Chapter 3: Characterization of mutants show pleiotropic defects in Touch Receptor Neurons

3.1) Introduction: Isolation of mutants

Molecular players involved in cell biological processes can be identified by screening methods. Forward and reverse genetic screens utilize mutations to identify function of the mutated gene in specific biological phenomenon or phenotype of interest. The forward genetic approach enables to identify allelic series of a gene giving broad phenotype and allow looking for the function of genes whose absence give lethality. The genetic lesions in single gene facilitates studying protein interaction and the role of specific domain in the protein.

Mutagenesis is done by various chemical reagents and radiations at a concentration that can cause $\sim 10^{-3}$ to $10^{-4}$ mutations in single gene per generation (Gengyo-Ando and Mitani, 2000), without much damage to the genome. The Ethyl methanesulfonate (EMS) mutagenesis leads to single nucleotide polymorphisms and small deletions in the genome compared to radiation mutagenesis which causes large deletions. The most common approach of forward genetic screening involves looking at F2 after mutagenized P0 as showed in the figure a below. F2 is screened for defects in the phenotype of interest.

![Forward screening mutagenesis](image)

**Figure a: Forward screening mutagenesis (adapted from Kutscher et al., 2014)**

We are interested in understanding the regulation of mitochondrial transport and distribution in the neurons. In *C. elegans*, the factors known to be involved in mitochondrial trafficking
are motors: Kinesin-1 and Dynein for anterograde and retrograde transport respectively (Hollenbeck and Saxton, 2005). Unlike higher model organisms, adaptors involved in mitochondrial transport are unknown in C. elegans. Therefore, in order to find out molecular players that may be involved in mitochondrial trafficking in C. elegans neurons, we used jsIs609(mec7p:: MLS:: GFP) animals in which the mitochondrial matrix is targeted with GFP and expressed under a touch receptor neuron (TRNs) specific promoter.

A forward genetic screen was conducted where jsIs609 animals were mutagenized with EMS (see methods section) and the F2 generation was screened for defects in mitochondrial distribution in the touch receptor neurons (TRNs) compared to wild-type parent strain, jsIs609 (fig. 1a) (unpublished Guru’s Thesis work).

Isolated mutants were categorized as following:

1) Mutants with low mitochondrial number
2) Mutants with more mitochondria number
3) Mutants with more mitochondria in the minor neurite of TRNs

3.2) Results and discussion

3.2.1) Screening and selection of mutants for mapping

Among the three categories of mitochondrial distribution defective mutants isolated from the screen, I screened and selected six mutants that showed fewer mitochondria compared to jsIs609 (pmeC-7: MLS:: GFP). In jsIs609, the mitochondrial distribution could be visualized as fluorescent bodies along the neuronal process (fig.1a). Thus, a change in the distribution of mitochondria could easily be observed.
The mitochondrial phenotype of selected mutants are as follows:

1. **tb118, jsIs609**: Fewer mitochondria clustered near cell body. The animals are mild dumpy and fat. (fig.2a, 2f)

2. **tb132, jsIs609**: Few mitochondria in the neuronal process, mostly clustered near cell body in axon initial segment and minor neurite. They are not fat though slow growing. (fig.2a)

3. **tb133, jsIs609**: Mitochondria distributed throughout the neuronal process and clustered in minor neurite and axon initial segment. They grow and move slow compared to jsIs609. (fig.2a, 2h)

4. **tb134, jsIs609**: Low number mitochondria distributed throughout the neuronal process. The animals are dumpy. (fig.3a)

5. **tb117, jsIs609**: Low number of mitochondria distributed throughout the neuronal process. The animals are dumpy. (fig.3a)

6. **tb119, jsIs609**: Low number of mitochondria distributed throughout the neuronal process. The animals showed uncoordinated movement. (fig.3a)

Selected mutants were mapped to the X chromosome using genetic approach (described in chapter 4). Further, the non-complementation test among isolated mutants showed that *tb118*, *tb133*, and *tb132* belong to the same complementation group, thus are alleles of the same gene.

As having multiple alleles ease up finding interactions, and sequencing analysis, I proceeded with the three mutants forming a complementation group for further characterization and mapping.

**3.2.2) Mutants show defective mitochondrial trafficking exclusively in TRNs**

The mutants selected for further characterization were examined for the mitochondrial distribution as well as flux along the neuronal process.
3.2.2.1) Mutants show defects in mitochondrial distribution in TRNs:

Characterization of jsIs609 wild-type animals show mitochondria distributed throughout touch receptor neurons (TRNs) in both ALM and PLM (fig 1a, 1b). In wild-type, the mitochondria number increases with the development of the animal (fig 1b), however, the density is maintained across developmental stages (fig 1d).

