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Journal Title: Journal of Neuroscience Research
Volume: Volume 94, Number 9
Publisher: Wiley: 12 months | 2016-06-19, Pages 850-856
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1002/jnr.23756
Permanent URL: https://pid.emory.edu/ark:/25593/rwxtn

Final published version: http://dx.doi.org/10.1002/jnr.23756

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Accessed January 9, 2021 6:11 AM EST
Behavioral analysis of the Hap1 Ortholog, trak-1 in Caenorhabditis elegans

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Abstract

The precise role of Huntingtin Associated Protein 1 (HAP1) is not known but studies have shown that it is important for early development and survival. A Caenorhabditis elegans (C. elegans) ortholog of HAP1 (T27A3.1 also called trak-1) has been found and is expressed in a subset of neurons. Potential behavioral functions of three knockout lines of T27A3.1 were examined. Based on its suspected role in mice, we hypothesized that T27A3.1 might be involved in egg hatching and early growth, mechanosensation, chemosensation, sensitivity to osmolarity and synaptic transmission. Our studies show that the knockout worms are significantly different from the wildtype worms in only the synaptic transmission test which was measured by adding aldicarb, an acetylcholinesterase inhibitor. The change in function was determined by measuring the number of worms paralyzed. However, when the T27A3.1 worms were tested for egg hatching and early growth, mechanosensation, chemosensation and sensitivity to osmolarity, there were no significant differences between the knockout and wildtype worms.

Graphical abstract
A Caenorhabditis elegans ortholog of Huntingtin Associated Protein, HAP1 has been identified as trak-1 and is expressed in a subset of neurons. Behavioral tests were performed on the knockout worms and reveal that they have defects in synaptic transmission compared to wildtype worms.

**Keywords**

Huntingtin-Associated Protein-1; T27A3.1; neurons; trak-1

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**INTRODUCTION**

Huntington Associated Protein 1 (HAP1) was originally identified as a binding partner for Huntingtin, the protein mutated in Huntington’s disease (Li, 1996; Liao et al., 2010, McGuire, 2006). The precise role for HAP1 has not been determined but it is important for survival since its deletion in mice results in early postnatal death (Chan et al., 2002; Gorska-Andrzejak, 2003; Li et al, 2003, Lin et al., 2010). HAP1 is enriched in neuronal cells and is involved with axonal transport and intracellular trafficking (Liao et al., 2010; Lin et al., 2010; Mercer et al., 2009; Li et al., 2003; Sheng et al., 2008; Wu et al., 2010). HAP1 is also closely connected with microtubules and various types of membrane bound organelles, which include mitochondria, endosomes, vesicular bodies, lysosomes and synaptic vesicles (Li et al., 2003; Rong, 2007, Li and Li, 2007).

T27A3.1 is an ortholog of trafficking kinesin-binding protein 1 (TRAK1), trafficking kinesin-binding protein 2 (TRAK2), Huntington Associated Protein 1 (HAP1) and Drosophila Milton (Wormbase). The TRAK proteins are kinase and dynein adaptor proteins. They bind to the kinesin heavy chains while HAP1 has been reported to bind to the kinesin light chains (Randall, 2013). In human, TRAK1 and TRAK2 have been found in hippocampal and cortical neurons (Loss, 2015). In human and rodents, HAP1 is found in neurons throughout the brain and we have shown that the C. elegans ortholog of HAP1, T27A3.1, is expressed in chemosensory neurons (Mercer et al., 2009). A comparison of the amino acid sequence of HAP1 and trak-1 revealed high levels of similarity and conserved domains. The finding of conserved HAP1_N domain, a Prefoldin domain and several coil coiled domains suggests that these proteins might have similar functions such as specific protein-protein interactions, and/or have similar binding partners (Mercer et al., 2009).
C. elegans has been used to study genetics and behavior due to its small size, short lifespan, ease and low cost to grow and well defined nervous system. In addition, C. elegans can be classified as a model organism since similar results in various studies obtained in this worm were also obtained with other organisms. It has a simple anatomy of only 959 adult cells, and a small genome. This nematode has exactly 302 neurons, 32 of which are chemosensory (Hiller, 2005, Lanjuin, 2004, Troemel, 1995).

