EFFECT OF GPA-16 ON BILATERAL ASYMMETRY, NEURONAL CONNECTIVITY AND LEARNING BEHAVIOR IN *C. ELEGANS*

by

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A THESIS

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ABSTRACT

Visceral organs structures and brain functions show asymmetric patterns in vertebrates including humans. Under some conditions, the stereotypic asymmetric laterality may be broken which can lead to abnormal structures and functions. Left-handedness is a type of brain functional laterality breaking condition. Research has shown that left-handers have increased performance in associative memory tasks compared to right-handed population. Also, left-handers tend to occupy the opposite ends of the performance spectrum, being more frequently observed in gifted individuals and in persons with psychosis.

C. elegans is an excellent research model to evaluate to answer basic biological questions, including the influence of laterality variations. Bilateral asymmetry is observed in visceral organs and some of the neuron pairs of *C. elegans*. The stereotypic placement of anterior gut locates towards the left. Certain neuron pairs such as ASE-L and ASE-R display functional asymmetry. They express different kinds of genes and detect different kinds of water-soluble chemical cues. *gcy-5* can only express on ASE-R, and *gcy-5::*GFP was used as ASE-R fluorescence marker in research. GPA-16 protein plays an important role in establishing and regulating asymmetric cell division during early stage of embryogenesis. Previous results demonstrated that *gpa-16* mutants showing anatomical reversal in both embryos and adult worms. *gpa-16* mutants show defects in both non-associative learning and associative learning. We examined the placement of ASE-R sensory neuron in *gpa-16* mutants, and found out 22% ASE-R neurons were mis-localized. The mis-localized ASE-R has the potential to generate atypical neuronal circuitry and abnormal synaptic connectivity to downstream neurons, which could lead to the observed learning defects.

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ACD	Asymmetric Cell Division
ASE-L/R	ASE functional asymmetric sensory neuron pair in *C. elegans*
AWC-L/R	AWC functional asymmetric sensory neuron pair in *C. elegans*
AIY-L/R	AIY symmetric interneuron pair in *C. elegans*
BLAST	Basic Linear Alignment and Search Tool
C. elegans	*Caenorhabditis elegans*
CGC	*Caenorhabditis* Genetics Center
Del.gpa-16	Gpa-16 loss-of-function deletion mutation strain
E. coli	*Escherichia coli*
FRAP	Fluorescence Recovery After Photobleaching
Gαi	G-protein alpha inhibitory
Gcy-5	A gene expressed only in ASE-R neuron
GOA-1	G-protein O alpha-1
GPCR	G-protein coupled receptors
GPA-16	G-protein alpha-16
gpa-16Δ	*gpa-16* deletion mutant or *del.gpa-16*

del.gpa-16	gpa-16 deletion mutant or *gpa-16Δ*
NaN3	Sodium azide
N2	Wild type
NGM	Nematode Growth Medium
OD57	A *C. elegans* strain with integrated GFP::tubulin and mCherry::histone
OP-50	An *E. coli* strain
PCR	Polymerase Chain Reaction
pHluorin	pHsensitive green fluorescent protein-based sensor
PKA	Proteinase K
t.s.gpa-16	A temperature sensitive allele of *C. elegans*
ttx-3	A gene widely expressed in AIY interneurons

CHAPTER I: INTRODUCTION

Our laboratory investigates the developmental basis of behavior, while examining the effects of left-right laterality variation to behavior from genetic, development, and neuronal connectivity aspects in *C. elegans*. My thesis is specifically focused on *gpa-16,* which encodes a G-protein α subunit, in terms of how its deletion mutation generates neuron bilateral misplacement and atypical neuron interconnections in an effort to correlate the neurobiological bases of the observed atypical behavior of gpa-16 mutant animals.

1.1. Brief Background

Asymmetric cell division is an essential feature in the development of multicellular organisms, allowing specialization in form and function of daughter cells to yield a functionally complex organism. Most animals including humans display anatomical bilateral asymmetry in the visceral organs. Our nervous systems also demonstrate structural and functional asymmetry. Modern neuroimaging techniques are able to identify the metabolic activities changes in the brain, and shown nervous system asymmetry which correlates with specific cognitive behavioral tasks. For example, neuro-imaging experiments on patients with *situs inversus totalis* (reversal of visceral asymmetry of the lateral axis) has revealed reversal of functional asymmetry in the brain (Ihara et al., 2010).

Caenorhabditis elegans（*C.elegans*）is an ideal model to observe the left-right laterality variation due to its transparent body and comparable simple nervous system. It has been reported that conditional mutations in a Gα subunit coded by *gpa-16* causes reversal of laterality in about 40% of the progeny at the non-permissive temperature of 25^{o}C, while all animals are normal at the permissive 15^{o}C (Bergmann et al., 2003). In early development, GPA-16 has been shown to

affect mitotic spindle positioning and orientation at the 4-cell stage just prior to very first left-right decision (Bergmann,2003). Also, observations in our lab have shown that *gpa-16* mutants are defective in associative and non-associative learning (Dhillon lab, unpublished). Based on the above we hypothesize that anatomically reversed animals display the observed behavioral phenotypes as a result of atypical neuronal connectivity that rooted during initiated during early embryogenesis. The precise sensory neurons and interneuron connectivity changes in *gpa-16* mutant animals are not clear, and also the molecular mechanisms underlying this process are not understood.

To address these issues, we have examined the placement of neuronal circuits of the functional asymmetric sensory neuron pair ASE (amphid single-sensory endings) and interneuron AIY (amphid interneuron class Y) in both deletion *gpa-16* mutants and wild type N2 animals. ASE (Left/Right) are the main chemosensory neurons, and AIY (Left/Right) are the primary post-synaptic target interneurons for ASE, interconnected via chemical synapses.

1.2 Gaps in knowledge and our hypothesis:

The gaps in knowledge about *gpa-16* mutant generated laterality reversal:

1. The deletion *gpa-16* mutant remains molecularly undefined. This mutation was generated from a Gene Knockout Mutation Project at Oklahoma Medical Research Foundation. However, the molecular sequence of the deletion allele is not known and therefore, needs to be sequenced.

2. The mechanism/s underlying the functional consequences of laterality reversal remain unknown.

Our hypothesis:

1. A significant proportion of *gpa-16* mutant animals demonstrate visceral organ reversal, so they might also have functional reversal of asymmetric chemo-sensory neuron pairs.
2. If sensory neuron pairs reverse in *gpa-16* mutants, their connectivity with downstream interneurons may change, and result in atypical behavior.

1.3 Specific Aims of this Study

The specific aims of my study are:

I. Characterizing *gpa-16* deletion including backcrossing mutant strain with N2 (wild-type) to ensure that the mutant results from single *gpa-16* DNA sequence lesion, followed by the PCR/DNA and sequencing to identify the exact deletion.

II. Expressing fluorescent proteins fused to microtubules and chromatin in *gpa-16* mutant animals, and observing the spindle orientation variation in cell division of early stage embryonic development.

III. Marking the functional asymmetric chemo-sensory neurons and their downstream interneurons with fluorescent proteins in order to identify if the neurons demonstrate atypical bilateral placement or abnormal circuit routes. Also, using confocal microscope and confocal stack imaging to observe the left-right laterality variation in visceral organs of *gpa-16* deletion mutant animals. The goal is to clarify the relationship between visceral organ displacement and neuron displacement.

1.4 Importance of this Study

Asymmetric cell divisions (ACD) occurs in various developmental stages of an animal, and they are essential for cell diversity, visceral organ placements, brain cognitive functions, and behavior. ACD is an evolutionarily conserved process but it can be disrupted in specific environments. Dysregulated asymmetric division may result in abnormal cell development in many tissues, including the brain. Also the disruption of ACD has associated with cancer (Gómez-López, 2014).

Hemispheric laterality plays an important role in neuropsychological models of some mental disorders, including bipolar disorder and schizophrenia. It has been reported that in schizophrenia patients, there is an increased asymmetry in global gray matter compared to control. Our research indicates that there is a relationship between the asymmetrical neuron pair laterality variation and learning behavior in *C.elegans* model. This research will aims to understand basic principles of the neural mechanisms of neuropsychological pathologies.

CHAPTER II: REVIEW OF THE LITERATURE

2.1 Laterality

Most organisms display an overall bilateral symmetry as well as some specific bilateral asymmetries. We know the most body plans exhibit external bilateral symmetry, which can be seen in a variety of groups as diverse as worms, insects, vertebrates, and humans. Even though most of the body plans are bilateral symmetric, the symmetry can be broken in development to create LR bilateral asymmetry. The bilateral asymmetry exists together with bilateral symmetry in almost all kinds of organisms, and it happens at both the structural and functional levels. The chirality in lower organisms, such as snail shell coiling and chirality in some plants are examples of structural laterality. The human body demonstrates a number of examples for structural and functional lateralities: such as handedness, which is the preference for using one hand over the other for certain tasks; footedness, which is the tendency of using one side of the foot to take the first step; brain lateralization, which means some neural functions are specialized to one side of the left or right cerebral hemisphere; and viscera laterality, etc.

2.2 Laterality Research in Animal Models

Animal models provide opportunities for researchers to understand the mechanisms and function of laterality through experiments. *Xenopus* tadpole is a model species in which researchers have learned many details about left–right asymmetric patterning mechanisms. For example, drug inhibition or overexpression by microinjection of formin has a chirality-randomizing effect in early (pre-cilia) embryos (Davison, 2016; Lohr et al. 1997). Zebrafish was used as a model in behavioral studies associated with lateralized control of behavior (Millosi, 2006) Rats have been used to study the development of functional brain laterality. Rats

hippocampus, which is an important structure for spatial navigation, can be modified by many epigenetic factors of early environment (Cowell, 1997). Chimpanzees have been used to study handedness which connected with brain functional laterality (Schaafsma et al., 2009). The invertebrate lab model *C.elegans* is being used as a model to study functionally asymmetric gustatory neurons ASEL/ASER in our lab.

2.3 Brain Functional Laterality and Psychosis

The human brain displays functional asymmetry between the two hemispheres. Each hemisphere has certain functional specializations. For example, the left hemisphere is largely responsible for language and logic, and the right hemisphere processes our creativity and artistic abilities. Typically, the left hemisphere shows linear thinking, and right hemisphere shows holistic thinking. A striking example of brain functional lateralization is that of Broca's and Wernicke's areas where speech and language comprehension are processed, both of them are found exclusively on the left hemisphere. So, left hemisphere plays a dominant role in speech and language.

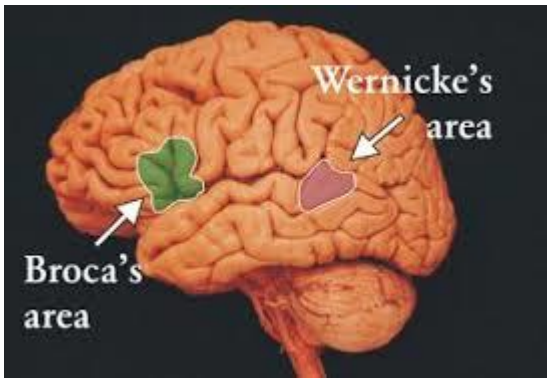


Figure 1: Broca's and Wernicke's areas are specialized for production and comprehension of language. Broca's area is located in the left inferior frontal gyrus and Wernicke's area is located in the left posterior superior temporal gyrus.

Human handedness is correlated with lateralization of cognitive functions. Relationship between handedness and language dominance has been studied. In most right-handed people the left hemisphere of the brain is dominant for language. But left-handed neurological patients demonstrated increased incidence of right-hemisphere dominance for language (Knecht, 2000).

