 2004a).

To carry out the electrotaxis assay on worms exposed to 6-OHDA, MPTP, and rotenone, we first optimized chemical exposure conditions (**see Materials and Methods**). Toxin-treated worms were placed inside the channel without any pre-selection and their electrotaxis responses were analyzed. We found that all three conditions resulted in

swimming behaviour defects. As mentioned in Chapter 2 these worms appeared visually normal but the electrotaxis phenotypes included slower speed, intermittent pauses, and reduced sensitivity. Exposure to 6-OHDA (for either 1 hour or 4 hour at 100 μM) caused a significant reduction in speed (40-60% slower, 126-174 μm/sec average speed) without altering the turn time (**Figure 15A**). Besides reduced speed, we also observed other defects in movement. Frequently, animals showed incoherent electrotaxis characterized by active sinusoidal motion followed by periods of slow responses or lack of activity. Partial paralysis was also observed where the posterior half of the body was rigid, such that the worm appeared to drag itself while moving. In addition, we detected phenotypes such as sudden freezing, tremors and a total lack of motion. Not all phenotypes were observed in every animal; furthermore, these were specific to the microfluidic channel environment. Animals grown on Petri plates did not show any such phenotype. In a few cases we also measured body bend frequency and found it to be lower in exposed worms when compared to controls (average frequency of 0.2 Hz for 6-OHDA 4 hours exposure, n = 11 animals and 1.8 Hz for N2, n = 10 animals).

Treatments with MPTP and rotenone for 8 hours caused similar defects (roughly 40% slower speed in each case, MPTP: 178 μm/sec, rotenone: 160 μm/sec) albeit the reduction in speed was somewhat less compared to 6-OHDA exposures at 1 hour (**Figure 15A**). This shorter duration of 6-OHDA exposure was determined as the time at which detectable defects were observed and animals were capable of swimming in the assay, in contrast to the maximum defect duration tested (4 hours), whereby most animals were rendered immobile. The turn time was also affected (up to 2-fold slower for each toxin;

MPTP: 19 sec; rotenone: 15 sec) (**Figure 15B**). Consistent with the slow electrotactic swimming speed, the body bend frequency of animals was also reduced (MPTP 8 hr: 1.0 Hz, n = 3, compared to 1.6 Hz, n = 4 for N2 control).

3.1.4. Dopaminergic neurons ablation causes defects in electrotactic swimming:

We also examined the response of *dat-1$_p$::ice* expressing transgenic animals. The dopaminergic neurons in *dat-1$_p$::ice* worms undergo cell death immediately after their birth due to the expression of the β-interleukin converting enzyme(Hills et al., 2004a). The loss of neurons can be confirmed by the disappearance of GFP fluorescence since the animals also carry *dat-1$_p$::GFP* plasmid. These animals demonstrated a significantly slow electrotactic swimming speed than the control N2 animals (**Figure 15C)**.

In summary, our experiments involving neurotoxins as well as *dat-1$_p$::ice* demonstrate the involvement of dopaminergic neurons in mediating the electrotactic swimming of worms. Additionally, the results show that the electrotaxis assay can be used to detect abnormalities in the dopaminergic neurons. Quantification of swimming defects allowed us to compare phenotypes in different conditions as well as between different sets of animals. We conclude that the electrotaxis assay using the microfluidics setup can be used to identify and study factors affecting dopaminergic neurons and neurodegeneration in worms.

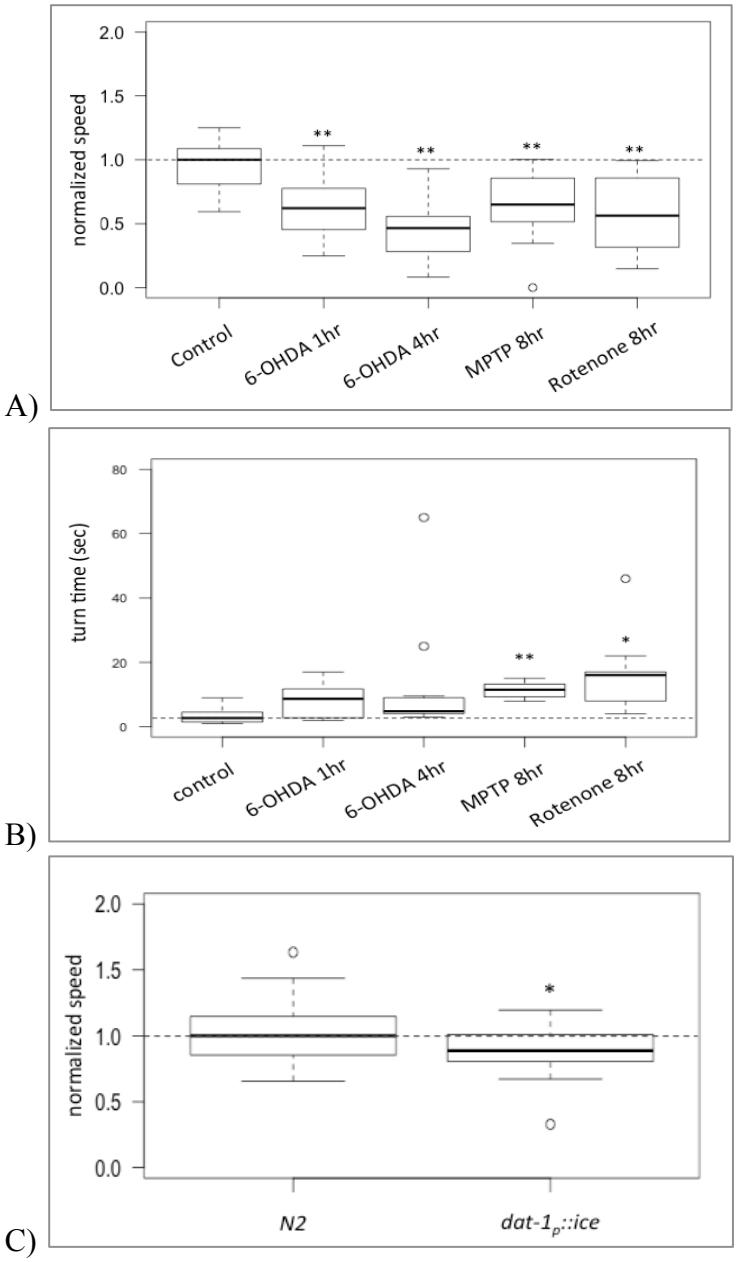


Figure 15. **A and B) Electrotaxis speed and turn time responses of toxin-treated worms inside the channel.** Control refers to non toxin-treated wildtype N2. 6-OHDA (n = 12 for 1 hr condition and 15 for 4 hr condition); MPTP and Rotenone: n = 12 and 13 worms, respectively. In all cases, the speed of exposed worms is significantly different from the control. C) Electrotaxis assay of genetically ablated DAergic neurons: n=30 and 27 respectively for N2 and *dat-1$_p$::ice*. Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

3.1.5. Plate-based phenotypic analysis of mutants and toxin-treated animals:

In addition to the electrotaxis phenotype, we examined gross morphology and the behaviour of animals on Petri plates. This was done in order to investigate whether movement defects are specific to the microfluidic device or observed on an open agar gel surface as well. For this we measured the speed of *osm-5* and *lin-11* mutants, following their tracks on bacterial lawns (**see Materials and Methods**). The average speed of *osm-5* was not significantly different from the wildtype N2 and *him-8* (**Figure 16A**). However, *lin-11* animals showed a lower speed when compared to N2 (40 hours stage), (**Figure 16B**). This agrees well with earlier studies showing that *lin-11* animals are weakly uncoordinated (Ferguson and Horvitz, 1985). Overall, our results provide support for the conclusion that abnormalities in the neuronal structure, such as the cilia, and developmental defects of neurons can cause abnormal electrotactic swimming responses. Furthermore, the spontaneous reversals in *osm-5* are unique to the microfluidics electrotaxis assay and not observed in the plate-based assay.

Next, we examined plate-level responses and cellular defects in toxin-treated worms. In general, worms were healthy and fertile with no obvious morphological defects at any stage. Except for a 4 hr 6-OHDA-exposure condition that caused weak uncoordinated movements (Unc) in roughly 40% of the population, in all other cases animals moved well and were fairly active (**Figure 16C**). In one case, 6-OHDA 1 hour, we determined the chemotaxis response to NaCl and found it to be similar to untreated N2 control animals (**Figure 16D**).

To correlate microfluidic behavioral defects in toxin-treated worms with dopamine neuronal function, the morphology of neurons was also investigated. For this, we used a *dat-1$_p$::YFP* transgenic strain *(bhEx120)* in which YFP expression is observed in dopaminergic neuronal cell bodies as well as their projections (**Figure 17A**)(Nass et al., 2002)(Jayanthi et al., 1998). The synchronized L1 stage *bhEx120* animals were exposed to 100 μM 6-OHDA for 4 hours and subsequently grown on a standard NG agar plate seeded with OP50 bacteria. The dopaminergic neurons were examined in adults, where we found an age-dependent increase in neurodegeneration in toxin-treated animals (18%, n = 81 on day 3 and 26%, n = 42 on day 6) (**Figure 17D**). The dendritic processes of CEPs showed variable degeneration such that YFP fluorescence had a spotty appearance (**Figure 17B**). In some cases the entire dendritic processes were missing (**Figure 17C**). These phenotypes are similar to those reported earlier (Nass et al., 2002) and are consistent with the electrotaxis defects in worms that we observed earlier.

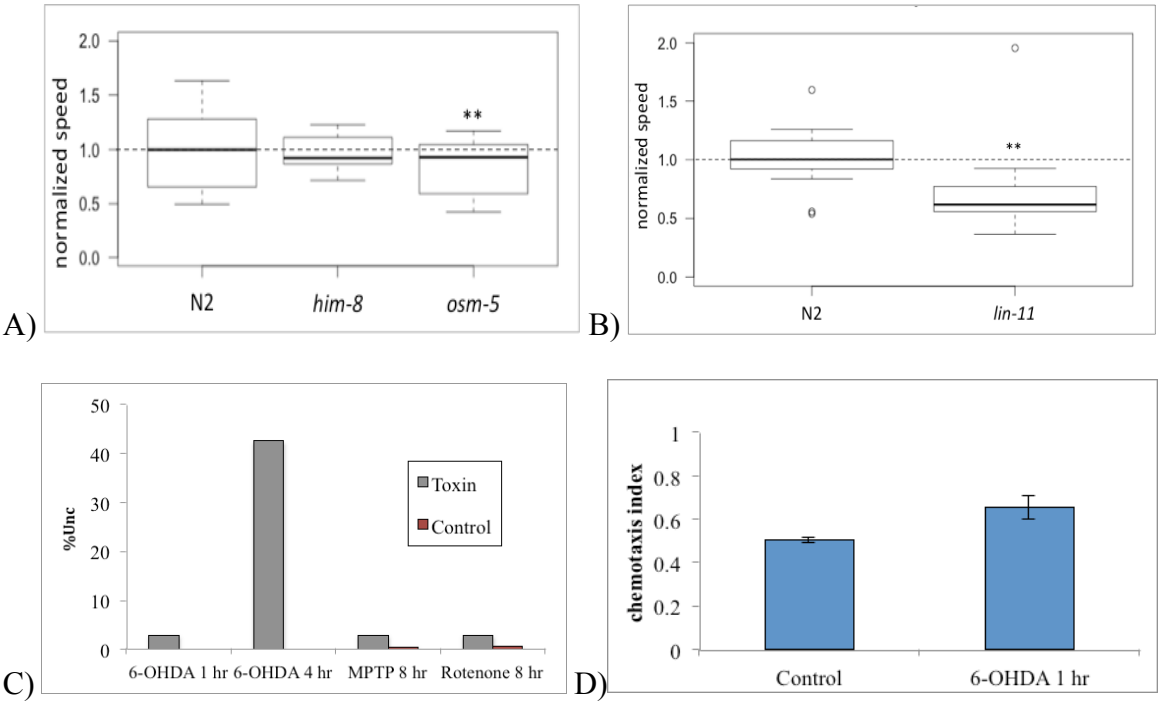
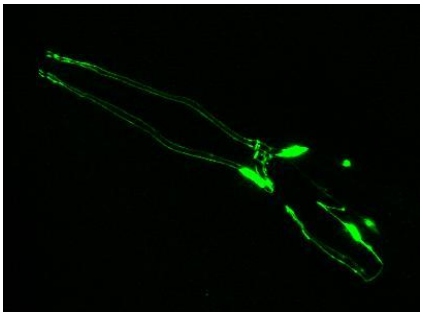