Genetic studies in C. elegans have shown that mutations in certain genes, many of which are from transcription factor families, have caused a loss of chemosensory functions (Lanjuin, 2004). C. elegans has been used in behavioral studies because they can sense and respond to both chemical and physical stimulation (Lanjuin, 2004, Melkman, 2004, Sengupta, 20007). For instance, they can recognize various water-soluble chemoattractants and odorants from the environment. Various chemotaxis studies have been performed with C. elegans. Chemotaxis is important for the worms because in their natural environment, it allows them to find their food source, which is bacteria, and to move away from toxic substances and harmful environments. Chemotaxis allows scientific investigators to analyze both sensory and motor functions of the worms. (Koga, 2004, Lino, 2009, Melkman, 2004, Sengupta, 2007, Yamada, 2009). Chemosensory ability also affects movement of the worms. The amount of food available to the worms affects their ability to move. For example, worms that are well fed will move more quickly than those that have been starved. This movement is mediated by chemosensory signals (Sengupta, 2007).

The nose touch test is another behavioral test frequently used in C. elegans. This response is mediated by three mechanosensory neurons: ASH, FLP, and OLQ that contain ciliated sensory endings (Kaplan, 1993). Normal C. elegans will respond to nose touch 70–80% of the time by initiating backward locomotion (Faber, 1999). Aldicarb is a chemical used to test for synaptic transmission. Aldicarb, an acetylcholinesterase inhibitor, causes an accumulation of acetylcholine in the synaptic cleft and subsequent over activation of cholinergic receptors results in muscle hypercontraction, paralysis and death of the worms (Mahoney et. al 2006).

In the current study, we used C. elegans to analyze the behavioral phenotype of three lines of T27A3.1 knockout worms (tm1572 A, B and C) in several tests that measured growth and development, chemical, sensory and motor functions and synaptic transmission. Control N2 worms as well as worms with known deficiencies for the specific behavior were also tested.

**MATERIALS AND METHODS**

**Worm lines**

Worm lines deficient in egg-laying (AA699 (din-1(hd36) II), nose touch (KP4 (glr-1(n2461)III), chemotaxis (che-2), and osmolarity (osm-5, osm-9) as well as N2 wildtype were obtained from the Caenorhabditis Genetics Center (CGC). A knockout line (tm1572) with a 706 bp deletion in the T27A3.1 gene was obtained from Dr. Shohei Mitani (from National Bioresource Project for the nematode, Tokyo Women’s Medical University, Japan). Due to the procedure used to generate this knockout line, other mutations may exist besides that in the T27A3.1 gene. Therefore, the worms were backcrossed 4 times to N2 worms.
It is standard procedure to backcross the worms 4–6 times to ensure that it is a pure knockout line (Ahringer, 2006, Rodenas et al., 2012). Another study indicated that 5 backcrosses resulted in a 96% pure line (Friedman and Johnson, 1988). Three lines of backcrossed tm1572 worms were maintained and are designated tm1572A, tm1572B and tm1572C.

*C. elegans* start off as eggs and then go through L1, L2, L3 and L4 molts before reaching the adult stage (Altun, and Hall, 2012). For our work, we used either eggs or L4 worms which ensure that worms compared are of the same stage (Stiernagle, 2006). L4 worms have a distinctive mark on their vulva (Porta-de-La-Riva, 2012).

### Materials

All worm lines were grown on standard NGM media, spotted with OP-50 *Escherichia coli* and kept at 20–25 °C. The behavioral procedures are explained below but materials needed for these assays in addition to the worms are ethanol, 1-octanol, benzaldehyde, ammonium acetate, sodium azide, sodium chloride, aldicarb and M9 media (Stiernagle, 2006).

### Procedures

For all of the behavioral assays, the investigators were blind as to which line of worms were being examined.

