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School of Biomedical Sciences

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Due Date	25/10/13
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Evidence of abnormal HSN function in an egg-laying defective *Caenorhabditis elegans* strain.

Joshua Deerain

Joshua Deerain 27/10/13 2:15 PM

Comment [1]: The order of the words in the title reflect the order of importance, from most important to least important.

Abnormal HSN function is the major finding, followed by where it was found.

Style: *The Journal of Neuroscience*, Brief Communications format

Number of words: 4479

Number of pages: 13

Number of figures: 4

Abstract

Caenorhabditis elegans is an important model organism for biomedical science and has been involved in a number of key discoveries. Short life cycle, transparency and amenability to genetic, molecular and pharmacological analysis are key features contributing to this. Mutations resulting in egg-laying defects have been the focus of extensive research and consequently we have a good understanding of this behaviour. In this study we characterised an unknown mutant strain of *C. elegans* using a DIC microscopy along with a number of pharmacological, behavioural, sensory response experiments. Characterisation of the unknown mutant has revealed that it releases significantly fewer eggs than wild type worms. The findings while not conclusive provide some evidence that the egg-laying defect may be a result of altered HSN function related to regulation of serotonin release.

Joshua Deerain 27/10/13 2:18 PM
Comment [2]: The abstract has been conducted using the model described in an earlier tutorial using the key sentences from each section. The Results/discussion represent a significant part of the abstract as they contain the most important and interesting information to a prospective reader.

Introduction

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One of biomedical science's most recognised and important animal models is *Caenorhabditis elegans*. The free-living nematode was first introduced by Sydney Brenner in 1974 and since then has been involved in a number of key discoveries including Alzhimer's disease, Diabetes type 2 and Depression (Trent et al., 1983; Sundaram and Greenwald, 1993; Koelle and Horvitz, 1996; Ogg et al., 1997; Ranganathan et al., 2001; Kaletta and Hengartner, 2006).

Short life cycle, transparency and amenability to genetic, molecular and pharmacological analysis are some of the features that make *C.elegans* an appreciated model organism. Furthermore the self-fertilisation observed in the hermaphrodite state contributes to the genetic tractability while the male state allows for cross-fertilisation (Kuwabara and O'Neil, 2001).

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Comment [4]: Each paragraph of the introduction (and discussion) contains only 1 topic.

Egg-laying is a particular phenotypic behaviour which has been the focus of research seeking to understand neuronal signal transduction mechanism. Much of this has to do with the well-characterised egg-laying apparatus in *C. elegans*. The egg-laying apparatus of *C. elegans* is made up of the vulva, 16 vulval and uterine muscles which are innervated by 2 hermaphrodite specific neurons (HSN) and less notably 6 ventral cord neurons (VC) (Lints and Hall).

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Comment [5]: Clear explanation of one of the key concepts

Self-fertilising, adult hermaphrodites produce sperm, of which is stored in the spermatheca and later oocytes (Schafer, 2005; Schafer, 2006). During the L4/adult molt the eggs are fertilised. Approximately 10-15 fertilised eggs are stored in the uterus of a young adult hermaphrodite (Schafer, 2005). Serotonin release from the HSN motor neurons innervates four vm2 vulva muscles, which are electrically coupled to the vm1 vulva muscles (White et al., 1986; Schafer, 2006). Innervation and consequently contraction of these muscles results in the release of eggs into the environment from the uterus via the vulva.

In a previous study by Trent et al. (1983), they used a forward genetic approach to identify and characterise a number of novel egg-laying defective (egl) genes. By utilising a number of genetic, behavioural and pharmacological assays, they were able to identify various mutations at each of the three levels of the egg-laying apparatus resulting in egg-laying defects.

We have received an unknown *C. elegans* mutant that displays an abnormal egg laying phenotype. Through use of microscopy, pharmacological, behavioural assays we aim to characterise the mutant strain and determine where the mutation has occurred. It is hypothesised that the isolated *C. elegans* strain has abnormal egg laying phenotype resulting from a mutation in genes that play a role in the formation or activity of the vulva, vulval muscles or upstream neurons.

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Comment [6]: The Hypothesis was written first and the introduction was constructed around it.

Materials and Methods

Worm strains and maintenance

N2 and an unknown mutant strain of *C. elegans*, were grown at 20°C on agar plates seeded with *Escherichia coli* OP50 according to standard cultivation techniques (Brenner, 1974).