Figure 16. Plate level phenotype of mutants and toxin-treated animals. (A) and B) N2(n=19), *osm-5* (n=18) and *him-8*(n=9). The *him-8* and *osm-5* animals are comparable to the N2 control but *lin-11* (n=16) animals show reduced speed. **(C) Exposure to 6-OHDA, MPTP and rotenone causes weak Unc phenotype.** Except for 6-OHDA 4 hr cases that caused roughly 40% of animals to become Unc, all other treatments affected less than 3% of animals. The numbers of animals examined for each condition are as follows: 6-OHDA - 605 for 1 hr and 420 for 4 hours, and N2 control - 220; MPTP - 502 and control - 192; Rotenone - 353 and control - 163. **D) The chemotaxis responses of N2 (n=188) and 6-OHDA(n=237) 1hr-exposed animals towards NaCl chemoattractant.** The toxin-treated worms show normal behaviour. Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

A)



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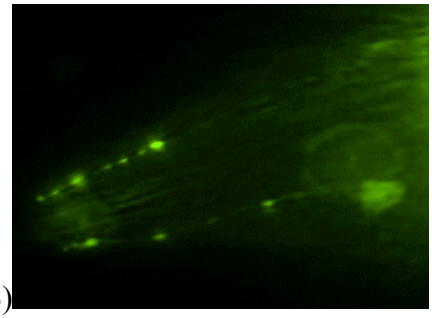

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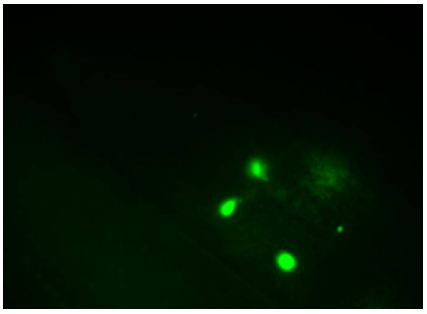

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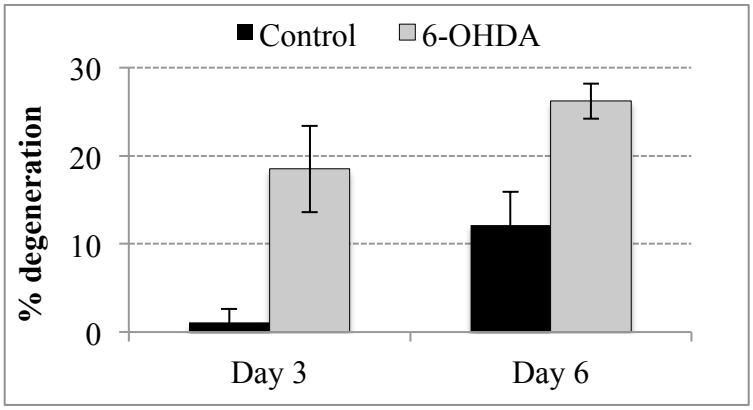


Figure 17. Degeneration of dopaminergic neurons in 6-OHDA-exposed worms visualized by *dat-1*$_p$*::YFP* expression. In all cases, posterior is towards the right. (A) In wildtype *bhEx120* animals, YFP fluorescence can be observed in CEP cell bodies and their processes. (B) Exposure of 6-OHDA causes a spotty appearance of CEP neuronal processes indicating degeneration of the neurons. (C) Another 6-OHDA-treated animal. The neuronal processes are almost completely missing. (D) Quantification of neuronal defects in 3 day and 6 day old controls (untreated) and 6-OHDA-treated animals (sample size: 81 6-OHDA and 88 controls for day 3 set; 42 6-OHDA and 33 controls for day 6 set).

3.1.6. Electrotaxis defects caused by neurotoxins can be rescued by treatment with Acetaminophen:

If toxin-induced dopaminergic neuron damage affects the electrotaxis behaviour of worms, then the phenotype could be suppressed by protecting the neurons. To examine this possibility, we tested an analgesic, acetaminophen, that possesses neuroprotective properties. Studies in rats and *C. elegans* have shown that acetaminophen protects against MPP^+, 6-OHDA and glutamate toxicity in dopaminergic neurons (Maharaj et al., 2004)(Casper et al., 2000)(Locke et al., 2008). We found that pre-treatment of acetaminophen conferred significant protection to dopaminergic neurons against all three neurotoxins. The pre-exposed worms were phenotypically normal and had significantly faster speeds when compared to neurotoxin-treated worms (**Figure 18**). However, the turn time of animals showed no improvement (data not shown). Because acetaminophen protects dopaminergic neurons (Maharaj et al., 2004)(Locke et al., 2008), these results further support the involvement of dopaminergic signalling in controlling the electrotaxis behaviour of animals. Additionally, our findings show that the microfluidic channel-based assay can be used as a screening tool to identify new chemicals with neuroprotective properties.

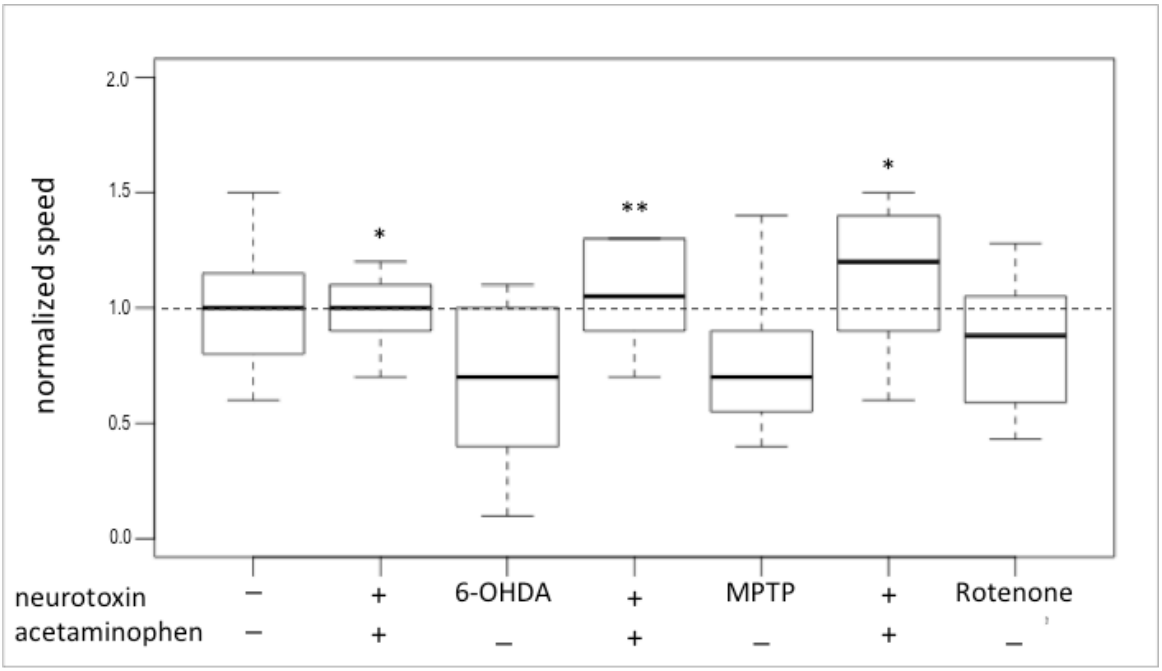


Figure 18. **Electrotactic responses of worms treated with toxins and acetaminophen (para acetylaminophenol, APAP).** Control refers to untreated wildtype N2 animals. The speed of APAP-treated groups is comparable to the control and higher than the corresponding toxin-treated group. Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

3.2. CHARACTERIZING THE PHENOTYPE OF DOPAMINE PATHWAY MUTANTS IN THE ELECTROTACTIC SWIMMING ASSAY:

Preface:

The results of experiments described in this chapter demonstrate the role of dopamine signalling in the electrotactic swimming response of *C. elegans*. It was found that dopamine in the synapse is required to maintain normal swimming speeds. Both D1 and D2 type receptors are involved in the process. While the D2 type recepor DOP-3 inhibits the electrotactic swimming speed, the D1 type receptor DOP-4 augments the speed.

3.2.1. Dopamine synthesis mutants are defective in electrotactic swimming behaviour:

As described in section 2.1 defects in dopamine neurons can alter the electrotactic swimming speed. Therefore, we set out to examine the electrotactic swimming response of dopamine pathway mutants in our microfluidic channel assay.

Animals having a mutation in the *cat-2* gene, the tyrosine hydroxylase required for dopamine biosynthesis (Lints and Emmons, 1999), showed normal responses however dopamine transporter mutants *dat-1(ok157*) showed defective swimming when compared to control animals (**Figure 19A and B**). Since DAT-1 mediates the reuptake of dopamine from the synaptic cleft back into the presynaptic terminal (Jayanthi et al., 1998), it is possible that in the absence of DAT-1 function, extracellular dopamine alters the activity of certain post-synaptic neurons thereby causing reduced electrotactic swimming speed in

the microfluidic channel. Additionally, dopamine could stimulate certain dopamine receptors on motor neurons resulting in the slower speed of animals. The lack of electrotaxis phenotype in *cat-2(e1121)* animals may be attributed to the fact that dopamine levels are not completely abolished (40% residual dopamine compared to wildtype (Sanyal et al., 2004b)). Because *dat-1* mutants show reduced speed but no impact on sensing the electric field polarity, it suggests that dopamine signalling modulates locomotion without affecting the electrosensory response of animals. This conclusion is supported by the normal ETI response of *dat-1* animals (p=0.1253) (**Figure 19C**). We also examined the speed of *dat-1(ok157)* on an open agar gel surface by measuring its tracks on bacterial lawns. We found the speed of *dat-1* similar to that of wildtype (**Figure 20**).

To further understand the role of dopamine in electrotactic swimming, other dopamine synthesis and trafficking mutants were examined in the electrotaxis assay. The loss of function alleles *bas-1(ad446)* and *cat-1(ok411)* that encode for aromatic amino acid decarboxylase and vesicular monoamine transporter respectively, exhibited a significantly faster speed by showing 52 % and 27% increases in speed as compared to wildtype (**Figure 21**). Because of the differences in observed speed amongst the dopamine mutants, we wanted to see if the speed phenotype correlated with dopamine levels in the mutants.

In collaboration with Asim Siddiqui and Dr. Ram Mishra (Department of Psychiatry & Neuroscience, McMaster University) we measured the dopamine levels of these mutants using HPLC analysis coupled with electrochemical detection **(Figure 22)**.

cat-1 and *dat-1* showed significant elevations in dopamine concentration, with 71.5 % and 146 % dopamine synthesis increases respectively. In contrast, *cat-2* and *bas-1* showed significant reductions in dopamine concentrations in comparison to wildtype strains, with 68% and 81% dopamine synthesis decreases respectively.