For the brood size assay, eggs were isolated by bleaching (Stiernagle, 2006). This procedure helps synchronize the growth of the worms and maximizes the number of L4 worms obtained. Confluent plates were washed with 2 ml of sterile water and then treated with a mixture of 1ml of bleach and 0.5 mL of 5M sodium hydroxide. After centrifuging two times for 30 seconds at 1300 rpm, the eggs were collected and resuspended in water. From the egg sample isolated, 100 μL were placed on the edge of a 60 mm plate previously spotted with 10 μL of OP-50 bacteria in the center of the plate. Three days post plating, one L4 worm was transferred to a new plate. There were 31 plates setup for the N2 worms, 29 for tm1572A worms, 32 for both the tm1572B and the tm1572C worms and 31 for the AA669 control worms. Three days after transferring the L4 worms, the plates were divided into four quadrants and the total brood per plate was counted.

In the body bend assay, worms were observed as they moved freely on an agar plate. The worms move by generating sinusoidal waves along the length of their body. The worms move forward when these movements occur from head to tail. If the waves go in the reverse direction, then the worms move backward (Zhao et al., 2003). Ten L4 worms were picked and transferred to NGM agar plates without food and the number of forward locomotion waves was counted for a period of 30 seconds. This assay was performed on three consecutive days on different L4 worms.

For the nose touch assay, 100 μL of the eggs released after bleaching were placed on the edge of a 100 mm plate. The worms were assayed two days after plating the eggs. A worm pick (an eyebrow hair) was sterilized with 70% ethanol for one group of worms. For the other groups, the pick was first sterilized in ethanol and then, placed in concentrated solutions of 1-octanol (Sigma-Aldrich) or benzaldehyde (Sigma-Aldrich). The worm pick...
was placed perpendicular to the origin of movement of the organism. Once the organism came in contact with the worm pick, sensory neurons (ASH, OLQ, and FLP) transmit a signal, which causes the *C. elegans* to reverse their direction of movement (backwards) (Zhao et al., 2003). The response of movement was categorized as a 1 if the worm responded after it was touched by the worm pick. The worms either responded almost immediately or they were unresponsive and did not respond. In the latter case, they were assigned a score of 0 (Hart, 2006). Except for the KP4 worms, 50 worms were tested for each line. For the KP4 line, 40 worms were tested. The assay that we used is a modified form of the nose touch response where we tested both mechanical and chemosensory responses.

For the chemotaxis assay, 10 μL of either 7.5M ammonium acetate (Sigma-Aldrich, NH₄Ac, an attractant) or 10 μL of sterile water was placed at diametrically opposed locations on a 10 cm plate of agar. An additional 10 μL of sodium azide (Sigma-Aldrich) were placed on each dot. Worms were washed with 1mL of M9 solution and collected in a centrifuge tube. They were then centrifuged for one minute on the lowest setting, refilled with de-ionized water, and centrifuged again. The supernatant was drawn off without disturbing the pellet of worms. Approximately 10 μL of worms were placed in the center of the plate, ensuring that at least 150 worms were present. The solution with the resuspended worms was allowed to dry. After one hour, plates were placed in the cold room and the number of worms at each dot was counted the following day. The chemotaxis index (CI) was calculated as follows: CI = ((number of animals at the test odorant) – (number of animals at the solvent or control odorant))/(total number of animals tested) (Hart, 2006, Lino, 2009, Yamada, 2009). The chemotaxis assay was performed on N2 (n=19), tm1723A (n=12), tm1723B (n=15), tm1723C (n=13) and che-2 (n =7) worms.

For the osmotic avoidance assay, the end of a test tube was dipped in a 4M NaCl solution with added bromophenol blue dye (Fisher Biotech) and used to form a ring at the center of a small agar plate. Forty L4 worms from each of the lines were individually picked and rinsed with M9 solution and placed in the center of the osmotic ring. After the M9 solution dried, the worms were allowed to move about for 30 minutes. The number of worms in and out of the ring was recorded. Each worm line was tested 12 times using different worms each time (Hart, 2006).

For the aldicarb assay (Sigma-Aldrich), the protocol followed was as previously described except that the concentration of aldicarb used in the plates was 2 mM (Mahoney et al., 2006). Briefly, worms were placed on the aldicarb plates and touched with an eyelash every 10 minutes for 3 hours to determine if they were paralyzed. In this assay, 20 N2, 25 tm1572A, 20 tm1572B and 35 tm1572C worms were tested.