Figure 1: Mitochondria number in TRNs increases linearly with the development

Montage of jsIs609 (mec7p::MLS: GFP) representing mitochondria in ALM and PLM (a) with marked cell body and scale bar=30µm. mitochondria in jsIs609 PLM at 60X, scale=10µm . Mitochondrial number increase in both ALM and PLM with the development (c) and mitochondrial density remains constant (d). Larval stage 3 (L3), larval stage 4 (L4),1day adult (1D). Number of animals, n=30 for each stage. Data represented as mean ± SEM. p-value (*) <0.05

Compared to jsIs609, the mutants show less number of mitochondria that are distributed only up to a certain length of the neuronal process (fig.2a and b). In stronger mutants, viz,
*tb118*, and *tb132*, neuronal process end near the synaptic branch (fig.2a), whereas in *tb133* the process extends beyond it although the branch does not form synapses at its end. Annotation showed a low mitochondrial number present in the neuronal process (fig.2b and c). Due to variation in neuronal processes length, we compared the density of mitochondria that represents number of mitochondria per unit length of the neuronal process. Stronger mutants have a lower density of mitochondria compared to *jsIs609* (fig.2c). Heterozygous mutants do not show a significant difference in mitochondria number in the neuronal process suggesting that the mitochondrial distribution defect in these mutants is recessive in nature (fig.2d). However, these heterozygous mutants show mitochondrial accumulation near cell body which suggest that mitochondrial accumulation in proximal region is a semi-dominant phenotype. Compared to wild type (fig.2e), strongest mutant *tb118* is little fat and dumpy (fig.2f), *tb132* is mild dumpy but not fat (fig. 2g) and *tb133* is bit lethargic and thinner (fig.2h)(for methods, see section 2.20.2). In the remaining three mutants, i.e. *tb134*, *tb117* and *tb119*, the mitochondria are distributed throughout the neuronal process in both ALM and PLM like in wild type, without any defects in the length of the process with respect to body length (fig.3a). The mitochondria are also fewer in number (fig.3b) with their low density in *tb134* and *tb117* mutants while *tb119* show no significant change (fig.3c).
Figure 2: Mitochondrial distribution in jsIs609 and mutants; and their external appearance

Mitochondrial distribution in PLM in wild-type jsIs609 and isolated mutants (a), scale bar=10um. Image contrast has been set to visualize the branch. (b) Graphical representation of mitochondria number and c) density in homozygous mutants compared to jsIs609. (d) mitochondria number in heterozygous in PLM of L4. ( ) shows cell body, ( ) marks mitochondria, ( ) marks extra branches and ( ) marks neuronal process end. Data represented as ± SEM, n=25 animals; e) f) g) and h) show bright field images of wild type and mutants on agar plate taken under stereo microscope at 5X magnification, scale bar=250 µm/ 0.25mm.
Figure 3: Mitochondrial distribution in the second set of mutants

Mitochondrial distribution in mutants compared to wild-type *jsIs609 (a), scale= 10µm.

Mitochondria number (b) and density (c) in TRNs of the second set of mutants, n=15, data represented as ± SEM. (˅) shows cell body and (˅) marks mitochondria.
3.2.2.2) **Mutants show reduced mitochondrial flux in TRNs**

To see the effect of mutations on mitochondrial dynamics, I looked at the fraction of moving mitochondria and rate of mitochondrial entry and exit to and from the neuronal process. For this, proximal region of the neuronal process was imaged (see methods, entry, and exit mitochondrial rate), time-lapse was converted to kymograph and were analyzed (fig.4). The movement was observed across a bleached stationary mitochondrion (bleach and recovery approach, chapter2). The wild type *jsIs609* show higher mitochondrial dynamics (fig.4c) compared to mutants (fig.4d, g, h).

A low mitochondrial density in the neuronal process could arise from two possibilities:

a) Less number of mitochondria entering the process from cell body in the given time,

b) Mitochondria entering into the neuronal process at a rate equivalent to wild-type but returning at a higher rate.

Mitochondrial flux calculation showed that in comparison to wild type, mitochondria enter at a slower rate in the mutants (fig.5a). Mutants show very low mitochondrial flux in the TRNs compared to wild type (fig.5b). In addition, they show an anterograde bias viz. more mitochondria move in an anterograde direction as observed in wild type in L4s and adults.
Figure 4: Mitochondrial flux in TRNs of wild type and mutants

Proximal region of PLM in jsIs609 (a), tb118 (b), tb132 (e) and tb133 (f) and corresponding kymographs (c), (d), (g) and (h) respectively, representing the position of mitochondria along X axis with respect to time in Y axis. The time lapse was acquired for 500 frames at 3 frames/sec and converted into kymographs. Scale=10μm
Figure 5: Mutants show fewer moving mitochondria

Mitochondrial entry to and exit from the neuronal processes (a), Fraction of moving mitochondria including anterograde and retrograde flux (b), Data represented as ±SD. Number of animals, n=25 for each.

In the neuronal process, mitochondrial pool show variation in size - small or big and round or tubular population. Defects in mitochondrial trafficking could be due to mutation of any factor that is involved in mitochondrial distribution, fission-fusion or transport. The presence of a high fraction of large mitochondria could represent a defect in fission and more small mitochondria could reflect a problem with fusion process (Yang et al., 2008). Defects in fission and fusion are also known to affect the number of mitochondria in the cell and change the stationary and mobile mitochondrial fraction (Varadi et al., 2004). Mitochondrial fission or fusion mutants in *C. elegans*, drp-1(IV) and eat-3(II) showed phenotype different than the isolated mutants. Former showed very large tubular mitochondria along the neuronal process and distal end whereas latter showed smaller, mostly round mitochondria throughout the neuronal process without any effect on the length of the neuronal process.