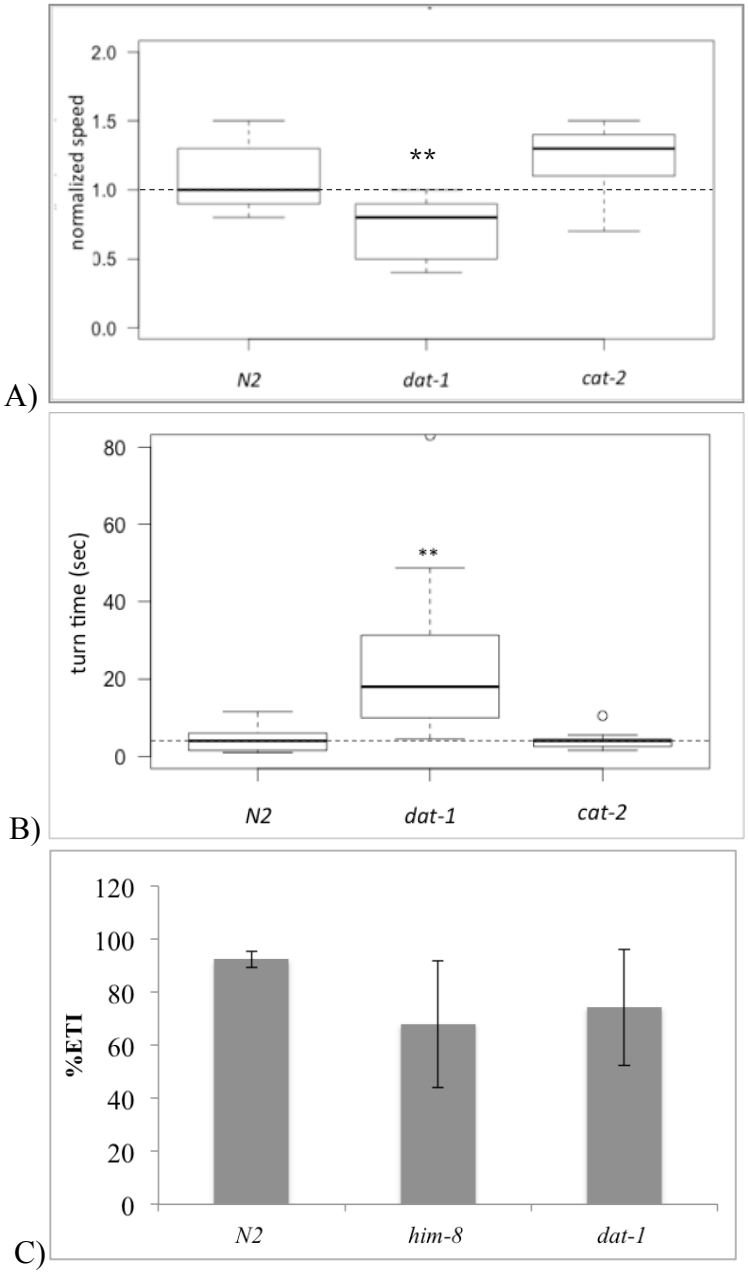


Figure 19. **A and B) Electrotaxis speed and turn time of *dat-1* and *cat-2 mutants*:** *dat-1* (n =10) *cat-2*(n =10). *dat-1* is electrotactic swimming defective but *cat-2* is similar to control. C) ETI of *dat-1*: ETI of *dat-1* animals is not significantly different from N2. Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

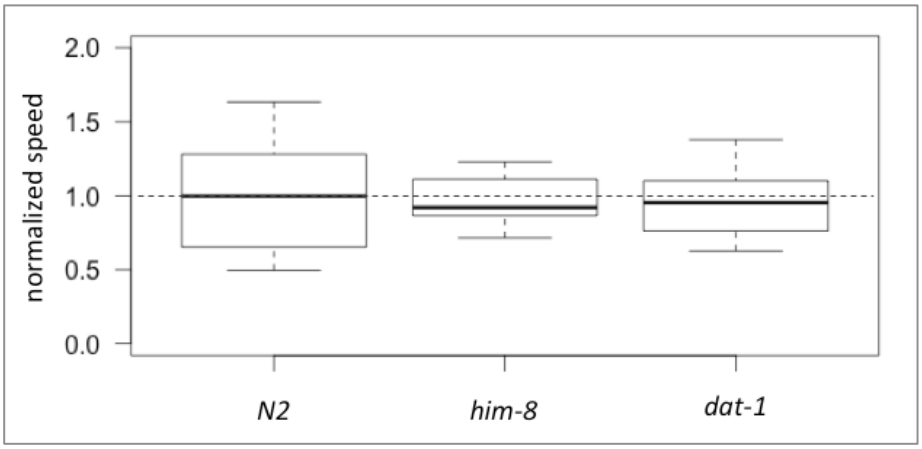


Figure 20. **Plate assay: N2(n=19), *dat-1* (n=23) and *him-8*(n=9).** The *dat-1* animals are comparable to the N2 control. Statistically significant responses are marked with stars (*: p<0.05, **: p<0.01, ***: p<0.001).

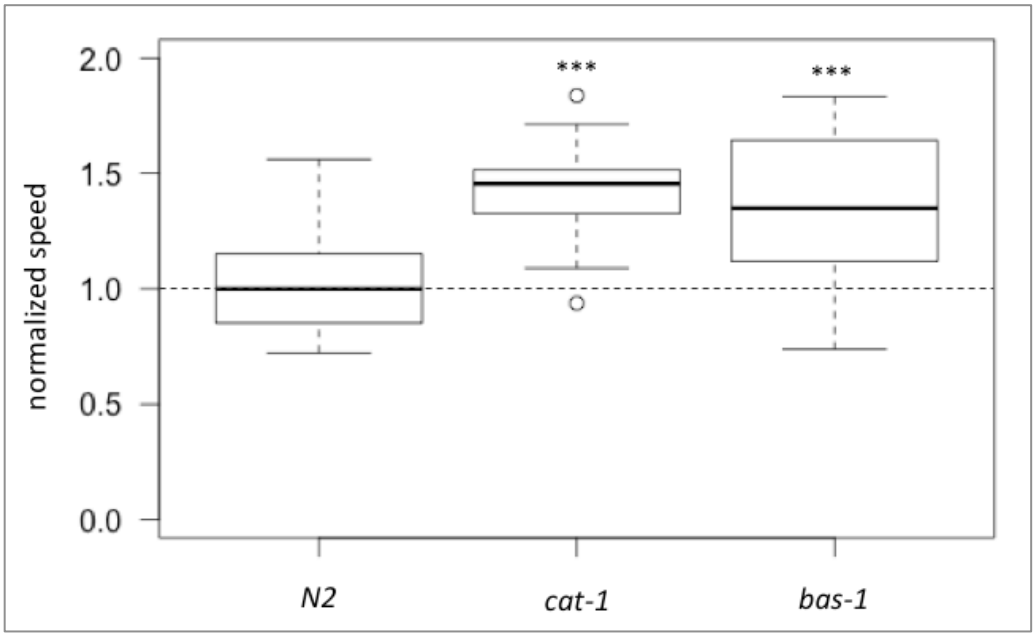


Figure 21. Dopamine synthesis mutants *bas-1* and transport mutant *cat-1* show faster electrotaxis speed: *bas-1*: control (n=50) and *bas-1*(n=32); MW p = 0.0018, *cat-1* (n=21). Statistically significant responses are marked with stars (*: p<0.05, **: p<0.01, ***: p<0.001).

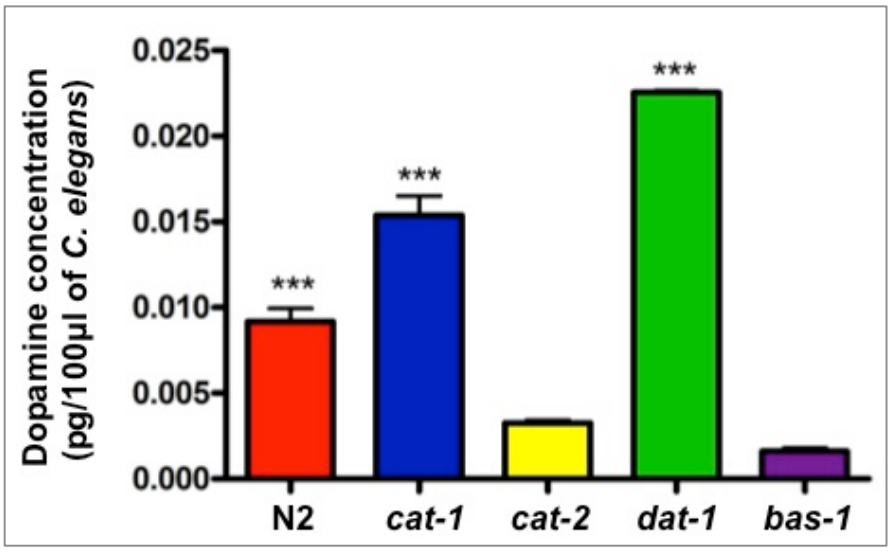


Figure 22. Quantification of Dopamine levels in dopamine pathway mutants using HPLC: Mass of dopamine is express in picograms per 100µl of worms, ANOVA, $F(4,10)=193.4$,****$p<0.0001$. Statistically significant responses are marked with stars (*: $p<0.05$,**:$p<0.01$,***:$p<0.001$).

3.2.2. EXOGENOUS DOPAMINE RESCUES SPEED DEFECTS IN A BAS-1 MUTANT:

The gene *bas-1* is involved both in dopamine and serotonin (5-HT) synthesis (Sawin et al., 2000)(Loer and Kenyon, 1993). To examine if dopamine alone can rescue the fast speed of *bas-1*, electrotaxis assays were performed on worms treated with exogenous dopamine. Since high concentration of exogenous dopamine can cause locomotor inhibition (Schafer and Kenyon, 1995b), we examined the effect of different dopamine concentrations on movement (**Figure 23A**) and determined that a 1 mM concentration did not impede movement in wildtype animals. Exposure to 1 mM dopamine rescued the fast speed of *bas-1* by 20% (**MW p *bas-1* vs *bas-1*+DA = 0.000) (Figure 23B).** A similar treatment in wildtype had no significant impact. We also performed the electrotaxis assay on serotonin (5-HT) treated *bas-1* however we did not observed any rescue effect **(Figure 23C)**. Thus, these results suggest that the faster speed of *bas-1* animals is due to a lack of endogenous dopamine.