Age synchronisation

L4 stage worms were identified by the translucent semi-circle morphology present in the middle of the worm. L4 stage worms were isolated and plated on a seeded agar plate for approximately 24 hours prior to experimentation.

Egg-laying assay

Approximately ten synchronised N2 and Mut worms were individually placed in seeded wells of a 24-well plate. The worms were left at room temperature for 3 hours with the number of eggs laid recorded at 1hour intervals. The total number of eggs over the 3hour period was used for analysis. A total of 5 replicate experiments were conducted. One of these replicates was conducted under blind conditions.

DIC microscopy

Differential Interference Microscopy was carried out over 5 different events, with a total of approximately 35 N2 hermaphrodite adults, N2 L4 stage hermaphrodites, Mut hermaphrodite adults and mutant L4 stage hermaphrodite worms observed. Standard mounting techniques described by Shaham (2006) were used to prepare slides with the exception that levamisole was used as the paralytic agent. An *Olympus Bx61* with a *Nikon DSR:1* camera was used in conjunction with *NIS elements F* program for image capture.

Pharmacological assay

For each of the three replicate experiments 8 synchronised hermaphrodite adults from both the N2 and mut groups were submerged in 20µL of 0.75mg/ml imipramine, 0.3mg/ml serotonin or M9 vehicle control. Experiment was conducted in a 96-well plate with individual worms in each well. The cumulative number of eggs laid was recorded at 30min intervals for 1.5 hours. The total number of eggs laid at 1.5hours was taken for analysis. Worms with no movement observable were not included in the final results.

Pharyngeal pumping assay

10 synchronised adult hermaphrodites were isolated on a seeded agar plate. The number of pharyngeal pumps was recorded over a 20sec period. Only worms on the bacterial lawn and actively feeding were recorded. Pumps were observed under a dissecting microscope and identified according to description outlined by Raizen et al. (2012).

Defecation assay

Results for the defecation assay were collected over 2 replicate experiments. Six mutant and six N2 synchronised adult hermaphrodite worms were used in the first experiment and 5 of each were used in the second. The worms were left for at least 5 minutes on a seeded agar plate prior to start of the count and only actively feeding worms recorded. The number of defecation events, as described by Hart (2006) was counted over a 10-minute period.

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Body bend assay

Nine synchronised N2 and mut adult hermaphrodite worms were used in this experiment. The worms were isolated on a seeded agar plate and observed under the dissecting microscope for 3minutes. The number of body bends in that time was recorded as described by Hart (2006).

Touch response assay

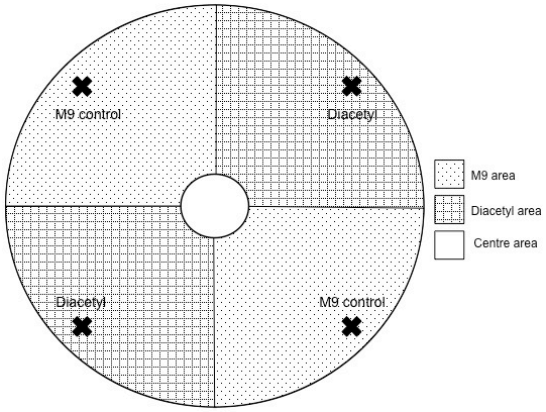
Two replicates of this experiment were conducted. In the first 10 N2 and 10 mut, adult hermaphrodite worms were used. The worms in this fist replicate were not age synchronised and the test was not performed as a blind experiment. For the second replicate 15 synchronised adult hermaphrodites of each strain were used and the test was carried out under blind conditions. In both replicates the worms were isolated onto seeded agar plates. A light touch, using a hair was applied to the midbody of worms moving in a forward direction in order to induce a reversal. The response was recorded as the duration of time from initial stimulation with the hair till cessation of reversal or resumption of forward directional movement.

Thermal avoidance assay

The thermal nociception response of 11 N2 and 10 mut synchronised adult hermaphrodite worms was tested. A metal rod was heated in flame until red and exposed as close as possible to the nose of the worm without touching it. The response of the worm was scored as the number of body lengths the worm reversed in in the 5 seconds following exposure.

Diacetyl chemotaxis assay

The diacetyl chemotaxis assay was conducted over 6 replicates, with 15 worms used within each replicate. An unseeded agar plate was marked as shown below. A 5µL drop of diacetyl was placed in two opposing quadrants and 5µL of the M9 control was placed in the remaining two quadrants. Before the worms were placed on the chemotaxis plate, they were moved to a transfer plate in order to reduce residual bacteria. The worms were placed in the centre circle and counts of how many worms present in each area was taken at 30 minutes.



