Developmental Perturbation in Early Embryogenesis Persist to Impair Neuronal Function in Adults

By

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DEDICATION

To my parents and my sister who have been through it all with me. Thank you!

Hulu Bersu Hone !

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To my parents, thank you for your never-ending love and unwavering support. I would be nothing without you. To my sister, you make my world go around. Thank you for your support and love. The rest of my family and friends that have become my family, thank you! You have filled my life with love and joy. Dr Dhillon thank you for always believing in me. Without your support I wouldn't be here today.

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PART I

ABSTRACT

Animal body plans tend to display external symmetry; however, their internal organs can be anatomically and/or functionally asymmetrical. Visceral organs such as liver, heart, and pancreas show clear anatomical asymmetry in a bilateral fashion. The right and left cerebral hemispheres are functionally asymmetric and small deviations have been correlated with pathologies such as schizophrenia and bipolar disorders. The bilateral asymmetry is genetically and developmentally defined as a third axis as are the other two axes: anterior/posterior and dorsal/ventral. The lab model *Caenorhabditis elegans* is particularly suited to study left/right (L/R) asymmetry. Like most other animals, *C. elegans* shows predominantly bilaterally symmetric external anatomy, but clear bilateral asymmetry in the viscera, a key feature being the placement of the anterior gonad towards the right. In addition to anatomical asymmetry certain neuronal pairs such as AWC-L and AWC-R also display functional asymmetry. The anatomic bilateral asymmetry is discernible during the initial cell divisions of the fertilized egg. Previous studies have suggested that PAR proteins along with Gα proteins associated with spindle positioning that play a role in anterior/posterior and dorsal-ventral are are likely to underlie the first symmetry–breaking step as well. The absence of *gpa-16,* a Gα protein, has been shown to yield up to 50% sinistral worms. On the contrary, wild type N2 animals invariably lead to dextral embryos. We have investigated the direct effects of disrupted asymmetry on embryonic lethality and adult behavior. Here, we show that the absence of *gpa-16* results in not only sinistral embryos but also randomly dividing embryos. Surviving adults with the *gpa-16* mutation are impaired in both, associative and non-associative learning. We are examining if the reversed asymmetry manifests its functional effects on behavior due to potentially atypical

neuronal circuitry and looking at synaptic connectivity of *gpa-16* mutants with the goal of unraveling anatomically atypical circuits.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

ABBREVATIONS

α - Alpha

β - Beta

δ - Gamma

µl - MircroLiter

AP – Anterior-Posterior

BPA – Bisphenol A

BPS – Bisphenol S

C. elegans – Caenorhabditis elegans

CGC - *Caenorhabditis elegans* Genetics Center

C.I – Chemotaxis Index

del – Deletion

DV – Dorsal- Ventral

E_2 – Estradiol

EDC – Endocrine Disrupting Chemicals

ER – Estrogen Receptor

G_α – G alpha subunit

$G_{\beta\delta}$ – G betta-gamma complex

LR – Left Right

mM – Millimolar

MRI – Magnetic Resonance Imaging

NaCl – Sodium Chloride

NGM – Nematode Growth Media

OP50 – *E. coli* Strain

PAR – Partition proteins

PCR – Polymerase Chain Reaction

ppm – Parts per million

t.s – Temperature sensitive

CHAPTER I: INTRODUCTION

Our research lab located at Delaware State University focuses on understanding the developmental basis of behavior. Specifically, my research has focused on the Gα protein GPA-16 and understanding its role in asymmetric cell division and learning and memory. I also looked at how early exposure to environmental toxins affect fecundity and learning and memory in adult worms

1.1 Brief Background

Most animal body plans tend to display external symmetry. This asymmetry is initiated as early as the first cell division and maintained through life. Viscerally, most animals display both structural and functional asymmetry. The brain is the hallmark example of functional asymmetry. This is exemplified through the commonly observed dextral bias in the population (90-95% of people are right handed). Disruption of this asymmetry has been shown to result in various atypical behaviors including disorders such as schizophrenia.

Asymmetric cell division is observed among different animal models. It is the result of carefully orchestrated events during the very early stages of development. Even though several model organisms have been used to study asymmetric cell division, the nematode *Caenorhabditis elegans* is one of the most suitable organisms to study asymmetry at the cellular and genetic levels. In *C. elegans*, anatomical bilateral asymmetry is discernible during the initial cell division of the fertilized egg. Previous studies have shown that PAR (partition) proteins along with (a) Gα protein(s) associated with spindle positioning play key roles in the first symmetry-breaking step.

C. elegans , a nematode is a preferred model for studying asymmetric cell division and laterality. Specifically its clear body makes it possible to study laterality at various stages of development.

1.2 Purpose of the Study

Previous studies have shown that spindle positioning is critical for asymmetric cell division. In *C elegans*, the Gα protein GPA-16 has been shown to be play a role in spindle positioning. Although in temperature sensitive *gpa-16* mutants, a 70% lethality rate has been reported, the work did not examine the non-viable embryos. The first goal of my research was to comprehensively examine both deletion and temperature sensitive *gpa-16* embryos and adult worms. The second goal of my research was to examine the direct effects of disrupted asymmetry on adult behavior and neuronal connectivity.

1.3 Research Questions

- GPA-16 plays a significant role in asymmetric cell division. The absence of functional GPA-16 causes embryonic lethality and high number of sinistral embryos. However, not much is known about embryos that are neither dextral nor sinistral. In addition, it is not clear if these embryos survive into adulthood. *C. elegans* was used as a model to study disrupted asymmetric cell division in both embryos and adults caused by the absence of functional GPA-16.
- Various factors affect behavior. So far, no work has been done to study the effects of disrupted asymmetric cell division (during the very early stages of embryogenesis) on adult *C. elegans* behavior. We used associative and non-associative tests in order to

investigate any aberrant behavior caused as a result of disrupted asymmetric cell division. In addition, we used confcal microscopy to investigate if there is atypical neuronal connectivity in *gpa-16* worms

1.4 Importance of the Study

Asymmetric cell division is required for creating and maintaining structural and functional asymmetry in living organisms. Anatomically, the two different hemispheres of the human brain are grossly symmetrical. However, functionally, they are asymmetrical. In patients with schizophrenia and dyslexia, brain images have shown less asymmetry in the planum temporale. Loss of asymmetry has also been associated with different forms of cancer. Therefore, it is crucial that we understand the basic molecular mechanisms that regulate asymmetric cell division in order to generate more effective forms of treatment and/or ways to better manage these diseases.

CHAPTER II: REVIEW OF THE LITERATURE

2.1 Laterality

Bilateral symmetry is very common among biological organisms, whether it is the radial symmetry of starfish or the bilateral symmetry of humans. However, once we get past the external symmetry, these living beings tend to display visceral asymmetry-both structurally and functionally. Structurally, major organs, both paired (lung, ovaries, kidneys, etc) and unpaired (heart, stomach, liver etc.), are positioned asymmetrically. Although the direct benefits of an asymmetric body plan is not known, researchers have proposed that in paired organs, asymmetry increases complexity that can be achieved with division of labor, compartmentalization, and specialization (1). In addition, the asymmetric placement of organs may have physiological advantages since it increases compaction form coiling and maximizes surface area (2) . Functionally, these major organs can also be asymmetrical. Perhaps the best example for functional asymmetry is the human brain.

Despite the apparent structural symmetry of the cerebral hemispheres, the brain is functionally asymmetrical. Language, speech, logic, and analysis are predominantly regulated by the left hemisphere, while spatial abilities like face recognition are localized in the right hemisphere (3).

2.2 Handedness, Language, and Brain Laterality

Since the 1800s, it has been known that the brain exhibits functional laterality via the localization of speech and language. The French neurologist Marc Dax is credited for being the first to suggest that damage to the left hemisphere results in speech disorders. A few decades

later, another French physician, Paul Broca, showed that patients with aphasia have damage in the left hemisphere, further proving that the brain's "language center is situated in the left hemisphere" (4). A century later, with the aid of improved technology, Roger Sperry's lab split-brain experiments have shown the brain's functional lateralization (1, 5, 6).

Additional studies have shown that the lateralization of speech areas strongly corresponds to the handedness of a person (7). Although there is no universal definition for handedness, some researchers define it as the hand one prefers to use while some define it as the hand that performs more precisely on manual tests (8, 9). More than 90% of the world's population is right handed with the number being slightly lower in western countries, where left-handedness is culturally accepted (3, 10). In overwhelming majority of right-handed people, the left hemisphere is the language-processing center. This exhibits the link between handedness and brain laterality. In fact it has been shown that the stronger right handedness is expressed in a person, the more likely it is that language is represented in the left hemisphere (11). However, 5-6% of right-handers show right hemisphere language dominance compared to 30-35% of left handers (12).

About 1% of the world's population shows mixed handedness where frequent change of hand preference is exhibited in between tasks (13) but surprisingly, natural ambidexterity is extremely rare. In some cases, ambidexterity can be taught allowing the individual to perform tasks with equal preference for each hand (14). Only 1 in ~20,000 individuals are known to display *situs inversus totalis in which there is* reversal of visceral asymmetry of the lateral axis as well as reversal of functional cerebral asymmetry (15).

In the past, several studies have been done to better understand the purpose of left-handedness and brain lateralization in general. The fact that brain lateralization is conserved or independently acquired during evolution suggests that it is necessary and has its own benefits

(16). Indeed when this asymmetry is disrupted, the possibility of increased chances of various neurological disorders like dyslexia, autism and schizophrenia are exhibited(10, 17).

Although left-handed individuals make up less than 10% of the world population, researchers have shown higher rates of psychosis and other disorders in left-handed individuals. It has been observed that 40% of individuals with schizophrenia are left-handed (10, 18). Furthermore, magnetic resonance imaging (MRI) studies have shown that patients with schizophrenia show less asymmetry in the planum temporale (19, 20). The same phenomenon was also seen in dyslexic individuals. Although right handed individuals have slightly larger planum temporale in the left hemisphere, MRI stuides of dyslexic children show same sized planum temporale in both hemispheres (16).

Some studies have indicated that brain lateralization might be sex dependent. While females have been shown to use both sides of the brain during language related tasks, males show an increased level of laterality (11, 21). However, this finding remains highly controversial.

Despite the research being done and the reported consequences of disrupted asymmetry on various neurological disorders, the biological mechanisms responsible for establishing and maintaining lateralization and asymmetry remain poorly understood.

2.3 Laterality in Animal Models

The study of asymmetry and laterality raises many different questions, including whether it is a common feature in the animal kingdom. As reviewed by Michael Levin, asymmetry is seen in both vertebrate and invertebrate models including but not limited to mollusks, sea urchins, zebrafish, flatfish, *C. elegans , Xenopus,* chicks, mice and rabbits (22).

Although the development of asymmetry during development varies from one model organism to another, it almost always begins shortly after gastrulation. The Left-Right (LR) axis is probably specified after the Anterior- Posterior (AP) and Dorsal- Ventral (DV) and is determined with respect to them (22). In the case of *Xenopus* and chick, the LR axis is established very early through gap junction dependent cell communication. In other species such as mouse and zebrafish, asymmetry in the LR axis is propagated, reset or initiated at the gastrulation stage (16).

Ever improving neuroimaging and molecular techniques such as genome-wide analysis have made it possible to study various factors associated with brain asymmetry in humans (16). However, it is still impossible to use humans to study the cellular and molecular mechanisms associated with the development and the initiation of laterality. Considering the well-conserved nature of laterality among various model organisms, it is beneficial to use these model organisms to gain insight into the development and the function of laterality.

2.4 C. elegans as the ideal model organism

Ever since the description of *Caenorhabditis elegans* (*C. elegans*) as a potential multicellular lab model in 1974 by Sydney Brenner, the worm has been widely used for biological research. *C. elegans* is a transparent, soil dwelling, nematode that feeds on bacteria. Their entire genome of 100.3Mbp was the first completely sequenced genome of a multicellular organism in 1998 and it has been estimated that it has 19,735 protein coding open reading frames(23).

C. elegans has two sexes, hermaphrodite and male. Although hermaphrodite is the predominant sex form, males compromise 0.05% of the population. After the hermaphrodites

lay the eggs, it goes through four cycles (L1-L4). The normal life cycle of *C. elegans* lasts for approximately 3 days when grown at 20°C making it the ideal model for studying development and the aging process. On average, each hermaphrodite lays about 300 eggs. In the laboratory, the organism is maintained on solid agar with *E.coli* as a food source.

The adult hermaphrodite contains 959 somatic cells and the developmental fate of each of these cells has been completely traced. *C. elegans* has a simple and compact nervous system, containing 302 nerve cells, which have all been identified and whose connectivity has been determined (24). More importantly, its clear and transparent body and eggs make it possible to use various techniques of imaging to study cell division and other aspects of development in living embryos.