Size analysis for moving mitochondria in wild-type *jsIs609* shows that the maximum average size of moving mitochondria is ~40 pixels in size (marked in fig.6b). I looked at the distribution of the size of mitochondria in the mutants to see a fraction of mitochondria that could move. Mitochondrial size analysis does not show any gross change in mutants, though we could see in *tb134* and *tb119*, the distribution shifts towards larger mitochondrial fraction (fig.6). The fraction of mitochondria capable of moving viz. up to the size of 40 pixels show no gross increase or reduction in mutants.
We do observe the rare events of big tubular mitochondria moving in L4 and young adults.

![Figure 6: Mutants do not show any gross change in mitochondrial size distribution](image)

The average size of mitochondria measured in pixels (a), Distribution of mitochondrial size in mutants compared to *jsIs609* in L4 (b). arrow marks the average cut-off size of moving mitochondria~40 pixels. Data represented in ± SEM, number of animals, n=20. P-value (*) <0.05

3.2.2.3) PLM show severe phenotype in mutants

Characterization of mitochondrial number and density in strong mutants, viz. *tb118* and *tb132* shows that the PLM is more severely affected than the ALM neuron (fig.7). Although mitochondria are fewer in both PLM and ALM (fig.7a, b) compared to wild type, the PLM in mutants has a lower density than ALM (fig.7c, d). Occasionally, in the mutants, few mitochondria are distributed along the ALM process, while PLMs rarely show mitochondria distributed along the process, instead, they remain accumulated in or near cell body.
Figure 7: Mutant phenotype is severe in PLM

The mitochondrial number is lower in PLM (a) than ALM (b), mitochondrial density shows a significant change in PLM (c) compared to ALM (d). Data represented as ±SEM. Number of animals, n=30 for each genotype. P-value (*) <0.05

3.2.2.4) Mitochondria in DA9 are present along the neuronal process

To find if the mitochondrial distribution is affected in neurons other than TRNs, I looked at wyex1709(mitoGFP) where mitochondrial matrix in motor neurons DA9 have been targeted with fluorescent marker GFP. DA9 are motor neurons that innervate dorsal muscles and release neurotransmitter acetylcholine. DA9 send output signal via VD neurons to move animal in backward direction on receiving signal from interneurons AVA, AVD, and AVE modulator neurons (Teichmann & Shen, 2011).

In wild-type animal, we could see the DA9 cell body, axon, and dendrite. Mitochondria were present along the axon as well as dendrite. DA9 has en-passant synapses along the axon where mitochondria were distributed. In mutants, tb118; wyex1709 and tb132; wyex1709, mitochondria are present along the neuronal processes, axon and dendrite similar to wild-type (fig.8). Mutants do not show any accumulation of mitochondria near DA9 cell body unlike in TRNs. Moreover, mitochondria are distributed at the synapse locations of DA9, whereas in
TRNs mitochondria are not present beyond a certain distance in neuron. The presence of mutant phenotype in the strain was confirmed by combining with jsIs609, that showed neuronal morphology and mitochondrial trafficking defects in TRNs without any effect on DA9 in genotypes wyex1709; tb118, jsIs609 and wyex1709; tb132, jsIs609. The phenotype was also confirmed to rule out any fluorescent markers effect by using tagRFP in TRNs along with DA9 mitoGFP, jsIs1073; tb118; wyex1709, it showed mitochondrial accumulation in TRNs without affecting DA9. In addition, the neuronal process morphology of DA9 in mutants is unaffected.

**Figure 8: Mutants do not show mitochondrial distribution defects in DA9 neurons**

Schematic shows position and structure of DA9. Mutants tb118 and tb132 show mitochondria along the neuronal process of DA9 as in wild-type wyEx1709. Images show marked DA9 Cell body (↑), and mitochondria (▼). Images captured at 60x magnification. Scale bar=10um
3.2.3) Mutants show defects in PSVs trafficking exclusively in TRNs

The molecular players involved in transport including microtubules and molecular motors, Kinesin and Dynein are common for multiple cargoes. Considering this, we hypothesized that there could be more than one cargo that is affected in the mutants under study. We chose to look at presynaptic vesicle trafficking in TRNs which also rely upon Kinesin and Dynein for their long-distance microtubule-based transport (Hirokawa and Takemura, 2005). I looked at presynaptic vesicular distribution using two specific markers: **rab-3** and synaptobrevin (**snb-1**).

3.2.3.1) Mutants show PSVs localization and transport defects in TRNs

In wild-type neurons as seen in **jsl1263 (mec-7p:: rab-3:: tagRFP)**, where **rab-3** is tagged with tagRFP, presynaptic vesicles are distributed throughout the neuronal process and clustered near the synaptic branch and synapses (fig.9). In contrast, the strongest allele, **tb118; jsl1263**, shows accumulation of PSVs near cell body and branches, with the process visible only up to a certain distance. Weakest allele **tb133; jsl1263** also showed accumulation of vesicles near cell body along with vesicles clusters throughout the neuron (fig.9). Further, in these animals, the synapses are not visible with vesicle marker (not shown). These vesicular phenotypes in the mutants were also confirmed using another marker, synaptobrevin in **jsl37 (mec-7p::snb-1:: GFP)** (Fig 10). In addition, mutants showed variation in neuronal process length and the extent of ectopic branches along the neuron.