Statistics

For the assays involving direct comparison between N2 and mut worms (Egg-laying, pharyngeal pumping, defecation, body bend, thermal response and touch response assays), an unpaired, parametric, two-tailed t-test was conducted. A P-value <0.05 is considered a significant result. For the drug and chemotaxis assay, a two-way ANOVA with multiple comparisons test was used. A P-value <0.05 is considered a significant result.

Results

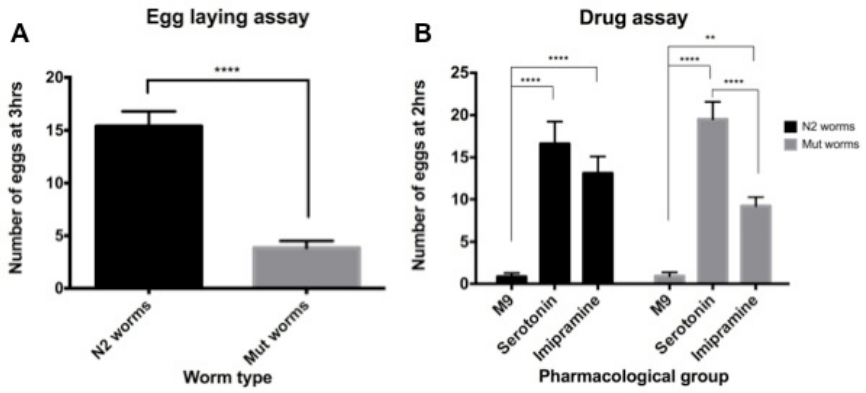


Figure1. Mutant egg laying phenotype. **A)** Cumulative number of eggs laid in a 3 hour period by individual *C. elegans* adult hermaphrodites. Bars represent average number of eggs ± SEM over 5 replicates. Wild type, N2 (*n*=50) worms laid significantly higher number of eggs than the Mut worms (*n*=55) P= < 0.0001. **B)** Cumulative number of eggs laid in 2 hour period by individual *C. elegans* adult hermaphrodites in the presence of a pharmacological agent. Bars represent average number of eggs laid ± SEM for each of the drug test groups. Stars indicate the level of significance with 4 stars indicating P = < 0.0001 and 2 stars indicating P= <0.01. N2 strain: M9 *n*=21, Serotonin *n*=23, Imipramine *n*=21. Mut strain: M9 *n*=24, Serotonin *n*=22, Imipramine *n*=22.

Confirmation and initial characterisation of an egg-laying mutation

Initial confirmation of egg-laying mutation in unknown mutant *C. elegans* was achieved by an egg-laying assay. Results of this revealed that compared to the wild type (N2) worms, the number of eggs laid by the mutant strain over three hours was significantly less (P= < 0.0001) (Figure 1). Furthermore it was observed that the eggs from the mutant worms were regularly laid at the comma stage or later while the N2 strain worms predominantly laid gastrula stage eggs. Further preliminary observations identified the mutants as being less active and sluggish in movement compared to the wild type worms.

Responses to pharmacological agents

The response of the unknown mutant to pharmacological agents that stimulate egg-laying in wild type worms was assessed. As illustrated in Figure1, the N2 strain worms are sensitive to both serotonin and imipramine. Exposure to both drugs resulted in significantly increased egg laying with respect to the M9 vehicle control (P= <0.0001 for both drugs). The mutant worms were also found to be serotonin and imipramine sensitive. Significantly higher numbers of eggs were laid in

the presence of serotonin and imipramine compared to the M9 vehicle control (P= <0.0001 and P= <0.01 respectively).

In the N2 worms there is no significant difference detected between the number of eggs laid during exposure to serotonin or imipramine. However in the mutant worms the response to serotonin was much higher than the response to imipramine with a significance of P= <0.0001. Suggesting the mutant worms are more sensitive to serotonin than imipramine.