**Data analysis**

Differences between strains were analyzed using GraphPad using one way ANOVA tests for the brood size, body bends, osmolarity, nose touch and chemotaxis assays. Posthoc analyses were performed with the Dunnett test. A two-way ANOVA was performed to analyze the data for the aldicarb assay. Posthoc analyses for this test were performed with the Bonferroni test.
RESULTS

Brood size and gross movement assays

We first examined the effect of the T27A3.1 knockout on brood size. Three days after placing a single L4 worm on plates, the total number of worms resulting on each plate was counted (Figure 1A). Brood size was assessed from 31 N2, 29 tm1572A, 32 tm1572B, 32 tm1572C and 31 AA699 worms. A one-way ANOVA revealed a significant effect of worm line on brood size at the p<0.05 level [F(4, 145) = 19.24, p<0.0001]. Post hoc comparisons using the Dunnett’s multiple comparison test indicated that the mean brood size for the AA699 was significantly different than the N2 line at p<0.0001. However, none of the other lines were significantly different than the N2 control at p<0.05. These results suggest no effect of the T27A3.1 knockout on brood size.

Next, a locomotion assay was performed on 30 L4 worms from each line (Figure 1B). A one-way ANOVA revealed no significant effect of worm line on body bend at the p<0.05 level [F(3, 116) = 2.151, p=0.977].

Test for sensory deficits (nose touch assay, osmotic avoidance and chemotaxis)

We next tested the knockout worms for potential gross sensory deficits. In the nose touch assay (Figure 2A), worms from each line were tested for their reaction to being touched with an eyelash that had been either sterilized with ethanol or dipped into the repellents 1-octanol or benzaldehyde. Except for the KP4 worms, 50 worms were tested for each line. For the KP4 line, 40 worms were tested. Percent response to touch was compared between lines using a one way-ANOVA test. There was a significant effect of worm line at the p<0.05 level for the reaction to ethanol [F(4, 235) = 43.90, p<0.0001], benzaldehyde [F(4, 235) = 59.07, p<0.0001] and 1-octanol [F(4, 235) = 24.22, p<0.0001]. Post hoc comparisons using the Dunnett’s multiple comparison test showed only KP4 worms to be significantly impaired in the reaction to all 3 stimulants compared to N2 controls at p<0.0001. These results suggest no effect of T27A3.1 knockout on reaction to the stimulants tested.

In the osmotic avoidance assay (Figure 2B), the T27A3.1 knockout lines, the N2 control lines and two osmotic deficient lines were tested for their ability to escape a ring of 4M NaCl. For each test, 40 L4 worms were placed in the center of the NaCl ring. The test was performed 12 times for each line using different worms for each test. A one-way ANOVA identified a significant effect of worm line at p<0.05 on the percentage of worms escaping through the ring over a 30 minute period [F(5, 66) = 8.423, p<0.0001]. Post hoc comparisons using the Dunnett’s multiple comparison test showed only osm-5 and osm-9 worms to be significantly less affected by the osmotic barrier compared to N2 controls at p<0.0001 and p<0.01. These results suggest no effect of T27A3.1 knockout on osmotic avoidance assay.

In the ammonium acetate (NH$_4$Ac) chemotaxis assay (Figure 2C), ~ 150 worms were used for each test. The chemotaxis assay was performed on N2 (n=19), tm1723A (n=12), tm1723B (n=15), tm1723C (n=13) and che-2 (=7). A one-way ANOVA identified no significant effect of worm line on the attraction to NH$_4$Ac at p<0.05 [F(4, 60) = 1.269, p=0.2921].
Alicarb assay to test for synaptic transmission