In addition to the favorable laboratory traits mentioned above, mutant *C. elegans strains* can be easily acquired from The *Caenorhabditis* Genetics Center (CGC). WormBase and WormAtlas a *C. elegans* online database, also provides extensive information about the *C. elegans* genome, neuro-anatomy, and development. It is available for scientists to consult and use for research studies (25).

2.5 Laterality in *C. elegans*

Asymmetric cell division plays a significant role in generating and maintaining cellular diversity. Previously, it was believed that mitotic cell division gave rise to two identical daughter cells. However, various studies have shown that this is not the case. Even though various model organisms have been used to study asymmetric cell division, *C. elegans* remains one of the widely studied organisms. In addition, *C. elegans* is especially useful when studying neuronal laterality. Although mostly bilaterally symmetric, the C. elegans' nervous system displays a

variety of bilateral asymmetry (26). It also has also visceral asymmetry, which proves crucial when studying laterality in adult animals. In addition, previous studies have shown that wild type worms are always dextral (27). This mirrors the dominance of right-handedness seen in humans making them an idle model organism for studying laterality.

In *C. elegans*, asymmetric cell division starts as early as the first cell division. During the first few divisions, the three principal axes (AP, DV and LR) are established (28). Initially, the sperm enters the egg from the opposite side of the oocyte. It is important to note that the sperm entry point establishes the posterior portion of the embryo. However, it does not serve as a bilateral symmetry-breaking cue (28, 29). Once the sperm and oocyte come together, the whole cortex undergoes surface contractions. The acto-myosin contraction causes the cytoplasm to move to one pole, leading centrosomes to form physical contact with the posterior cortex. This breaks the symmetry of the oocyte and initiates AP polarity establishment (Figure 1) (28). The establishment of this polarity initiates the polarized distribution of a group of proteins present in the zygote called the PAR proteins (par titioning-defective), which are a conserved group of proteins that function in establishing cell polarity during development (30). Further contraction causes the PAR proteins to be distributed asymmetrically (Figure 2).

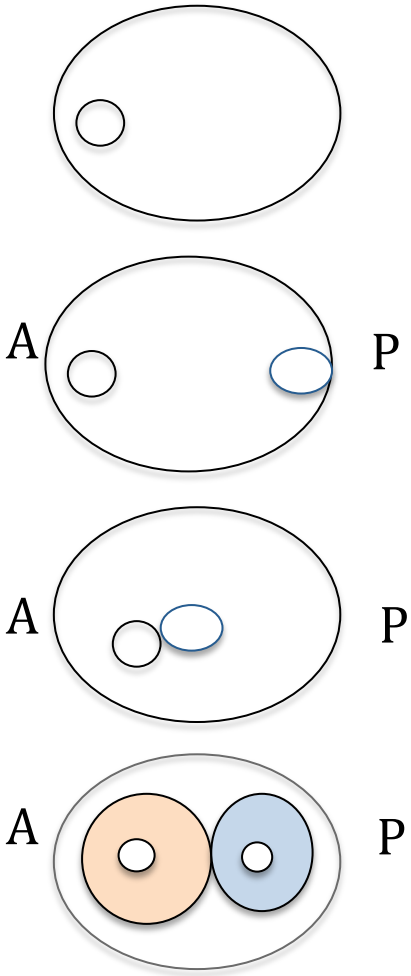


Figure 1: First symmetry breaking point: the sperm (blue circle) enters the embryo establishing the posterior end of the embryo. The oocyte (black) and the sperm (blue) then come together initiating the first symmetry breaking cell division. This results in two asymmetric daughter cells

During the one-cell stage, PAR-3 and PAR-6 proteins are found in the anterior cortex. Meanwhile, the PAR-2/PAR-1 complex moves towards the posterior pole while PAR-4 and PAR-5 are distributed equally. While the previously stated movements are taking place, the centrioles are duplicating. Once the migration is completed, the two pro-nuclear complexes come together along with the newly duplicated centrosomes. After reaching the center, they rotate 90°C to align with the anterior –posterior (AP) axis. The presence of PAR-1 in the posterior

pole (P) prevents the zing fingers MEX-5/6 from accumulating in the posterior pole. In turn, the presence of MEX-5/6 in the anterior pole prevents P granule formation. These steps ensure the establishment of polarity (28-32).

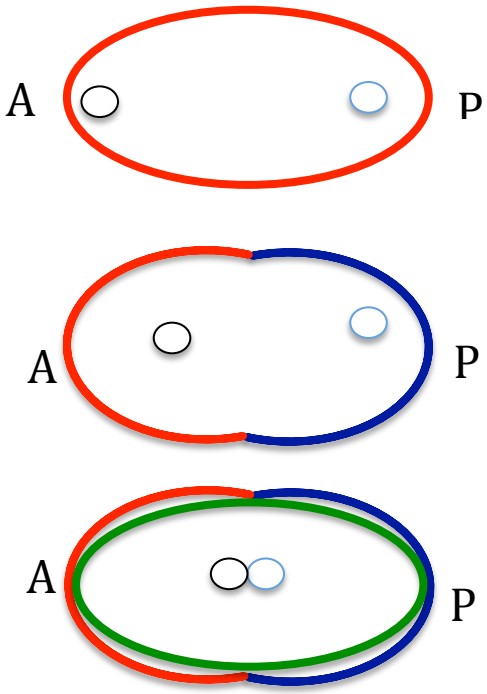


Figure 2 Distribution of PAR proteins**:** PAR-3 and PAR-6 are represented by the red color, blue represents PAR-1 and PAR-2 while PAR-4 and PAR-5 are represented by the color green; A= anterior P= posterior. Sperm entry point establishes (p), As a result, PAR proteins start to be distributed unevenly. PAR-3 and PAR 6 (red) become enriched in the anterior cortex while PAR-1 and PAR-2 (blue) become enriched in the posterior cortex. PAR-4 and PAR-5 (green) are distributed equally.

Spindle positioning plays a significant role in making sure that everything is segregated to daughter cells accordingly. Although not much is known about the spindle positioning mechanism, studies have shown that it is highly dependent on PAR proteins (33). Although the direct mechanisms of how PAR proteins regulate polarity remains unclear, it has been suggested that heterotrimeric G-proteins play a role (27, 34). RNAi work done by the Ahringer lab showed that asymmetric spindle elongation in the one cell embryo is dependent on the G-proteins GOA-1

and GPA-16 (35). When goa-1 and gpa-16 are inhibited, symmetrical cell division occurs. Based on amino acid sequence, GOA -1 belongs to Gαi/o group. However, GPA-16 doesn't belong to any of the known G-protein families but is closely related to Gαo (36).

2.6 GPA-16 in asymmetric cell division

C. elegans gpa-16 gene is involved in spindle and centrosome orientation that plays a role in the determination of handedness (27). A temperature-sensitive *gpa-16* mutant allele (*it143*), when reared at non-permissive (25° C), about 70% of the embryos are unviable, but of the survivors, 40% of those are sinistral. When reared at the permissive temperature of 16°C, about 98% of the embryos are viable and of that percentage, a negligible amount are sinistral (27) Previous studies have clearly shown the reversed spindle orientation of sinistral animals during their 4-6 cell developmental stage. The 70% embryonic lethality observed at non-permissive temperature has not been examined, though it may hold a clue to genetic mechanisms governing L/R establishment.

In addition, the inactivation of $G_{\beta\gamma}$ has also been shown to produce spindle-positioning defects in one-cell embryos (28, 35, 36). Surprisingly, the loss of Gα activity completely suppresses the spindle positioning defects seen in $G_{\beta\gamma}$ mutants, suggesting that $G_{\beta\gamma}$ plays a significant role as the negative regulator of the Gα proteins (28)

Although various studies have investigated the mechanisms of asymmetric division, the field remains unexplored. More importantly, the direct effect of disrupted asymmetric cell division (during early embryogenesis) on adult behavior remains unknown.

2.7 Asymmetric cell division and neuronal laterality

Although most of the neurons in *C*. elegans exhibit bilateral structural symmetry, few GABAergic neurons are known to be structurally asymmetric. RIS and AVL (both GABAergic) are expressed only in one part of the worm. Functionally, the chemosensory neurons ASE and AWC exhibit asymmetry. Work done by Johnston and Hobert identified *lsy-6* as the first microRNA that has a role in neuronal patterning through a loss-of-function mutation where ASEL expression is lost, causing a gain of ASER (37)

In *t.s gpa-16* worms, it has been shown that reversed laterality also causes reversal in the left and right ASE gustatory neurons, ASEL (ASE-left) and ASER (ASE-right) (27, 38). This study found that it is only the sidedness of the ASE neurons that are dependent upon *gpa-16* and not the function of the ASE lineages on the left or right side. A link has been established between the AP and LR axes in which an "asymmetry mark" is present during AP axis development used to control postmitotic neuronal asymmetry, acting as a prepatterning mechanism. The "asymmetry mark" may trigger a cascade of transcription factors required for a specific stage or initiate a heritable epigenetic mark, possibly on the chromatin level in the ASEL or ASER cell lineages (38). This neuron pair provides a link between asymmetrical gene expression and functional lateralization (37, 38). This provides headway for the possibility that very early patterning during embryogenesis may provide a blueprint for later LR development (38)

The olfactory AWC neuron pair is also affected by asymmetrical cell division. In *C. elegans*, there is a specific mechanism that controls the initial determination and management of the olfactory neuron identities, AWC^{ON} and AWC^{OFF}, which have similar morphologies, but different functions and patterns of gene expression. This neuron pair is identified as the right or left AWC neuron, where the neuron would be found 'on' on the left side and 'off' on the right.

In *C. elegans*, the identification of each neuron depends on the transmembrane protein NSY-4 and the embryonic gap junction network created by NSY-5, which induces one AWC to switch from the AWC^{OFF} identity and become AWC^{ON}, which subsequently regulates the stabilization of the AWC^{OFF} identity (39).

2.8 Learning and Memory

Many organisms are born with some essential innate information they need to survive already hardwired in their brain. Human babies are born with limited information required for survival, and most of our new skills are acquired through experience. Learning is generally defined as the process of acquiring new knowledge or modifying the knowledge that already exists (40, 41) . In order to survive and reproduce, living things must not only learn to adapt to their ever-changing environment, but they must also store the information gathered for future recall. This process of storing and recalling information is known as memory.

In non-associative learning, the simplest form of learning, the organism's response towards a harmless but repeated stimulus, is tested (40). Habituation and sensitization are classified as non-associative learning. During habituation, the organism is exposed to a repeated stimulus. Exposure to this harmless stimulus will result in reduced response towards the stimulus. This phenomenon explains why a person learns to ignore the sound of a pendulum clock after a few minutes of exposure. However, in sensitization, the organism exhibits an increase of attention towards the stimulus. In the more complex associative learning, the animal learns to associate one stimulus with another (40). Perhaps the most famous example of associative learning was presented by Ivan Pavlov, in which dogs were trained to associate the

sound of a bell with food. Whenever the dogs heard the sound of the bell, they salivated in anticipation of food.

Eric Kandel using the marine sea slug Aplysia californica studied the details of habituation. Stimulus was delivered to the siphon resulting in the withdrawal of the siphon and gills. After repeated exposure to this stimulus, the organism eventually stopped withdrawing. This experiment indicated that repeated stimulation causes excitatory interneurons to produce weaker synaptic potentials, thereby causing the motor neurons to drastically reduce and eventually stop their firing This decrease in synaptic potentials is caused by reduction of the number of transmitter vesicles released into the synapse (40)

C. elegans, behavior is strongly influenced by changes in the environment. As a result, it has the ability to associate change in the environment with the presence or absence of food. Its ability to learn through association makes *C. elegans* an ideal organism to study the molecular changes that take place during learning and memory.

C. elegans' ability to learn can be tested through habituation (non-associative learning) and chemotaxis (associative learning). Administering repeated mechanical tap stimuli could test habituation by counting the number of taps it takes for the animal to stop responding. Associative learning, such as classical conditioning and differential conditioning, is tested by using assays in which worms are conditioned to specific chemicals that are paired with the presence or absence of food and then assaying the worms for changes in chemotactic response towards the conditioned chemical (42-44)

2.9 Asymmetry and learning and memory

Behavioral consequences of laterality have also been studied in another invertebrate experimental model *Drosophila* is also used to study laterality. *Drosophila* has an asymmetrically placed brain structure near the fan body which connects the right and left hemispheres. Studies have shown that in few wildtype animals, this structure is found symmetrically. Animals with this symmetry have been shown to have a lack of long-term memory, demonstrating that asymmetry is important for the formation and revival of long term memory (45). Functional laterality and its correlation with learning and memory was also shown in honey bees. During olfactory learning, the left hemisphere was shown to be responsible for long term memory while learning and short term memory was controlled by the right hemisphere (46). Based on these findings, we also anticipate learning and memory defects in worms with reversed laterality.

Hypothesis

We hypothesized that *gpa-16* mutant worms with atypical laterality will have atypical neuronal connectivity and thereby resulting in aberrant behaviors.