Abnormalities in neuronal functional laterality have been widely documented in studies of schizophrenia in recent years. For example, neurochemical and neurophysiological asymmetries were found in patients with schizophrenia. Dopamine concentrations in the left amygdala of patients with schizophrenia were elevated compared with controls (Reynolds 1983; Reynolds and Czudek, 1987). A higher proportion of men than women with schizophrenia were found to use their left-hand for writing (Fleminger et al., 1977). It has been reported that lateralization of cortical gray matter structures is reduced at the onset of schizophrenia. (Vita 2012)

2.4 Laterality and Early Development

Left-right laterality may have an evolutionary advantage for organisms, because the breakdown of the asymmetric patterning is connected with severe malformations of organs as well as psychosis in humans. Because of the importance of left-right laterality to vertebrates' health, researchers need to explore a number of basic questions, such as how early is laterality initiated; how does the information propagate to cell fields to generate L and R identity; what is the biological basis of human brain functional laterality, etc. Exploration of genetic and molecular mechanisms in animal models and in humans have greatly advanced the understanding of the initiation and establishment of left-right asymmetry in vertebrate embryos. The origins of laterality are highly conserved and established during early embryonic development stage, as

early as the initial breaking of bilateral symmetry (Komatsu, 2014). Mouse model studies have indicated that the activation of nodal signaling cascade is necessary to the establishment of LR asymmetry in mouse embryos (Belo, 2017). Nodal-related ligands of the transforming growth factor-beta (TGFβ) superfamily play central roles in patterning the early embryo during the induction of mesoderm and endoderm and the specification of left-right asymmetry (Shen, 2007). Research on adult male rat indicated that functional asymmetry of spatial navigation behavior is sensitive to environmental influences during early development (Cowell PE, 1997). Human lateralization study also found that hand preference for pointing and language asymmetries are developed early in toddler stage (Cochet, 2013). These studies indicate that laterality establishment is initiated in early development.

2.5 *C. elegans* as an Ideal Model System to Explore Laterality

In 1965, Sydney Brenner initiated experiments with *Caenorhabditis elegans* (*C. elegans*) as a model organism for pursuing research in developmental biology and neurology (Brenner, 1973). Ever since its introduction by Brenner, *C. elegans* has been widely used in research laboratories (Wood, 1988). Our lab uses the well-established *C. elegans* model to carry out genetic, behavioral and neurological studies due to its many advantages. The small 1 mm long adult *C. elegans* is a non-pathogenic free-living nematode existing in soil, feeding on microbes such as bacteria. They can be maintained on petri dishes seeded with bacteria, and their average life span is merely 2-3 weeks. Its genome was the first completely sequenced eukaryotic genome, consisting of 100.3 Megabase pairs (Mbp), with 19,735 predicted protein-coding genes (Hillier et al., 2005). *C. elegans* has 959 somatic cells, 302 neurons that include 282 somatic neurons and

20 pharyngeal neurons. It has two sexes: self-fertilizing hermaphrodite (XX) and male (XY). Hermaphrodites produce haplo-X gametes (sperms and eggs), and give rise to XX hermaphrodite self-progeny. Rare males are generated through spontaneous X chromosome loss (Herman, 2005). 35-37°C heat shock can increase frequency of males in population.

C. elegans worms can be frozen in 15% glycerol solution and preserved in -80°C freezer for years. The frozen worms can be revived when they are warmed to room temperature. Hundreds of transgenic and mutant strains that available from *C. elegans* Genetics Center (CGC) can be preserved and maintained in the lab using this freezing method.

C. elegans genetic studies are relevant to understanding fundamental biology of human function as well as disease processes. Most orthologues of human developmental genes and genes implicated in disease are well conserved in *C. elegans*. These well conserved genes regulate embryogenesis, morphogenesis, development, nerve function, behavior and aging. Our lab is using *C. elegans* as a study object in neuronal functional laterality and psychosis research. Studies using *C. elegans* model may solve the fundamental mysteries of modern biology and neurology.

2.6 Left/Right Asymmetry in *C. elegans*

C. elegans shows predominantly external symmetry but clear bilateral asymmetry in the viscera. In early *C. elegans* embryo, a series of asymmetric divisions are established in the three principal axes of the body plan anterior-posteriot (AP) axis, dorsal-ventral (DV) axis, and left-right (LR) axis (Rose, 2005). Cell polarity is crucial for asymmetric cell division, and it is controlled by the highly conserved PAR (PARtitioning defective) proteins (Noatynska, 2012).

Polarized distribution of PAR proteins and associated downstream components on the cell cortex mediate asymmetric segregation of AB and P1 blastomere cells on AP axis. (Noatynska, 2012, Rose, 2005). DV laterality is established at 2-cell to 4-cell stage, Following the asymmetric division between EMS and P2 (Liro, 2016)**.**

The LR asymmetry is established during 4-cell to 6-cell stages, shortly after the division of ABa and ABp. As the ABa/p spindle elongates, it pushes against the eggshell surrounding the embryo and is forced to rotate. This rotation of the mitotic spindles causes ABa and ABp to make asymmetrical placements in their daughter cells. The left daughters of ABa and ABp (ABal and ABpl) localize more anteriorly than the right daughters ABar and ABpr. (Mickey,1996; Sulston et al., 1983; Wood, 1997), *C. elegans* chiral L/R asymmetric embryos will ultimately develop into a fixed L/R asymmetric adult body plan. The most apparent visceral organ asymmetry is the location of the intestine and gonad, which run longitudinally along almost the whole length of the worm.

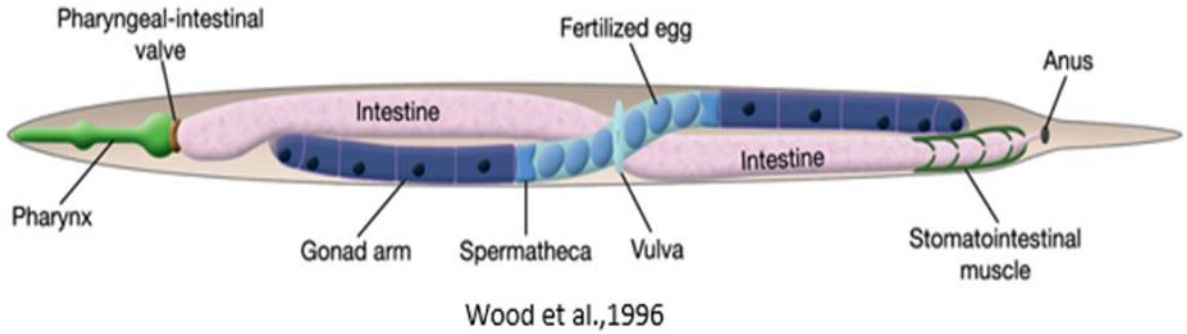


Figure 2: Left-Right asymmetry in *C. elegans* adult hermaphrodite. The anterior intestine places to the left, and anterior gonad places to the right.

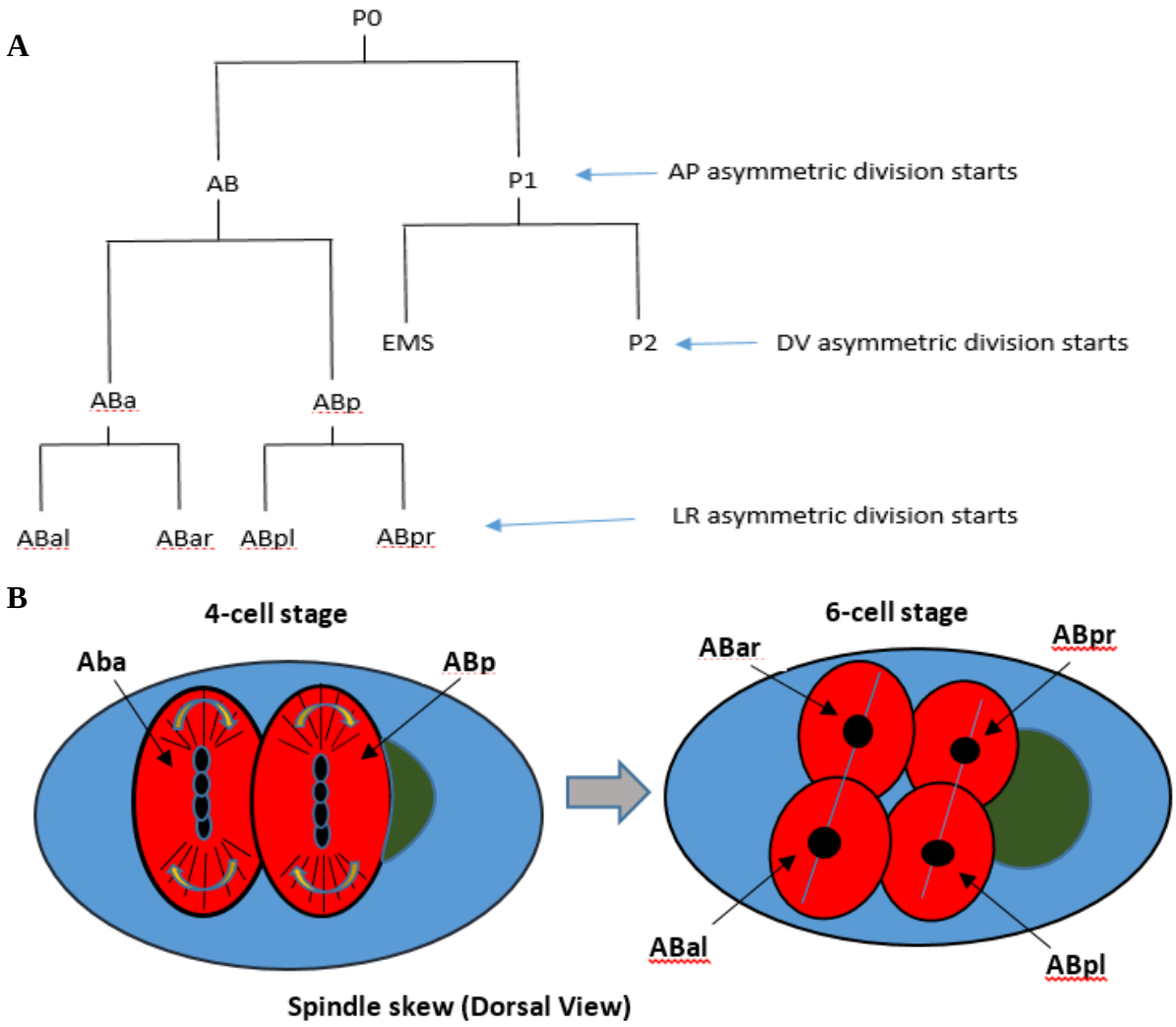


Figure 3: (A) The embryonic development stages in which the AP, DV, and LR asymmetric division initiate. **(B)** Left-Right asymmetry is initiated at the 4-cell stage to 6-cell stage of *C. elegans* embryos. ABal and ABpl localize more anteriorly than ABar and ABpr.

2.7 Nervous System Left/Right Asymmetry in *C. elegans*

The two hemispheres of human brain are anatomically symmetric, but they are functionally asymmetric. Analogously, the nervous system of *C. elegans* also displays a variety of bilateral asymmetries and functional lateralization (Hobert et al., 2002). *C. elegans* has 302

neuron cells, 198 of these neurons appear in pairs which make up 99 neuron pairs. 63 of these 99 neuron pairs exhibit left-right symmetry regard to their placement, axon anatomy, synaptic connectivity, and neuronal function, while the remaining 36 pairs exhibit left-right asymmetry (Bowerman, 2006). For example: ASE (L/R) gustatory neuron pair display distinct types of functional asymmetry.

In the case of humans, L/R asymmetrically expressed genes have not yet been linked to specific L/R asymmetric brain functions, but in *C. elegans* some essential structural genes (e.g. chemoreceptor genes) express asymmetrically in the ASE (L/R) neurons endow distinctive chemoreceptive properties in left and right ASE sensory neurons (Bertrand, et al., 2011). The ASER neuron preferentially detects chloride and potassium ions, while the ASEL neuron preferentially detects sodium ions (Pierce-Shimomura et al., 2001). The cells of another olfactory sensory neuron pair AWC(L/R) are also functionally distinct from each other: one detects butanone, and the other detects 2,3-pentanedione, and both detect benzaldehyde and isoamyl alcohol (Wes and Bargmann, 2001).

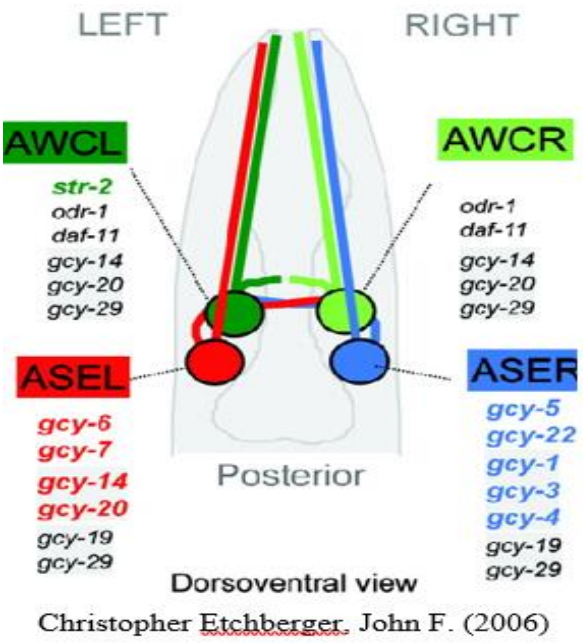


Figure 4: Different genes express asymmetrically in ASE (L/R) chemosensory neurons and AWC (L/R) olfactory neurons.