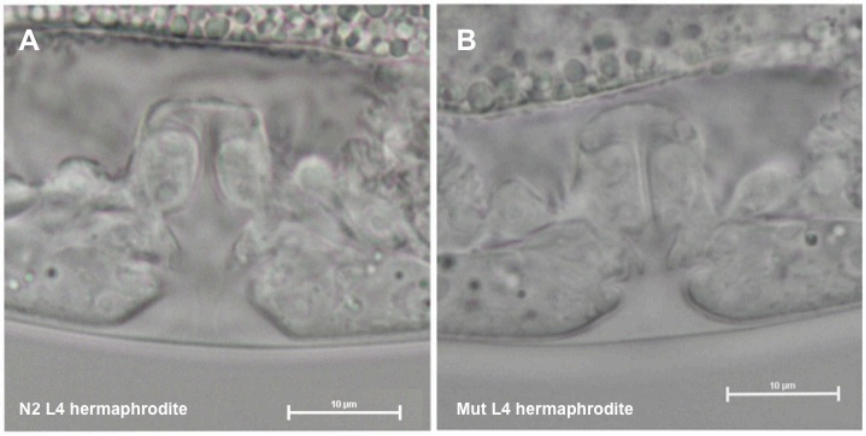


Figure2. Normal formation of the vulva in unknown mutant. **A)** Wild type (N2) hermaphrodite *C. elegans* at L4 stage of development. **B)** Unknown mutant hermaphrodite *C. elegans* at L4 stage of development. Both images captured using n *Olympus Bx61* with a *Nikon DSR:1* camera.

Excluding anatomical abnormalities of vulva formation.

Differential Interference Microcopy (DIC) was used in order to determine if the unknown mutation resulted in abnormalities in vulva formation. Observations of the vulva from L4 stage hermaphrodites of both groups (N2 and Mut) revealed no distinct abnormalities. In total, approximately 35 worms from each group were observed. While a number of distinct morphologies were observed across all the worms, examples of each were identified in both the N2 and Mut groups. This suggests any variation was due to different stages of L4 development. Figure2 illustrates the similar 'Christmas tree'-like structure of the vulva in both the N2 and Mut worms.