The presence of vesicles along the neuronal process could mean that they are moving anterogradely and retrogradely like in wild-type but are unable to move beyond a certain distance due to short neuronal processes. Flux analysis of these presynaptic vesicles marked with RAB-3 or SNB-1 shows that the mutants show a gross reduction in the number of vesicles along the process, with most vesicles showing jiggling movement as shown in the kymographs and their annotation (fig.11 and 12). The mutants showed vesicles moving with short run length viz., more pause during movement. The number successively reduced from the proximal to the distal region. A similar accumulation of vesicles near cell body and reduction in the number are observed with synaptobrevin marker (fig.12). Occasionally RAB-3 marked vesicle clusters in the proximal neuronal process were seen moving as thick lines as shown in kymograph of **tb133; jsl1263** (fig 11); however, this could be because of tagRFP...
marker accumulation rather than vesicle movement, as such events were not observed with other fluorescent vesicle markers.

These observations suggest that the mutants have a generalized trafficking defects in TRNs affecting both, mitochondrial and vesicle transport.

Figure 9: Mutants show disrupted RAB-3 presynaptic vesicles distribution in TRNs

Localization of RAB-3::tagRFP vesicles in mutants (tb118 and tb133) compared to jsIs1263 PLM, Mutants show a similar accumulation of vesicles in cell body in ALM. Below two images also show variation in PLM in weakest allele tb133; jsIs1263. scale=10µm.

Figure 10: Mutants show accumulation of synaptobrevin vesicles in proximal neuronal process
Presynaptic vesicles localization in jsIs37 (*mec-7p::snb-1::GFP*) and mutant *tb118; jsIs37 (mec-7p::snb-1::GFP)* L4 ALM. Similar localization observed in PLM. Scale bar=5µm

**Figure 11: Mutants show defects in presynaptic vesicles trafficking in TRNs**

Kymographs of jsIs1263 (*rab-3::tagRFP*) with mutants *tb118; jsIs1263* and *tb133; jsIs1263* in columns a, b and c respectively. Time-lapse was acquired of the proximal region including PLM cell body (CB). CB is at left in kymographs. Annotation of total mobile vesicles (d) and anterograde and retrograde moving vesicles (e). Time lapse acquired at 5fps and converted to kymographs. Scale=10um. Data represented as ± SD. Number of kymograph analysed, n=5
for each. P value >0.05<0.25 for antero
grade and >0.2 for retrograde

Figure 12: Presynaptic vesicles flux reduced in mutants

Localization of synaptobrevin (snb-1: GFP) in wild-type jsIs37(a) and mutant tb118; jsIs37 (b) and corresponding kymographs (c) and (d) respectively. Time lapse acquired at 5fps and converted to kymographs. Scale=10µm.

3.2.3.2) PSVs are not accumulated near cell bodies in other neurons

Mutants show presynaptic vesicles accumulated near cell body in TRNs. Mitochondrial trafficking defects are restricted to TRNs, therefore we proceeded to see whether PSVs trafficking defect is exclusive in TRNs or affect other neurons.

I looked at the distribution of vesicles in pan neurons using two markers: RAB-3 and Synaptobrevin. In jsIs682[rab-3p:: GFP:: RAB-3; pJM23], rab-3 is tagged with GFP and expressed under endogenous promoter that is expressed in all neurons including TRNs. In jsIs1[pSB120 (snb-1:: GFP); pRF4 (rol-6(su1006))], synaptobrevin with endogenous promoter have been tagged with GFP. Both markers showed grossly similar phenotype.

NonTRNs in mutants do not show vesicles accumulation in cell bodies rather have vesicles distributed along the neuronal process and at synapses like in wild-type (fig.24,15). TRNs showed reduced morphology and trafficking defects under a pan-neuronal promoter.
Reduction in branching and cargo presence along the TRNs under pan-neuronal promoter indicates the possible significance of TRN promoter in worsening the phenotype. In addition, I looked at flux of the vesicles in TRNs under pan neurons compared to TRN promoter (fig.13,14,15). Under pan-neuronal promoter, TRNs show vesicles throughout the neuronal process with more fraction of mobile vesicles compared to that with TRN promoter, in jsIs1263 or jsIs37 (fig.11). In addition to vesicle accumulation in TRN cell body, PLMs in the pan-neuronal background show extra branching phenotype (fig.14). This indicates that the two phenotype, cargo trafficking, and neuron morphology, might be separable.

Figure 13: Mutants show reduced defects in vesicle dynamics in TRNs with endogenous Rab-3 promoter

Samples of Kymographs of jsIs682 (upper) and tb132, jsIs682 (lower). PLM images with pan-neuronal RAB-3:: GFP background Images correspond to the first kymograph of the corresponding genotypes. Time lapse acquired at 5fps and converted to kymographs. Scale=10µm.
Figure 14: Mutants exclusively show defects in TRNs with endogenous vesicle promoter

*jsIs1*(*snb-1::GFP*) do not show branching in PLM and PLN (a, b); *tb118; jsIs1 (snb-1:: GFP)* show branching in PLM (c), but not in PLN (d). Schematic shows SAB process in the head. No defects observed in mutant SAB processes (f) compared to wild-type (e). Scale bar=10µm.
Figure 15: Mutants do not show vesicles accumulation in cell body in other neurons

Schematic shows position of PLM and PLN in the tail. Mutant *tb118* show accumulated vesicles in PLM CB (d), vesicles not accumulated in PLN (e) and other cell bodies in tail compared to wild type PLM (a), PLN (b) and other cells in the tail (c). Images are of different planes from z section to look at cell bodies. Scale bar= 10µm.