2.8 GPA-16 in Asymmetric Cell Division

The gene *gpa-16* was originally designated as *spn-1* which encode the Gα protein GPA-16. Loss-of-function mutation of this gene can cause randomization of spindle handedness at the 4-cell to 6-cell stage (Wood, 1998). Since *spn-1* is the same as a molecularly defined gene gpa-16, and later on this gene was referred as *gpa-16* (Jansen et al., 1999).

There are two *gpa-16* mutant alleles: temperature sensitive mutant allele (*t.s.gpa-16*) and loss of function deletion allele (*del.gpa-16* or *gpa-16Δ*). t.s.gpa-16 animals result in spindle orientation defects in early embryos at restrictive temperatures, which is probably to underlie the first symmetry-breaking steps. Overall 75% embryos exhibited failure of P1 rotation when worms were grown at 25° overnight (Liro, 2016). It has been demonstrated that *t.s.gpa-16* mutation leads to randomization of the ABa/p spindle skew headedness (Pohl, 2011). The loss-

of-function *gpa-16* gene knock out mutation *del.gpa-16* (*gpa-16Δ*) verified the early mitotic spindle orientation defect in embryonic development. Our study with embryonic laterality and visceral organ laterality in adult worms showed that *del.gpa-16* mutants also produced more sinistral embryos compared to the wild type embryos (Mersha, 2017).

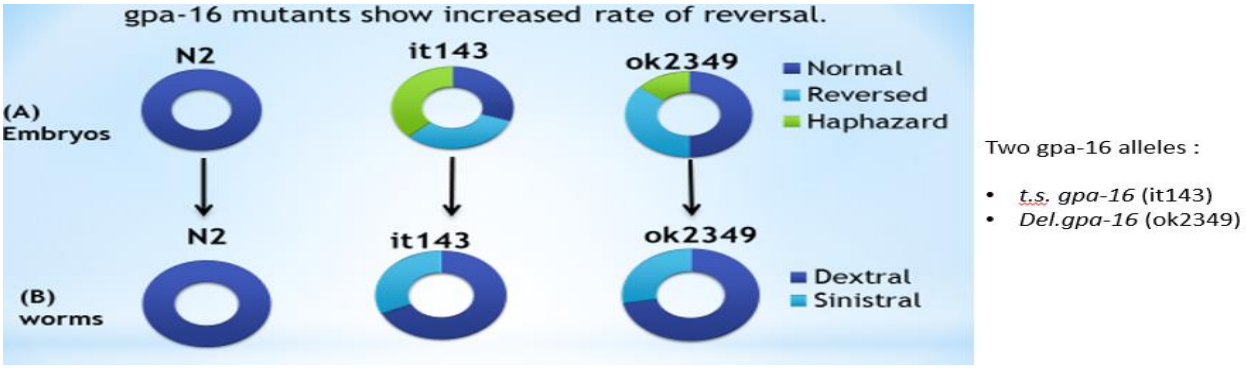


Figure 5: The reversal situation of two *gpa-16* mutant alleles. (A) all wild-type embryos divided normally while both *t.s.gpa-16* and *del.gpa-16* embryos show increased (about 1/3rd) **reversal.** (B) All wild-type embryos developed into stereotypic dextral adult worms while both gpa-16 surviving embryos showed increased (about 1/3rd) visceral organ reversal in adult worms.

The Gα proteins GOA-1 and GPA-16 act in a partially redundant manner to generate pulling forces along astral microtubules (Afshar, 2005). Two G-protein activators GPR-1 and GPR-2(GPR1/2 show asymmetric location in cells), function together with GOA-1 and GPA-16 to generate asymmetric spindle pole elongation during cell divisions in the P-lineage (Tsou et al., 2003).

Randomization of ABa/p spindle skew handedness will generate sinistral embryos and sinistral adult worms though the lethality rate of the *gpa-16* mutant embryos is high. The way to identify the sinistral worms with N2 worms is through observing the position of the anterior gonad relative to the gut. Gonad handedness is a reliable predictor of overall handedness

(Wood,1991; Wood et al., 1996). The embryonic spindle orientation in ABa and ABp were scored for handedness using Olympus IX71 DIC microscope (Mersha, 2017).

The *gpa-16* gene is highly conserved throughout species and human. GNAi is the human homolog of GPA-16. The alignment of *C. elegans* GPA-16 amino acid sequence with human GNAi amino acid sequence showed that these two protein products have 79% similarity. Study on GPA-16 may help us understand more molecular and cellular basis of human brain functional laterality variations.

```
GPA-16 1     GCIMSQEDEAAKRRSKKIDRLLKEDGENSMRTIKLLLLGAGESGKSTILKQMRIIHDVGY  60
             GC +S ED+AA  RSK IDR L+EDGE + R +KLLLLGAGESGKSTI+KQM+IIH+ GY
GNAi   1     GCTLSAEDKAAVERSKMIDRNLREDGEKAAREVKLLLLGAGESGKSTIVKQMKIIHEAGY  60

GPA-16 61    TTEERKVFRGVVYGNIILSLNAIIHAMEQLKISFTTLDHESDARKLLMFSTTGEEDELPE  120
             + EE K ++ VVY N I S+ AII AM +LKI F       DAR+L + +   EE  +
GNAi   61    SEEECKQYKAVVYSNTIQSIIAIIRAMGRLKIDFGDSARADDARQLFVLAGAAEEGFMTA  120

GPA-16 121   ELVVLMKSVWSDSGIQKALERSREYQLNDSAGYYLSQLDRICAPNYIPTQDDILRTRIKT  180
             EL  ++K +W DSG+Q    RSREYQLNDSA YYL+ LDRI  PNYIPTQ D+LRTR+KT
GNAi   121   ELAGVIKRLWKDSGVQACFNRSREYQLNDSAAYYLNDLDRIAQPNYIPTQQDVLRTRVKT  180

GPA-16 181   TGIVETQFVYKDRLFLVFDV GQRSERKKWIHCFEDVTALIFCVALSEYDMVLVEDCQTN  240
             TGIVET F +KD  F +FDVGGQRSERKKWIHCFE VTA+IFCVALS+YD+VL ED + N
GNAi   181   TGIVETHFTFKDLHFKMFDVGGQRSERKKWIHCFEGVTAIIFCVALSDYDLVLAEDEEMN  240

GPA-16 241   RMRESLKLFDSICNNKWFVETSIILFLNKKDLFEEKIVRSPLTHCFPEYTGANNYEEASA  300
             RM ES+KLFDSICNNKWF +TSIILFLNKKDLFEEKI +SPLT C+PEY G+N YEEA+A
GNAi   241   RMHESMKLFDSICNNKWFTDTSIILFLNKKDLFEEKIKKSPLTICYPEYAGSNTYEEAAA  300

GPA-16 301   YIQQQFEDMNKRTTGEKNQEIYTQFTCATDTNNIRFVFDAVTDIIIRDNLRTCGLY  356
             YIQ QFED+NKR   +  +EIYT FTCATDT N++FVFDAVTD+II++NL+ CGL+
GNAi   301   YIQCQFEDLNKR---KDTKEIYTHFTCATDTKNVQFVFDAVTDVIIKNNLKDCGLF  353
```

Figure 6: GPA-16 comparison with human GNAi protein product. Identities: 237/357 (66%), Positives: 283/357 (79%). *t.s.gpa-16* allele has a G to D replacement at site 202 (dark blue). *Del.gpa-16* allele has a deletion from amino acid residue 23 to 79 (red).

2.9 Asymmetric Cell Division and Neuronal Laterality

The asymmetric cell division generates neuronal bilateral asymmetry in *C. elegans* (Hobert, 2005). There has been some exploration of mechanisms initiating functional asymmetries of nervous system. Previous research on an asymmetric neuron pair ASEL and ASER has shed some light on the functional asymmetry initiation in early embryonic development. ASEL/R's terminal cell fates develop from a hybrid precursor state. Some ASE cell markers co-express in ASEL/R precursor cells, but later become restricted to either ASEL or ASER neuron(Richard J. Poole, 2006). a bi-stable regulatory circuit, composed of several miRNAs and transcription factors, will ensure that ASER fate is turned off in ASEL and ASEL fate is turned off in ASER (Richard J. Poole, 2006). *Gpa-16* plays an important role on ASEL/R asymmetry. It encodes Gα protein GPA-16, and GPA-16 has a partially redundant function with GOA-1, both of them are involved in generating pulling forces along astral microtubules through working together with asymmetrically located GPR1/2 (Afshar, 2005). Mutants *of gpa-16* can generate visceral organs asymmetry reversal animals.

2.10 Laterality Variation and Cognitive Ability

Previous research provides key insight into the the link between brain asymmetry and the lateralized behavior, such as eye use preference, handedness, etc., but there are limited studies showing the link between asymmetry variation and cognitive ability. One studywas done on *Xenopus* tadpoles, where three groups of tadpoles (wild-type, left-right-randomized and left-right-reversed tadpoles) were tested for their ability to learn color cues in an automated assay. Results indicated that animals with either randomization or reversal of somatic left-right patterning learned more slowly than wild-type siblings (Blackiston,2013). Several different

associations between hand laterality and cognitive ability have been observed. Average cognitive ability increases monotonically with increasing strength of laterality (Nettle, 2003). Other research in mouse models indicated that environments induced handed behavior would generate cryptic genetic variation in learning ability. Cryptic genetic variation may be exposed to selection by developmentally plastic responses that alter trait performance. (Hallgrimsson, 2011; Palmer,2011). Little research has been done on how *gpa-16* mutation induced laterality variation could influence the learning and memory ability in *C. elegans*.

Hypothesis

We hypothesize that since *gpa-16* mutant animals have high rate of visceral organ reversal, therefore they will also have asymmetric sensory neuron pairs reversal which leads to atypical neuronal connectivity and atypical learning behavior.

CHAPTER III: MATERIALS AND METHODS

3.1 Strains

The following *C. elegans* strains were obtained through the *Caenorhabditis* Genetic Center (CGC), University of Minnesota, Minneapolis, MN.

Wild-type strain (N2),

RB1816 deletion *gpa-16 (ok2349),*

BW1809 *t.s.gpa-16(it143); him-5 (e1490),*

OH3192 *gcy-5::GFP (ntIs1),*

OH7193 *otIs181;him-8 (e1489),*

And OD 57 [GFP::tubulin, histone::mCherry].

The following strains were generated from Dr. Dhillon's lab:

The *gpa-16 (ok2349)* deletion mutant strain was backcrossed three times with N2 before crossing with other transgenic strains or carrying out the behavior test.

Del.gpa-16: gcy-5::GFP: ttx-3::mCherry crossing strain

Gcy-5::GFP:ttx-3::mCherry crossing strain

And *del.gpa-16*: OD57

The above strains were used throughout the study.

3.2 Maintenance

Worms were maintained following standard procedure described by Brenner (1974). All strains were cultured on nematode growth medium (NGM) plates at 20°C, and the NGM plates were seeded with *Escherichia coli* (OP50) as food source.

3.3 Generating Males

We generated *C. elegans* males by heat shock. Three *E. coli* OP 50 seeded NGM plates with 5 x L4 hermaphrodites per plate were set up, and sealed with parafilm. These plates were heat shocked at 34º C for 4 hours. After heat shock, the temperature was shifted back to 20°C. We could get a few males per plate in F1 generation in about 3-4 days. In order to get more males, three males obtained through heat shock were picked and transferred to a plate with two hermaphrodites, so as to generate males at higher rate (up to 50%) by mating.

3.4 Generating Mutant Crossing Strains

3.4.1 Generating *gcy-5::GFP: ttx-3::mCherry* crossing strain

Five OH3192 [gcy-5::GFP] males were transferred and allowed to mate with two OH 7193 [ttx-3::mCherry] hermaphrodites. Once their progeny became young adults, 10 hermaphrodites were transferred to a new plate and allowed to lay eggs. After laying eggs, the single hermaphrodite/parent worm was picked to do single worm PCR for checking if both GFP and mCherry DNA are there in the crossing progenies. Confocal microscopy also helped to check if both of the fluorescence proteins are expressed in *gcy-5::GFP:ttx-3::mCherry* crossing strain.