CHAPTER III. MATERIALS AND METHODS

3.1 Strains

All *C. elegans* were obtained through the Caenorhabditis Genetic Center, University of Minnesota, Minneapolis, MN. The strains N2 (wild type Bristol isolate), RB1816 *gpa-16 (ok2349),* BW1809 *gpa-16(it143); him-5 (e1490)*, DR 466 *him-5 (e1490)*; OH3192 *gcy-5::GFP (ntIs1)* and OH7193 *otIs181;him-8 (e1489)* were used throughout the study.

3.2 Maintenance

Worms were grown on nematode growth media (NGM) plates as described in (Brenner, 1974; Hope, 1999). The *Escherichia coli* strain OP50 was used as a food source. Depending on the strain, worms were raised at 15°C, 20°C or 25°C

3.3 Embryonic videos

A small drop of M9 was place in the center of a glass-bottomed dish(MatTek Corporation, P35-G-0-14-C). Then 3 well-fed adult worms with visible eggs were placed in the M9 drop . Under dissecting microscope worms were cut in half using hypodermic needles (KENDALL MONOJECT 1ml 27G x ½") as shown in figure (3). One-cell embryos were then chosen and observed under Olympus IX71 DIC microscope. Using Metamporph (version 7.8.3.0 , Molecular Devices Corp, Sunnyvale, CA) time lapse images of the dividing embryos were obtained

3.4 Adult laterality

Using a dissection microscope, well fed (8-10) 3 days old worms with single rows of eggs were picked and transferred to a glass bottom dish with small drop of M9 and sodium azide. A Drop of DAPI (Prolong ^{TM} Gold antifade reagent with DAPI, Invitrogen P36941) of placed before

transfering the plates into the confocal microscope (Olympus Fluoview TM FV10i). Z- stack images (approximately 36 slides) were taken.

3.5 Behavioral Assays

For each experiment, well fed, three-day old synchronized young adult worms were used. Special attention was given to make sure that the worms were not over crowded or starved.

3.5.1 H*abituation assays*

In preparation for the assay both non-seeded and seeded Nematode Growth Media (NGM) plates were prepared fresh the night before the assay and left overnight at room temperature. Approximately 10 worms were transferred to the new NGM plates. Using an eyelash hair, the worm was tapped on the head. In response to this stimulus, the worms typically move backwards. The number of times the animal moves backward until it no longer responds to the stimulus was counted.

3.5.2 *Chemotaxis assay*

chemotaxis plates were prepared the night before the assay and kept at room temperature for 1 hour before use as described by (Bargmann et al, 1991). Animals were collected with M9 buffer and washed two more times before the assay. For conditioning, the animals were exposed to 3 µl of isoamyl alcohol for 90 minutes. In order to immobilize the animals 2µl of 1M sodium azide (NaN_3) was placed on the trap and gradient points 10 minutes before the start of the assay. Worms were placed at the starting point equidistant to both the trap and gradient points. 1/1000 isoamyl alcohol diluted in 100% ethanol was placed on the gradient while 100% ethanol was placed on the trap point. Plates were left undisturbed for one hour and then put at - 10°C for 10 minutes. Chemotaxis index was calculated by subtracting the number of worms at the trap point

from the number of worms found at the gradient point and dividing it by the total number of worms found on the plate. Worms located at the starting point were excluded from counting, as these worms were most likely dead or severely injured during the washing process.

3.6 Generating Males

Five well-fed L4 worms transferred to NGM plates seeded with OP50. They were then heat shocked at 34º C for 4 hours. After 2-3 days, three males were picked and transferred to a plate with at least two hermaphrodites.

3.7 Generating Mutant Strains

Five *gcy-5::GFP* males were transferred and allowed to mate with one *gpa-16* hermaphrodite. Once the progenies become young adults, 5-10 worms were transferred to a new plate and allowed to lay eggs (1 worm per plate). After laying eggs, a single hermaphrodite worm was removed and its mutation was confirmed by PCR. Same procedure was followed when generating *OH7193;gpa*-16:: *gcy-5::GFP* strains

3.8 Single Worm PCR

DNA from a single worm was extracted using proteinase K. 1 µl 20mg/ml proteinase case was dissolved with 95 µl 1x PCR buffer. Each worm was lysed in 5µl proteinaseK-buffer solution. After freezing and thawing for five minutes, worms were lysed in the PCR machine using the following setting.

65˚C for 60 min

95˚C for 15 min

Once DNA was extracted, PCR was used to amplify 3.3kb N2 and 1.6kb of the coding regions of *gpa-16.* The following primers were used:

Fwd: 5' – AGC GAA ACG AAFG ATC CAA GA-3'

Rev: 5'-ATT CGT GAT CGA GTG TGG TG- 3'

The following settings were used for PCR:

1. 95°C, 2 min
2. 95°C, 30 sec
3. 55°C, 30 sec
4. 72°C, 4 min
5. Steps 2, 3 and 4 repeated for 31 cycles.
6. 72°C, 5 min

For *gcy-5::GFP,* PCR was used to amplify 650bp of the GFP coding regions. The following primers were used:

Fwd: 5'- GTC AGT GGA GAG GGT GAA GG- 3'

Rev: 5'- TTG AAC GCT TCC ATC TTC AAT-3'

The following settings were used for PCR:

1. 95°C, 2 min
2. 95°C, 30 sec
3. 55°C, 30 sec
4. 72°C, 1 min
5. Steps 2, 3 and 4 repeated for 34 cycles.
6. 72°C, 5 min

PCR products were confirmed by gel electrophoresis.

For *mCherry* amplification in OH7193*,* PCR was used to amplify 300bp of the *mcherry* coding regions. The following primers were used:

Fwd: 5'-AGA TCG AGG GAG AGG GAG AG - 3'

Rev: 5'- CCC ATG GTC TTC TTT TGC AT-3'

The following settings were used for PCR:

1. 95°C, 2 min
2. 95°C, 30 sec
3. 55°C, 30 sec
4. 72°C, 1 min
5. Steps 2, 3 and 4 repeated for 34 cycles.
6. 72°C, 5 min

3.9 Sequence analysis

The DNASTAR Laser Gene seqbuilder software was used to analyze deletion sequences.

3.10 Statistical analysis

For behavioral assays, data was recorded in Microsoft Office 2007 Excel software (Microsoft Corporation, Redmond, WA). All statistical analysis was done using GraphPad Prism7 (GraphPad software, La Jolla, CA). The Student unpaired *t*-test was used (with a significant p value > 0.05) to analyze habituation results. For chemotaxis data, two way ANOVA was used.

CHAPTER IV: LOSS OF GPA-16 AFFECTS LATERALITY IN BOTH ADULTS AND EMBRYOS

4.1 Introduction

Overwhelming number of species display some form of asymmetry with a highly favored bias for one enantiomer body plan over the other. However, there is no obvious benefit to have this asymmetry be maintained in one specific direction. The human brain hemispheres are generally symmetrical at the anatomical level yet display functional asymmetry (10). This is exemplified through the commonly observed dextral bias in the population (90%-95%). It has been observed that 40% of individuals with schizophrenia are left-handed (10, 18, 47). Behavioral scientists have attributed this bias to the fact that the left cerebral hemisphere (which controls the right side of the body) is the language hemisphere in over 90% of all humans, and language, in conjunction with visual perception are considered to play major roles in consciousness (2, 48). Both cerebral asymmetry and handedness are heritable traits based on studies of families of concordant twins and adopted individuals. However, because the Mendelian mode of inheritance has not been demonstrated for handedness, biologists as well as psychologists have not accepted genetic explanations employing alternate mechanisms. Anatomically and functionally precise 3-dimensional body plans of multicellular organisms/animals are based on asymmetric cell divisions that take place in early development. Establishment of anterior-posterior and dorsal-ventral axes is well understood with similar explanations are usually extrapolated towards explaining the left-right lateral axis (22). In order to better understand the L/R inheritance and the molecular basis of asymmetric cell division, the use of simpler organisms where individual asymmetric cell divisions can be tracked proves useful.

The nematode lab model *Caenorhabditis elegans* shows predominantly bilaterally symmetric external anatomy with clear internal L/R asymmetry that is established during early embryogenesis. The point at which the sperm enters the embryo during fertilization becomes the posterior end of the embryo (29-31, 49). Dorsal/ ventral polarity is established during the second cleavage (Figure 1), defining the dorsal-ventral axis of the animal. This division yields two spindles which are initially parallel to the L/R axis and shift at an angle of 20° in an anti-clockwise manner, when viewed ventrally. This leads to dextral laterality in virtually all wild-type animals (27, 50) and it has been proposed that the foundations of neuronal LR asymmetry in adult worms are laid in early embryonic decisions (38).

The *C. elegans gpa-16* gene is involved in spindle and centrosome orientation that plays a role in the determination of handedness (27, 51). Previous studies have shown that temperature-sensitive *gpa-16* mutant allele (*it143*), when reared at non-permissive temperature (25° C), about 70% of the embryos are unviable, but of the survivors 40% of those are sinistral. When reared at permissive temperature 16°C about 98% of the embryos are viable and of that percentage a negligible amount are sinistral (27, 52). It has also been reported that there is reversed spindle orientation during the 4-6 cell stage in sinistral animals (27). The 70% embryonic lethality observed at non-permissive temperature has not been examined. In order to unravel the fate of these embryos at the critical stage for L/R axis establishment we carefully examined both *temperature sensitive gpa-16 and* deletion mutants' embryos at their 4-6 cell stages. We then looked at the laterality of the surviving adults.

4.2 Methods (For full methods see Chapter III)

4.2.1 Animals and preparations

The strains N2; *gpa-16 (ok2349), gpa-16(it143); him-5 (e1490)* were obtained through the Caenorhabditis Genetic Center, University of Minnesota, Minneapolis, MN. Worms were grown in nematode growth media (NGM) plates as described in (53, 54). The *Escherichia coli* strain OP50 was used as a food source. Depending on the strain, worms were raised at 15°C, 20°C or 25°C

4.2.2 Collecting and photographing embryos

Well-fed adults were picked and transferred into glass bottom dish with a drop of M9. They were dissected towards the middle as shown in Figure 3 and one to two cell embryos were chosen and photographed. Worm raised at 15°C, 20°C or 25°C were used

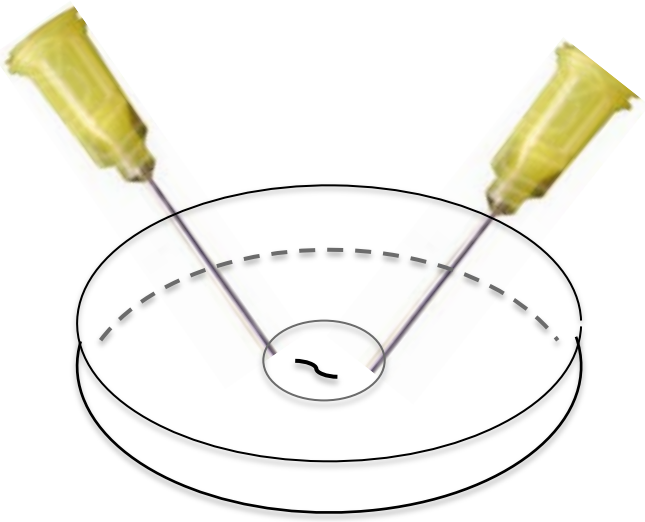


Figure 3: a representative picture of worm dissection

4.2.3 identifying sinistral, dextral and ambiguous embryos

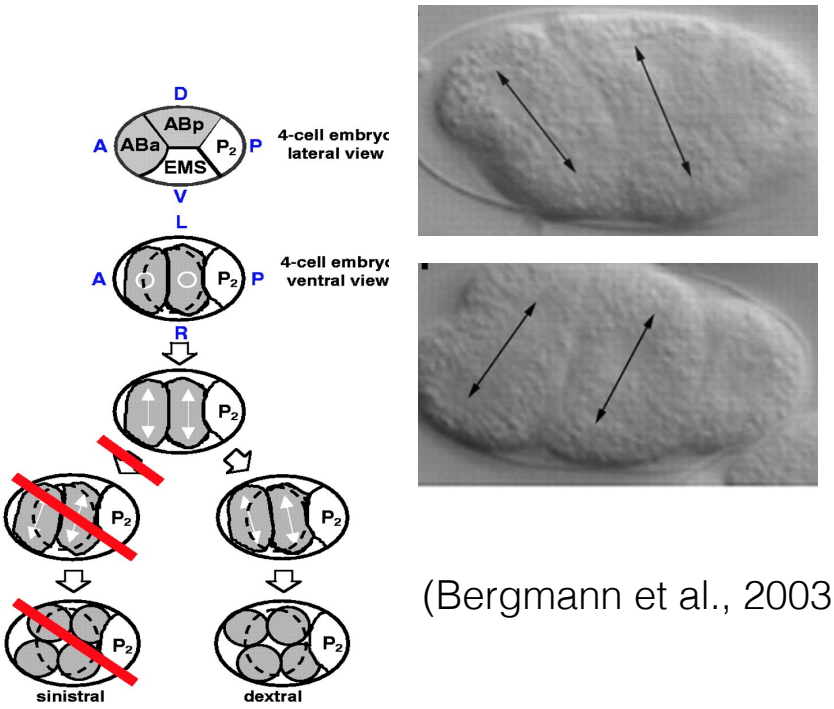


Figure 4: 4- cell embryos used to identify sinistral or dextral embryos. The picture (adopted from Bergmann et al, 2003 or (27)) shows a four-cell embryo. In the ventral view, the EMS (represented by the dashed lines) is found dorsally. If the embryo rotates counter clockwise as it is dividing from 4-cell embryo to 5-cell embryo, the embryo will be dextral. However, if the EMS remains in the same location but the embryo rotates clockwise, this embryo will be sinistral.