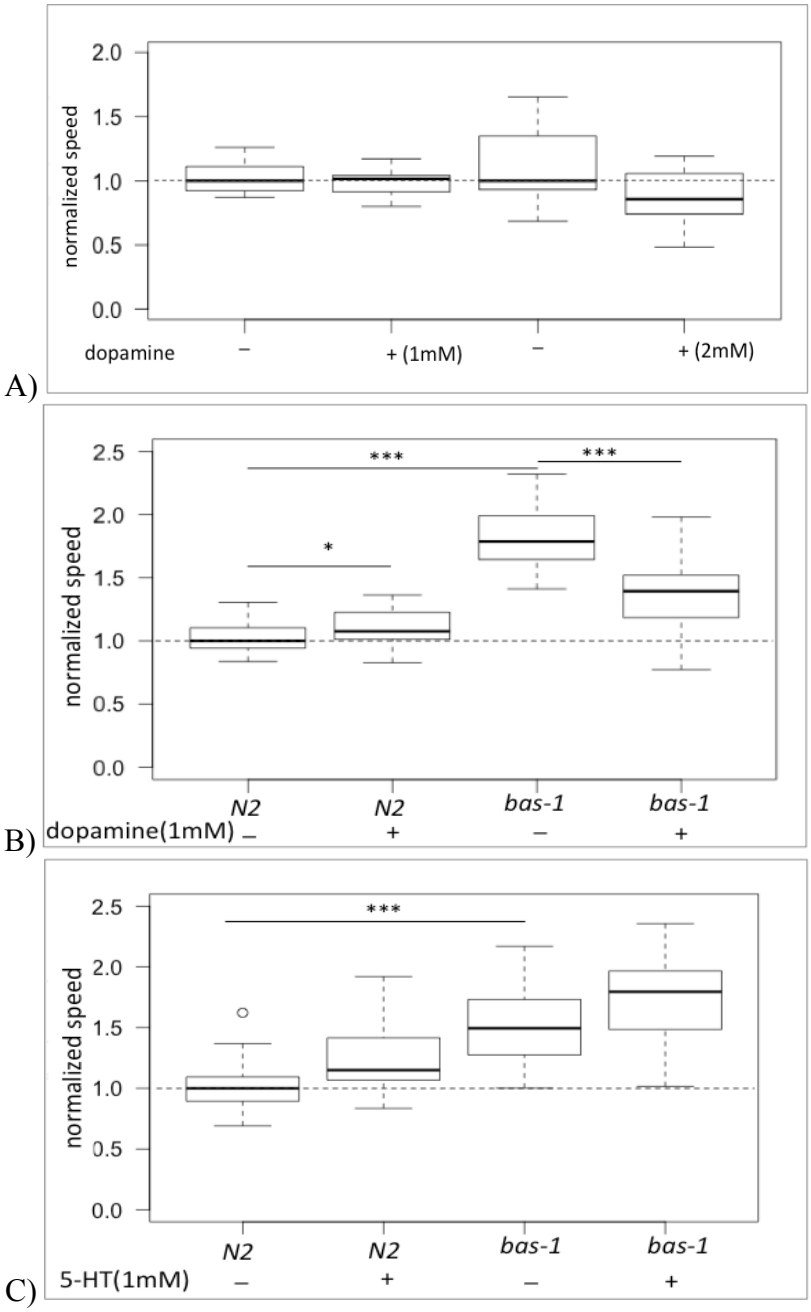


Figure 23. A) Determination of dopamine concentration: N2(n=9), N2+DA1mM(n=9), N2(n=10) and N2+2mM (n=10). **B) Dopamine treatment rescues the faster speed of *bas-1*:** control (n = 18), control+DA (n = 19), *bas-1*(n = 20); *bas-1*+DA (n = 20). **C) 5-HT(serotonin) treatment of *bas-1*:** N2 (n= 18), N2 + ser (n=18), *bas-1* (n=20), *bas-1* + ser (n=20). Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

3.2.3. RECEPTOR MUTANTS SHOW DEFECTS IN ELECTROTACTIC SWIMMING:

C. elegans dopamine receptors are directly involved in regulating movement as determined by assays such as the basal slowing response and swimming induced paralysis (Sawin et al., 2000)(McDonald et al., 2007). We examined the electrotactic swimming phenotype of the dopamine receptor mutants. The D1 type receptor mutants *dop-1(vs100)* and *dop-4(tm1392)* were not significantly different in speed from wildtype (**Figure 24**). **(MWp= 0.91)** and 0.97 **(MWp=0.17)**. The D2 type receptor mutant *dop-2(vs105)* also showed normal speed (**MWp=0.95**), while the *dop-3(vs106)* mutant was significantly faster than wildtype (20.3%) **(MWp=0.002) (Figure 25A**). The *dop-2(vs105);dop-3(vs106)* double mutant was also significantly faster (41%, **MWp=0.000**) than wildtype **(Figure 25B**).

The DOP-1 and DOP-3 mutants are known for their paralytic activity in response to exogenous dopamine as well as in SWIP behaviour (McDonald et al., 2007). Therefore we can expect that excess extracellular dopamine in *dat-1* mutants will activate DOP-3 receptors, leading to a reduction in speed, and that *dop-3(vs106)* mutants will suppress such a phenotype observed in *dat-1*. Therefore we examined the response of *dat-1(ok157); dop-3(vs106)* double mutants in the electrotaxis assay and found it to be 36.8% faster in speed (**MWp=0.002**) (**Figure 26**). This result is consistent with *dop-3* and *dat-1* genetic interactions as previously reported by others (McDonald et al., 2007), demonstrating that *dop-3* is epistatic to *dat-1* in mediating electrotactic swimming. Since

the D1 type receptors *dop-1* and *dop-4* showed normal speeds, we expected that these receptor mutants would not alter the *dat-1* slow speed phenotype. The results of our electrotaxis assays involving *dat-1(ok157); dop-4(tm1392)* double mutants confirmed this prediction (**MWp=0.002**) (**Figure 26**).

Overall, the experiments involving dopamine synthesis and receptor genes have demonstrated that dopamine signalling plays a pivotal role in modulating the electrotactic swimming response of worms.

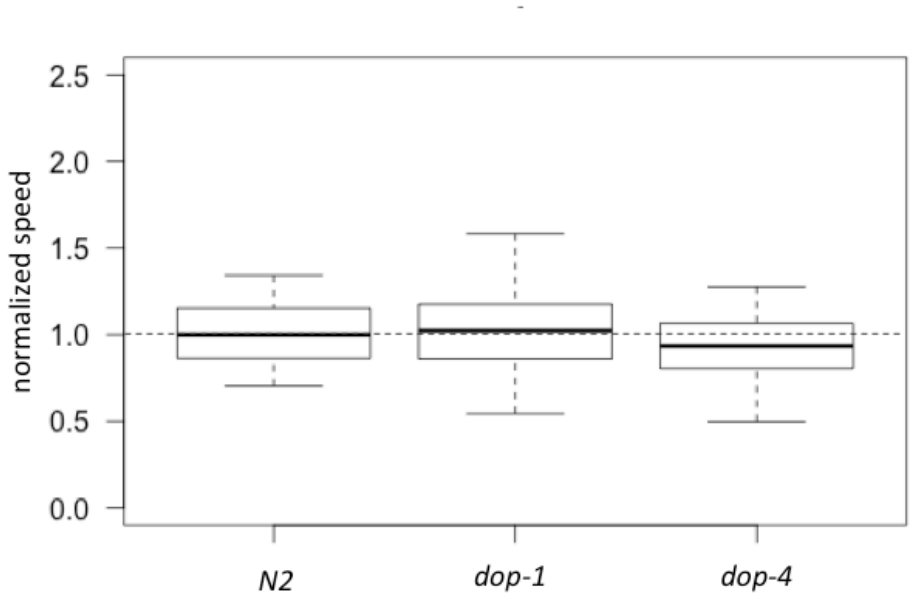
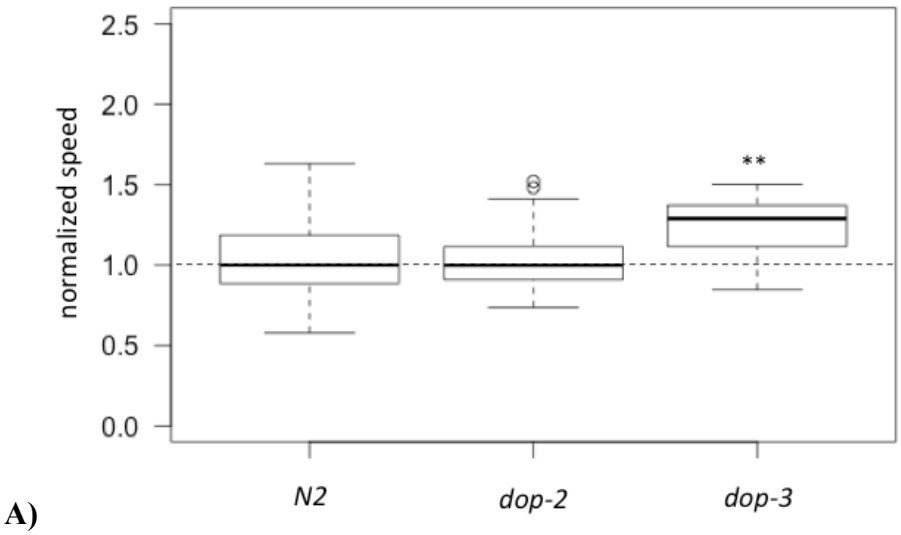
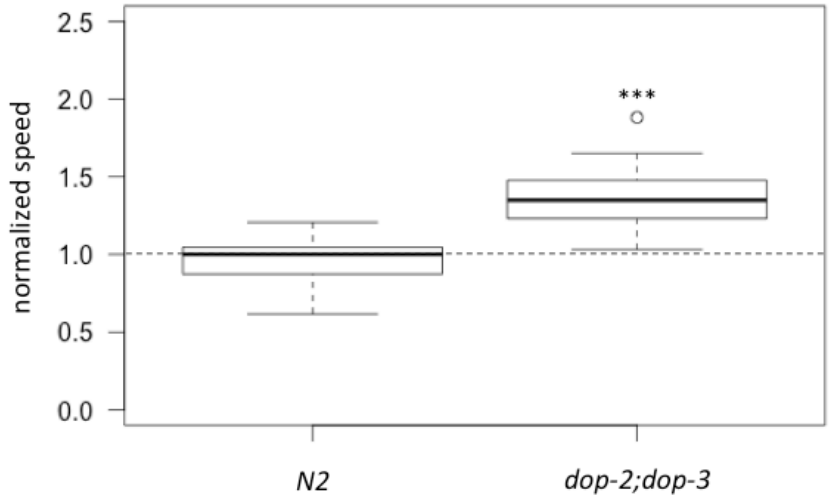


Figure 24. Electrotaxis phenotype of dopamine receptor mutants. *dop-1* and *dop-4* receptor mutants have normal speed. *dop-1* (n=20) and control (n= 20), MW p = 0.84; *dop-4* (n = 20) and control (n = 22). Statistically significant responses are marked with stars (*: p<0.05, **: p<0.01, ***: p<0.001).



A)



B)

Figure 25. **A) Electrotaxis phenotype of dopamine receptor mutants**. *dop-2* and *dop-3* receptor mutants have normal speed. *dop-2* is also normal but dop-3 animals swim faster than controls. *dop-2*(n = 33) and control (n = 31); *dop-3*(n=22), p= 0.002 and control (n= 21). **B) D2 type receptor double mutant:** The *dop-2;dop-3* double mutant is faster and similar to the *dop-3* single mutant; *dop-2;dop-3* (n=21) and control (n= 18). Statistically significant responses are marked with stars (*: p<0.05, **: p<0.01, ***: p<0.001).

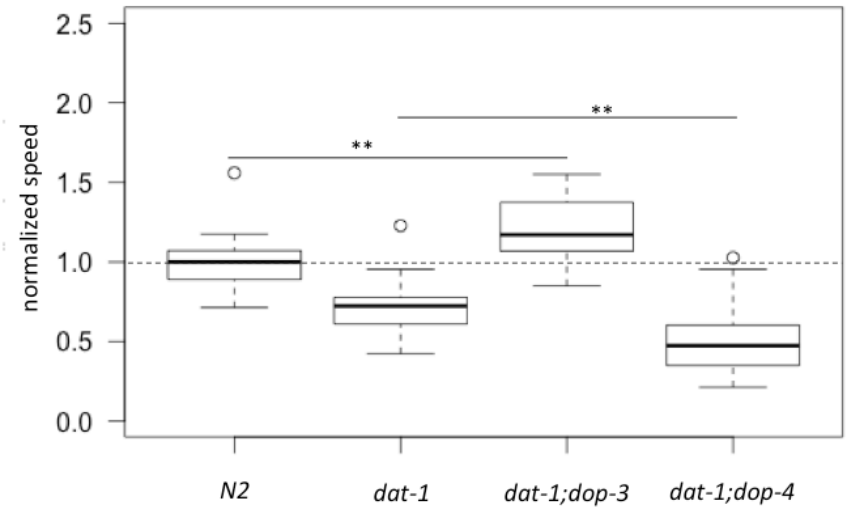


Figure 26. Dopamine transporter and receptor mutant combination electrotaxis assay: ***dat-1;dop-3*:** N2 (n=20), *dat-1* (n=20) *dat-1;dop-3* (n=22) *dat-1;dop-4* (n=21). MW p value for N2 vs *dat-1;dop-3* and *dat-1* vs *dat-1;dop-4* is 0.002 and 0.002 respectively. Statistically significant responses are marked with stars (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

3.3. CHARACTERIZING THE PHENOTYPE OF DOPAMINE PATHWAY MUTANTS IN PROLONGED EXPOSURE TO AN ELECTRIC FIELD:

Preface:

During the course of the electrotaxis experiments, we observed that prolonged exposure to an external electric field causes a gradual reduction in the swimming speed of worms. Given our findings in previous sections, that dopamine signalling modulates electrotaxis, we speculated that a longer exposure to the electric field may alter dopaminergic neuron-mediated signalling leading to a gradual decline in the swimming speed. It is worth pointing out that in 2012, Ires Chen *et al.* had reported that in rats, electrical forepaw stimulation could restore dopamine homeostasis in dopamine deficient models (Chen et al., 2012). Thus, electric field exposure could implicate the dopamine system of *C. elegans*, which can modulate behaviour.