Finally, aldicarb, an acetylcholinesterase inhibitor, was used to assess synaptic neurotransmission (Figure 3). In this assay, 20 N2, 25 tm1572A, 20 tm1572B and 35 tm1572C worms were tested. A two-way analysis of variance revealed a main effect of worm line \[ F(3, 306) = 4.56, p=0.0028 \], as well as a main effect of time \[ F(18, 306) = 77.80, p<0.0001 \] on the reaction of the worms to aldicarb. The test also revealed a significant interaction effect of worm line and time \[ F(54, 306) = 3.58, p<0.0001 \]. Two out of three of the tm1572 knockout lines (B and C) became paralyzed and unresponsive to touch with an eyelash when exposed to aldicarb compared to the N2 worms. A Bonferroni post hoc test revealed that this sensitivity occurred at earlier time points for the tm1572 B and C worms compared to the N2. For example, at 60 minutes after the addition of aldicarb, 47% of tm1572B \[ D=41.75, t=4.040, p<0.01 \] and 70% of tm1572C worms \[ D=58.00, t=6.429, p<0.001 \] were paralyzed compared to only 10% of N2. The tm1572B worms were also more sensitive to aldicarb than control N2 worms at 70 \[ D=31.75, t=3.072, p<0.05 \] and 80 minutes \[ D=32.75, t=3.169, p<0.05 \]. The tm1572C line was also more sensitive to aldicarb than control N2 worms at 50 \[ D=36.29, t=4.022, p<0.01 \], 70 \[ D=40.86, t=4.529, p<0.001 \], 80 \[ D=51.86, t=5.748, p<0.001 \], 90 \[ D=43.43, t=4.814, p<0.001 \], 100 \[ D=38.29, t=4.244, p<0.001 \] and 110 minutes \[ D=33.14, t=3.674, p<0.01 \] after the addition of aldicarb (Figure 3). It also took less time for the tm1572 B and C lines to be completely paralyzed compared to the N2. All of the tm1572 B worms were paralyzed by 160 minutes. The tm1572C worms were all paralyzed after 120 minutes but it took 180 minutes for all of the N2 worms to be paralyzed.

DISCUSSION

*C. elegans* is a model organism used extensively to study the effect of gene mutations on behavior (Hobert, 2003, Li, 2006, Lipton, 2005). *C. elegans* is a useful model system for studying the function of human genes for which a *C. elegans* ortholog or homolog has been identified. *C. elegans* has been studied to study neurodevelopmental diseases (Bessa, 2013) and diseases such as spinal muscular atrophy that cause degeneration of motor neurons (Briese, 2009). In addition, *C. elegans* models of the neurodegenerative diseases Huntington’s Disease, Parkinson’s Disease and Alzheimer’s disease have been generated and are been used for drug screening (Kaletta, 2006). In the present study, we analyzed the effect of deleting the T27A3.1 gene, an ortholog of Hap1, on several well established *C. elegans* behavioral tests. According to wormbase.org, T27A3.1 and HAP1 are also orthologs of human HAP1 and HAP21. The rationale for our study was that mice that have the HAP1 gene knocked out do not survival past a few days postnatal (Li et al., 2003; Chan et al., 2002; Gorska-Andrzejak, 2003) implying that this gene plays an important role in early development. Since these knockout mice only live to be 3–4 days of age, it is not known whether the knockout of Hap1 causes adult behavioral deficits. In a previous study, in which the N2 strain was fed T27A3.1 RNAi, there were no differences in the number of laid oocytes, number of dead embryos, egg hatching rate, and morphology. It was, however, argued that sometimes neurons are resistant to RNAi (Mercer et al, 2009). In this current study, the knockout *C. elegans* lines used had the T27A3.1 gene ablated by mutagenesis and
not by utilizing RNAi. The creation of knockout lines is often more reliable than RNAi in decreasing the function of genes when dealing with neurons that may be resistant to RNAi.

Similar to what we had previously shown by RNAi, the knockout worms hatched normally and did not show any visible early developmental delays. The T27A3.1 knockout lines showed no significant deficit in gross locomotion, nor did they show differences in their ability to sense osmotic changes, or being repelled or attracted by certain chemicals compared to control N2 worms. The only significant difference seen in two out of the three T27A3.1 knockout lines compared to controls was revealed by the aldicarb assay suggesting impairment of synaptic transmission.