4.2.4 preparing adult worms for laterality experiments

As mentioned earlier, well-fed adults were picked and transferred to a glass bottom dish with a drop of M9, sodium azide and DAPI. Z stack images were taken using confocal microscope. The experiment was repeated using worms were raised at 15°C, 20°C or 25°C.

4.2.5 identifying adult worm laterality

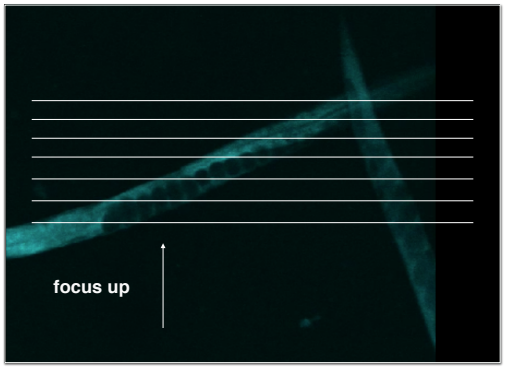


Figure 5: Z stack image of a worm: in order to establish if a worm is either right handed (normal laterality) or left handed (reversed laterality), adults worms with eggs were selected and z-stack images were taken. We also identified the position of the vulva and the head. Based on that, we looked to see if either the gut or gonad region comes into focus first. Using our model, we were able to identify if it is a right-handed or left-handed worm.

4.3 Results and Discussion

4.3.1 Results – embryonic laterality

A

	Dextral	Sinistral	Ambiguous
15°C	84.80%	9.10%	6.10%
20°C	64.70%	17.64%	17.64%
25°C	28%	48%	24%

B

	Dextral	Sinistral	Ambiguous
15°C	46.10%	46.10%	7.70%
20°C	53.80%	38.40%	7.69%
25°C	43.75%	37.50%	18.75%

Table 1: Embryonic laterality: (A) *t.s gpa-16* (B) *del gpa-16* at non-permissive temperature, *t.s gpa-16* worms showed higher percentage of sinistral embryos while *del gpa-16* worms showed elevated percentage of sinistral embryos at all temperatures.

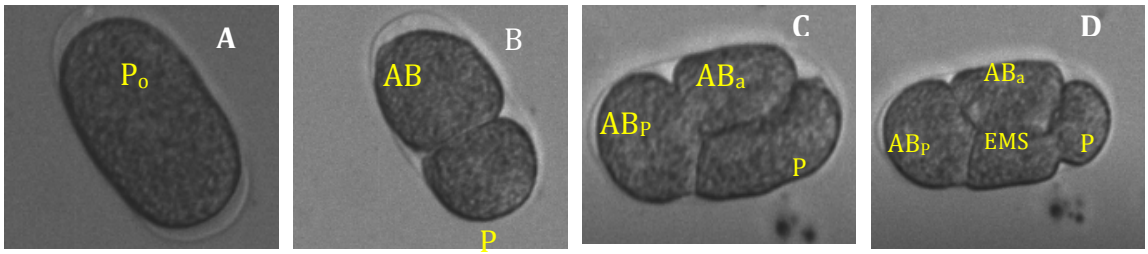


Figure 6 wild type cell divisions during the very early stages of embryogenesis. (A) 1-cell (B) 2-cell (C) 3 cell (D) 4 cell

The G-protein GPA-16 has been shown to regulate spindle position and thereby maintain asymmetric cell division. Previous work done with *t.s gpa-16* has shown that at the non-permissive temperature of 25°C, there is a 70% lethality. In addition, significant numbers of the surviving embryos were found to be sinistral compared to the 100% dextral wild type embryos (27). In our study, we focused on the embryos that do not survive into adulthood. We also looked at *gpa-16* deletion mutants. As shown in Table 1A, *t.s gpa-16* raised at 20°C and 25°C had higher number of sinistral embryos. Although not as significant as the 20°C and 25°C embryos, we saw sinistral embryos at 15°C. In *del gpa-16* mutants, we also saw high number of sinistral embryos. Perhaps the most interesting finding here was the ambiguously dividing embryos. Figure 6, shows wild type embryos dividing from the 1 cell stage to the fourth cell stage. The first symmetry breaking division (giving rise to the P and AB cells) is seen in figure 6B. After that, the embryo goes through another division (figure 6C) where the AB cells divide again to create AB_a (AB anterior) and AB_p (AB posterior). Finally, figure 6D shows the division of the P cell to give rise to the EMS cell. Our pictures are inline with the various pictures produced from

previous studies. This ensures that our dissection technique is accurate and is not damaging the eggshell, which in turn causes abnormally dividing cells (27).

In addition to increased number of sinistral embryos, some of the embryos from both *t.s gpa-16* and *del gpa-16* worms divided in a very haphazard way. Figure 7 shows such embryo. Initially, the cell starts out dividing normally. However, at the 4 cell stage,

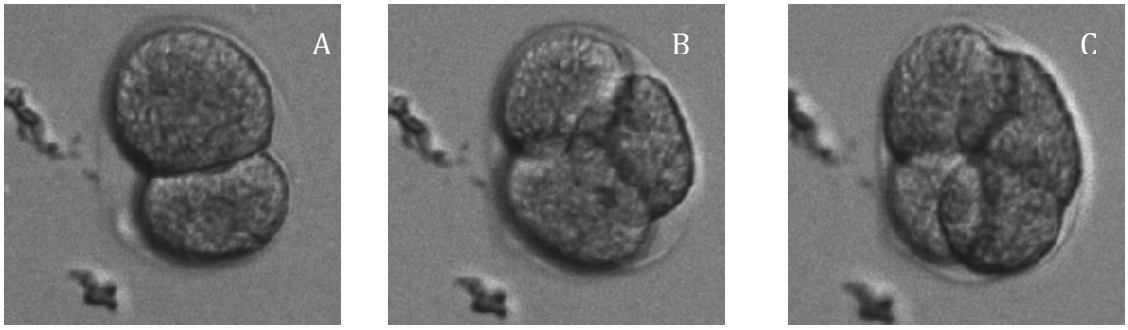
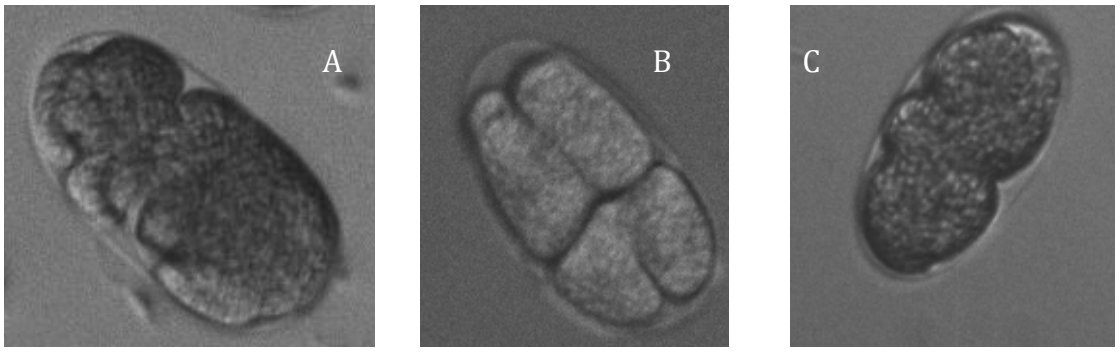


Figure 7 ambiguous cell division in *gpa-16* t.s worms at non-permissive temperature : (A) the first cell division resulted in asymmetrical to cell embryo (B) during the second cell division, the embryo starts to display abnormality (C) a haphazard 4 cell embryo (n≥13 for each temperature).

the cell takes a very ambiguous shape compared to a normal 4 cell stage embryo (figure 6A). This phenomenon was seen in both strains. Figure 8 also shows other forms of unusual embryos we encountered. Although most of them divided randomly, figure 8B shows a symmetrical 4-cell stage embryo. This is highly unusual and extremely rare in wild type worms raised under normal conditions.



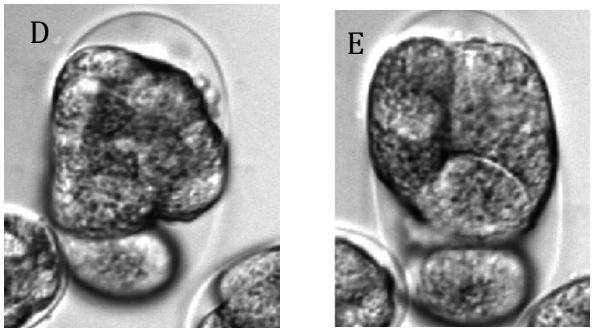


Figure 8 : ambiguous embryos: (A,C) shows embryos with no distinct definition of the cells. (B) shows a symmetrical 4 cell stage embryo (D, E) a very abnormal 4 cell stage embryos

4.3.2 Results – adult literality

A

	Normal	Reversed	Ambiguous
15°C	78.00%	22%	0
20°C	60%	40%	0
25°C	54%	45.90%	0

B

	Normal	Reversed	Ambiguous
15°C	78.50%	21%	0
20°C	66%	33%	0
25°C	70%	30	0

Table 2: Adult laterality: (A) *t.s gpa-16* (B) *del gpa-16* at non-permissive temperature, *t.s gpa-16* worms showed higher percentage of sinistral embryos while *del gpa-16* worms showed elevated percentage of sinistral embryos at all temperatures (n≥25 for each temperature).

Here, we used both deletion and temperature sensitive adult worms raised at the three different temperatures mentioned above in order to score them for handedness. We used a confocal microscope to take Z stack images of worms stained with DAPI. Using the vulva and the head as markers (figure 9), we were able to determine if the worm was left-handed based on which organ (gut or gonad) we encountered first when analyzing the Z stack images.

Similar to the embryos described above, *t.s gpa-16* worms raised at 25°C had more left handed worms (table 2). In fact, at this non-permissive temperature, close to half of the *t.s gpa-16* worms were left handed. *del gpa-16* worms also had an increased rate of left handed worms. Although not reported, our work with N2 wild-type worms resulted in no left handed worms. This has also been reported in previous studies where the researchers have looked at over 10,000 N2 worms and found no left-handed worms (52).

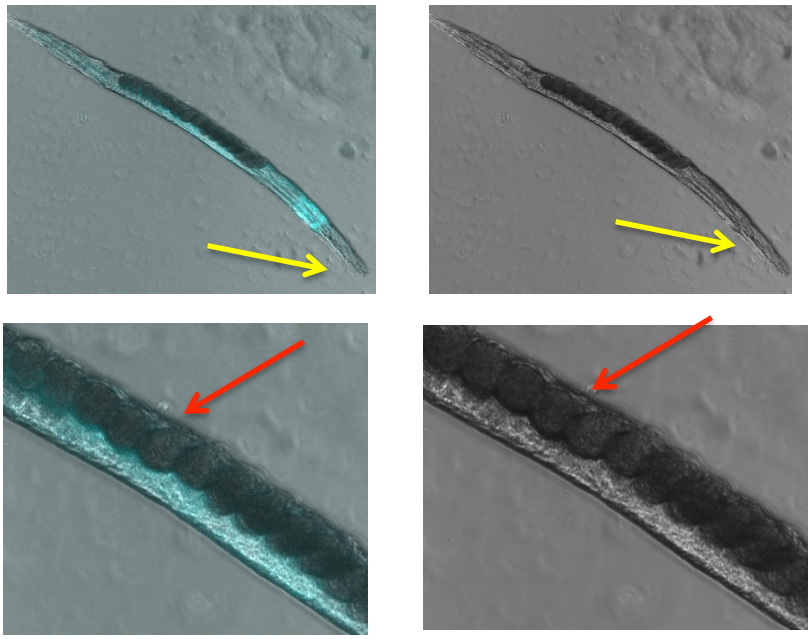


Figure 9: adult worms used to identify laterality using organ placement. Top panel a whole worm (right-DAPI, left –DIC). Bottom panel shows the placement of vulva (red arrows). The vulva and the positioning of the head (yellow arrow) is used as landmark when establishing organ placement.