To test the electrotaxis behaviour of worms in prolonged exposure to external electric field, we chose ten minutes duration. This exposure time is similar to that used in SWIP assay which is also a dopamine-regulated behaviour in *C. elegans*(McDonald et al., 2007). Furthermore, ten minutes duration is a reasonable compromise between the time taken to perform the assay and the longer exposure to electric field stimulus. While the decline in electrotaxis swimming speed could be observed in as little as 5 minute, ten minutes exposure was enough to see a consistent and reliable change in the swimming speed.

3.3.1. Quantification of swimming speed reductions due to prolonged exposure to the eletric field:

To characterize the impact of prolonged exposure to a DC electric field, we quantified and compared the speed of wildtype animals during the first and last minute of exposure in a 10 minute long electrotaxis assay. The results showed a decrease in speed of 37% ±4.36 by the end of the electrotaxis assay **(Figure 27)**. To determine whether the reduction in speed is an electric field induced phenomenon, and not a non-specific effect of swimming in the channel environment, two control experiments were performed. In one case, wildtype animals were allowed to swim for 10 minutes inside the channel in the absence of the electric field. Since there is no control of worm movement without the use of an electric field, speed was quite variable. Nevertheless, analysis of the data revealed that the speed at the end of 10 minute was 28% ±24.67 higher than in the beginning. In the other case, worms were exposed to an electric field only during the first and the last minute of 10 minutes of swimming. In between, worms were allowed to swim for 8 minutes without the influence of the electric field (**Figure 27**), hence only controlling the movement during the first and last minute of swimming. If indeed there is a reduction in speed due to exhaustion from swimming, we should observe a slower speed in the last minute as compare to the speed of the first. The observed reduction in speed was only 6%.±8.62 Together, these experiments demonstrated that a continuous prolonged (10 min) exposure to an electric field causes more than a one-third reduction in the swimming speed of *C. elegans*. In the next several sections we have described experiments to

examine the basis of this phenomenon and demonstrate that it is mediated by dopamine signalling.

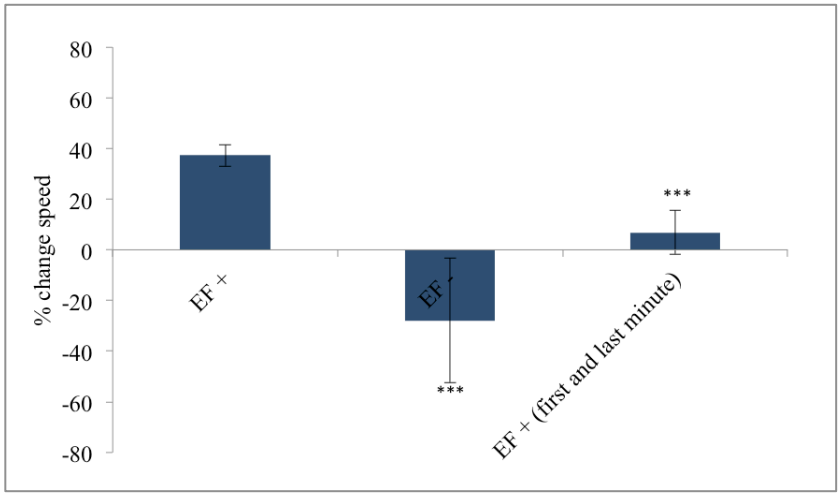
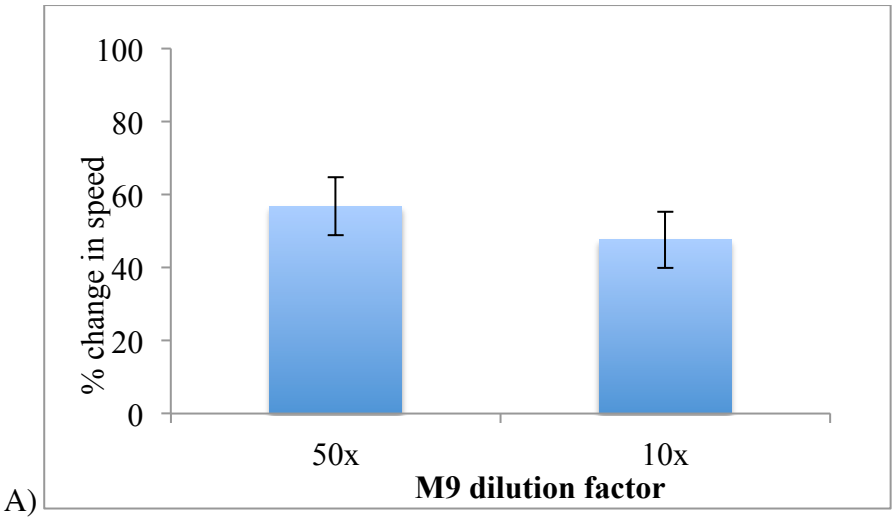


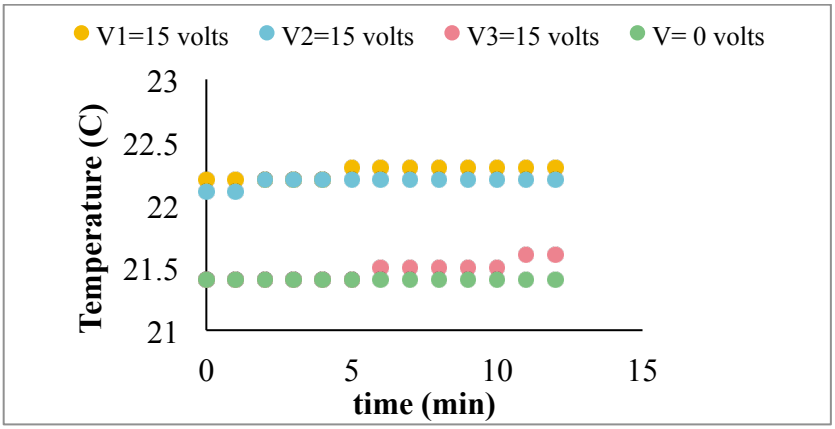
Figure 27. Wildtype 10 minutes electrotaxis assay: The presence of an electric field (EF +) (n = 16), absence of electric field (EF -) (n = 6) and electric field only during the first and last minute (EF + first and last minute) (n = 12), Student's t-test between EF+ and EF - = 0.0003 (***p ≤ 0.001), EF+ and EF+(first and last minute) = 0.0015(***p ≤ 0.001)

3.3.2. Effect of temperature on electric field induced swimming in the microchannel:

The M9 solution contains salts that could generate heat due to ionic movement during prolonged exposure to the electric field. If the heat is sufficiently high, then it might affect the movement of worms inside the channel. To test this possibility we designed two experiments (Ali Shahid from Mechanical Engineering, McMaster University performed these experiments and was assisted by Cory Richman from Biology, McMaster University). First, 10 minute long electrotaxis assays of wildtype animals were done using M9 solution that was diluted 50 times and 10 times with de-ionized water. We reasoned that if a reduction in speed is indeed caused by a change in temperature, then by reducing the ionic concentration the phenotype should be reversed. The results showed that it was not the case. Thus, worms demonstrated a 57% ±7.91 and 48% ±7.71 reduction in speed (50x and 10x diluted M9, respectively) (**Figure 28A**). Secondly, temperature change inside the microchannel was measured using an embedded thermocouple. At 15 volts, an increase of 0.1 degree in temperature was observed in 10 minutes when pure M9 solution was used (maximum 0.2 degree in 15 min) (**Figure 28B**). There was no change in temperature in the absence of the electric field. We conclude that temperature increase is not a significant factor causing the worms to slow down during prolonged electric field exposure.



A)



B)

Figure 28. Temperature measurement experiments: A) Long electrotaxis in diluted M9: 50x n=16, 10x n=9. B) Temperature measurements using a thermocouple (This experiment was kindly done by Ali Shahid).

3.3.3. Effect of the electric field potential on swimming speed:

Another factor that may affect the speed of worms during prolonged electric field exposure is the potential of the field itself. Rezai et al. had shown earlier that worms start to behave abnormally in high electric fields and fail to maintain a sinusoidal pattern of motion (Rezai et al. Lab Chip). To examine if the reduction in swimming speed is dependent on the magnitude of the electric field applied, a 10 minute electrotaxis assay was performed at 4V/cm (33% ±9.36 increase over the normal potential). The reduction in swimming speed was found to be similar to that of the 3V/cm condition, (**Figure 29**). This suggests that the reduction in swimming speed is not dependent on the electric field potential.

3.3.4. Characterizing the swimming speed reduction phenotype of dopamine ablated animals:

Dopaminergic neurons are sensory and synapse with 41 other neurons (White et al., 1986). We examined whether dopamine neuron ablation causes altered electrotactic swimming in the 10 minute electrotaxis assay. We used worms expressing *dat-1$_p$::ice* in which dopamine neurons are genetically ablated (Hills et al., 2004b). In *dat-1$_p$::ice* worms, 1b interleukin converting enzyme, an apoptotic enzyme, is expressed specifically in dopamine neurons and kills the neurons by apoptosis (Hills et al., 2004a). The animals are phenotypically normal on standard NG-agar plates, however when exposed to the electric field for a long duration, we observed that unlike wildtype animals *dat-1$_p$::ice* worms had no reduction in swimming speed (**Figure 29**).

3.3.5. Characterizing the swimming speed reduction phenotype of dopamine synthesis mutants:

To further characterize the involvement of dopaminergic neuron signalling in the swimming speed reduction following prolonged electric field exposure, dopamine deficient mutants *cat-2(e1112)* and *bas-1(ad446)* were examined during the 10 minute electrotaxis assay. As expected, we observed very little reduction in the speed of *cat-2* and *bas-1* animals, 1% ± 4.64 and 9% ± 3 decreases respectively (**Figure 29**). These results show that dopamine is involved in reducing the swimming speed during prolonged exposure to an electric field. This phenotype was able to be rescued with 1mM external dopamine treatment in *cat-2(e1121)* and *bas-1(ad446)* mutants. Since *bas-1* is also involved in serotonin synthesis, we did a rescue experiment with *bas-1(ad446)* by exogenously supplying 1mM serotonin, however serotonin treatment did not rescue (**Figure 29**) the phenotype.

3.3.6. Characterizing the swimming speed reduction phenotype of dopamine transport mutants:

Dopamine transporter DAT-1 is required for normal clearance of dopamine from the synaptic cleft and is required for normal thrashing behaviour in L4 wildtype N2 animals. As thrashing is required for normal electrotactic swimming, we examined the *dat-1(ok157)* mutant for its response during 10 minutes of electrotaxis. Since the mutant has a higher extracellular dopamine concentration, it is expected to show a reduction in swimming speed during the assay. We observed a 50% reduction in the speed, with few animals (2/20) demonstrating paralysis (**Figure 30**). Next, to examine if the reduction in

speed is enhanced in the *dat-1* background, as the mutants are impaired of clearing extracellular dopamine, we analysed the speed at the midpoint (i.e., 5 min). Results showed a gradual change in speed both in control and *dat-1* mutants.