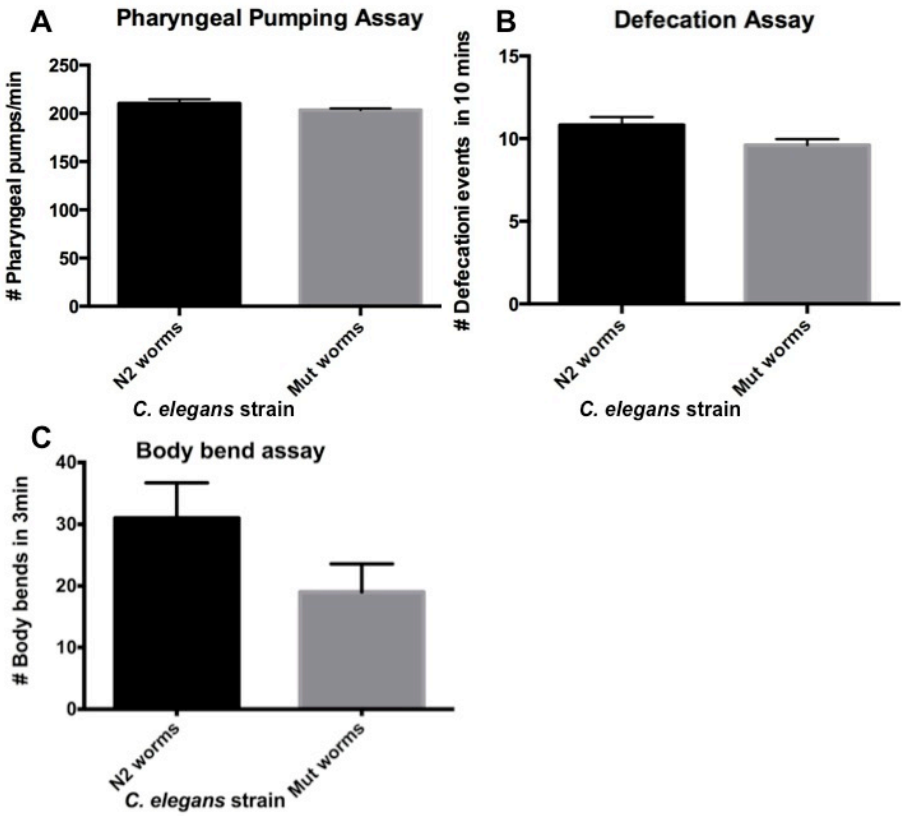


Figure3. Mutant strain presents with phenotypically wild type behaviour. **A)** Feeding behaviour of adult *C. elegans* hermaphrodites. The average number of pharyngeal pumps was recorded over a 20sec period for N2 (*n*=19) and Mut (*n*=18) worms and standardised for 1minute. Bars represent the mean pharyngeal pumps per minute ± SEM. No significant difference between N2 and Mut worms could be identified (P=0.1595). **B)** Defecation behaviour of adult *C. elegans* hermaphrodites. Number of defecation events in 10 min was recorded for N2 (*n*=15) and Mut (*n*=15) strains. Bars represent mean number of defecation events ± SEM. No significant difference between N2 and Mut worms could be identified (P= 0.0589). **C)** Locomotion behaviour of adult *C. elegans* hermaphrodites. Number of body bends in a 3-minute period was recorded for N2 (*n*=9) and Mut (*n*=9) was recorded. Bars represent mean number of body bends in 3 minutes ± SEM. No significant difference between N2 and Mut strain with P= 0.1199.

Feeding, defecation and locomotion behaviours

To test if the unknown mutant strain suffers from other mutant phenotypes we conducted a number of behavioural assays. The rate of pharyngeal pumping is a measure of feeding behaviour (Avery and You, 2012). The results from the pharyngeal pumping assay (refer to figure 3) show no significant difference between the two groups (P=0.1595). The mean pharyngeal pumping rate for the N2 and mut worms was 210.3 ± 4.334 and 203.5 ± 1.667 pumps per minute respectively. The frequency of pharyngeal pumping made accurate recordings difficult. Therefore we cannot be completely confident in the results.

The defecation assay looked at the number of defecation events in a 10 minute period. No significant abnormalities in defecation could be observed between the wild type (N2) and mutant worms (P = 0.0589) (refer to figure3). The mean number of defecation events in 10 minutes for the N2 and mutant strains was 10.80 ± 0.4899 and 9.600 ± 0.3625 respectively.

The frequency of body bends is used as a measure of locomotion (Riddle, 1997). In this study we used the number of body bends in a 3-minute period to test for abnormalities in the locomotion of the mutant strain. The mean number of body bends in the 3 minute period for the N2 and Mut worms was 31.00 ± 5.706 and 19.00 ± 4.561 respectively. While the mutants show a lower mean number of body bends this result was not significant with the current *n* of 9 (P = 0.1199). A larger sample size is required to determine if the results are significant.