3.2.4) Mutants show TRNs specific neuronal morphology defects

Since the cargo markers (both mitochondrial and vesicle) could be tracked only to a certain length in TRNs of mutants, we decided to see if the TRNs show any neuron morphology defects that possibly allow the cargos to travel only a short distance. Further, in mutants, the ventral synapses were also not visible using either cargo marker in TRNs suggesting an absence of synapses. To rule out the possibility that the visible neuron
morphology phenotype could be an artifact of cargo marker, we used diffused marker to see if mutants have short neuronal processes. Diffused marker proved that the neuronal processes are indeed short, and the ventral synapses are not visible in mutants (fig 16 and 17). The extent/severity of the phenotype follows the allelic strength as \( tb118 > tb132 >> tb133 \).

3.2.4.1) Mutants show short neuronal processes in TRNs

In the wild-type strain, PLM and ALM extend through the entire animal length. Both PLMs extend from tail to vulvar region. On their way, the straight neuronal process forms a synaptic branch at the ventral side of the body before vulva. ALM starts where the PLM ends and extend straight till the mouth and forms one synaptic branch towards the nerve ring.

Both strong alleles, \( tb118; tbIs222 \) \((mec-4p::mcherry)\) and \( tb132; tbIs222 \) show a short neuronal process for both ALM and PLM with mcherry accumulated in the proximal region (fig.16). The PLM process in mutants extends till PVM in 70% of the animal population, rest 30% show process extending beyond PVM but end before the synaptic branch. In mutants, unlike wild type, the PLM ends much before the ALM starts. The neuronal processes in mutants frequently show a branched or curved structure at the end (fig.16). Further, in the wild-type strain, \( tbIs222 \) \((mec-4p::mcherry)\), with soluble mcherry in the neuron, synapses are visible as clusters whereas in mutants synapses are not seen.

Mutants with another soluble marker, \( zdIs-5 \) \((mec4p::GFP)\), show consistent phenotype as a short neuronal process with few extra branches along the TRNs (fig.17).

[Note: \( zdIs-5 \) was imaged in the heterozygous state as homozygous animals show strong GFP accumulation along the neuronal process]
Figure 16: Mutants show neuron morphology defects in TRNs
PLM with the soluble mcherry marker in mutant *tb118* compared to wild type show extra branching (†), a curved neuronal process (‡), and the short neuronal process as the distance between PLM end and ALM cell body (¶). Scale=10µm

Figure 17: Mutants show short neuronal process
Soluble *zdIs-5*(me4-4p::GFP) in PLM of mutants showing extra branches and absence of synaptic branch as marked (†) in wild-type *zdIs/+* and PLM schematic. Scale=10µm
3.2.4.2) Mutants show extra branches in TRNs

Although the mutants do not show synaptic branch, they show several ectopic branches along the neuronal process in both PLMs and ALMs. In strong alleles, the extent of branching was analyzed based on the presence of primary, secondary and tertiary branches. Annotation showed that extra branching is present only in mutants and the extent of branching enhances with the development of the animal (fig. 18a, b, c). The neuronal processes in strong mutants’ end either as branched or curved structure (fig. 18c).

![Figure 18: Extent of Branching in mutants increase with the development](image)

Percentage of animals showing extra branching in strong mutants in L2 and L4, wild type do not show any branching (a), histogram of *tb118, jsIs609* branching phenotype with development (b), (c) fraction of animals of *tb118* and *tb132* showing curved ending in mutants compared to wild type *jsIs609*. Number of animals annotated:
- *jsIs609* L2, L3, L4 = 15, *tb118, jsIs609* L2, n = 15; L3, n = 15; L4, n = 20; *tb132, jsIs609*, L2, n = 23; L4, n = 25; Similar pattern seen for ALM
Further, the severity of the defects for both trafficking and neuron morphology in the mutants was found to be greater in the PLM neuron as compared to the ALM.

3.2.4.3) Mutants do not show branching defects in nonTRNs

Mutants do not show any gross mitochondrial and vesicle trafficking defects in other neurons. Using vesicle markers, viz. rab-3 and synaptobrevin, under endogenous promoter, mutants showed extra branching phenotype exclusively in TRNs as marked in the figure in *tb118; jsIs1* (fig.14). Neuron morphology defect was not present in DA9 when observed using a mitochondrial marker (fig.8). To rule out the possibility of an effect of using cargo marker, I looked at neuronal morphology in other neurons using a soluble fluorescent marker.

To look at other neurons, I built *tb118* with following transgenes: (i) *uIs59 [unc-119p:: YFP]* where soluble YFP is present in all neurons and (ii) *wdIs51 [F49H12.4:: GFP + unc-119(+)]* where PVD neurons express GFP. Other neurons in mutants viz. *tb118; uIs59* and *tb118; tbIs222; wdIs51* do not show any extra branching phenotype compared to controls viz. *uIs59* and *wdIs51; tbIs222* respectively, ectopic branches exclusively observed in TRNs (fig.19 and 20).