3.3.7. Characterizing the reduction in swimming speed phenotype of dopamine receptor mutants:

The dopamine receptor mutants were also examined in prolonged electrotaxis assays in order to understand the role of receptor signalling. The D2 type receptor mediates inhibitory signals, therefore *dop-2(vs105); dop-3(vs106)* was expected to demonstrate a smaller reduction in speed. The results showed a 9% ± 3.79 reduction in swimming speed, significantly different from the control ($p \leq 0.001$) (**Figure 29**). Furthermore, the D1 type receptor double mutant *dop-1(vs100)dop-4(tm1392)* demonstrates a 20% ± 7.85 reduction in speed, which is also significantly different from the control ($p \leq 0.05$). As a sum, this suggests that the swimming speed phenotype is mainly due to D2 type signalling.

To further understand the role of D2-mediated signalling, single mutants *dop-2* and *dop-3* were examined during 10 minutes of electrotaxis assay. *dop-3(vs106)* mutants demonstrated only a 5% ± 8 reduction in speed whereas *dop-2(vs105)* mutants demonstrated a 35% ± 7.85 reduction, showing that DOP-3 is the primary receptor mediating the inhibition of speed during electrotactic swimming.

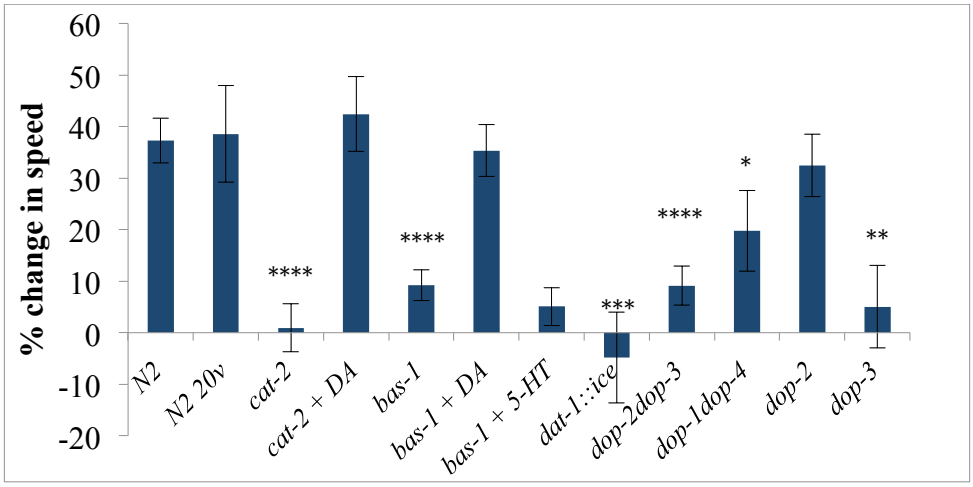


Figure 29. **Long electrotaxis assay: 10 minute electrotaxis assay for dopamine mutants, different electric field and dopamine neuron ablated strains:** N2 (n=20); N2 20V (n=23); *cat-2* (n = 20); *cat-2*+DA (n = 19); *bas-1* (n = 20); *bas-1* + DA (n = 18); *bas-1* + 5-HT (n = 20); *dat-1::ice* (n=24); *dop-3* (n=23); *dop-2*(n=16); *dop-2dop-3* (n = 17); *dop-1dop-4* (n=21).

All the electrotaxis assays were conducted at 15V except for the 20V condition. P values for Student's t-test between N2 and N2 15V = 0.91, N2 and *cat-2* = 1.43645E-06 (****p ≤ 0.0001), N2 and *bas-1* = 5.15657E-06 (****p ≤ 0.0001), N2 and *dat-1::ice* = 0.0002 (***p ≤ 0.001), N2 and *dop-3* = 0.002 (**p ≤ 0.01), N2 and *dop-2;dop-3* = 0.0004 (***p ≤ 0.001).

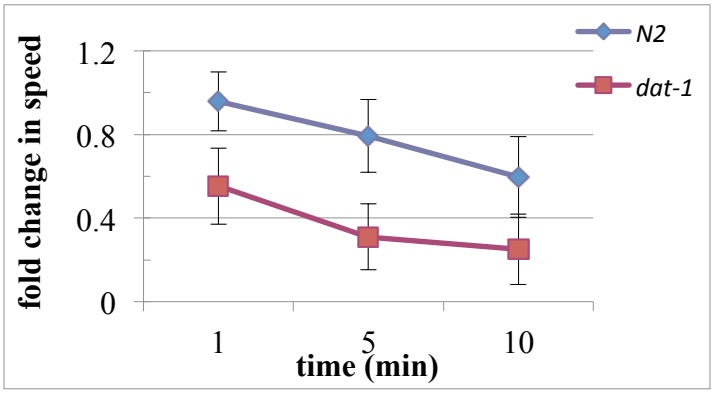


Figure 30. ***dat-1(ok157)*** **10 minutes electrotaxis assay:** All the speeds are in the order of first, fifth and ninth minute respectively. N2 (n=20), *dat-1(ok157)* (n=20).

3.3.8. Electrotaxis phenotypes of worms following treatments with the D1 agonist SKF38393 and D2 antagonist Haloperidol:

Although mutant analysis has demonstrated the role of dopamine receptor signalling in mediating the reduction in speed phenotype during prolonged exposure to an electric field, the D1 type receptor double mutant *dop-1(vs100)dop-4(tm1392)* showed a significant change in speed from the control. To determine the specificity of dopamine receptors in mediating the behaviour, a pharmacological approach involving dopamine receptor specific agonist and antagonists in N2 animals was taken. We expected to observe a lower reduction in speed of N2 animals upon exposure to Haloperidol, a known D2 receptor specific antagonist (Kimura KD et al., 2010). Different concentrations of Haloperidol (100uM, 200uM, 300uM and 500uM) were initially tested and we observed the expected phenotype at 500uM (**Figure 31A**).

N2 animals exposed to the D1-like receptor agonist SKF38393 (K.-X. and J.R., 1994) was expected to show less of a reduction in speed due to increased signalling of D1-type receptors. Different concentrations of SKF38393 were initially examined, 100uM, 300uM and 500uM, but the expected phenotype was not observed at these concentrations (**Figure 31B**). This could be because of low affinity of the agonist to the *C. elegans* receptor. However**,** we did observe the expected phenotype following co-exposure to lower concentrations of Haloperidol (300μM) and SKF38393 (300μM) (**Figure 31C**). This suggests that both D1 and D2 type receptors function together to cause a reduction in speed during prolonged exposure to an electric field.

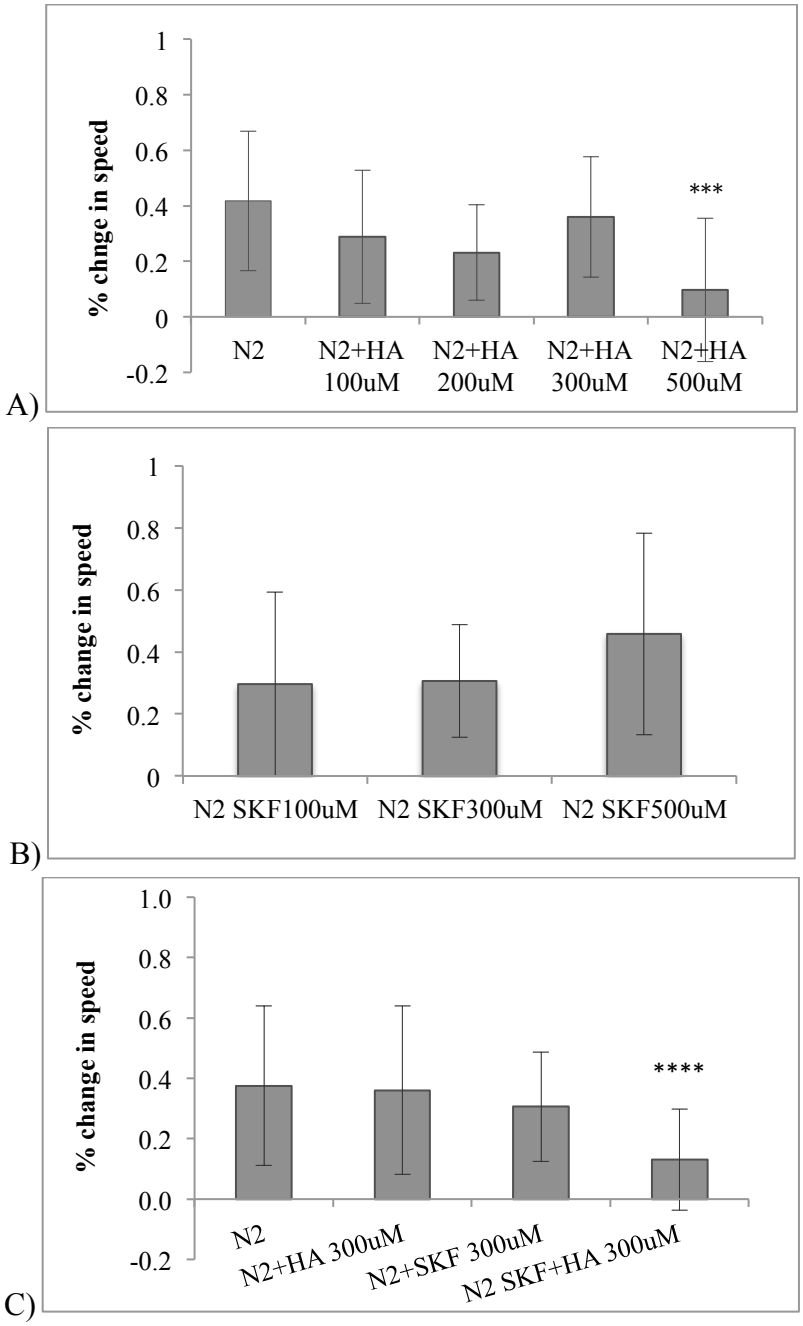


Figure 31: Agonist and antagonist treatment: A) Haloperidol(HA)/ antagonist treatment of N2: N2 n=15, 100uM n=18, 200uM n=13, 300uM n=5, 500uM n=15; B) SKF38393 /agonist treatment of N2: 100uM n=19, 300uM n=10 and 500uM n=19; C) Haloperidol(HA) and SKF38393 co- exposure of N2: N2 n=17, HA 300uM n=10, SKF 300uM n=10 and SKF+HA 300uM n=21. ANOVA test 0.0057**, Tukey's test = 0.0071.

4. DISCUSSION

4.1. DOPAMINE SIGNALLING MODULATES THE ELECTROTACTIC SWIMMING BEHAVIOUR OF WORMS

4.1.1. Electrotaxis is mediated by neuronal signalling:

We have characterized the electrotaxis phenomenon in *C. elegans* using a custom designed microfluidics device and shown the involvement of sensory and dopaminergic neurons in mediating this behaviour. The microfluidics system has allowed us to indirectly control motion inside the channel. In the absence of an electric field, worms show random swimming behaviour and cannot be guided in a desired direction. However, when exposed to the field, they move towards the cathode in a stereotypical manner with constant speed (Rezai et al., 2010). Previous findings (Rezai et al., 2010)(Gabel et al., 2007) and results presented in this paper demonstrate that this behaviour depends on the function of neurons and muscles. The electrotaxis response is highly stereotypic, reproducible, and quantifiable (Rezai et al., 2010)(Rezai et al., 2011). This makes it possible to precisely assess the impact of alterations on a worm's behaviour by observing its swimming characteristics.

We have shown that the normal electrotaxis response relies on an intact electrosensory system. In the case of *osm-5* (human IFT88 homolog) and *lin-11* (LIM homeobox family) mutants that affect amphid neurons, animals showed significantly reduced speed and frequently failed to detect the electric field polarity. While *osm-5*,

required for sensory cilia function, was reported earlier to mediate the electrosensory behaviour on an open gel surface (Gabel et al., 2007), our work has revealed novel turning and paralysis phenotypes in *osm-5* animals. This suggests that *osm-5* is needed in sensory neurons for precise sensing of electric field polarity and strength. We have also shown for the first time the important role of *lin-11* transcription factor in amphid neuron differentiation in mediating electrotaxis.