Interestingly, we did not find any ambiguous adult worms during our experiments. In few cases, we observed worms that were too hard to call due to the arrangement of their organs/guts. However, we did not count these worms as ambiguous. Their unusual look was mainly caused as a result of old age and had more than 1 row of eggs or no eggs. Therefore, these worms were discarded.

Based on our findings, we infer that the abnormally dividing embryos are responsible for the increased rate of lethality seen in this *gpa-16 t.s* and *gpa-16 del.*

From our studies, we have noted that these embryos do not survive into adulthood. This also explains why we do not see any ambiguous adult worms.

CHAPTER V: EFFECTS OF DISRUPTED ASYMMETRIC CELL DIVISION ON ASSOCIATIVE AND NON-ASSOCIATIVE LEARNING

5.1 Introduction

In *C. elegans,* behavior is influenced by various external and internal changes. As a result, it has the ability to associate changes in the environment with the presence or absence of food. Its ability to learn through association makes *C. elegans* an ideal invertebrate to study the molecular changes that take place during learning and memory.

C. elegans' ability to learn can be tested through habituation (non-associative learning) and chemotaxis (associative learning). Administering repeated mechanical touch stimuli can be used to test habituation by counting the number of gentle anterior touches it takes for the animal to stop responding. Associative learning, such as classical conditioning and differential conditioning, can be tested by using assays in which worms are conditioned to specific chemicals that are paired with the presence or absence of food and then assaying the worms for changes in chemotactic response towards the conditioned chemical(42-44).

In *C. elegans*, out of the 98 sensory neurons, 63 of them are bilaterally symmetric and only four neurons are unilateral (55). AWC, chemosensory neurons are both structurally and functionally symmetric. However, only one of the two AWC neurons expresses the G protein coupled olfactory receptors (GPCR) gene *str-2*. This asymmetric expression of *str-2* in either AWC-Left or AWC-Right is suggested to play a significant role in odor sensing (55). In experiments where the asymmetry of *str-2* is affected, the worms exhibited odor discrimination defect (56).

The olfactory neurons ASER and ASEL are structurally symmetric but functionally asymmetric. When this functional asymmetry is disrupted, their ability to distinguish between

two water-soluble odorants diminishes. As mentioned in Chapter II, in *temperature sensitive gpa-16 (it143)* strains there is an increased reversal of left and right ASE neurons (27, 38, 55).

In *Drosophila*, it has also been shown that when asymmetry is disrupted, the formation and retrieval of long-term memory is affected. Based on these findings, we hypothesized that *gpa-16* worms would have aberrant response in isoamyl alcohol based conditioned chemotaxis assay.

In the case of non-associative learning, very few studies have been done to examine the effects of disrupted asymmetric cell division and reversed laterality on habituation. In one study it was reported that reduced habituation is a common feature in schizophrenic patients (57). Here, we performed habituation assays on *C. elegans* in order to study the effects of disrupted asymmetry in habituation.

5.2 Methods (For full methods see Chapter III)

5.2.1 Animals and preparations

The strains N2; wild type Bristol isolate, RB1816 *gpa-16 (ok2349),* BW1809 *gpa-16(it143); him-5 (e1490)* were obtained through the Caenorhabditis Genetic Center, University of Minnesota, Minneapolis, MN. Worms were grown in nematode growth media (NGM) plates as described in (53, 54). The *Escherichia coli* strain OP50 was used as a food source. Depending on the strain, worms were raised at 15°C, 20°C or 25°C

5.2.2 Conditioned chemotaxis assay

Synchronized young adult worms were collected with M9 buffer. For conditioning, the animals were exposed to 3 µl of isoamyl alcohol for 90 minutes. They were then washed and transferred to testing plates. In order to immobilize worms sodium azide was used. Worms were placed at

the starting point equidistant to both the trap and gradient points. 1/1000 isoamyl alcohol diluted in ethanol was placed on the gradient while ethanol was placed on the trap point (Figure 3). The plates were left undisturbed for one hour. The number of worms found in each point was counted. Chemotaxis index was calculated by subtracting the number of worms at the trap point from the number of worms found at the gradient point and dividing it by the total number of worms found on the plate. The experiment was repeated with worms raised at three different temperatures (15°C, 20°C and 25°C)

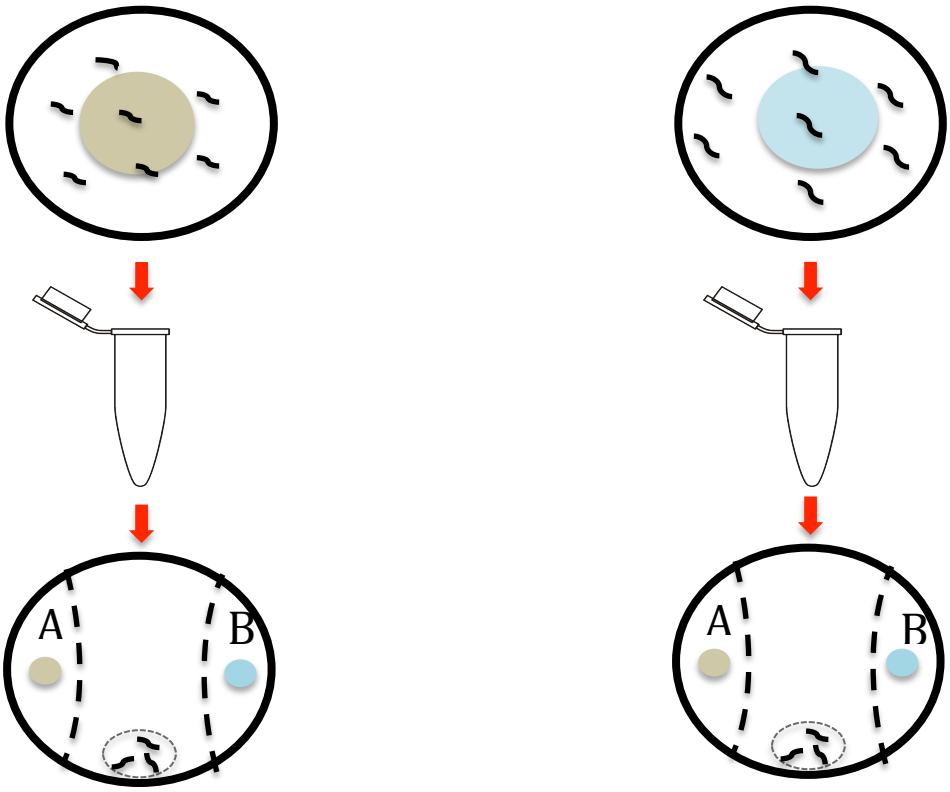


Figure 10 *C. elegans* chemotaxis assay with isoamyl alcohol**:** worms were either conditioned with pure (attractant) isoamyl alcohol (beige) or ethanol used a control (blue). They were then

transferred to small tubes and washed once. Finally, worms were transferred to testing plates. In order to immobilize worms, sodium azide was placed on both testing points (A and B). Diluted isoamyl alcohol was placed on one side of the testing plate (A) and isoamyl alcohol on the other side (B). Worms were placed in the middle and allowed to move to direction of choice. Chemotaxis index was calculated using the formula $\mathrm{C.I} = \frac{B-A}{total}$

5.2.3 Habituation assay

Approximately 10 synchronized worms were transferred to new unseeded plates. Using an eyelash hair stuck at the end of a toothpick, a gentle touch to the anterior region was applied (Figure 5). The number of times the animal moves backward until it no longer responds to the stimulus was recorded.

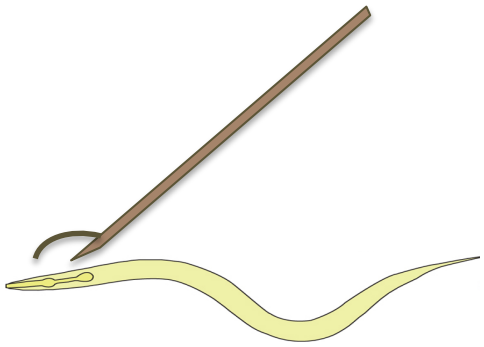


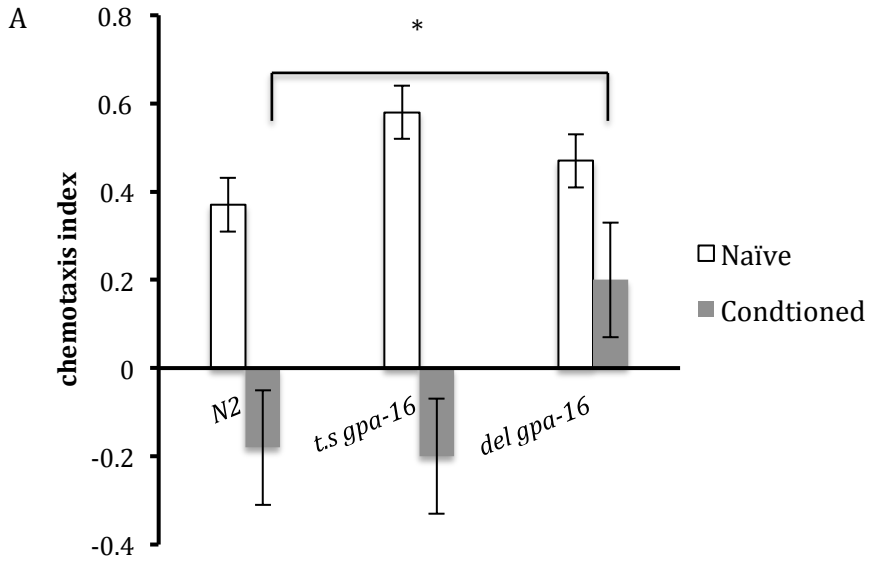
Figure 11: schematic representation of habituation assay to test non-associative learning

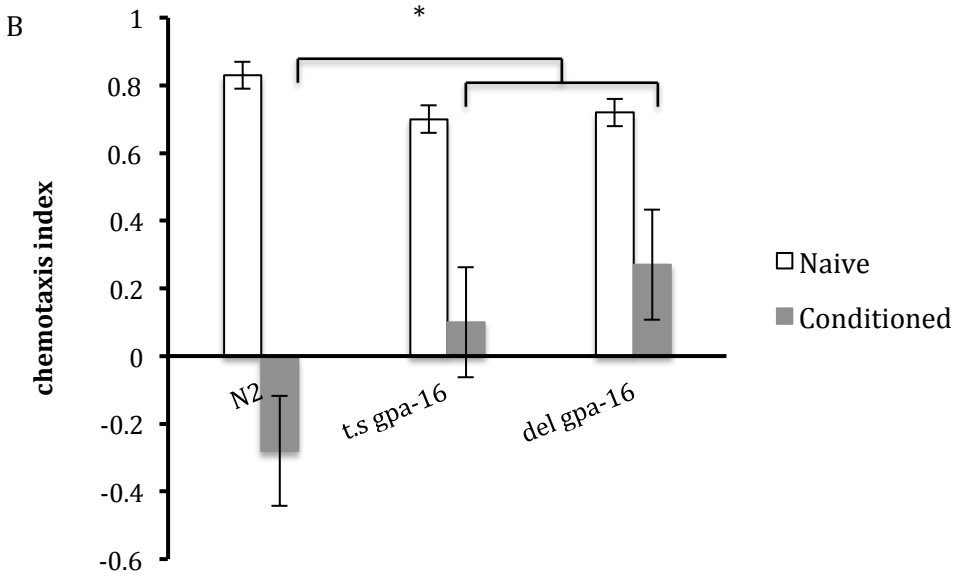
5.2.4 Data Analysis

Data were analyzed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA). Histogram bars represent mean while error bars denote standard error of mean (SEM). All Statistical analysis was done using GraphPad Prism7 (GraphPad software, La Jolla, CA). Unpaired student's t-test was

done to analyze habituation data and two-way ANOVA was done to analyze chemotaxis data. Statistical significance $p<0.05$