Thus, these observations together show that the mutation affects touch receptor neuron-specific phenotype and functioning.
Figure 19: Mutants do not show neuron morphology defects in PVD

No defective branching observed in PVD in *tb118; tbIs222(mec4p::mcherry); wdIs5 (F49H12.4::GFP)* compared to *tbIs222(mec4p::mcherry); wdIs5(F49H12.4::GFP)*. scale=10um
Figure 20: Other neurons do not show gross defect in mutant

SAB in the head, TRNs and motor neurons in mutant *tb118* compared to wild-type animal with soluble YFP in pan-neurons. (♀) show other processes, (♂) mark ALM and PLM processes. scale=10um

3.2.5) Touch receptor neurons of mutants show defects in microtubule dynamics

The defects in neuron morphology could be an outcome of any defect in microtubule growth or stability along neuronal processes. Microtubules are the backbone of any cell structure and become more important for a complex long cell such as neuron which has a defined polarity along the process. Defects in microtubules dynamics or stability affect multiple cell biological processes and cargo distribution along the neuron (reviewed in Dubey et al., 2015). TRNs have a specific alpha (*mec-12*) and beta (*mec-7*) tubulins that polymerize to form thicker and stable microtubules of 15 protofilaments compared to other neurons in *C. elegans* that have 11 protofilaments microtubules (Savage et al., 1989).

Microtubule stability is directly proportional to the acetylation level of tubulins (reviewed in Bass et al., 2016). Thus, I looked at microtubule acetylation level of TRNs. In wildtype, the TRNs were acetylated almost uniformly along the length of the neuronal process (fig 21). However, the mutant shows gaps in acetylation along the neuronal process (fig.21) suggesting the presence of less acetylated microtubules and thus fewer stable microtubules along the TRN process. Acetylated microtubules in TRNs of mutants also show short neuronal processes and extra branches (fig.21. b and d).
Figure 21: Mutant show defects in microtubule stabilization in TRNs

Immunostaining for acetylated tubulin in jsIs609 ALM (a), PLM (c) and mutant tb118, jsIs609 ALM (b) and PLM (d). Defects observed in L3, L4, and adults. Scale= 10µm. Marked cell body (●) and branch in tb118 (●)

Further, we looked at transgenes tbIs213 (mec7p: ebp-1:: CFP) and juEx2843(mec7p:: ebp-2:: GFP) that are known to bind the growing plus end of microtubules (Van de et al., 2016). With both ebp-1:: CFP and ebp-2:: GFP markers, the wild type animals show uniform distribution along the process while mutants show accumulation in the proximal region and extensive branching (fig.22 and 23). This distribution indicates defects in the growth and stability of microtubule along the neuron. We also looked at EBP-2 dynamics in TRNs to see the direction and growth of microtubules and whether the growth is affected in mutants. In wild-type TRNs, shown in PLM, most EBP-1 and EBP-2 puncta show movement towards synapse or away from the cell body (fig.23), whereas in mutants more fraction of EBP show retrograde, accumulation and disrupted movements. The microtubule movements observed in
mutants were similar to more shrinking than growth (Yoge et al., 2016). Together, the microtubule dynamics data suggest defects in the mutant’s TRNs microtubules movement, polarity, and stability along the neuronal process.

Figure 22: Mutant show defects in microtubules end binding protein (EBP-1) distribution

EBP-1 localization in the tb118 neuron compared to wild-type. EBP-1 is accumulated in proximal region in the mutant. Short neuronal process and ectopic branches also observed. Scale=10µm

A)
Figure 23: Mutants show defects in microtubule dynamics in TRNs

A) Neuronal morphology in mutant *tb118* and wild-type with EBP-2:: GFP in TRNs, scale bar =10um. B) Kymographs of wildtype *juEx2843* (EBP-2:: GFP) (a, b) and mutant *tb118; juEx2843* (EBP-2:: GFP) (c, d, e, f). scale= 10µm
3.2.6) Mutants are insensitive to gentle touch

The benefit of using TRNs as a paradigm is that one can easily examine the defects for behavioral output. Defects in gentle touch sensitivity indicate malfunctioning of TRNs (Savage et al., 1989; Goodman, 2006). In gentle touch assay, when the animal is touched with eyelashes at the anterior body it moves backward and on touching at the posterior body, it moves forward (chalfie et al., 2014.Wormbook). ALM and AVM respond to the anterior touch whereas PLM neurons respond to posterior touch.

Beside neuron morphology and cargo trafficking defects, mutants do have behavioral defects.

I did gentle touch assay for 6 touches, 10 touches, and 20 touches, each touch applied alternatively to the anterior and posterior sides. For the 6 touches assay, the wildtype jsIs609 and tb134 responded to all touches, whereas other mutants show mec behavior with tb117 mild mec (fig.24 a). Strong mutants are dominant mec as heterozygotes also were insensitive to gentle touch by eyelashes (fig.24b). Similarly, 10 and 20 touch assays were done with the three mutants forming a complementation group. For the 10-touch assay wild-type animals respond to all 10 touches while the mutants respond to anterior 2-3 touches without responding to any posterior touch (fig.24c, d). Response to anterior touch near the head is also mediated by the sensory circuit in the head of the animal (chalfie et al., 2014), which could be the reason of mutant’s anterior response.