3.4.2 Generating *del.gpa-16:gcy-5::*GFP*:ttx-3*::mCherry crossing strain

Crossing OH3192[*gcy-5::GFP*] with *del.gpa-16* (or *gpa-16Δ*) animals to get *del.gpa-16:gcy-5::GFP* mutant strain first, and then crossing *del.gpa-16:gcy-5::GFP* with OH7193[*ttx-3::mCherry*] to get *del.gpa-16:gcy-5::GFP:ttx-3::mCherry* mutant strain. The detailed process of crossing is similar as the process to generate *gcy-5::GFP:ttx-3::mCherry* crossing strain. But the PCR step in this crossing needs to detect if *gpa-16* deletion band being amplified in the crossing strains.

3.4.3 Generating *del.gpa-16:OD57* crossing strain

The process of generating *del.gpa-16:OD57* is similar as the one to generate *del.gpa-16:gcy-5::GFP*. All of the detailed steps are showed in the following diagram:

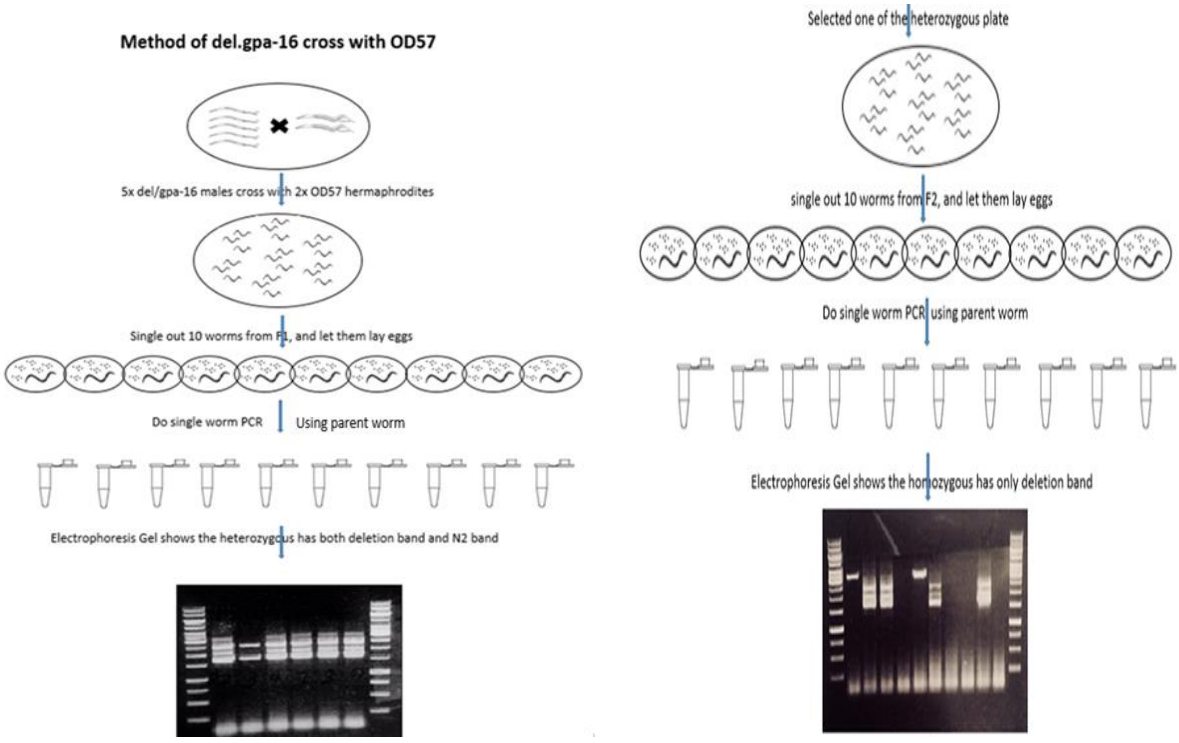


Figure 7: The process of generating *del.gpa-16:OD57* mutant strain.

3.5 Single Worm PCR Reaction

PCR Amplification	Primer Sequences
A 1.687 kb sequence was amplified from *del.gpa-16* mutant animal genomic DNA using *gpa-16* outer primers.	Fw:5'-AGC GCA ATG GGG TGT ATT AT-3' Rv: 5'-CGA ATC GGA CCA AAC ACT AT-3'
A 600 bp GFP sequence was amplified from *C. elegans* genomic DNA using the GFP primers.	Fw: 5'-GTC AGT GGA GAG GGT GAA GG-3' Rv: 5'-TTG AAC GCT TCC ATC TTC AAT-3'
A 350 bp mCherry sequence was amplified from genomic *C. elegans* DNA using the mCherry primers.	Fw: 5'-AGA TCG AGG GAG AGG GAG AG-3' Rv: 5'-CCC ATG GTC TTC TTT TGC AT -3'

Table 1: Single worm PCR reactions used to amplify *gpa-16* deletion band, GFP band, and mCherry band.

The following single-worm PCR reaction program was used to effectively amplify *gpa-16* deletion band, this program can also be used to amplify GFP band and mCherry band.

1. 95°C, 2 min
2. 95 °C 0.5 min
3. 55 °C 0.5 min
4. 72°C 3 min
5. Repeat for 31 cycles
6. 95°C 5 min
7. 12°C infinite

PCR product was confirmed by gel electrophoresis purified and stored at -20°C for future use.

3.6 Sequence Analysis

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

3.7 DNA Cloning and Sequencing of *gpa-16 deletion*.

3.7.1: PCR amplify *gpa-16* genomic DNA fragment from deletion mutants.

- *del.gpa-16* single worm PCR using Go-taq Flexi DNA polymerase and J19/J20 primers.
- PCR product (1687 bp) was purified using "Illustra GFX PCR DNA and gel band purification kit"

3.7.2: Cloning of desired *gpa-16* DNA fragment.

- TA Cloning Reaction using TOPO TA cloning kit (pCR™2.1-TOPO® vector).

TA cloning reaction (total vol:5µl)	
DD water	2µl
5 x buffer	1µl
Vector	1µl
Ligase	0.5µl
PCR product	0.5µl

- incubate the ligation reaction at room temperature for 15 minutes.

3.7.3: Transformed the cloning product into chemically competent cells Mach1™ T1R.

- Gently add 2.5µl of the ligation product to the vial of competent cells (Mach1^{TM} T1R).
- Put the vial on ice for 30 mins.
- Incubate the vial at 42°C water bath for 30 seconds.
- Add 250µl pre-wormed S.O.C. to the vial.
- Incubated 1 hour at 37°C shaking at 225 rmp.
- Spread 100 µl on LB X-gal Amp Plates.
- Incubated the plates at 37°C overnight.

3.7.4: Selection of transformed cells.

- Prepare 36 x LB ampicillin tubes (2ml) to cultivate single white colony.
- Circle 36 white colonies from LB X-gal Amp plates, mark them with numbers.
- Gently picked the marked single colonies using tips of toothpicks, and transferred into the 36 x LB Amp tubes (2ml).
- Incubated the tubes at 37°C for 1-2 days shaking at 225 rmp.

3.7.5: Plasmid DNA purification and restriction analysis.

- Wizard Plus SV minipreps DNA Purification system was used to extract and separate plasmid DNA from rest of the cellular components, including membranes, proteins and enzymes.

- Miniprep Digestion used EcoRI.

Digestion Reaction (total vol:15µl)	
DD water	6.4µl
10x NEB EcoRI buffer	1.5µl
EcoRI	0.1µl
Plasmid DNA	7µl

- Running agarose gel electrophoresis.

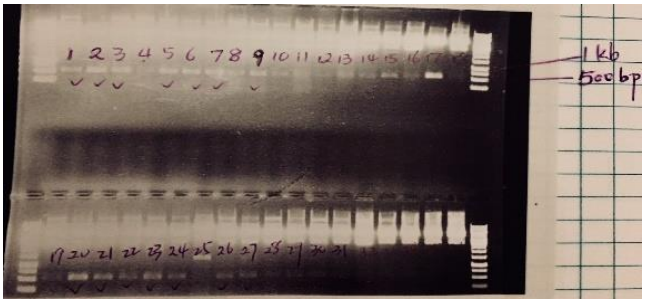


Figure 8: Restriction reaction results in two sizes of expected bands in electrophoresis. The band of the vector was 3.9kb, and the band of inserted DNA was about 500bp.

3.7.6: Sequencing of DNA insert was carried out at the sequencing core service through plant-microbe genomics facility in the Ohio State University (https://pmgf.osu.edu/)

3.8 Adult Internal Organ Laterality

In normal N2 hermaphrodites, the adult body plan has fixed L/R positioning of internal organs. Gut resides on the left and gonad on the right, when viewed the anterior section of the worm body from the dorsal aspect. We can observe the position of gut and gonad by Zeiss LSM 510 laser scanning confocal microscope.

5% agarose was dropped onto a plain glass microscope slide, gently pressed it into a thin slice using another slide. Dispense ~10 μl of M9 and sodium azide onto the slice, then picked ~20 adult hermaphrodites onto the center of slice, covered the agarose slice using No. 1.5 thickness cover slips, sealed the coverslip with nail polish. Under transmitted light channel of the microscope, we could take Z-stack images which could cover all image planes going through the worm body, so the position of gut and gonad could be identified by analyze the Z-stack images.

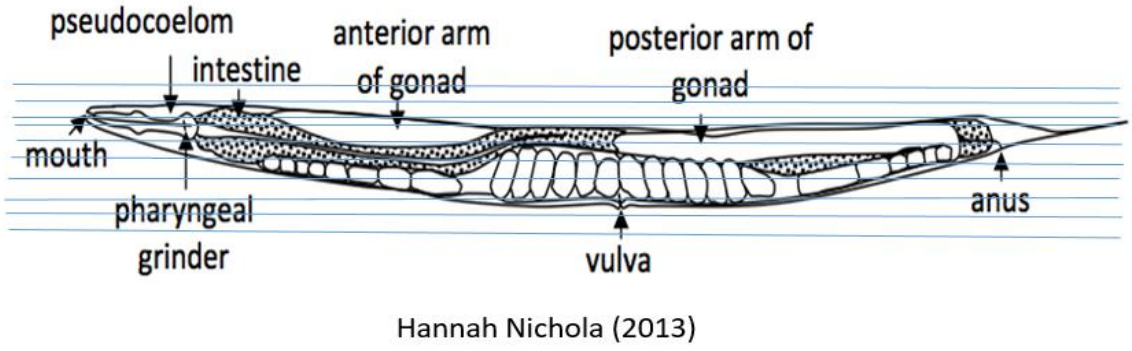


Figure 9: Z-stack images taken by Zeiss LSM510 confocal microscope helps to figure out the position of visceral organs. The focus of microscope will go through the worm body from bottom to top. The anterial organ appears first locating to the right, and appears later locating to the left in the case when volva shows a down-lateral view.

3.9 ASE Neuron Laterality

The method for preparation of slides for determining ASE neuronal laterality was the practically identical to that used for visceral organ laterality observation detailed above. 488nm laser line and 633 nm laser line were used to observe GFP marked ASE neuron and mCherry marked AIY neuron. The shape and position of AIY neuron can help determine the

ventral/dorsal side of the worm, and the relative position of ASE and AIY can help determine whether the ASER neuron is L/R mis-localized.

3.10 Confocal Microscope Images Analysis

Zen software was used to analysis the images taken from Zeiss LSM510 confocal microscope.

3.11 Statistical Analysis

Data was recorded in Microsoft Office 2007 Excel software (Microsoft Corporation, Redmond, WA). Statistical analyses were done using the above software or through GraphPad Prism7 (GraphPad software, La Jolla, CA).

CHAPTER IV: LOSS OF GPA-16 CAN CAUSE VISCERAL ORGAN REVERSAL AND NEURONAL REVERSAL WHICH CAN LEAD TO LEARNING DEFECTS

4.1 Introduction

The *C. elegans* nervous system displays a variety of bilateral asymmetries (Hobert et al., 2002). For example, the ASE (L/R) amphid neuronal pair is unique in that ASE-L and ASE-R display functional asymmetry in chemosensory modalities. Specific genes including chemoreceptor genes express asymmetrically in the ASE (L/R) neurons generating distinctive chemoreceptive properties accordingly (Hart and Chao, 2004) ASEL and ASER each sense a characteristic set of chemical cues. the ASER neuron preferentially detects chloride and potassium ions, while the ASEL neuron preferentially detects sodium ions (Bargmann, 2005). ASE L/R neurons show ON/OFF asymmetry when they detect certain chemicals, underscoring their functionally distinctiveness.