Besides *osm-5* and *lin-11*, another gene involved in electrotaxis behaviour is *trp-4*. TRP-4 protein regulates calcium entry into the cell in response to mechanostimulation and is expressed specifically in dopamine and stretch neurons (Li et al., 2006). To our knowledge this is the first report of *trp-4* playing a role in the electric field-induced movement of worms. The involvement of both *trp-4* and *osm-5* in cilia formation suggests that ciliated neurons play an important role in the electrotaxis behaviour of worms.

Our work has provided the first evidence of the involvement of DAergic neuron signalling in modulating the electric field-induced swimming of *C. elegans*. This is based on two complementary sets of experiments in the microfluidic channel device, one involving neurotoxins that damage DAergic neurons and the second utilizing mutations that affect dopamine synthesis and transport. Exposure of worms to low doses of neurotoxins 6-OHDA, MPTP and rotenone at a 25-700 mM range (for a maximum duration of 8 hours) caused electrotaxis defects without seriously impacting the growth and viability of animals. Likewise, mutations in the dopamine synthesis genes *bas-1* and *cat-1*, dopamine transporter *dat-1*, and D2-type receptor *dop-3* cause defects in the

electrotaxis assay (**see Section 4.2** for a discussion on dopamine pathway genes). Thus, in addition to investigating the genetic basis of the electrosensory response, the microfluidic platform can also be used as an effective and non-invasive tool for the detection of neuronal abnormalities.

4.1.2. The effect of neurotoxins on *C. elegans* electrotaxis:

Previous studies and our own work have shown that the neurotoxins 6-OHDA, MPTP and rotenone cause movement and other abnormalities in *C. elegans* (Braungart et al., 2004b)(Ved et al., 2005). Some of the phenotypes, such as lethality, could be non-specific and may result from exposure to high doses of toxins. Because dopamine neurons are not required for survival, the viability defect could result from disruption of other cellular processes. Consistent with this, we found that reducing toxin doses eliminated lethality.

Since worms exposed to low doses of toxins appear generally healthy and active on plates, a sensitive assay is needed to monitor dopamine signalling defects. While one could directly visualize neurons using a GFP reporter, such an approach is slow and subjective. Therefore, it is unlikely to be a sensitive measure of dopamine neuronal activity. Our work establishes microfluidic electrotaxis as a rapid and sensitive assay to monitor movement and its neuronal basis in worms. The quantification of movement parameters allows for a comparison between different groups of animals in a reliable manner and eliminates any bias associated with manual counting and judgment.

Aside from reliable quantitative analysis, our assay provides a unique opportunity to investigate some of the phenotypes caused by dopamine neuronal loss. During electrotaxis experiments we observed that toxin-treated worms often failed to coordinate the swimming of different parts of their body relative to one another. This lack of synchrony translates into an overall gait abnormality that is characterized by short, staggered movements. We did not observe such a phenotype on the plate level. 6-OHDA induced gait problems have been observed previously in the rat model but most importantly, this phenomenon relates closely with the shuffling gait that is a classical symptom of human Parkinson's disease (Cenci et al., 2002). The execution of synchronized voluntary movement requires not only proprioceptive feedback from peripheral receptors but also higher-level supraspinal processing that allows for kinesthesia (Maschke et al., 2003). Defects in kinesthetic ability in toxin-treated worms could explain the etiology behind the short, staggered movements observed during electrotaxis. The gait abnormality observed in toxin-treated worms may result from a loss of proprioceptive ability due to the loss of dopaminergic neurons.

The sudden freeze, tremor and partial paralysis phenotypes of toxin-treated worms in the channel assay may be reminiscent of bradykinesia in Parkinsonian subjects. In *C. elegans*, sinusoidal movement requires the out of phase contraction of dorsal and ventral musculature (Driscoll and Kaplan, 1997). In Parkinson's patients, the D1 and D2 class of dopamine receptors are proposed as major contributing factors to the development of bradykinesia (Korchounov et al., 2010). The corresponding receptor family members in *C. elegans*, *dop-1* (D1) and *dop-3* (D2) are expressed in the ventral cord neurons and are

primarily involved in controlling locomotion. In the electrotactic swimming assay, *dop-1* and *dop-4* (D1) and *dop-2* (D2) mutants are normal while *dop-3* is faster. These findings demonstrate the important role of D2 receptors in *C. elegans* electrotaxis and show that the microfluidics-based assay can be used to investigate the conserved mechanism of dopamine signalling in regulating the movement response in animals.

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4.2. DOPAMINE SIGNALLING PATHWAY COMPONENTS MODULATE THE ELECTROTACTIC SWIMMING BEHAVIOUR

4.2.1. Defects in dopamine synthesis, release and transport alters the electrotaxis swimming speed:

Our initial findings on the *dat-1* mutant showing a slower electrotaxis speed (see Chapter 2) provided the first genetic evidence that dopamine signalling is involved in mediating the electric-field induced swimming response. Subsequently, we examined other dopamine synthesis and trafficking genes. The *bas-1* mutant that lacks dopamine and serotonin showed a faster electric-field induced swimming speed. The treatment of *bas-1* mutants with external dopamine, but not serotonin, rescued the phenotype. The *cat-1* mutant, which is disrupted in the loading of presynaptic vesicle monoamines also showed faster electrotactic swimming. The slower speed of *dat-1* mutants and the faster speed of *bas-1* and *cat-1* mutants suggest that an optimum level of extracellular dopamine in the synaptic cleft is needed to maintain the normal electrotactic swimming speed of *C. elegans*. Inhibition of the locomotor activity in the *dat-1* mutant is similar to the SWIP phenotype, as expected. The lack of a phenotype in the *cat-2* mutant in our electrotaxis assay is similar to other locomotor behaviour assays such as the basal slow response where the worm demonstrates a reduction in body bends upon finding a bacterial lawn. The faster electrotactic swimming phenotypes of *bas-1* and *cat-1* are also similar to that of the basal slowing response, where both the mutants do not slow down upon encountering the bacterial lawn (Sawin et al., 2000)(Duerr et al., 1999). In mammals, heterozygous VMAT2 knockout mice demonstrate reduced locomotor responses (Hall et

al., 2014). Thus, our findings of dopamine synthesis, transport and trafficking mutant phenotypes in electrotactic swimming is consistent with other dopamine regulated behavioral paradigms.

4.2.2. DOP-3 receptor sinalling inhibits swimming speed:

In mammals, dopamine receptor signalling regulates vital functions such as reward processing, learning and memory formation. The D1 type and D2 type receptor functions are best studied in locomotor activity. The D1 receptors expressed in postsynaptic neurons have moderate stimulatory effects on locomotor activity. Activation of D2 type receptors in the presynaptic neurons causes a reduction in locomotor activity by reducing dopamine synthesis via a feedback mechanism, whereas activation of postsynaptic D2 type receptors stimulates locomotion. In *C. elegans*, dopamine signalling regulates locomotion such as the basal slowing response, SWIP and electrotaxis (Sawin et al., 2000)(McDonald et al., 2007)(this work). The expression of the D1 type receptors DOP-1 and DOP-3 does not overlap, except in the cholinergic neurons. DOP-1 and DOP-3 are both expressed in the head, ventral cord and tail neurons. Within the ventral cord motor neurons which innervates the body wall muscles to control locomotion, DOP-1 is expressed mainly in cholinergic motor neurons and DOP-3 in GABAergic neurons (strongly) and cholinergic motor neurons (weakly).

Signalling through the D2 type receptor DOP-3 inhibits locomotion whereas the D1 like receptor DOP-1 (co-expressed in the same cholinergic motor neurons as DOP-3) antagonized DOP-3 activity. Therefore, we expected *dop-3* mutants to demonstrate a faster electrotactic swimming speed. The faster speed of *dop-3* mutant is consistent with

the D2 type receptor function, suggesting that DOP-3 acts to lower the electrotactic swimming speed. In cholinergic neurons, the D2 type receptor signals through GOA-1, which inhibits acetylcholine release (Chase et al., 2004). The same mechanism could explain the electric field induced swimming phenotype in *C. elegans*. The D1 type receptor DOP-1 single mutant does not have any effect on locomotion, as demonstrated by the basal slowing response and SWIP (McDonald et al., 2007). The DOP-4 mutant also does not have an effect on locomotion as shown in SWIP (McDonald et al., 2007). Therefore we expected to see normal electrotactic swimming in *dop-1* and *dop-4* mutants. Our observations were according to our expectations, suggesting that these receptors have less of an effect on the swimming speed.

4.2.3. Spill over dopamine acts on DOP-4 receptor to enhance the swimming speed:

Dopamine neurons synapse with 41 other neurons and there is only one of the 58 ventral cord neurons in this set (Ezcurra et al., 2011). Thus, there is very little overlap between DAT-1 transporter expressing neurons (dopamine neurons) and receptor-expressing neurons (**section 4.2.2**), signifying that dopamine signals via some of its receptors extrasynaptically. In the *dat-1* mutant background, the synaptic dopamine clearance mechanism is disrupted resulting in abnormal receptor signalling due to excess extracellular dopamine. DOP-4 is expressed in the amphid neuron ASG and polymodal neuron AVL which acts as an interneuron as well as motor neuron (Sugiura et al., 2005b). AVL is also a GABAergic neuron and excitatory in function. In *dat-1;dop-4 double* mutant animals, impaired DOP-4 signalling causes slower electrotactic swimming speed compared to that of *dat-1* alone, suggesting the stimulatory DOP-4 signalling is opposing

the inhibitory signalling of DOP-3 in a higher extracellular dopamine environment. Thus, DOP-4 acts to enhance the swimming speed, which was not observed in the *dop-4* single mutant. This observation is in agreement with D1 type receptor function, that it signals via Gαq to enhance locomotion, and EGL-30 may be one possible downstream target that DOP-4 might interact with. Thus, this study provides the first evidence of the role of DOP-4 in locomotor activity. Further, since DOP-4 is expressed in the GABAergic AVL neuron, it implicates the possible involvement of this interneuron/motor neuron in electrotactic swimming.

4.3. NOVEL FEEDBACK CONTROL BY DOPAMINE SIGNALLING IN PROLONGED EXPOSURE TO AN ELECTRIC FIELD:

4.3.1. The electrotaxis speed of worms is reduced to half when exposed to 10 min long electric field:

Like other animals, the *C. elegans* sensory system not only responds to cues but also demonstrates complex plasticity. For instance, *C. elegans* has the ability to sense a bacterial lawn and move towards the food source and reduce its locomotion speed (Sawin et al., 2000). Similarly, *C. elegans* demonstrates locomotor plasticity during prolonged exposure to an electric field by slowing its swimming speed. Gabel et al. had hinted that the electrosensory behaviour in *C. elegans* may have evolved as a host finding strategy in parasitic nematodes (Gabel et al., 2007). Evidence suggests that certain plants generate an electric potential at the root tip, which serves as a cue to attract worms. Interestingly, certain nematodes of the genus *Steinernema* (Shapiro-Ilan et al., 2009b)*, Tylenchus* and *Pratylenchus* and other species *Panagrellus redivivus* (Caveness and Panzer, 1960) and *Heterodera schachii* (Jones, 1960) respond to electric fields. During the process of host finding and invasion, the parasitic nematodes attracted by the cues congregate around the root tips by modifying or inhibiting their movement. This mechanism prevents the nematode from leave the rhizoshphere (narrow region of the soil directly influenced by root secretion). An attractive possibility could be that the dopamine system of parasitic nematodes is involved in the inhibition of movement rate after reaching the host root (Prot, 1980).

The altered swimming response of the dopamine deficient mutant *cat-2*, which was rescued upon external dopamine treatment, suggests that dopamine is involved in mediating the slow swimming in prolonged exposure to electric fields. Exogenous application of dopamine to the *bas-1* mutant also rescued the speed defect, similar to the *cat-2* mutant.