Defects in PLMs are severe as compared to ALMs, this could also lead to an anterior response to some extent by the animal. Insensitive to gentle touch suggests defects in functional touch receptor neurons.
Figure 24: Mutants are unresponsive to gentle touch

Homozygous (a) and heterozygous (b) animals responding to gentle touch assay. (c) show fraction of animals corresponding to the number of touches. (d) An average number of touches responded by mutants compared to jsIs609. n=20. Data represented as ±SD

3.2.7) Behavioral assay from other neurons

NonTRNs did not show any visible defects, therefore, I did behavioral assays to see any effect on the function of other neurons that might not show a gross change in phenotype of the neuron but affects behavioral output.
3.2.7.1) Mutants are little sensitive to aldicarb

First, I looked at the output of motor neurons via aldicarb assay (for methods, refer to chapter 2, Section 2.17.4). Mutants are slight sensitive compared to controls jsIs609 and jsIs1073 (fig.25). The experiments were done in two batches, both showed consistent sensitivity. Mutants showed delayed recovery (fig.25 b) after the assay. This suggests that though we do not see vesicles at TRN synapses, this is affecting motor neurons as well. The severity of phenotype may be high in TRNs, there could be slight defects in other neurons. There is a possibility that synapses are active to a lesser extent.

![Graph showing ratio of animals paralyzed](image)

![Graph showing recovery after aldicarb test](image)

![Graph showing animals paralyzed](image)

![Graph showing ratio of animals paralyzed](image)

**Figure 25: Mutants are sensitive to aldicarb**

(a) and (b) represent first set of aldicarb assay and recovery of the animals after the assay respectively. Mutants showed delayed recovery. (c) and (d) show the response of animals in second aldicarb assay. N=2, n=15 for each set.
3.2.7.2) Mutants are sensitive to plate tapping

Animals responded well to plate tap assay (for methods, refer to chapter 2, section 2.17.3), thus are sensitive to mechanical stimulus (table 1). Experiments were done in two sets, one with pan-neuronal YFP and another with TRNs mitochondrial marker.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of animals</th>
<th>if moved</th>
</tr>
</thead>
<tbody>
<tr>
<td>uIs59</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>tb118; uIs59</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>jsIs609</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>tb118, jsIs609</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>tb132, jsIs609</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>tb133, jsIs609</td>
<td>20</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1: Response of animals to plate tap

First column of the table shows Genotype used, second column shows number of animals taken and third column is the response of animal- whether they moved on plate tap or not.

3.2.7.3) Mutants respond to harsh touch:

Response to harsh touch is mediated via PVD and FLP neuron circuits that sense stimuli along the body and head respectively. PVD and FLP mechanosensory neurons have an axon and extensive branched dendritic structure that envelope the animal body and sense external thermal and mechanical stimuli. In harsh touch assay, TRNs are ablated to avoid their contribution to the response. As mutants are insensitive to gentle touch response, there was no need to ablate TRNs. Mutants show unnoticeable difference from wild type in harsh touch response (fig. 26). They are sensitive to harsh touch by platinum wire and responded by backing up or moving forward (for method, refer to chapter 2, section 2.17.2).
3.2.8) TRNs have not changed fate to nonTRNs: PVD and FLP

*Caenorhabditis elegans* hermaphrodites have 30 and males have 52 mechanosensory neurons. Mechanosensory neurons have been further subgrouped based on their origin. *Unc-86* and *mec-3* express in all mechanosensory neurons, albeit with different expression levels. Further, fate determination and differentiation of the neurons occur as an outcome of the interaction of multiple molecules and downstream components which are poorly understood (Smith et al., 2010; Albeg et al., 2011). TRNs have simple unbranched morphology, whereas other sister mechanosensory neurons, PVD and FLP show branched dendrites.

PLMR and PLML arise from migrated Q cells at the right and left sides of the body (Sulston and Horvitz, 1977). Later PVD, FLP, and TRNs differentiate to respond to different stimuli. TRNs respond to the gentle touch assay whereas PVDs and FLP neurons respond to harsh touch along the entire body and head.

Since TRNs in the mutants show extra branching along the neuronal process, I hypothesized that this could be due to defect in maintenance of TRN fate or due to fate change into nonTRNs such as PVD or FLP, which are closely related with respect to upstream fate-determining transcription factor.
To find out if the fate of TRNs has changed to FLP or PVD, I looked at both homozygous and heterozygous mutants, *tb118; tbIs222/+; cx11872 kyEx3233, tb118; wdIs59; tbIs222 and tb118/+; tbIs222/+*, and controls *cx11872 kyEx3233, wdIs59; tbIs222* where GFP was expressed under FLP or PVD-specific promoters while soluble mCherry was expressed under TRN-specific promoter.

The mutants neither express FLP nor PVD marker in TRNs (fig.27 and 28). Additionally, the TRNs marked with soluble mCherry could be distinctly seen separate from the FLP or PVD neurons (fig.27 and fig.28). The absence of PVD marker in TRNs was confirmed in additional transgenic with mutant viz. *tb118; tbIs222; wdIs59* (fig.28). Thus, the TRNs in mutants have not changed their fate to their sister neurons.