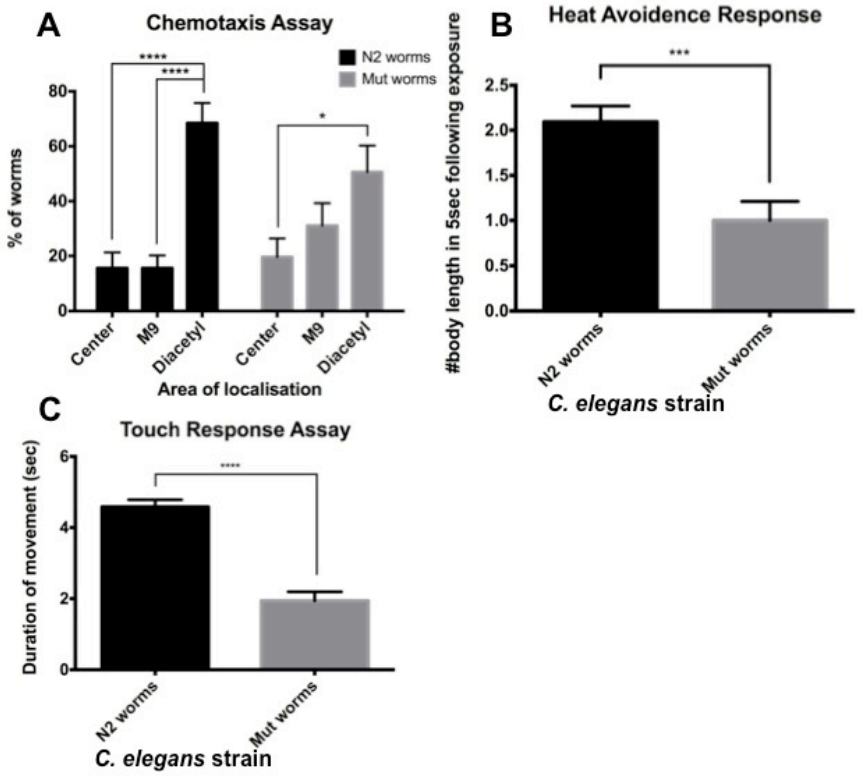


Figure4. Mutant *C. elegans* strain show decreased stimulus response. **A)** Adult *C. elegans* hermaphrodites' response to the chemoattractant, Diacetyl. The percentage of N2 and mutant worms that localised to the centre region or the areas containing diacetyl or M9 was recorded. Bars show mean percentage of worms localised to each area over 6 replicates ± SEM. Stars indicate the level of significance with 4 stars indicating P = < 0.0001 and 1 star indicating P= <0.05. **B)** Adult *C. elegans* hermaphrodites' response to thermal nociception. The number of body lengths the N2 (*n*=11) and the mut (*n*=10) strain worms reversed following exposure to heated rod was recorded. Bars show mean number of body lengths ± SEM. The difference in response in the mut worms was significantly less with P-vaule of 0.0008. **C)** Adult *C. elegans* hermaphrodites' mechanosensory response to a soft eyelash touch. Following a soft touch to the midgut of N2 (*n*=25) and mut (*n*=25) worms, the duration of reversal was recorded. Bars show mean duration of reversal ± SEM. The difference in mechanosensory response in the mut worms was significantly less (P=<0.0001).

Sensory response to stimulus

To determine the sensory response of the mutant a number of stimulus response assays were conducted. In a chemotaxis assay with diacetyl (refer to figure4), the N2 worms show significant localisation to the areas with the attractant compared to the M9 and centre areas (P=<0.0001). From

the mutant worms approximately 20% of the worms remained in the centre, which was found to be significantly less than those in the diacetyl (P=<0.05). However the number of worms that localised in the diacetyl areas was not significantly higher than the M9 area.

In the thermal response assay (refer to figure 4) N2 worms were found to reverse for 2.091 ± 0.1760 body lengths following exposure while the mutant worms revered for 1.000 ± 0.2108 body lengths. The mutant response was determined to be significantly less (P= 0.0008).

In response to a soft touch to the midgut the N2 worms were found to reverse for a longer duration than the mutant worms (P=<0.0001) (figure4). The mean response duration in the N2 worms was 4.580 ± 0.2035 seconds and 1.940 ± 0.2522 seconds in the mut worms.

Discussion

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In this study we have been able to confirm that the unknown *C. elegans* mutant strain has an egg-laying defect characterised by a reduced egg-laying activity. While our characterisation is not conclusive the findings indicate a potential abnormality in the neurons involved egg-laying. We have some evidence which points to mutations in neuronal regulatory elements as a possible cause. However current results make it impossible to definitively distinguish between neuronal and muscular abnormalities.

Egg-laying events in *C. elegans* have been reported to occur in short 1-2minute bursts under favourable conditions (Waggoner et al., 1998; Schafer, 2005). These bursts are then followed by periods of about 20minutes with no/minimal egg-laying events (Waggoner et al., 1998; Schafer, 2005). The egg-laying assay conducted in this study looks at the cumulative number of eggs laid over a 3-hour period. This timeframe should therefore have encompassed a number of these short burst events. Indeed a significant number of eggs were recorded over the time course by the N2 (wild type control) worms. The mutant strain of worms did show evidence of egg-laying however the number of eggs laid over the 3-hour period was significantly less. This suggests that the mutants do not have a completely ablated egg laying behaviour as seen some mutants with abnormalities in HSN, vulva and muscle development (Trent et al., 1983). The retention of eggs also provides an explanation why later stage eggs were regularly observed.

A number of *C. elegans* egg-laying defective mutants which have deformities in vulva development have been described (Trent et al., 1983). However in this study, DIC microscopy failed to identify any abnormalities, suggesting a muscular or neuronal basis for the egg-laying defect. Unfortunately fluorescent transgene crosses could not be produced. This has prevented a clearer observation of not only the vulva structure but also the HSN and vulval and uterine muscles

In a study conducted by Trent et al. (1983), they used serotonin (5HT) and imipramine to categorise *C. elegans* egg-laying mutants into 4 categories based on their responses. The HSN is a serotonergic motor neuron that innervates the vulval muscles, while imipramine acts on the 5HT reuptake transporter to inhibit serotonin reuptake at the neuromuscular junction. (Trent et al., 1983; White et al., 1986; Dempsey et al., 2005).