5.3 Results and discussion





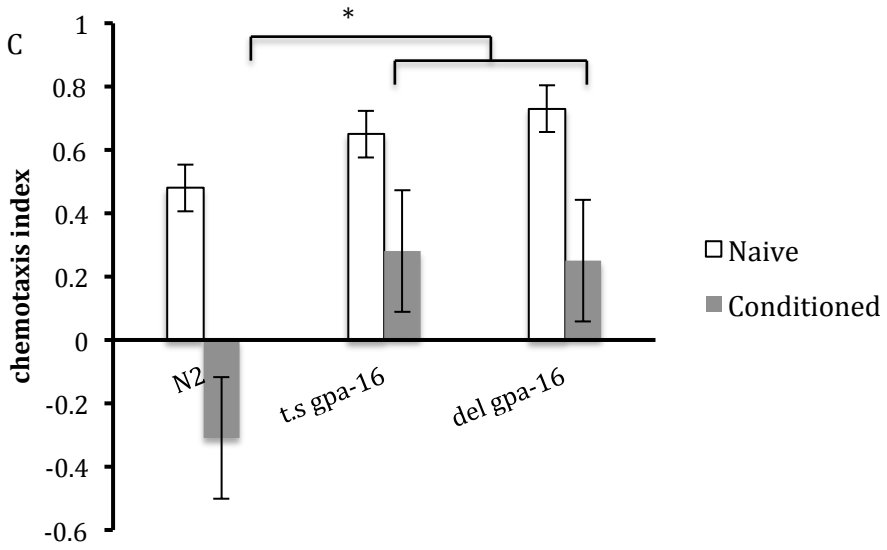
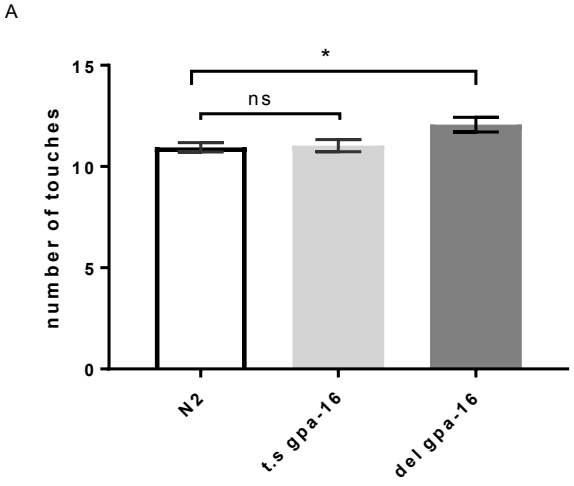


Figure 12 *gpa-16* mutants show limited associative learning*:* synchronized worms were conditioned using pure isoamyl alcohol and tested with 1:1000 isoamyl alcohol in the absence of food. Chemotaxis index measures attraction strength. (A) Worms were raised in 15°C. While conditioned N2 and del gpa-16 were able to break their attraction to isoamyl alcohol, *t.s gpa-16* worms maintained their attraction. (B, C) Both at 20°C and 25 °C, both *gpa-16* strains show significantly higher chemotaxis index when compared to conditioned N2 worms (*p<0.05,two way ANOVA). Error bars represent SEM values n=7



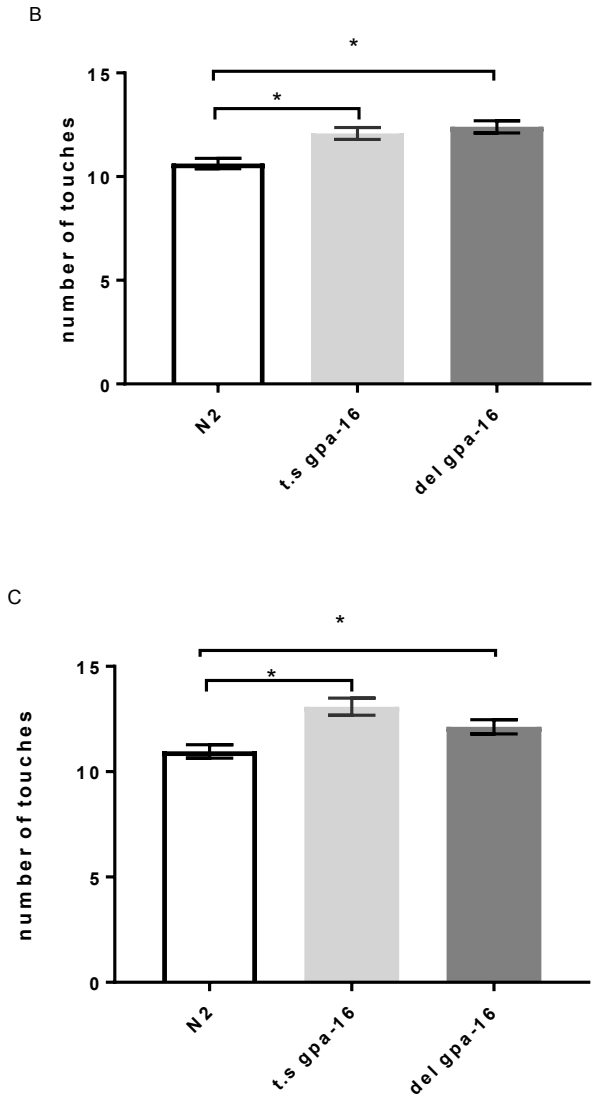


Figure13 *t.s gpa-16* and *gpa-16 del* exhibit abnormal learning: Worms were gently touched on the anterior until they stop responding to a stimulus. The number of times the animal moved backwards until it no longer responded to the stimulus was taken as the habituation point. (A) at 15℃ only *del gpa-16* showed a significantly higher habituation rate (*p=082 for del gpa-16*). (B,C) both deletion mutants required significantly more touches in order to habituation (B) 20℃ and (C) 25℃. *p=<0.05, unpaired t-test). Error bars represent SEM, n=60

Although previous studies have examined the importance of asymmetric cell division, no work has been done to study the effects of *gpa-16* in behavior and behavioral plasticity. As stated in Chapter II, associative learning is influenced by brain asymmetry. In addition, *t.s gpa-*

16 worms have been shown to exhibit reversal in the left and right ASE gustatory neurons, ASEL (ASE-left) and ASER (ASE-right) along with the chemosensory AWC neurons (27, 38). Therefore, we conducted the associative learning assay in order to determine if the reversal of the right and left ASE and AWC neurons will interfere with their ability to associate isoamyl alcohol with the absence of food.

Before transferring them to the testing plates, the worms were conditioned with pure isoamyl alcohol in the absence of food. Under normal conditions, worms are highly attracted to isoamyl. When transferred to the testing plates, wild-type worms conditioned with isoamyl alcohol move as far away as possible from the area where diluted isoamyl alcohol was placed (Figure 3). Meanwhile, control worms conditioned with ethanol in the absence of food maintained their attraction to isoamyl alcohol when placed in the testing plates. The test was done in three different temperatures. At 15°C*,* a non-permissive temperature for *t.s gpa-16, the t.s gpa-16* worms were able to break their attraction towards isoamyl alcohol after conditioning (Figure 12 A). In the contrary, *del gpa-16* worms still exhibited attraction to isoamyl alcohol after conditioning. *del gpa-16* maintained this aberrant behavior in both 20°C and 25°C (Figure 12 B, C). As the temperature increases, *t.s gpa-16* worms start to exhibit defect in associative learning. Although wild-type worms avoided isoamyl alcohol at 20°C and 25°C after conditioning, *t.s gpa-16* worms were unable to do so.

Habituation assays were performed to test non-associative learning in worms with gpa-16 mutants. So far, the effects of disrupted asymmetric cell division in non-associative learning in *C. elegans* remains poorly understood. However, GPA-16 is expressed in key neurons associated with habituation. Therefore, we were interested in investigating the effects of loss of functional GPA-16 on habituation Similar to the chemotaxis assay, the habituation assays were also conducted in three

different temperatures (15°C, 20°C and 25°C). Compared to wild type, *del gpa-16* required significantly more touches in order to habituate (Figure 13). This behavior was exhibited in all three temperatures. At 15°C, *t.s gpa-16* showed no significant difference in the number of touches required to habituate when compared to wild type N2 worms (Figure 13 A). Similar to the associative learning data presented earlier, at 20°C and 25°C, higher habituation rates were recorded for *t.s gpa-16* worms (Figure 13 B, C).

Taken together, these results suggest that the disruption of stereotyped asymmetric cell division interferes with the worm's ability to form and recall memory.

It has been reported that the neurons ALM, AVM, PLM, and PVD along with the interneurons AVD, AVA, AVB, PVC, DVA and PLM regulate the worm's tap withdrawal response. Ablating any one of these neurons results in aberrant habituation including but not limited to always reversing or always accelerating in response to tap (58). *gpa-16* is expressed in a significant sub-set of these neurons (AVM and PLM) and interneurons (PVC). Considering these neurons play a big role in habituation, the aberrant habituation results we saw in both deletion and temperature sensitive *gpa-16* mutants are reasonable. The absence of GPA-16 in deletion mutants and the lack of functional GPA-16 protein in temperature sensitive mutants has the potential to interfere with the proper functioning of neurons that were identified to mediate habituation.

In the case of associative learning, previous studies have shown that ASE neurons play a significant role in sensing water-soluble chemicals (26, 58). It has also been reported that in *gpa-16* mutants, a reversal of the ASEL and ASER has been reported. This interferes with their functional lateralization and affects their ability to discriminate between different water-soluble odorants (27, 37, 38, 58). Although these studies mainly focused on the worms' response to

NaCl, our finding shows that it also applies to the non water-soluble odorant isoamyl alcohol. When there is no functional GPA-16, the worms' ability to break their attraction to isoamyl is affected. Wild type worms avoid isoamyl alcohol after being conditioned in a plate with no food. However, both *del gpa-16* and *t.s gpa-16* worms at 20°C and 25° C failed to break their attraction. This shows that the lack of functional GPA-16 hinders the functional laterality of the ASEL and ASER neurons disrupting their ability to form and recall memory as seen by their attraction towards isoamyl alcohol even after conditioning in the absence of food. In addition, ASE neurons have a very strong communication with the AIY interneurons (Figure 14). Laser ablation of AIY interneurons has been shown to result in reduced avoidance of isoamyl alcohol (59). If ASE expression in reversed, it is possible that their connection with the AIY neurons is also affected. Thus, our abnormal chemotaxis assay response seen in *gpa-16* mutants could have been caused as a result of atypical communication between the ASE sensory neurons and the AIY interneurons.

While conducting our associative learning behavioral assays, we were interested in scoring the laterality of worms that stayed in the side of the plate with isoamyl alcohol (after conditioning). These worms were counted as learning deficient. However, we were unable to score them for laterality. Considering that all our behavioral assays are done with young adult worms, the lack of eggs in these worms made it difficult to tell if they had reversed laterality. We also tried to reduce the amount of sodium azide used in the testing plates (for the purpose of immobilizing the worms) from 2µl to 1 µl (to make sure that they do not die). We then picked these worms and transferred them to seeded plates after the chemotaxis assay was completed. We tried to let them grow for 24 hours in order to make sure they will have eggs in the gonad.

However, we were unable to score the worms. It is possible that during the transfer process the worms were too damaged to develop eggs.

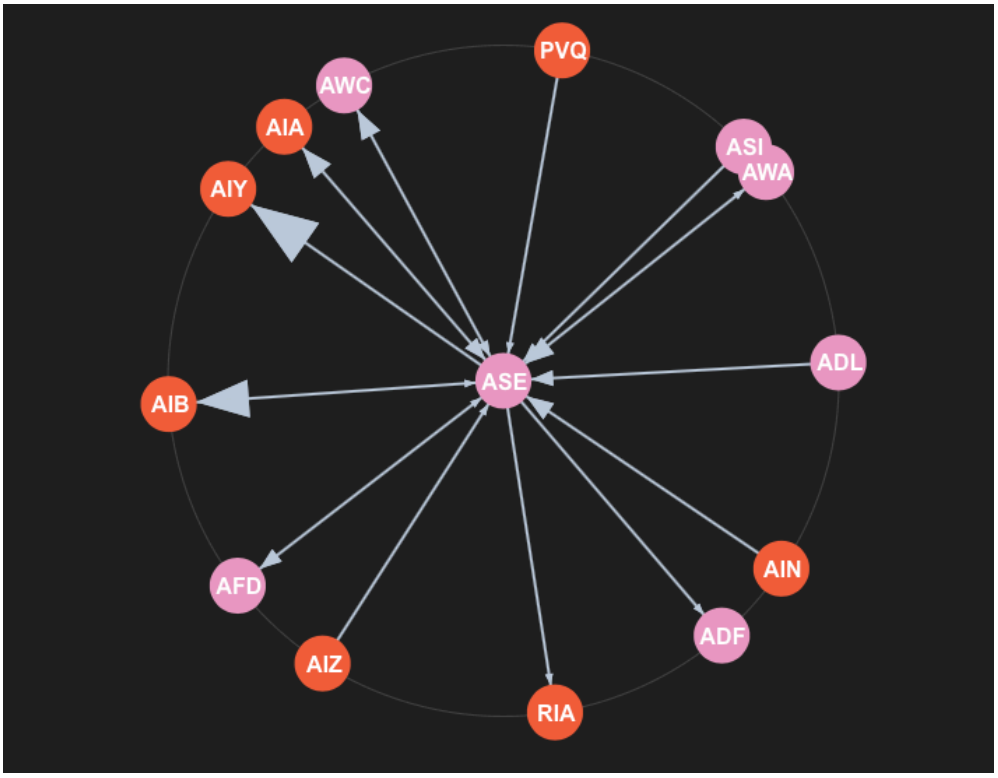


Figure 14 Neuronal connections between the ASE sensory neurons and various interneurons and sensory neurons. The picture is taken from www.wormweb.org A bigger arrow head indicates a very strong connection.