The ASE sensory pair post-synaptic target includes another pair, AIY-L and AIY-R interneurons which are known to be bilaterally symmetric. AIY (L/R) cell bodies are located in the ventral head ganglion, right below the terminal bulb of pharynx. AIY axons enter nerve ring from the ventral side, and running dorsally till left and right AIY dendrites meet at the dorsal midline. ASE (L/R) cell bodies locate at the intermediate level of terminal bulb of pharynx, ASE axons projects into nerve ring from ventral ganglion, making synaptic connections in ring neuropil. ASE (L/R) neurons make strong connections with AIY (L/R) interneurons via chemical synapses in ring neuropil. Signal transduction between ASEs and AIYs are not clear yet, but theoretically the transduction goes through the classical neurotransmitters because serotonin,

dopamine, octopamine, and tyramine have all been shown to modulate the animal's chemosensory responses (Chao et al. 2004; Ferkey et al. 2007; Wragg et al. 2007)

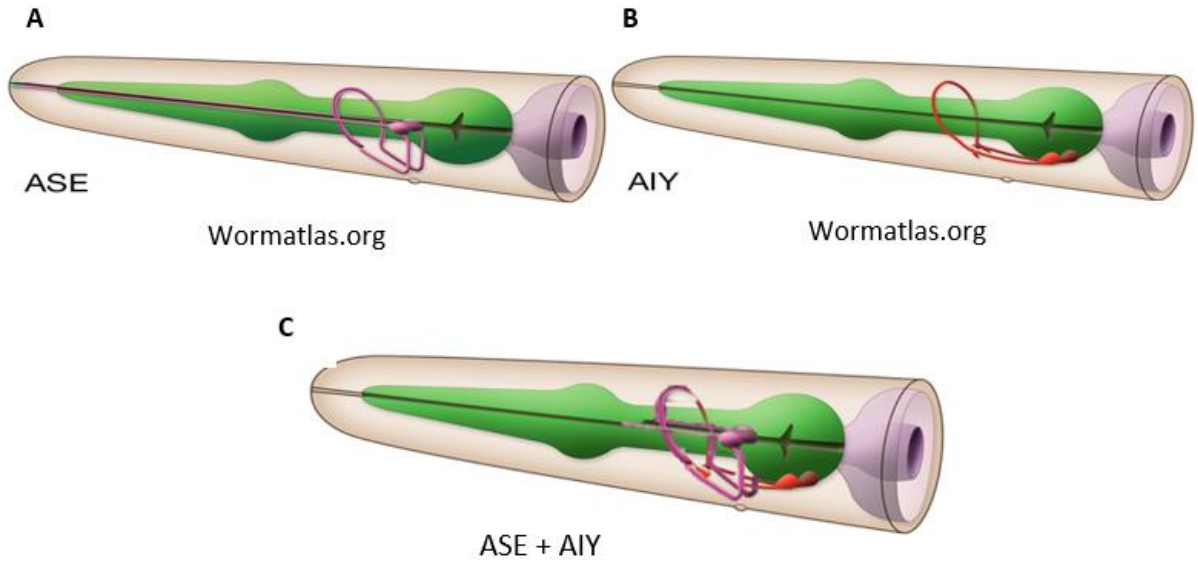


Figure 10: The position of ASE chemosensory neurons (A) and AIY interneurons (B). the axons of ASE neurons and AIY neurons make strong connection in circumpharyngeal nerve ring (C).

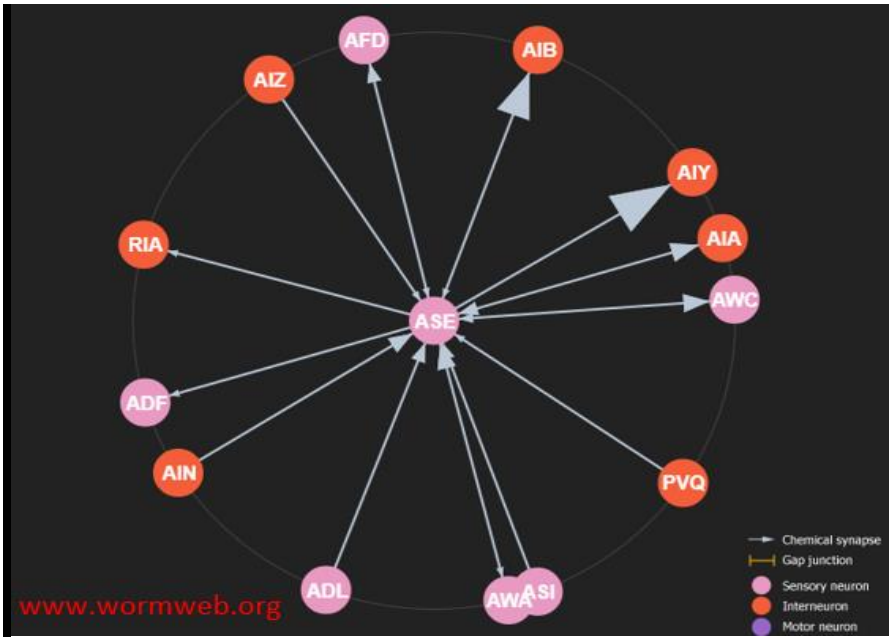


Figure 11: Neuronal connection between the ASE sensory neurons and various interneurons and sensory neurons. Bigger arrow head indicates stronger neuronal interconnection, and indicating major output to AIY.

A mutant strain, *t.s.gpa-16(it143),* was found to have mitotic spindle orientation defect in early embryonic development which can generate anatomically reversed embryos and adult worms. *gpa-16* encodes GPA-16, G-protein α-subunit, which is a key component in the upstream effectors of the laterality determination pathway. GPA-16 is involved in generating forces to pull and position the astral microtubules asymmetrically (Gotta and Ahringer, 2001; Colombo *et al*., 2003; Srinivasan *et al*., 2003; Tsou *et al*., 2003). At non-permissive temperatures, the conditional mutation in gpa-16 causes reversal of laterality in about 40% of the progeny (Bergmann et al., 2003).

In order to record the spindle orientation and the laterality establishment in more detail, we used fluorescence proteins to mark the cell microtubules and chromatins in both of the wild-type and *gpa-16Δ* mutant animals, so the atypical spindle orientation in early embryonic development and the pathway of how laterality variations being established in *gpa-16Δ* mutant can be easily tracked. A transgenic strain OD-57, which is the wild-type strain but expressing both GFP::tubulin and mCherry::histone H3, was used to cross with *gpa-16Δ* mutant (Allard and Colaiacovo, 2010). We generated a cross strain *gpa-16Δ:OD57*, so *gpa-16Δ* mutant also express GFP::tubulin and mCherry::histone. OD57 was generated by particle bombardment of a pie-1 promoter vector (Praitis et al., 2001) engineered to express a histone H2b fusion to a version of mCherry. Comparing time-lapse images of wild-type embryos and *gpa-16Δ* embryos expressing

GFP::tubulin and mCherry::histone with confocal microscope can reveal the mitotic spindle orientation changes during cell division.

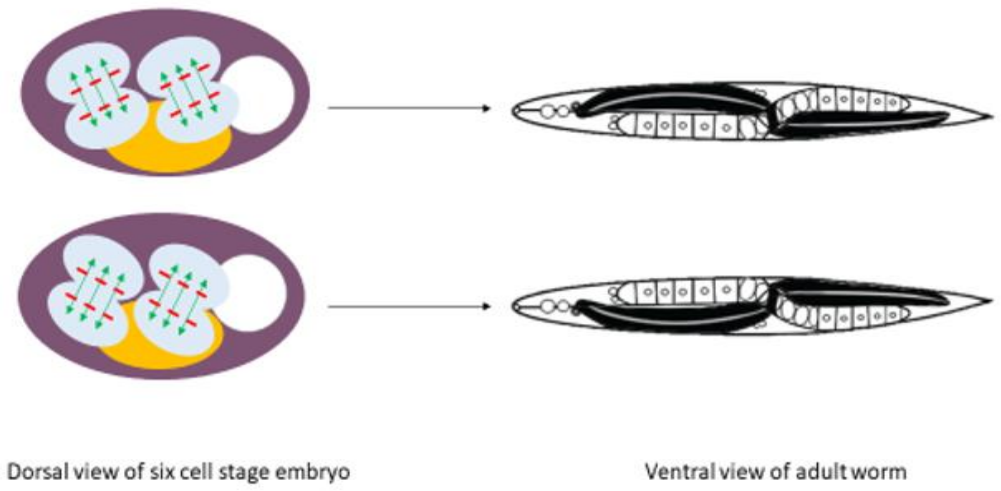


Figure 12: Hypothesized sinistral spindle orientation in six-cell embryonic developmental stage produce gut/gonad reversal adult worms in *gpa-16Δ:OD57* animals (upper: dextral, lower: sinistral).

Both *t.s.gpa-16* and *del.gpa-16* mutants were showing some learning defects at restrictive temperature (Mersha, 2017). Associative learning and non-associative learning were conducted in our lab, the study indicated that absent of GPA-16 showing significantly higher habituation rate and conditioned chemotaxis index in both *gpa-16* mutants (Mersha, 2017).

4.2 Hypothesis

We hypothesize that the learning disabilities may be related to some atypical neuronal inter-connections caused by the laterality reverse of neurons. Both of the *gpa-16* mutants have high rate of visceral organs reversal, they may also have sensory neuron pairs reversal which contribute to learning behaviors.

4.3 Methods (for full method see chapter III)

4.3.1 Mutant strain *gpa-16Δ* preparation

The strains N2 (wild type Bristol isolate) and RB1816 *gpa-16Δ (ok2349)* were obtained from the *Caenorhabditis* Genetic Center, University of Minnesota, Minneapolis, MN. But the strain RB1816 *gpa-16Δ (ok2349)* hadn't been backcrossed, and the accurate location of the deleted sequence was not clear. First, we backcrossed the mutant strain with N2 for three times, and then we did the DNA cloning and sequencing for identifying the precise location of the genomic DNA deletion fragment (cloning and sequencing methods see chapter III).

Worms were grown in nematode growth media (NGM) plates at 20°C. The *Escherichia coli* strain OP50 was used as a food source.

4.3.2 Crossing strains preparations

In order to test our hypothesis, we selected two transgenic strains OH3192 *gcy-5::GFP (ntIs1)* and OH7193 *otIs181;him-8 (e1489)*, which are wild-type strains but have GFP marked ASER sensory neuron and mCherry marked AIY interneurons in each of them. The gene *gcy-5* is expressed in ASER only, so *gcy-5::GFP* works as reporter gene in ASER neuron. The *ttx-3* gene is expressed in AIY interneuron pair, so *ttx-3::mCherry* works as reporter gene in AIY(L/R) interneurons. We crossed the backcrossed *gpa-16Δ (ok2349)* with both of OH3192 and OH7193, so the *gpa-16Δ* mutant strain also have fluorescence marking ASER and AIY neurons. We also crossed *gpa-16Δ* mutant with OD57, so *gpa-16Δ* mutant also express *GFP::tubulin and mCherry::histone*. The detailed crossing methods were recorded in chapter III.

4.3.3 Slides making and photographing by confocal microscope

As mentioned earlier, L4 to adult hermaphrodites were picked and transferred to a slide with 10μl of M9 and sodium azide. Covered and sealed with nail polish. Z stack images were taken using Zeiss LSM510 confocal microscope

4.3.4 Identify visceral organs laterality

Details method see chapter III 3.8

4.3.5 Identify ASER sensory neuron laterality

Details method see chapter III 3.9

4.4 Results

4.4.1 DNA sequence of the deletion section in *gpa-16Δ* mutant animals

The gpa-coding sequence from deletion mutants was PCR amplified and cloned into pCR™2.1-TOPO® vector using TA cloning, and sequenced. Four clones were sequenced to confirm the deletion size of 1687 bp.