The unknown mutant in this experiment was able to lay eggs in response to both serotonin and imipramine, a class C mutant as described by Trent et al. (1983). This suggests that the vulval muscles of the mutant strain are functional. The sensitivity to imipramine suggests that there is at least some serotonin present in the neuromuscular junction. We believe that the HSN is present and able to release at least some serotonin. This corresponds with the results of the egg-laying assay that showed the mutant worms are able to release some eggs.

It was also noted however that the mutant strain had a significantly higher sensitivity to serotonin than imipramine not seen in the wild type worms. This evidence provides an indication that there is some defect of the HSN functioning (Trent et al., 1983). A possibility is that there is an abnormality with the regulation of serotonin release in the HSN. Egg-laying can be regulated in response to the environment and a number of genes which confer this have been identified (Trent et al., 1983; Koelle and Horvitz, 1996; Schafer, 2005). Mutations have been found in subunits of the G_o and G_q G protein homologues that have been shown to regulate egg-laying behaviour (Bastiani et al., 2003). Mutations in the RGS proteins that regulate the G-proteins have also been reported an example of which is *egl*-10 (Koelle and Horvitz, 1996; Hajdu-Cronin et al., 1999).

Behavioural assays were conducted in order to determine if the mutation was isolated to the egg-laying apparatus. Pharyngeal pumping, defecation and locomotion are all behaviours tested with relative ease in *C. elegans*. Pharyngeal pumping is mediated by coordinated contraction-relaxation cycles of the radially-oriented muscles of the corpus, anterior isthmus, and terminal bulb (Kwok et al., 2006). The pharyngeal pumping nervous system has been reported to be non-essential for pumping, however the MC and M4 motor neurons are believed to influence the rate of pumping (Avery and Horvitz, 1989; Riddle, 1997). According to our findings there was no difference in pumping rate between the mutant and wild-type strains. This could suggest one of a number of possibilities; The mutation does not present in the muscles or neurons responsible for pharyngeal pumping, the mutation is present in the neurons but is not implicated in the regulation observed in pharyngeal pumping or that the assay is not sensitive enough to observe a difference.

Similarly no obvious altered phenotype was observed in the defecation assays. 3 motor steps, utilising a number of different muscles innervated by two GABAergic motor neurons make up the defecation motor program. Defecation is reported to be controlled by an endogenous clock with events occurring every 45sec (Liu and Thomas, 1994). If the mutation is involved in regulation this may provide a reason why no difference is observed here. However like the pharyngeal pumping assay this could also suggest that the mutation is not shared between defecation an egg-laying apparatus.

Locomotion abnormalities are another behaviour, which are often seen in egg-laying defective worms. Observations of 'sluggish' movement led us to conduct a body bend assay. We expected to see a significant decrease in frequency of body bends consistent with some other findings concerning egg-laying defective worms with muscular or neuronal mutations. However the results of this experiment were found to be insignificant and only a small sample size was observed. While no conclusive results could be drawn there is small evidence of a decreased locomotion in the mutant strain group. Further replicates of the experiments are required to confirm this. Decreased locomotion can be a result in a number of mutations in *C. elegans* muscles, neurons and the RGS protein *egl*-10 (Trent et al., 1983; Koelle and Horvitz, 1996).

With the results from the pharmacological assay indicating a potential neuronal defect and some evidence indicating a regulatory element a number of response assays were undertaken. These assays aimed to quantify the mutant *C. elegans* response to mechanosensation, thermal nociception, and chemosensation. Each of the responses is mediated by different sensory neurons to produce some sort of locomotive response (Bargmann et al., 1993; Kaplan and Horvitz, 1993; Mori and Ohshima, 1995; Bargmann, 2006).

In each case there was a significant decrease in response observed in the mutant worms. For the Thermal response and touch response assay the response of individual worms was recorded. In both instances when the worms were subjected to the stimulus there was an initial avoidance response but the duration of this response was minimal. This could suggest a defect in regulation of motor neurons but does not rule out the possibility of other neuronal or muscular mutation.

The findings of this study appear to suggest a neuronal basis for mutation. However because most of these assays are testing phenotypes that have a neuronal and muscular component it is impossible to draw definitive conclusions. Further experiments need to be conducted that are able to distinguish between the two. Fluorescent staining or antibody probing techniques like those described by Koelle and Horvitz (1996) could be used to determine the presence and functioning of the neuronal regulatory elements in the mutant strain.

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