In order to investigate the possibility of atypical neural connectivity between ASE and AIY, we crossed *del gpa-16* worms with ASER:: GFP worms (fluorescence is driven by the gcy-5 promoter) . We then crossed this new strain with AIY::mCherry worms. The ttx-3 promoter drives the AIY mcherry expression. Unfortunately, the cross with the AIY:mCherry was not successful (Figure 15) . Therefore, we were unable to identify a possible a typical neuronal connectivity. We believe that the male AIY::mCherry worms might be poor at mating.

In the very near future, we anticipate to make a new strain expressing AIY:mCherry using microinjection.

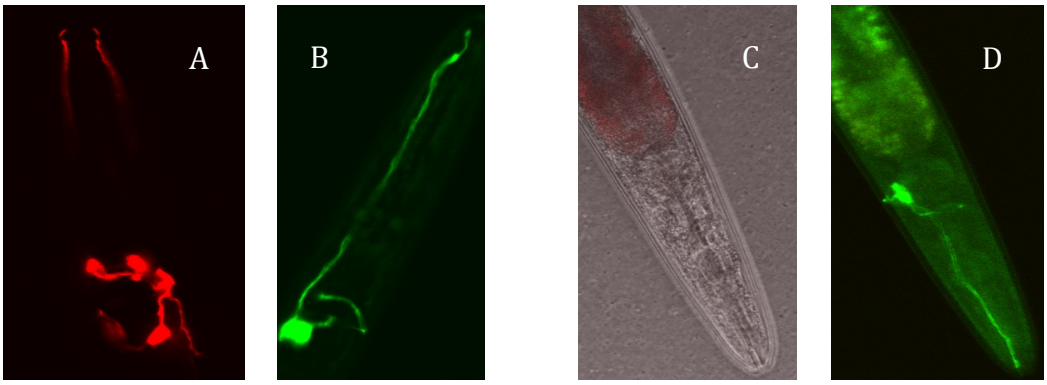


Figure 15 del gpa-16::ASER:GFP::AIY:mCherry generated in our lab. The triple cross failed to show mcherry expression but maintained its GFP expression. (C,D) . However, we were able to see fluorescence in both original AIY::mCherry worms (A) and ASER::GFP worms (B).

CHAPTER VI: CONCLUSION

Asymmetric cell division provides the building blocks required for generating the functional and structural asymmetry/laterality required for the proper functioning of living things. As far back as the earliest part of the 19th century, scientists have been studying the functional asymmetry of the human brain. In fact, the French physicians Marc Dax and Paul Broca were the first to identify the functional laterality of the brain (2, 4). Additional studies have since shown that the lateralization of speech areas strongly corresponds to the handedness of a person (7).

More than 90% of the world's population is right handed with a left hemisphere that is responsible for language processing(11). Even though left-handers only make up less than 10% of the world's population, disproportionate number of left handed people show different behavioral disorders. In fact, 40% of schizophrenic patients are left handed (10, 18). MRI studies have shown that these patients show less asymmetry in the planum temporale (20). Although several studies were done to show the effects of disrupted asymmetry and its effects, the biological mechanism responsible for establishing this asymmetry is poorly understood.

In our study, we used the model organism *Caenorhabditis elegans* to better understand how this asymmetry is created and maintained through out its life. In *C. elegans,* the first symmetry breaking point takes place during the first cell division. Previous studies have shown that PAR (partition) proteins play a significant role in asymmetric cell division. They are distributed unequally through out the embryo resulting in asymmetric cell divisions. In addition, G alpha proteins GOA-1 and GPA-16 have also been shown to play a role in asymmetric division by regulating spindle positioning (28, 31). Work done by Bill Wood has established

that in *temperature sensitive gpa-16* mutants, there is an increased rate of lethality and in the surviving embryos, there is an increased rate of left handedness (27, 50, 52). Using this study as our starting point, we proceeded to study laterality in the *del gpa-16 mutants.* Specifically, we were interested in the embryos that do not survive. We were also interested in examining the effects of disrupted asymmetrical cell division during early embryogenesis have on adult behavior.

Our work with embryonic laterality showed that both *temperature sensitive* and *deletion mutants* produced more sinistral embryos compared to the wild type embryos (which had neither sinistral embryos or left handed adult worms). In addition, we encountered embryos that are neither sinistral nor dextral. These embryos showed very randomized division. In some cases, we also encountered symmetrically dividing embryos. These abnormal embryos die before the L1 stage. These embryos account for the increased lethality rate seen in the *gpa-16* mutants. In the adult worms, increase in number of left-handed worms (with reversed organ placements) was seen.

We also conducted both associative learning and non-associative learning assays in order to investigate if the lack of functional GPA-16 has any effect on learning and memory. We were able to establish that both *gpa-16* mutant strains perform poorly in both types of assays.

Over all, our study has shown that the absence of GPA-16 has significant effect on the worms laterality along with its ability to form and recall memory possibly due to atypical neuronal connections created as a result of disrupted asymmetric cell division

CHAPTER VII: REFERENCES

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PART II

This part of the dissertation includes work that has already been published

Mersha, M. D., et al. (2015). "Effects of BPA and BPS exposure limited to early embryogenesis persist to impair non-associative learning in adults." Behav Brain Funct **11**: 27.

ABSTRACT

Bisphenol-A (BPA) is a highly prevalent polymerizing agent found in commonly used items like plastic bottles, dental sealants, sales receipts, etc. It is classified as an endocrine disrupting chemical with Estradiol-like hormonal properties. BPA has been shown to have various adverse effects ranging from increased risk of miscarriages and cancer to impaired chromosomal double-strand break repair mechanism. Various studies have also shown that exposure to BPA affects neuronal development. Recently, manufacturers have made the switch to Bisphenol-S (BPS), a supposed safer replacement for BPA. However, not many studies have been done to prove this claim. Using the nematode *Caenorhabditis elegans*, we investigated the effects of low dose BPA and BPS exposure on egg laying properties and habituation, a form of non-associative learning. We established that embryonic exposure to low doses of BPA and BPS has effects that persist into adulthood including decreased fecundity and abnormal learning and memory. We show that BPS, a proclaimed safer substitute to BPA has similar adverse effects as BPA. In addition, since BPA has been shown to affect chromosomal double-strand break repair mechanisms, we are also investigating its effects on chromosomal segregation and asymmetric cell division.

CHAPTER I: INTRODUCTION

1.1 Brief Background

Bisphenol-A (BPA) is a polymerizing agent used in plastic bottles and other routinely used consumer items. It is classified among endocrine disrupting chemicals suspected to cause adverse health effects ranging from infertility and cancer to behavioral disorders. Work with the invertebrate lab model *Caenorhabditis elegans* has shown that BPA affects germ cells by disrupting double-stranded DNA break repair mechanisms. The current study utilizes this model organism to provide insight into low-dose and long-term behavioral effects of BPA and Bisphenol-S (BPS) that has been presented as a safer alternative.

1.2 Purpose of the Study

Although FDA has labeled BPA as safe, various studies have shown that it is not the case. A plethora of studies have shown that BPA has been shown to be responsible for various neurological disorders, different types of cancers and infertility. Therefore, my research is focused on showing the effects of low dose exposure to BPA has on fecundity and learning and memory in the model organism *Caenorhabditis elegans*. My study also investigates the effects of BPS since it is presented as a safer replacement for BPA.

1.3 Research Questions

- BPA has been shown to have various adverse effects on various organisms. However, most of these studies used higher concentration of BPA when conducting their studies. In addition, these studies have also exposed their model organisms to BPA throughout their lifetimes. I am using a very low dose of BPA to study its effects on *c*

elegans. The worms will be exposed during the very early stages of embryogenesis (only) before being tested for non associative learning as adults. I am also studying how BPA affects the number of eggs laid by these worms

- Recently, there has been a push to replace BPA with a safer chemical. Manufacturers have introduced BPS as a much safer alternative. However, not enough studies have been done to prove this claim. I am repeating the same experiments as mentioned in the previous point with embryos exposed to BPS and evaluating its effect on fecundity and non associative learning

1.4 Importance of the Study

BPA is found in almost everything we use. It is found in water bottles, dental sealants, the lining of food cans, store receipts, etc. Therefore it is not surprising that it is found in the urine of 90% of Americans. In addition, numerous studies have shown that BPA has very adverse effects ranging from infertility to cancer and neurological disorders. It is essential that we further investigate its adverse its effects. More importantly, manufacturers have introduced BPS as a safer alternative despite the lack of evidence to back up their claim. Therefore, it is crucial that we carefully investigate and show if BPS is indeed a safer alternative.

CHAPTER II : REVIEW OF THE LITERATURE

The human health impact of Bisphenol-A (BPA or 4,4'-isopropylidenediphenol), which has been in commercial use since the 1960s, has been under scrutiny in recent years. BPA is used in the polymerization process of polycarbonate plastics and resins as well as in the manufacture of commonly used products ranging from thermal paper used for sales receipts to flame retardant precursors, dental sealants, and the inside coating of beverage and food cans including those used for infant formula (1, 2). Considering its widespread use, it is not surprising that 90% of Americans have traceable amounts of BPA in their urine (3). BPA is suspected to induce pre-term birth in pregnant women (4), along with adverse health effects including nervous system disorders in children as well as in adults (5, 6).

BPA exhibits hormone-like properties, mimicking 17-β Estradiol (E_2) and is classified as an endocrine disrupting compound (EDC) (2). E_2 is known to act through different members of the estrogen receptor family including ER-α, ER-β and ERR-δ, which play critical roles in the regulation of embryonic development including neuronal survival and plasticity (7). Considering the critical roles of E_2 in development and the EDC properties of BPA, a number of recent studies have focused on the biological effects of exposure to BPA. Exposure to BPA affects nervous system function with chronic exposure leading to an increase in dopamine D_1 receptor expression in mouse limbic forebrain, which can result in hyperactivity, attention deficits and heightened sensitivity to drugs of abuse (8, 9). Furthermore, in mouse embryos exposed to BPA, the long-term neuronal defects that persevere into adulthood have been shown to be epigenetically mediated through DNA methylation (10).

With the trickle of reports incriminating BPA in contributing to adverse health effects and a considerable increase in public awareness, some industries in the United States have initiated self-regulatory measures towards voluntary replacement of BPA with a purported safer substitute Bisphenol-S (BPS or 4,4'-sulfonlydiphenol) which shares remarkable structural similarity to BPA and estradiol (Figure 16). Recent studies have shown that BPS has comparable anti-androgenic effects and has been shown to regulate estrogenic transcription at a level comparable to estrogen itself (11, 12).

Considering the conserved nature of genes of *Caenorhabditis elegans* with mammals, including its steroid hormone-receptor genes (13), researchers have begun utilizing this genetically tractable lab model to understand the effects of EDCs to obtain foundational insight on the mechanisms of BPA action (14, 15). One key report has linked increased sterility and embryonic lethal effects of BPA to genomic instability caused due to breakdown of double-stranded DNA break repair mechanisms (14, 15). However, it is important to note that this study is based on internal BPA concentrations at par with those used in commonly used mammalian models equivalent to occupational exposure levels of 2 ppm (14, 15). Newer and alternate approaches to chemical safety determination indicate that low doses of toxic chemicals are associated with distinct pathologies and that the observations at high doses may not necessarily predict low-dose toxicity (2, 16). A low-dose, based on US Environmental Protection Agency and US National Toxicology Program panel guidelines, (16) may be defined as any dose below the level of one which has been reported to cause an observable biological change or damage (2). Our focus is on studying the low-dose effects of BPA on the functional integrity of the nervous system. A diverse range of behavioral effects attribute to BPA exposure in mammals (6), however, its behavioral effects have not been studied in the *C. elegans* model.

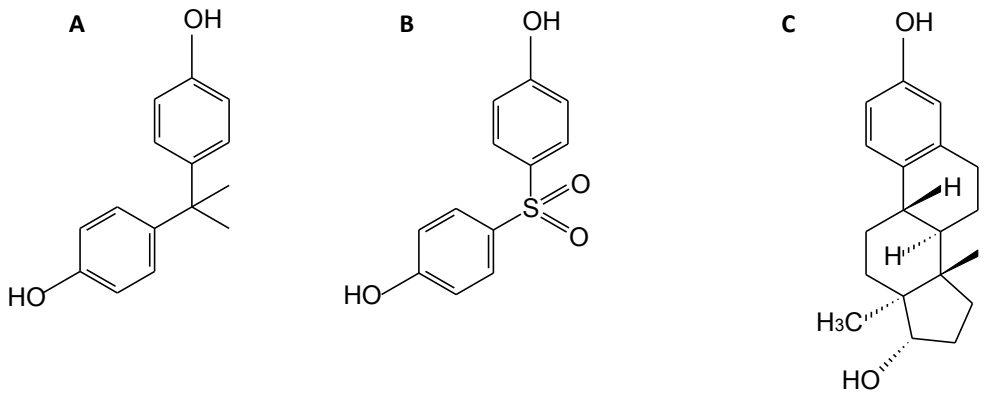

Figure 16: Bisphenol-A (A) and Bisphenol-S (B) are classified as endocrine disrupting compounds, which may act through receptors for the naturally occurring steroid hormone 17-beta estradiol (C).