------CGATTTTTGTGTGAGAAAACCTGAAAAATTA------1687 base deletion-------TCAAAAATTGTCTGTTTTTCA-----

Figure 13: The starting and ending loci of the deletion in *gpa-16Δ* mutant genome.

4.4.2 GPA-16 functional domains that being disrupted in *t.s.gpa-16* and *del.gpa-16* mutants.

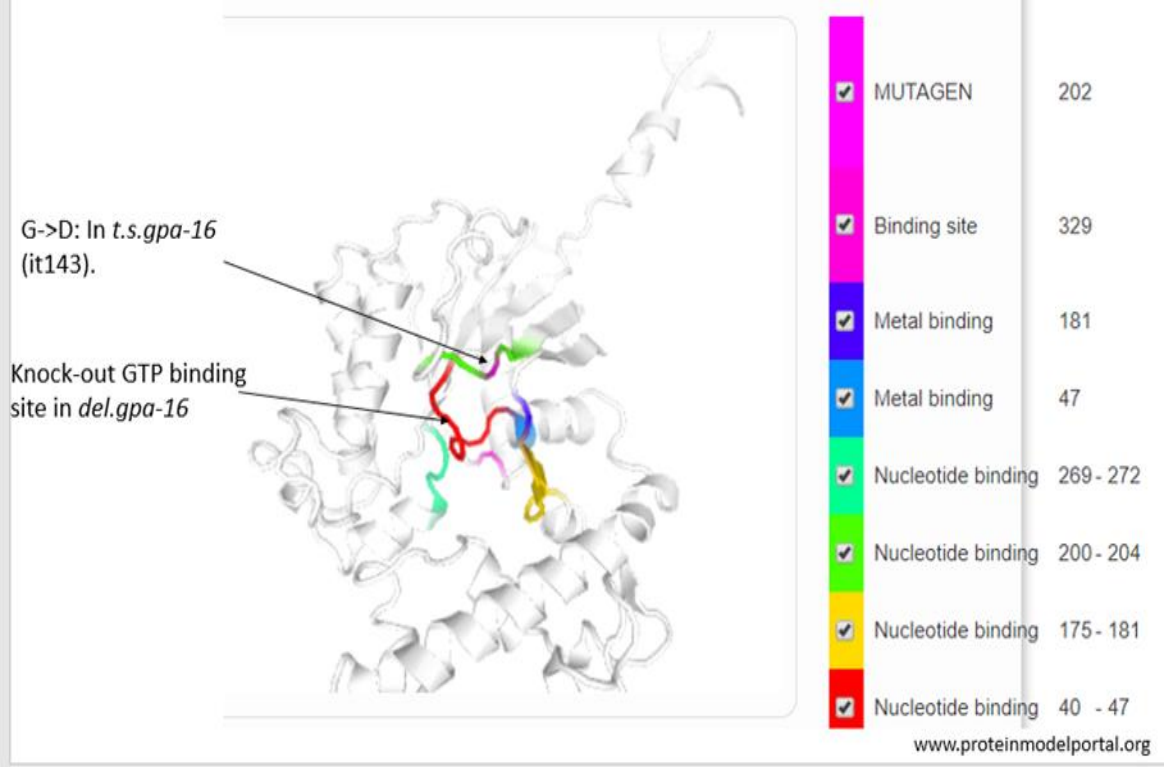


Figure 14: 3-D Model of GPA-16 subunit with functional domains. *t.s.gpa-16* allele has a G to D replacement in one of the Nucleotide (GTP) binding site (200-204). *Del.gpa-16* allele includes a knock-out GTP binding site (40-47).

4.4.3 Crossing to obtain transgenic mutant strain *gpa-16Δ:gcy-5::GFP:ttx-3::mCherry* and transgenic control strain *gcy-5::GFP:ttx-3::mCherry*.

We crossed *gpa-16Δ* mutant with *gcy-5::GFP* first to obtain *gpa-16Δ:gcy-5::GFP* (Figure 14A), then using this new strain to cross with *ttx-3::mCherry* to obtain *gpa-16Δ:gcy-5::GFP:ttx-3::mCherry* (Figure 14B). We crossed two transgenic strain *gcy-5::GFP* with *ttx-3::mCherry* to obtain the control strain *gcy-5::GFP:ttx-3::mCherry* (Figure 14C). All crosses were confirmed using single worm PCR.

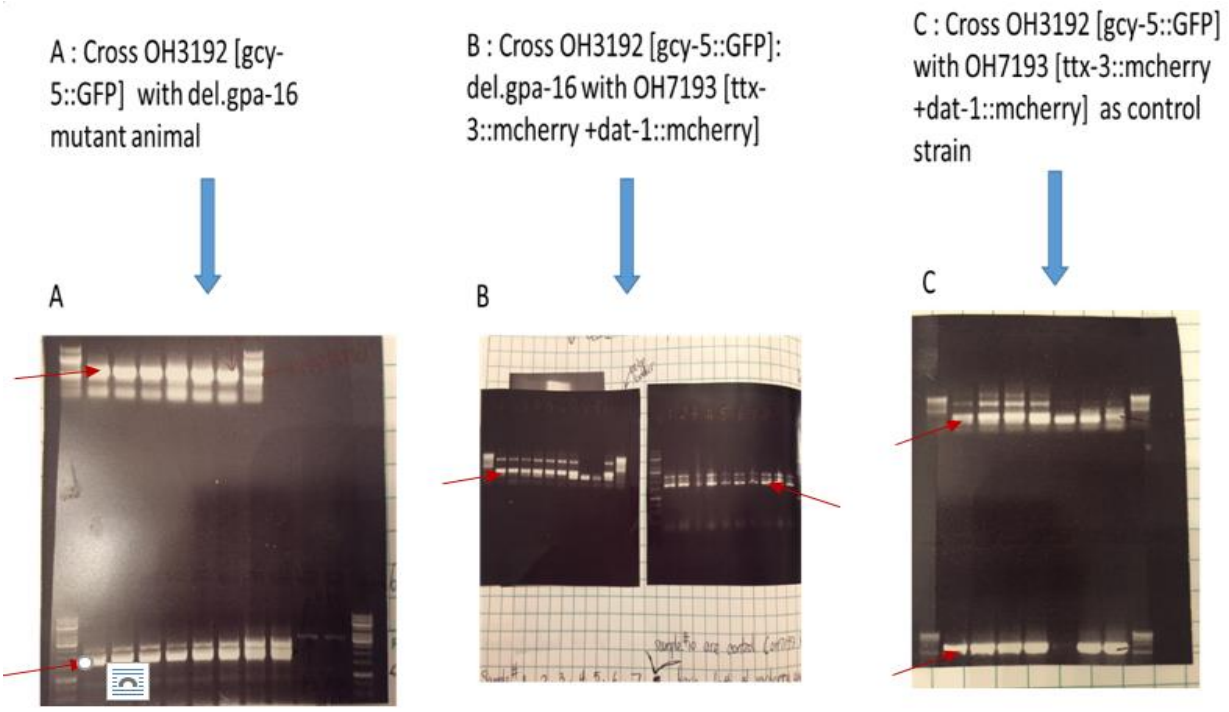


Figure 15: Crossings to get the *gpa-16Δ:gcy-5::GFP:ttx-3::mCherry* mutant strain and *gcy-5::GFP:ttx-3::mCherry* as control strain. **A**: Electrophoresis banding pattern shows the crossing strain is *gpa-16Δ:gcy-5::GFP* homozygous. The upper part of the image shows 600bp GFP bands and lower part shows 1.7kb *gpa-16* deletion bands. **B**: Electrophoresis banding pattern shows the crossing strain is *gpa-16Δ:gcy-5::GFP:ttx-3::mCherry* homozygous. Left picture shows 300bp mCherry bands, and right picture shows 1.7kb *gpa-16* deletion bands. **C**: Electrophoresis banding pattern shows the crossing strain is *gcy-5::GFP:ttx-3::mCherry*. Upper image shows 300bp mCherry bands and lower image shows 600bp GFP bands.

4.4.4 GFP marked ASER sensory neuron and mCherry marked AIY interneurons were able to see in *gpa-16Δ* mutant animals and in wild-type animals.

We use Zeiss confocal microscope LSM510 to take images of ASER sensory neuron and AIY interneurons. ASER cell body locates at the front-middle of pharynx terminal bulb. Dendrite of ASER projects to the cilia in the mouth opening, and the axon of ASER projects into nerve ring from right to left. AIY cell bodies locate at ventral of pharynx terminal bulb, axons of AIY project into nerve ring from ventral, and fuse at the dorsal midline.

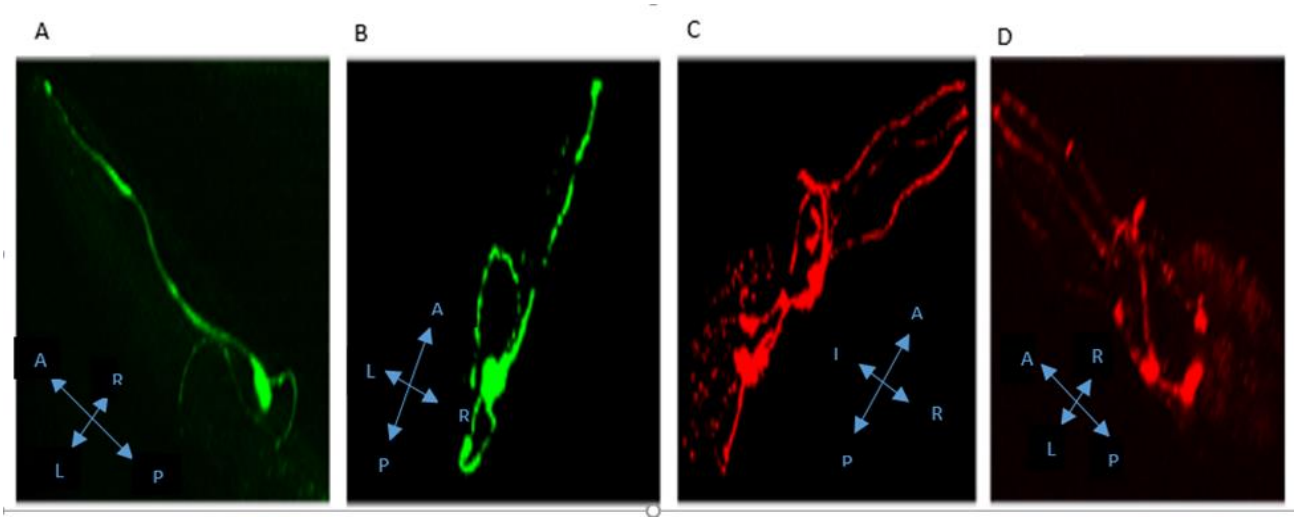


Figure 16: The transgenic mutant strain *gpa-16Δ:gcy-5::GFP:ttx-3::mCherry* was generated in our lab. We were able to see the fluorescence markers in both ASER sensory neuron (A) and AIY interneurons (C). The fluorescence markers were also able to see in original *gcy-5::GFP:ttx-3::mCherry* control strain (B) and (D).

4.4.5 ASE-R neuron cell body location is reversed (dislocated) in some *gpa-16Δ* animals.

In all of the wild-type animals and most of the *gpa-16Δ* animals, ASER neuron located to the right side of the body. But in certain percentage of the *gpa-16Δ* animals, ASER neuron dislocated to the left side of the body. The ASER axon projecting orientation is reversed

compared with wild-type animals, so synaptic connectivity between ASER sensory neuron and AIY interneuron varies accordingly in those ASER reversal mutant animals.

Figures below show ASER and AIY interneurons and their connections in wild type and *gpa-16Δ* animals. Images were taken using Zeiss confocal microscope (LSM510). The 3D images can help us tell ventral/dorsal and left/right orientations of the body, and which could help me identify the location of ASER neuron.