Several reasons can be given to explain differences between the results from published mouse studies and this present study. First, the effect of the T27A3.1 knockout on behavior may not be very strong and hence, these worm assays may not be sensitive enough to detect differences. The aldicarb assay specifically characterizes signaling at the *C. elegans* neuromuscular junctions (NMJs) (Locke, 2008) and we did see an effect in this test. Since T27A3.1 is an ortholog to trak-1 and since the TRAK1 and TRAK2 are motor proteins involved with transport, the aldicarb effects are reasonable (Randall, 2013). Second, another gene may compensate for the lack of the T27A3.1 in *C. elegans*. There are various genes that bind to the HAP1 protein and it is possible that some of them may have a similar function(s) to T27A3.1. For example, HAP1 interacts with the breakpoint cluster region protein (bcr) whose downstream regulators are ERK and p38 (Huang, 2015). HAP1 is also a binding protein for the tuberous sclerosis complex (TSC). This interaction regulates mTOR signaling (Mejia, 2013).

The deficit in synaptic transmission revealed by the aldicarb assay is of particular interest since Hap1 has been previously shown by us and others to be associated with synaptic vesicles and potentially involved in their transport along microtubules (Gutekunst., 1998; Martin, 1999). To further characterize the role of T27A3.1 in *C. elegans*, additional behavioral assays would need to be used. For example, isoflurane is another volatile anesthetic that inhibits synaptic transmission (Saifee, 2011). There has also been an optical method reported for studying neurotransmitter recycling genes but these studies are beyond the scope of this paper (Wabnig, 2015). It might also be worthwhile to look at double knockout lines where the worms have more than one binding partner of Huntingtin deleted.

**Acknowledgments**

Contract grant sponsor: This research was supported by an NIH grant (1R03NS058376), a linkage fellowship from the minorities affairs committee of the American Society of Cell Biology, funds from the Natural Sciences department at Clayton State University and the Neurosurgery department at Emory University.

We thank the *Caenorhabditis elegans* center for N2 and control *C. elegans* strains. We thank Dr. Shohei Mitani, Tokyo Women’s Medical University, Japan for supplying the original stock of worms used to generate the pure line of T27A3.1 worms. We thank Elliot Aronson, Miranda Holmes and Caitlyn Van Heest for technical assistance.

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*J Neurosci Res.* Author manuscript; available in PMC 2017 March 01.


*J Neurosci Res.* Author manuscript; available in PMC 2017 March 01.


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SIGNIFICANCE STATEMENT

This study examines the behavioral phenotype played by an ortholog of the Huntington Associated Protein 1 (HAP1) designated T27A3.1 in *C. elegans*. This protein is expressed prominently in neuronal cells. It has many potential roles relating to the nervous system including but not limited to survival, axonal transport and intracellular trafficking. In *C. elegans*, it is expressed in chemosensory neurons. Importantly, it is a binding protein for the Huntingtin protein which is the protein mutated in Huntington’s disease. This study attempted to examine the role of T27A3.1 in *C. elegans* by examining the behavioral phenotype of three knockout lines of worms.
Fig. 1.
Egg hatching (A) and body bend assays (B). In the egg hatching assay, the number of worms hatched from a single L4 were averaged for each line. The n values correspond to the number of broods analyzed for each line. In the body bend assay, the number of forward locomotion waves was counted. The n values indicate the number of worms tested per line. Bars represent the mean +/- SEM. *p<0.01. ***p<0.0001.
Fig. 2.
Nose touch (A), osmolarity (B) and chemotaxis (C) assays. In the nose touch assay, responsiveness of the worms to a pick dipped in ethanol, benzaldehyde or 1-octanol, was recorded. For the KP4 line 40 worms were tested. For all the other lines we used 50 worms. In the osmolarity assays, 40 worms were placed at the center of 4M NaCl. After 30 minutes the percent of the worms that had escaped the ring were determined. The mean percent escape was obtained by averaging the results of 12 different tests for each line using different worms each time. In the chemotaxis assay, ~150 worms were placed in the center of the testing plate and tested for their ability to move towards a 7.5M ammonium acetate attractant. The n values on the graph indicate the number of times the test was performed for each line using different worms. Bars represent the mean +/- SEM. #p<0.05. ***p<0.0001.
Fig. 3.
Aldicarb assay. For each test 5 L4 worms were placed on a plate containing aldicarb (2mM). Over a period of 3 hr each worm was touched with an eyelash every 10 minutes to determine if they were paralyzed. For each line this test was performed 4–7 times on a different set of worms. Symbols represent the mean +/- SEM. Statistics are discussed in the results section.