HYPOTHESIS

Short term and low dose embryonic exposure to BPA and BPS has adverse effects on *C elegans*.

CHAPTER III: MATERIALS AND METHODS

3.1 Strains

The strains N2 were obtained through the Caenorhabditis Genetic Center, University of Minnesota, Minneapolis, MN.

3.2 Maintenance

Worms were grown in nematode growth media (NGM) plates as described in (17, 18). The *Escherichia coli* strain OP50 was used as a food source.

3.3 Exposure to BPA/S

Bisphenol-A/BPS (obtained from Sigma-Aldrich, St. Louis, MO) was solubilized using 10% ethanol to make a 100 mM stock solution and subsequent dilutions were made in S-buffer (0.1 M sodium chloride 0.05 M potassium phosphate, pH 6.0). *C. elegans* embryos were isolated from gravid adults using basic hypochlorite solution and exposed to 0.1 to 10 mM BPA concentrations for four hours while being rocked gently at room temperature. After four hours, the worms were transferred to seeded NGM plates.

3.4 Behavioral assay

3.4.1H*abituation assays:* In preparation for the assay both non seeded and seeded Normal Growth Media (NGM) plates prepared fresh the night before the assay and left overnight at room temperature. Approximately 10 worms were transferred to the new NGM plates. Using an eyelash hair, the worm was tapped on the head. In response to this stimulus, the worms typically

move backwards. The number of times the animal moves backward until it no longer responds to the stimulus was counted.

3.5 Fecundity assay

Individual L3 larval stage worms were picked and transferred to fresh, seeded plates and the numbers of eggs laid were counted every 24 hours for 4 days

3.6 Statistical Analysis

All statistical analysis were done using one way ANOVA followed by Tukey's post-hoc analysis; GraphPad Prisim6 (GraphPad software, La Jolla, CA)

CHAPTER IV: THE EFFECTS OF BPA/S ON PROGENY SIZE AND HABITUATION

4.1 Adult animals exposed to BPA as embryos have decreased progeny

We observed a statistically significant decrease in the number of eggs laid at BPA concentrations of 1.0 µM and higher (Figure 17a). A dramatic decrease in the number of eggs laid by *C. elegans* that were continually exposed to higher BPA concentrations (≥1 mM) beginning from the embryonic period and continued throughout adulthood, has been reported previously (15). Our observations are based on exposure to lower doses that were limited to the embryonic period.

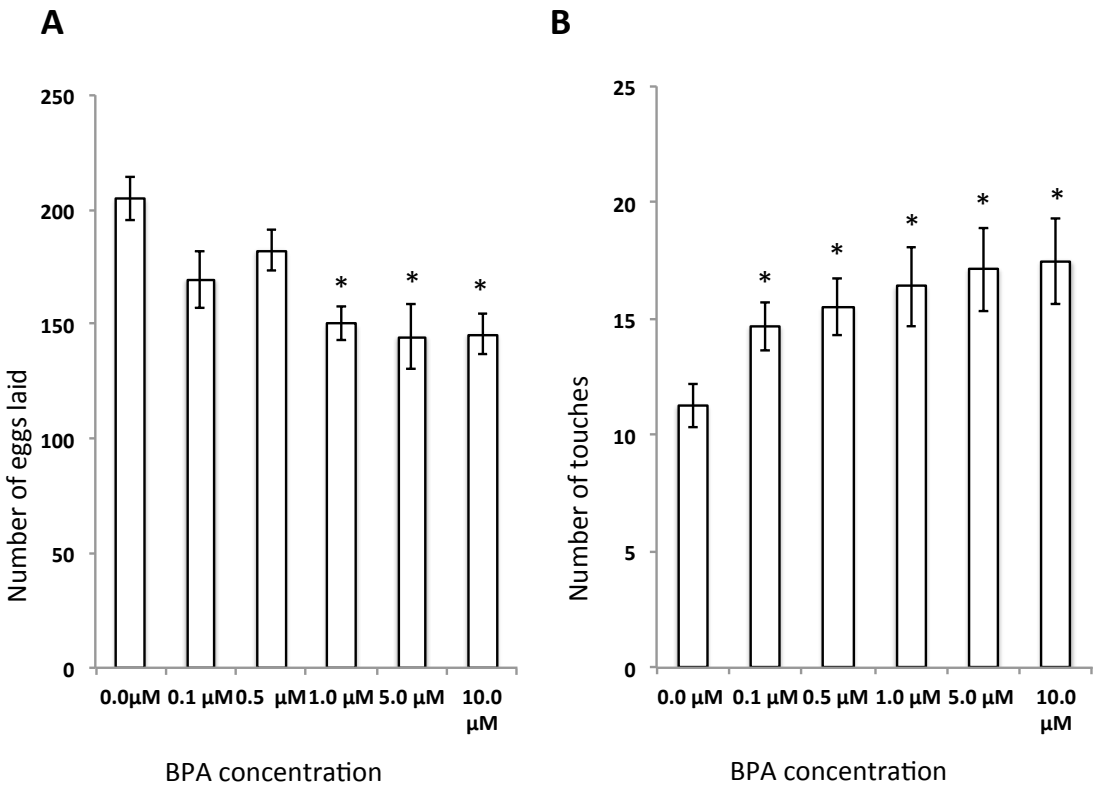

Figure 1**7.** The effects of BPA on egg laying and habituation (A) Synchronized worms exposed to BPA laid significantly fewer eggs (except for 0.1and 0.5µM groups). Eggs were collected using hypochlorite solution before being exposed to BPA for 4 hours at room temperature and

transferred to seeded NGM plates (n=10, *p< 0.05). (B) Worms exposed to BPA required more touches to habituate. 10 Synchronized and exposed worms were transferred to unseeded NGM plates. The number of gentle taps (on the head) required for the animal to stop responding was counted (n=60 , * p < 0.05). Graphs represent means while error bars denote standard deviation. All statistical analysis were done using one way ANOVA followed by Tukey's post-hoc analysis

4.2 Embryonic BPA exposure slows the habituation response of adult worms

Next, we used anterior touch sensory function to examine the habituation behavior of adult worms (19) exposed to BPA in their embryonic phase. BPA exposure of embryos was performed as described above, and the exposed embryos were transferred to seeded NGM plates without exogenous BPA. Well-fed 3 day old adult worms were assayed for habituation to anterior touch as described previously (19). Briefly, animals were given repeated gentle anterior touch stimuli with 10 seconds inter-stimulus-intervals until they no longer responded to the stimulus. The number of stimuli required by an animal until it no longer moved backward was recorded. We found that worms exposed to BPA at even the lowest concentration tested (0.1 μM) required more stimuli to become habituated, when compared to unexposed worms (Figure 17b)

4.3 Exposure to BPS causes effects similar to BPA

The above results with low-dose BPA led us to carry out a similar set of experiments with Bisphenol-S/BPS (Sigma-Aldrich, St. Louis, MO). Exposure to BPS and habituation assays were carried out using essentially identical protocols as described for BPA. As in the case of BPA, we found that exposure to BPS led to a significant decrease in the number of eggs laid at 0.5 mM and higher concentrations (Figure 18a). Additionally, adult worms, resulting from surviving embryos that were exposed to BPS (ranging from 0.1 to 10 mM) displayed a decrease in habituation when compared to animals to vehicle alone (Figure 18b). We did not observe any morphological differences in our exposed embryos or adults for either BPA or BPS, conceivably

due to our use of considerably lower concentration than those used in a previous *C. elegans* embryonic exposure study (15).

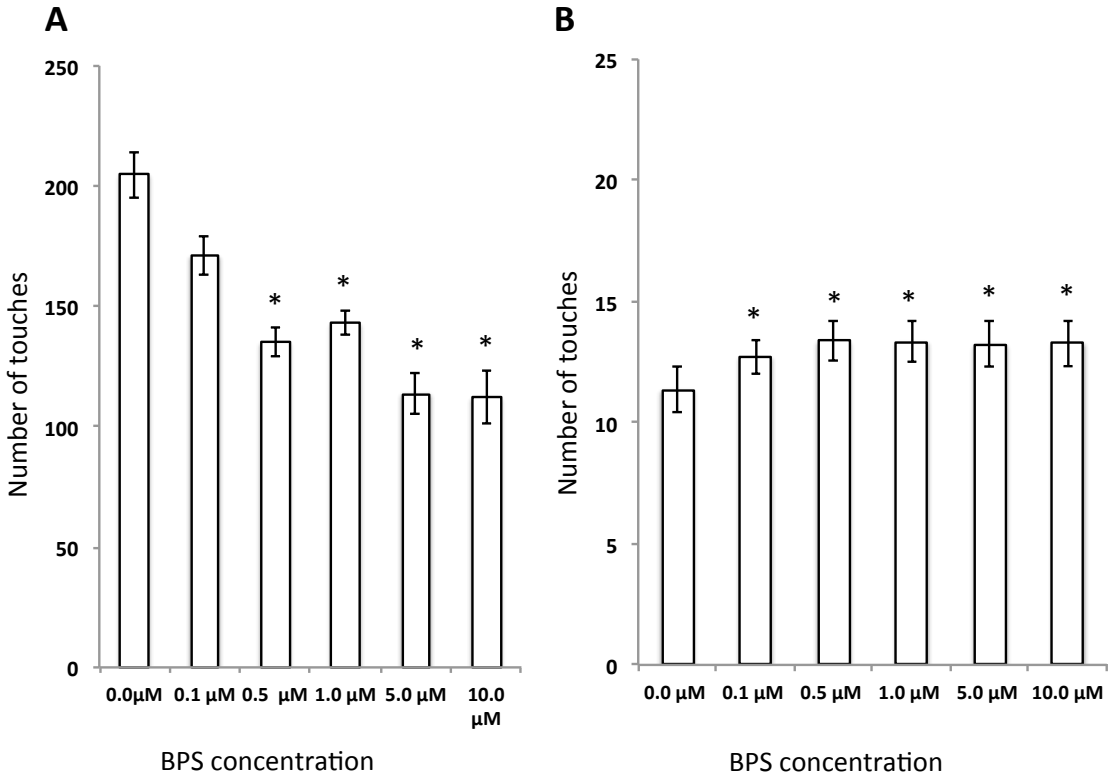

Figure 18 Habituation and egg laying properties were reduced following BPS exposure (A) Similar to BPA, worms exposed to BPS also laid significantly fewer eggs. Experiment was conducted following the steps described in the previous figure (n=10, *p< 0.05) (B) BPS exposure results in slower habituation (n=60 , * $p < 0.05$). Bars depict the mean number of eggs laid (A) and the number of gentle taps required for habituation (B). Error bars denote standard deviation. Analysis done with one way ANOVA followed by Tukey's post hoc analysis

CHAPTER V: CONCLUSION

Our results demonstrate that in *C. elegans* the effects of embryonic exposure to considerably low levels of BPA persist into adulthood, affecting their neural function as assayed by measuring their habituation to anterior touch sensory stimuli. Additionally, we found that BPS, intended to be a safer alternative to BPA, also caused decreased habituation suggesting that it is likely to exert its action in a similar manner as BPA. While confirming previously reported decreased egg-laying caused by continual exposure of *C. elegans* to BPA at ≥1 mM concentrations (15), our results extend these observations by demonstrating decreased fecundity at significantly lower concentrations (as low as 1 mM BPA and 0.5 mM BPS) with exposure limited solely to the embryonic period. Due to their hormone-like properties and structural similarity with estradiol, BPA and BPS may have the potential to interfere with estradiol's modulatory role in synaptic plasticity (20). It is notable that mammalian studies have shown that BPA exposure can increase levels of dopamine in the midbrain (21) as well as up-regulate dopamine D_1 receptor expression (8). Interestingly, postulated mechanisms of mechano-sensory habituation in *C. elegans* point to a central role for dopamine (19, 22-24). Considering the above reports, along with the results presented here, future studies on the effects of BPA and BPS on dopamine regulation may yield valuable information on the mechanisms by which these EDCs affect neuronal function. In conclusion, our study extends knowledge gained from previous reports by examining low-dose exposure in the *C. elegans* model by utilizing an evolutionarily conserved behavior as a surrogate for integrity of neural function. Extending the assay used with our model has the potential to uncover subtle behavioral effects of low-dose exposure to suspected neurotoxic compounds that may not cause phenotypically visible abnormalities.

CHAPTER VI: REFERENCES

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