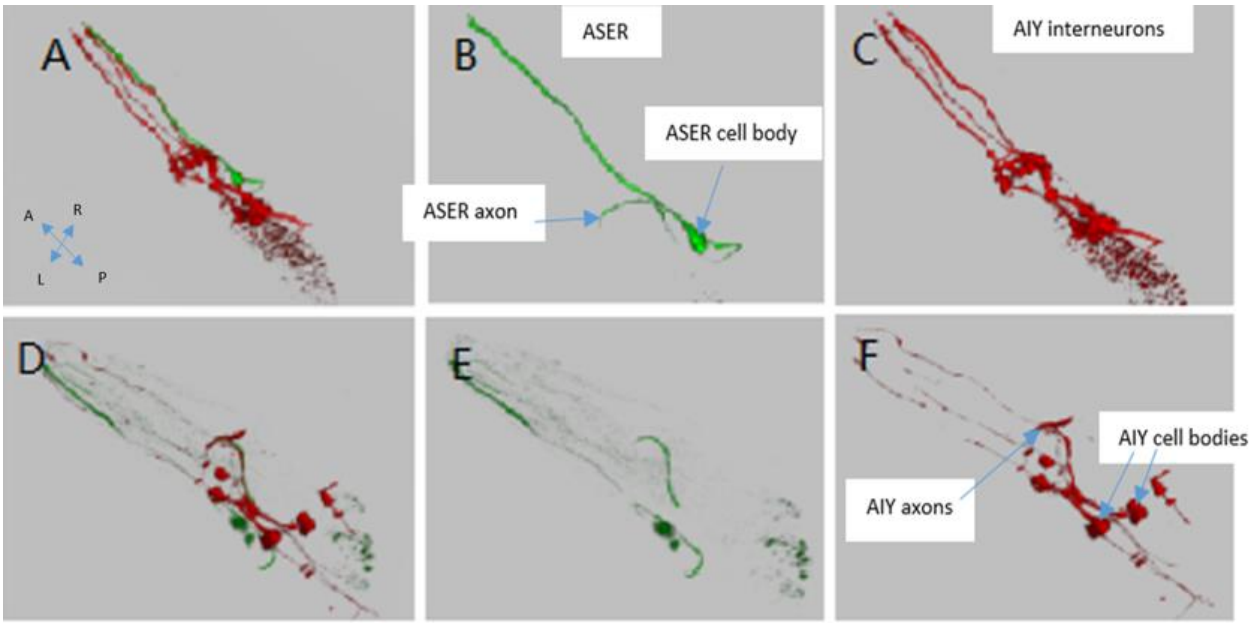


Figure 17: 3-D images show connectivity of sensory neuron ASER with interneuron AIY. Wildtype (N2) animals show stereotypic ASER location(A-C) while some of the *gpa-16Δ* animals showed reversed ASER neuron location(D-E). ASER sensory neuron and AIY interneuron in N2 animals (A). ASER located to the right of AIY, and axon loop projected to left (B). AIY interneurons in N2 animals (C). ASER sensory neuron and AIY interneuron in *gpa-16Δ* animals(D). ASER located to the left of AIY and axon loop projected to right (E). AIY interneurons in *gpa-16Δ* animals (F).

4.4.6 ASER sensory neuron laterality variation in *gpa-16Δ* mutant.

All of wild-type animals show stereotypic ASER placement, but 24.65% of *de.gpa-16* mutants show reversed ASER placement. In those ASER reversed animals, I haven't observed any gut/gonad reversal yet.

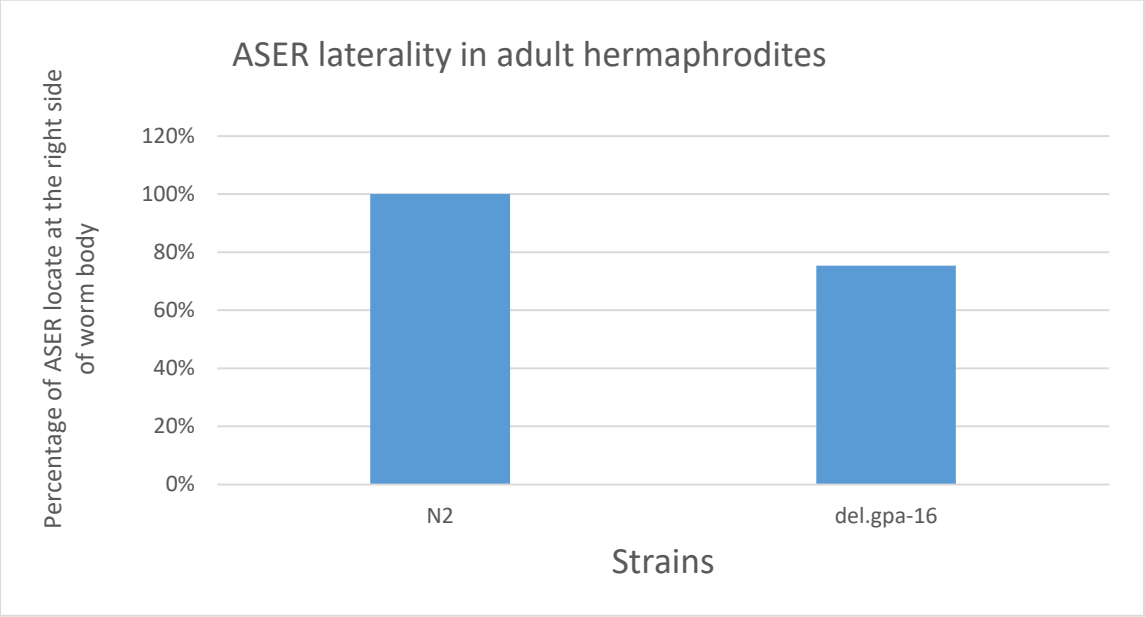


Figure 18: ASER sensory neuron locates 100% to the right in wild-type animals and 75.35% to the right in *gpa-16Δ* mutants. (Two sample student t-test. p-value=0.1501)(n=50 in N2, and n=36 in *gpa-16Δ*).

4.4.7 Visceral organs reversal in *gpa-16Δ* mutant animals.

All of wild-type animals show stereotypic gut/gonad placement, but 9% of *de.gpa-16* mutants show reversed gut/gonad placement. In the gut/gonad reversal animals, no ASER reversal happened simultaneously. The reversal of neuron and reversal of visceral organs could be independent.

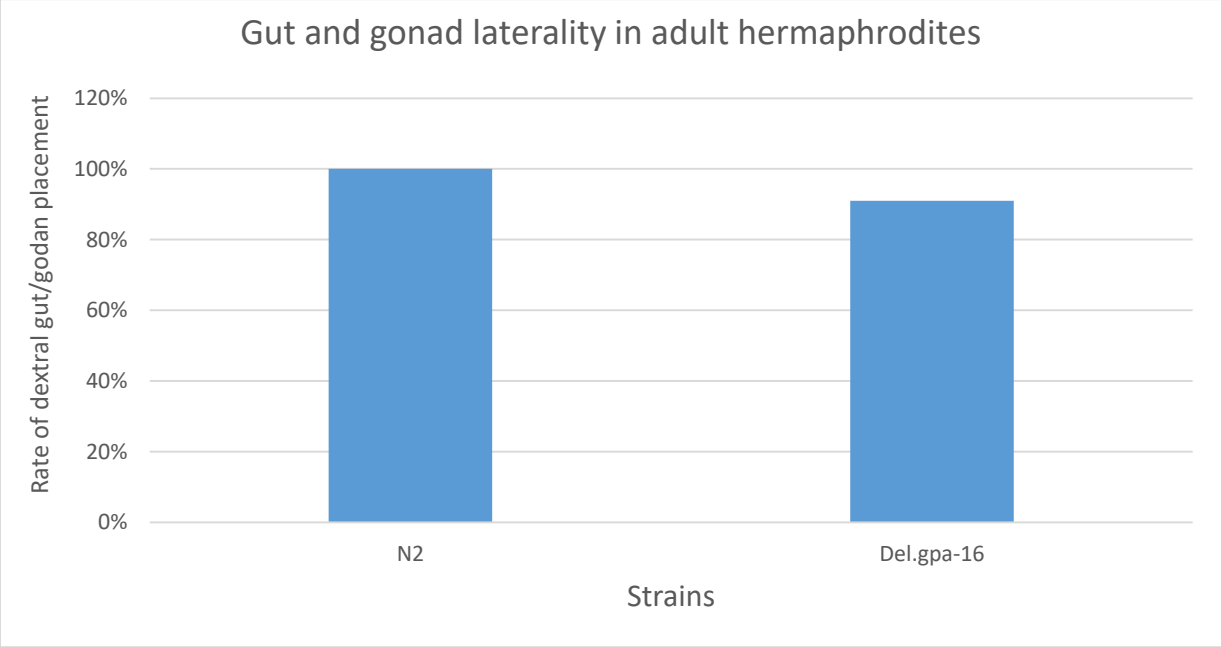


Figure 19: Wide-type animals show 100% dextral stereotypic gut and gonad placement, and *gpa-16Δ* mutant animals show 91% dextral gut and gonad laterality, 9% of the mutant animals have reversal gut and gonad laterality. (Two sample student t-test. p-value=0.3306) (n=39 in N2, and n=44 in *gpa-16Δ*).

4.4.8 GFP marked tubulin and mCherry marked chromatins demonstrate the spindle orientation in cell division.

The following images (Figure 20) show cell division in later stages of embryonic development. The L/R laterality of *C. elegans* was established as early as 4-6 cell stage, and that stage is a good window to monitor the laterality variation. But unfortunately, we didn't catch the

cell division image from 4-6 cell stage. This work will be done in the future study using Zeiss LSM880 confocal microscope.

A B

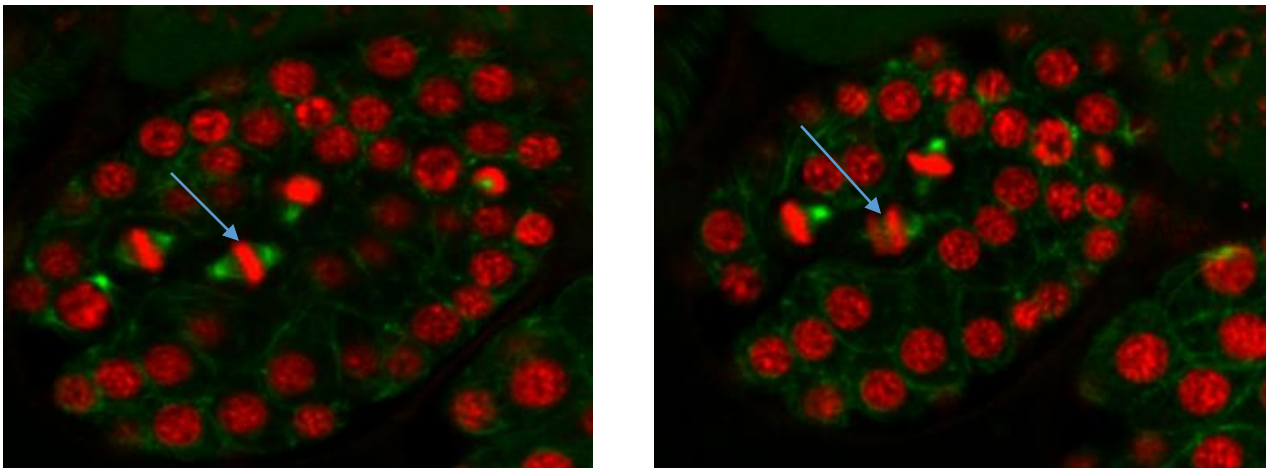


Figure 20: *gpa-16Δ:OD57* embryo expressing GFP::tubulin and mCherry::histone. The cell in the middle showed metaphase to anaphase transition. **(A)**Paired chromatids aligned in cell's equator, **(B)** Daughter chromosomes begin to separate and move to opposite of the poles. (time lapse images were taken by Zeiss LSM880 confocal microscope)

CHAPTER V: CONCLUSION, DISCUSSION AND FUTURE DIRECTIONS

5.1 Conclusion

gpa-16 is an important modulator for spindle orientation, and the loss-of-function mutation can increase the laterality reversal level. In *gpa-16Δ* animals, a very early embryonic cell division (four- to six-cell stages) defect generates significant influences on much later visceral organ development and neuronal development outcome. *gpa-16Δ* mutant demonstrates visceral organs reversal and ASE-R neuron reversal.

We found that the reversal of visceral organs and reversal of ASE-R sensory neuron did not always correlate with each other in *gpa-16Δ* mutant animals. Some mutant animals showed gut/gonad reversal might have stereotypic ASE-R placement, and some showed ASE-R neuron reversal might have normal gut/gonad orientation. This result matches the finding from Callander and Alcorn (2014) who reported that reversed gut/gonad orientation is independent of L/R asymmetric expression of *gcy-5* in the ASER neuron (Callander and Alcorn, 2014). Even though the asymmetry of the L/R axis is established during early embryogenesis; however, the downstream symmetry breaking procedure could be independent of the earlier L/R asymmetric body plan. It is likely that the downstream anatomical and neuronal bilateral asymmetries originated from multiple L/R symmetric breaking procedures at different development stages. Sensory neurons and interneurons differentiated from neuroblasts during the first larval development stage, while the gut is generated from gut-cell lineage at the 8-cell stage, and gonad formation is initiated from germ-cell lineage at the 16-cell stage. The mechanisms that mediate

and control of neuroblasts, gut-line cells and germ-line cells migration and polarization are likely to be distinct.