**Figure 27: TRNs have not changed fate to FLP or PVD**

Column 1 shows FLP in the head in GFP in both wild-type (a) and mutant (b). Mid Body and Tail represents PVD in GFP and TRN in mCherry. Scale= 10µm
3.2.9) Genetic interactions of the mutants with motors

Kinesin-1 is the known major motor in mitochondrial anterograde transport (Hollenbeck and Saxton, 2005). From prior observations, we know that mitochondrial transport in *C. elegans* is also mediated via kinesin-1. The defect in mitochondrial transport observed in the mutants could be due to a defect in the interaction of the motor with either cargo or microtubule. Therefore, I looked at the effect of kinesin-1 mutation on mitochondria number in the mutants. Further reduction of the mitochondrial number in mutants will suggest that the transport of mitochondria in mutants are still dependent on *kinesin-1* and the mutation has not affected the motors and cargo interaction.

I used kinesin-1 heavy chain, *unc-116 (e2310)* III, and a light chain, *klc-2 (km11)* V, hypomorph mutants where functional kinesin level is reduced. In stronger alleles, very few mitochondria (~2) are present in the neuronal process. Therefore, the change in mitochondrial number in heterozygous mutants were analyzed.

*Unc-116 (e2310)* III is a non-curated allele and codes for kinesin heavy chain. Deletion mutants are null so hypomorphs were used that show reduced levels of functional kinesin. We did not see a significant reduction in either mitochondrial number or density in *e2310*;
tb118/+, jsIs609 compared to tb118/+, jsIs609 (fig. 29 a). Double heterozygotes of km11/+; tb132/+; jsIs609 showed a significant reduction in mitochondrial number compared to km11/+; jsIs609 and tb132/+; jsIs609. Double heterozygotes of km11/+; tb118/+; jsIs609, however, did not show any significant difference compared to km11/+; jsIs609 and tb118/+; jsIs609 (fig. 30). This could be due to some effect on kinesin-1 mediated mitochondrial trafficking or another possibility could be the contribution of another anterograde motor in mitochondrial trafficking.

To rule out the possibility of defects in retrograde trafficking, I looked at the doubles with dynene, dhc-1(js121/hT2) I. Double mutants showed negligible change in the mitochondrial distribution and branching phenotype (not shown).

In addition to mitochondrial trafficking defects, the mutants also display vesicle trafficking defects in TRNs (results section 3.2.2 and 3.2.3). UNC-104 is the major motor responsible for the trafficking of PSVs (Hirokawa et al., 2009) and affects mitochondrial trafficking (Tanaka et al., 1998). To see if UNC-104 motors available to transport vesicles could lead to neuron morphology defects and thus motor over expression could rescue branching phenotype, tb118; tbls222 was built with transgene unc-104:: GFP. tb118; tbls222/+; unc-104::GFP/+ showed tb118 phenotype viz. showed ectopic branches (fig. 31). Though overexpression of unc-104 could not rescue branching phenotype but reduced the morphology defects in terms of neuronal process length. Thus, the phenotype is not mediated via unc-104, though there could be enhanced contribution of UNC-104 involved in mitochondrial transport in mutants.

Together, the mutants show TRN specific defects. As there are multiple monopolar neurons in different systems and its biology is not well understood, it would be interesting to explore how trafficking and microtubules are regulated in a neuron-specific manner.
Figure 29: Mutants did not show a reduction in mitochondria number in \textit{khc-1} mutant

Change in an average number of mitochondria in PLM (a),(b) and ALM (c) and mitochondrial density in PLM (b) and ALM (c). N=3, n=20 animals, Data represented with ±SEM. P<0.05.
Figure 30: Mitochondrial number in mutants is slightly affected in kinesin mutant

a) Mitochondria number in \( tb132/+ \) in \( klc-2(km11) \), (b) Mitochondria number in \( tb118/+ \) in \( klc-2(km11) \), \( n=15 \). Data represented as ± SD. \( P<0.05 \)

Figure 31: Overexpression of UNC-104 reduced the severity of neuronal morphology in mutants

Wild-type \( tbIs222 \) (1\textsuperscript{st} row) without neuron morphology defect, mutant \( tbIs222; tb118 \) (2\textsuperscript{nd} row) showing branching and short neuronal process. mutant with overexpressed \( unc-104 \): GFP (3\textsuperscript{rd} row) showing the long neuronal process with synaptic branch (\( \bigstar \)) and reduced
extent of branching. Marked PLM CB in column a ( ), neuronal process end ( ). Scale = 10µm

3.3) Conclusions:

1) Mutants isolated in the screen show TRN specific mitochondrial and vesicle trafficking defects.
2) In addition, mutants show short neuronal processes in touch receptor neurons with extra branches and do not form the synaptic branch.
3) Mutants show defects in microtubule dynamics and stability in TRNs.
4) Both trafficking and neuron morphology defects are severe in PLM compared to ALM and the extent of defects increases with the development.
5) Mutants are behaviorally mec, thus TRNs are non-functional.
6) NonTRNs in mutants grossly neither show any defects in mitochondrial and vesicles distribution nor in neuron morphology.
7) There seemed to be involvement of additional anterograde motor for mitochondrial transport because kinesin-1 defects cause insignificant reduction in mitochondrial number in TRNs.
8) UNC-104 overexpression reduced extra branches to some extent in tb118.
9) Touch Receptor Neurons in mutants have not changed fate to sister sensory neurons, PVD or FLP.