Although the worms appeared visually normal after prolong exposure to electric field, we did not carry out experiments to specifically examine any adverse effects. Future studies should be done to test the viability, brood size, life span as well as chemosensory response. It is worth pointing out that electrotaxis experiments performed earlier by Gabel et al involved much longer periods (1-3 hours) of continuous electric field exposure to worms (Gabel et al., 2007).

The *dat-1$_p$::ICE* strain lacks dopaminergic neurons, due to genetic ablation caused by the expression of the ICE protease (Hills et al., 2004b), and thus has impaired dopamine signalling. This strain shows no reduction in speed during prolonged exposure to the electric field. Since the dopamine receptors are expressed both in cholinergic and GABAergic neurons, disruption of dopaminergic neurons could create an imbalance in the remaining neurons expressing dopamine receptors. Thus, the faster speed of *dat-1$_p$::ICE* animals may suggest higher cholinergic neuron activity during prolonged exposure to the electric field.

4.3.2. DOP-3 mediates a reduction in swimming speed:

DOP-3 appears to be a primary receptor that is responsible for the slowness in prolonged electric field exposure. The wildtype like phenotype of the other D2 type

receptor, *dop-2,* could be accounted for by DOP-2 autoreceptor function. In *C. elegans dop-2* is expressed in dopamine neurons and there could be a conserved mechanism of DOP-2 receptor signalling. In mammals, the D2-like presynaptic receptor is known to act as a negative feedback mechanism (Beaulieu and Gainetdinov, 2011). It adjusts the neuronal firing rate as well as the synthesis and release of neurotransmitter in response to extracellular neurotransmitter levels. It may be that in wildtype *C. elegans*, DOP-2 is part of a negative feedback in response to prolonged exposure to the electric field. In this model the wild type movement phenotype of *dop-2* mutants could be explained by dopamine signalling acting via normal DOP-3 receptors. This is well supported by our data of the *dop-2(vs105);dop-3(vs106)* double mutant that fails to slow down in 10 minute long electrotaxis (**Figure 29**).

As discussed above, the D2 type receptor DOP-3 appears to be a primary receptor mediating the slowness of the swimming response. A pharmacological approach was taken to further investigate the specificity of the receptors. The D2 type receptor antagonist, Haloperidol and D1 type receptor agonist SKF38393 are known compounds that specifically target the receptors (Seeman and Tallerico, 1998)(Dubois et al., 1986). Haloperidol exposure (500μM) was able to cause a reduction in the swimming speed in wild type animals, which agrees well with the *dop-3* mutant phenotype. Exposure of wild type animals to SKF38393 (500μM) had no obvious effect. This could be because the compound has a lower affinity for the *C. elegans* D1-type receptor, as most of the receptor agonists are designed to target mammalian receptors. Co-exposure to haloperidol (300μM) and SKF38393(300μM) caused less inhibition in swimming speed at the end of

a 10 minute period, suggesting that DOP-3 receptors function to modulate the locomotor behaviour in response to electric an field. This supports the mutant data that D1 type receptors act to enhance swimming speed.

4.3.3. Longer exposure to electric field may induce dopamine-mediated habituation:

Dopamine in *C. elegans* is known to involved in behavioural plasticity such as habituation to mechanosensory (tap) and odor response (Sanyal et al., 2004a). It is likely that, the reduction in speed induced by the prolong exposure to external electric field is a dopamine dependent habituation response. Since, DOP-3 is the primary receptor involved in mediating this behavior, DOP-3 signalling may affect the kinetics of habituation to the electric filed environment. DOP-3 is expressed in GABAergic and cholinergic neurons (Chase et al., 2004) and dopaminergic neurons synapsed with the glutamatergic command interneurons AVD and AVE (White et al., 1986). It may be that dopamine signalling alters one of these downstream targets to cause habituation.

4.4. CONCLUSIONS AND FUTURE DIRECTIONS:

The work described in this thesis reports a novel phenomenon of electric field induced swimming behaviour in *C. elegans* and provides the first evidence for the role of dopamine in modulating such behaviour. The observations that support these findings involve the use of a custom-designed microfluidic device to precisely control the swimming response of animals in a channel-like environment and characterize defects in the electrotactic swimming response of mutants and drug-treated animals. The electrotaxis defects in *osm-5* and *trp-4* mutants provides support to the conclusion that neuronal cilia play important roles in modulating electrosensory behaviour.

Although there is no electrosensory defect upon ablation of dopamine neurons, it can be speculated that electrical cues cause physiological changes in dopamine neurons and might cause dopamine release. This speculation could be correlated to that of the external electrical stimulation of a rat's forepaw, which increases dopamine release in the brain (Chen et al., 2012).

My work provides a foundation to investigate the cellular and molecular basis of electric field induced swimming behaviour in animals. Future studies could focus on different aspects of electrotaxis such as neuronal circuits, pathways and the physiological relevance of the behaviour. Gabel *et al.* had reported ASJ and ASH as the primary electrosensory neurons and proposed the neuronal model for manoeuvring during electrotaxis locomotion (Gabel et al., 2007). My work has shown that in addition to these two, other amphid neurons as well as dopamine neurons are important in the regulation of

electrotaxis behaviour. It is not clear *how dopamine signalling interacts with the amphid neurons*.

The identity of neurons that sense the electric field remains to be understood. The worm strain *dat-1$_p$::GCaMP* could be used to examine whether dopamine neurons are activated following electric field exposure, or whether they represent secondary targets of other amphid neurons that sense the electric field and in turn activate dopamine neurons. This approach may involve measuring global changes in the neuronal activity following electric field exposure by analyzing GCaMP expression under a pan neuronal promoter such as *rab-3*. This analysis could be done better by using a new microfluidic device that allows for the immobilization of worms while exposing them to an external electric field and focusing on neurons in order to see changes in their activity.

It is likely that the signalling pathway involved in electrotaxis behaviour involves some components from other pathways. This is demonstrated by the epithelial cells of mammalian organs that shows similarities in signalling mechanisms but not a complete overlap with chemotaxis behaviour (Cortese et al., 2014)(Zhao et al., 2006). We speculate that a similar functional overlap may exist in *C. elegans* in genes that mediate both electrotaxis and chemotaxis behaviours. Thus, examining the role of chemotaxis genes and neuronal circuits may have the potential to uncover mechanisms resulting in electrotaxis behaviour. Since a large number of genes that mediate chemotaxis are already identified (e.g., chemoreceptors, etc.) (Bargmann, 2006a), one could begin by examining the electrotactic phenotype of a subset of these genes. Some of the amphid neurons, e.g., AFD, the primary thermosensory neuron (Chung et al., 2006), could also be involved in

regulating electrosensory behaviour. Therefore, it is possible that pathway components may be common between electrotaxis and thermotaxis. In the future, examination of genes mediating thermotaxis, e.g., *eat-16* (RGS), vesicular glutamate transporter *eat-4*, calcinurin A *tax-6* and guanylate cyclases *gcy-8, gcy-18, gcy-23* (Kimata et al., 2012), in electrotaxis behaviour may provide useful information.

Swimming demonstrates age dependent activity as younger worms thrash more than older ones (unpublished observations). During electrotactic swimming, younger worm swim faster than the older worms (Rezai et al., 2010)(from experience in the lab). This could be due to factors such as declining muscle strength and a decrease in neuronal activity, which may be the consequence of normal aging. Thus, *it can be hypothesized that aging associated genes affect electrotactic swimming activity*. Candidate genes that affect aging may be tested for their role in the electrotactic swimming response. Further, the role of such genes could be tested by localizing their expression in specific cell types, e.g., dopamine neurons and muscles or using heterologous promoters. These findings could help in understanding the molecular mechanisms of electrotaxis behaviour.

Another interesting direction to explore could be alterations in electrotactic speed following prolonged exposure to the electric field stimulus. An exposure of 10 min to a DC field reduces the swimming speed, a response mediated by dopamine signalling. It may be caused by either some kind of negative feedback mechanism to fine tune the movement response, or fatigue. Fatigue due to continuous physical activity is traditionally attributed to the occurrence of a metabolic end point. In humans, fatigue is linked to exhaustion of the peripheral system (muscle) or due to the involvement of the nervous

system (termed "central fatigue")(Davis, 1995). Neurotransmitters such as serotonin, dopamine and/or adrenaline are linked to changes in fatigue. An increase in serotonin and its interaction with dopamine has been observed in fatigue (Meeusen et al., 2006). In one study, fatigue has been shown to be associated with reduced connectivity between mesocorticolimbic structures(Finke et al., 2014)(Dobryakova et al., 2015). A similar observation has been made in working memory study (Dobryakova et al., 2015). Modulation of cognition behaviour by dopamine is explained by the gating hypothesis, which suggests that too much dopamine shuts down excitatory input from the glutamate neurons, while too little dopamine allows interference between different inputs (neurotransmitters)(Dobryakova et al., 2015). Furthermore, several studies have shown that too little dopamine is associated with higher degrees of fatigue (Dobryakova et al., 2015), however *there is no evidence demonstrating sustained activation of dopamine signalling contributing to fatigue*. My work suggests that dopamine may play a role in modulating the electrotactic swimming response during prolonged exposure to an electric field in *C. elegans*. This model could be used to understand the role of dopamine in exercise and fatigue. To validate the paradigm, key molecules associated with fatigue can be measured. One such molecule is the mitochondrial enzyme CoQ10 (WBGene00020093 in *C. elegans*)(Filler et al., 2014). Additionally, the CoQ10 model in worm could be used for further investigation of the mitochondrial role in relation to fatigue. These experiments may address key questions such as *how mitochondrial dysfunction relates to fatigue.* Further testing of the electrotactic swimming response of animals treated with pharmacological agents such as methylphenidate, a dopamine

transporter blocker that mimics high dopamine conditions, may provide useful hints to this paradigm.

Thus the study of electrotaxis behaviour has broad reaching applications, allowing for the analysis of sensory-motor, neurodegenerative, muscular and aging dependent phenotypes.

5. List of publications resulting from this and other work.

1. ***Salam, S.*** *et al*. Neuromodulatory role of Dopamine in mediating electrotactic induced slow swimming in C. elegans. (manuscript in preparation)

Peer reviewed Journal articles:

2. Tong J, Rezai P, **Salam S**, Selvaganapathy PR, Gupta BP. Microfluidic-based electrotaxis for on-demand quantitative analysis of Caenorhabditis elegans' locomotion. J Vis Exp. 2013 May 2;(75):e50226. doi: 10.3791/50226. PMID: 23665669; PMCID: PMC3670555.

3. **Salam S**, Ansari A, Amon S, Rezai P, Selvaganapathy PR, Mishra RK, Gupta BP. A microfluidic phenotype analysis system reveals function of sensory and dopaminergic neuron signaling in C. elegans electrotactic swimming behaviour. Worm. 2013 Apr 1;2(2):e24558. doi: 10.4161/worm.24558. Epub 2013 Apr 18. PMID: 24058875; PMCID: PMC3704449.

4. Rezai P, **Salam S**, Selvaganapathy PR, Gupta BP. Electrical sorting of Caenorhabditis elegans. Lab Chip. 2012 Apr 24;12(10):1831-40. doi: 10.1039/c2lc20967e. Epub 2012 Mar 30. PMID: 22460920.

5. Rezai P, **Salam S**, Selvaganapathy PR, Gupta BP. Effect of pulse direct current signals on electrotactic movement of nematodes Caenorhabditis elegans and Caenorhabditis briggsae. Biomicrofluidics. 2011 Dec;5(4):44116-441169. doi: 10.1063/1.3665224. Epub 2011 Dec 15. PubMed PMID: 22232698; PMCID: PMC3253587.

6. Pouya Rezai, **Sangeena Salam**, P. Ravi Selvaganapathy, Bhagwati P. Gupta. Microfluidic systems to study the biology of human diseases and identify potential therapeutic targets in *C. elegans.* 2011, Book: Integrated Microsystems: Electronics, Photonics and Biotechnology" CRC Press; (p581-604)

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