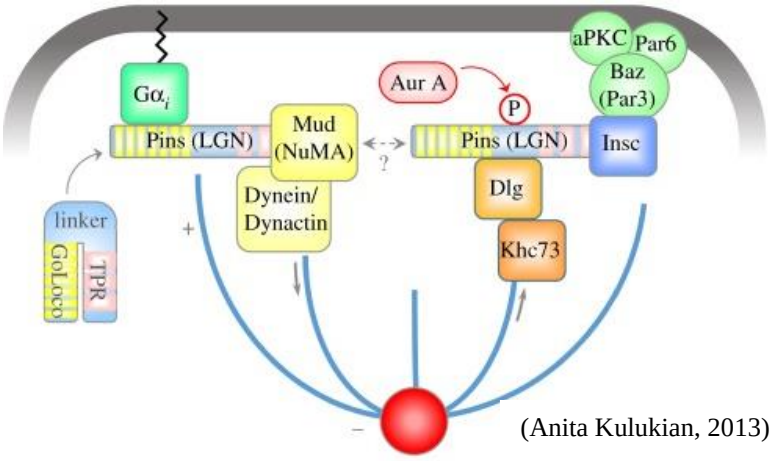
Anatomical reversal of neuron placement is expected to cause atypical synaptic transduction and inefficient neuronal connections that can lead to non-associative/associative learning defects. Preliminary results from our lab already indicated that *gpa-16* mutants show learning and memory defects in habituation and conditioned chemotaxis (Mersha, 2017).

The *gpa-16Δ* associated laterality variations might derive from the randomization of the spindle orientation in early stage cell division, and the bilateral asymmetric body plan is partially destroyed by the mutation. In order to confirm this speculation, we crossed the *gpa-16Δ* mutant animal with a transgenic strain OD57, which has GFP marked astral tubulins and mCherry marked chromatins. The crossing strain *gpa-16Δ:OD57* enabled us to monitor changes in spindle orientation during early embryonic development, the images we took displaying clear astral tubulins and chromatins in mitosis. However, we are still having some difficulties to catch the four- to six-cell division process which the left-right asymmetry starts to form. The cell proliferation speed is fast in early embryos, and the GFP marked microtubules only visible in cell division process. In future research, we can develop an appropriate time-lapse imaging assay to monitor the early stage cell division in *gpa-16Δ* mutant animals.

5.2 Discussion

The mechanism of *gpa-16* regulating spindle orientation in the *C. elegans* model has been explored previously with limited advancement. This Gαi sub-unit was initially identified as a spindle orientation factor in *C. elegans*, and Gαi is the mammalian orthologue of GOA-1 and

GPA-16 (Bergstralh, 2013; Gotta M,2001). The modulating mechanism of Gαi gives us some ideas of how GPA-16 modulating laterality determination in *C. elegans*. Gαi is required for initiating the force generating mechanism which exists in cell cortex to regulate astral microtubules (Zheng, 2013). Gαi interacts with the adaptor molecule LGN forming Gαi-LGN complex. This Gαi-LGN complex interacts with the NuMA adaptor to recruit the dynein motor complex to the cell cortex. The Gαi-LGN-NuMA-dynein complex located at the cell cortex can generate pulling forces to astral microtubules, the strength and orientation of the pulling forces will determine the orientation of cell division (Zheng et al., 2013).

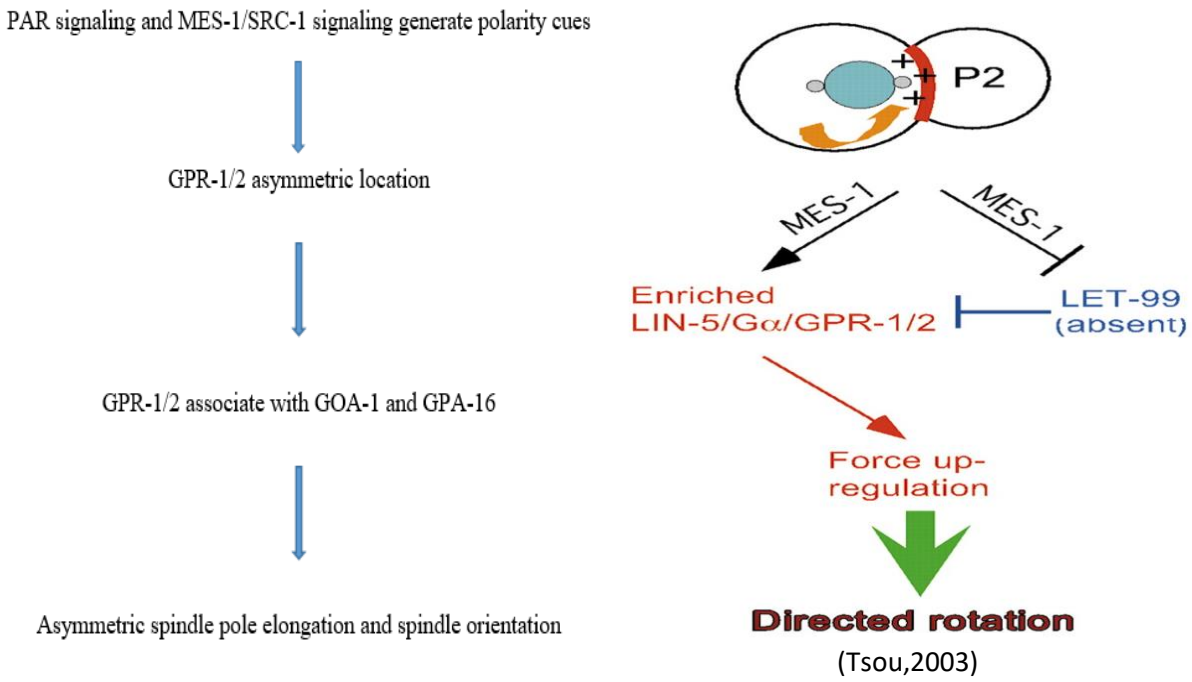


(Anita Kulukian, 2013)

Figure 21: The Gαi-LGN-NuMA-dynein complex located at the cell cortex can generate pulling forces to astral microtubules. The strength and orientation of the pulling forces will determine the orientation of cell division.

In *C. elegans* embryos, Gα is required for spindle orientation in cell division, also involved in spindle pole elongation. Two G-protein activators GPR-1 and GPR-2, function together with Gα subunit proteins GOA-1 and GPA-16 to generate asymmetric spindle pole elongation during cell divisions in the P-lineage. Gα/GPR-1/2 levels at the EMS/P2 boundary are

particularly high (Tsou et al., 2003). The asymmetric localization of GPR-1/2 depends on PAR-3 and MES-1/SRC-1 signaling which generate polarity cues. GPR1/2 associate with GOA-1/GPA-16 generating pulling force which regulate spindle elongation and asymmetric cell division (Tsou, et al.,2003)



(Tsou,2003)

Figure 22: Models for the roles of GPA-16 and GPR-1/2 in regulating asymmetric spindle pole elongation and spindle orientation. PAR signaling (left figure) and MES-1/SRC-1signaling (right figure) initiate GPR-1/2 asymmetric location.

These two model mechanisms explain how does the Gα subunit protein GPA-16 play an essential role in determining the orientation of cell division and forming laterality. In Gαi-LGN-NuMA-dynein complex model, GPA-16 initiates pulling forces generation to astral microtubules, and in PAR-3/MES-1/SRC-1---GPR1/2---GOA1/GPA16 signaling model, GPA-16 helps regulate spindle elongation and spindle orientation. In *gpa-16Δ* (ok2349), the GPA-16 involved

laterality establishment are perturbed, and the spindle orientation in 4-cell to 6-cell stage shows certain level of randomization. At 20°C, 53.8% of the *gpa-16Δ* embryos were dextral, around 38.4% of embryos were sinistral, and 7.7% of the embryos were haphazard (Mersha, 2017).

There are other factors that also play a role in regulating spindle orientation, such as Rho-GTPase (RhoA, a kind of small G-protein), formin, and other protein complex in Gα subunits, Gβ and Gγ subunits, ERM proteins (ezrin-radixin-moesin) (Mitsushima,2009, Minc,2012, Machicoane,2014). These spindle orientation modification factors might compensate the dysfunction of GPA-16, and this can explain why there are about 30% reversal demonstrating in embryos instead of 100%. Some epigenetic modification changes in other genes or post-protein-translation modification may also compensate the *gpa-16* mutation, generating certain level of phenotypic compensation in *gpa-16Δ* animals.

The neuronal functional laterality variation experiments performed on the *C. elegans* model in our lab provide some basic principles to understand certain psychiatric disease in human. It has been reported that atypical functional brain lateralization has been associated with neuro-psychiatric conditions, ranging from schizophrenia to autism (Reynolds 1983; Reynolds and Czudek 1987). In human, left-handers could be an example of brain functional lateralization and behavior lateralization. 90-92% of people are stereotypic right-handed, and the other 8-10% left-handed people has certain level of brain functional laterality variation, causing their right cerebral hemisphere becomes dominant over the left (Faurie and Raymond 2004). Left-handers show increased performance in associative memory tasks compared to right-handed population (Beratis et. al 2013, Coren 1995). But at the same time, left-handers are more frequently observed in gifted individuals on one hand, and in children with Down's syndrome or autism on

the other hand (Dane,2007, Dragovic, 2005, Deep-Soboslay, 2010). Atypical lateralization of ASE sensory neurons in *C. elegans* and its effect to learning behavior may help us to better understand the atypical lateralized situation in human brain.

5.3 Future Work

In future research, it will be useful to fuse another fluorescence protein YFP with a ASE-L specific receptor gene, for example *gcy-6,* and express *gcy-6::YFP* in the wild-type animals and *gpa-16Δ* mutant animals, to evaluate the placement reversal situation of ASE-L. The reversal situations of both ASE-L and ASE-R could be more complex than we imagine. We also can mark the other asymmetric neuron pairs to see if other neuron pairs also demonstrate reversal placement in *gpa-16Δ* mutant animals.

Testing the synaptic connection efficiency between ASE-R neuron and AIY interneurons using Fluorescence recovery after photobleaching (FRAP) could help in looking at the functional connectivity between these neurons. This technique can help evaluate the neurotransmitter releasing concentration in ASE-R axon synaptic terminals. The internal pH of the synaptic vesicles is about 5.5, but after the vesicles fuse with the cell membrane, the neurotransmitters will come to the extracellular synaptic cleft where the pH is about 7.25 (Michaelson and Angel, 1980; Fuldner and Stadler, 1982). pH-sensitive GFP (pHlourin) fused to the C-terminus of the synaptic vesicle protein SNB-1. The animals expressing pHlourin can produce fluorescence signal in neutral pH of extracellular space. We speculate that *gpa-16Δ* mutant animals result in an obviously different rate of fluorescence recovery after the photo-bleaching compare with the

wild-type animals, which can give us a clue that the interconnection between ASER and AIY is atypical in *del.gpa-16* animals (Hogue et.al., 2016)

Dopaminergic signaling is known to modulate various neuron functions, such as associative/non-associative learning through different types of neurons (Chase and Koelle, 2007). Therefore, we plan to examine whether *gpa-16Δ* mutant influence dopaminergic signaling. Previous work in our lab has already shown that dopamine is a major modulator of learning and memory in *C. elegans*. Interestingly, *gpa-16* gene is known to express in PDE pair of dopaminergic neurons. In future research, we will use confocal microscopy to identify the laterality variation of dopaminergic neurons, because it was reported that the CEP-D and CEP-V dopaminergic neurons were functional asymmetric (Tanimoto, 2016). Potential use of FRAP to demonstrate the dopamine releasing rates in dopaminergic neuron synapses in *gpa-16* mutants will be useful towards mechanistic understanding. Results from our research are expected to provide foundational information of developmental aspects of dopamine-mediated behaviors, which is a key yet not very well understood aspect in neurological research.

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