Comparison of wild-type and *unc-2* mutant *Caenorhabditis elegans* egg-laying rates

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Abstract

The *unc-2* gene of the soil nematode *Caenorhabditis elegans* encodes for a subunit in voltage-gated calcium ion channels found in the hermaphrodite-specific neurons and ventral cord neurons of the egg-laying muscles. We compared the egg-laying rate between wild-type N2 and uncoordinated *unc-2* VC854 mutant *C. elegans*. Five L4-stage worms were chosen from each strain and observed for a period of four days. After each 24-hour incubation period, we recorded the total progeny for each worm. We conducted a two-way ANOVA on the results and found that time from incubation has an effect on the rate of eggs laid (p-value = 0.006). However, the presence of the *unc-2* mutation does not affect the rate of egg-laying (p-value = 0.3) and time from incubation has the same effect on egg-laying rate in wild-type and mutant worms (p-value = 0.06). Our results suggest synaptic function and muscle contractions are still occurring even in the presence of mutated UNC-2 proteins. These findings give us a better insight into the synaptic function of neuromuscular junctions in the egg-laying muscles in *C. elegans*.

Introduction

Caenorhabditis elegans is a free-living nematode commonly found in soil habitats (Corsi, Wightman & Chalfie 2015) that feeds on *Escherichia coli* (Stiernagle 2006). It exists primarily as a self-fertilizing hermaphrodite (Lints & Hall 2009a), producing sperm until its last larval stage (L4) (McGovern et al. 2007). After the L4-to-adult molt, remaining germline cells differentiate into oocytes (Schafer 2006). The egg-laying muscles are controlled by two types of motor neurons: the hermaphrodite-specific neurons (HSNs) and ventral cord (VC) neurons (Schafer 2006). The *unc-2* gene encodes for the pore channel subunit in high-threshold voltage-gated calcium channels (VGCCs) expressed in the presynaptic region of neuromuscular junctions (NMJs) of motor neurons, including the HSN and VC neurons (Mathews et al. 2003). The UNC-2 protein functions in calcium-sensing in the NMJ, allowing for the release of neurotransmitters and thus muscle contraction or relaxation (Richmond 2007) (Figure 1).

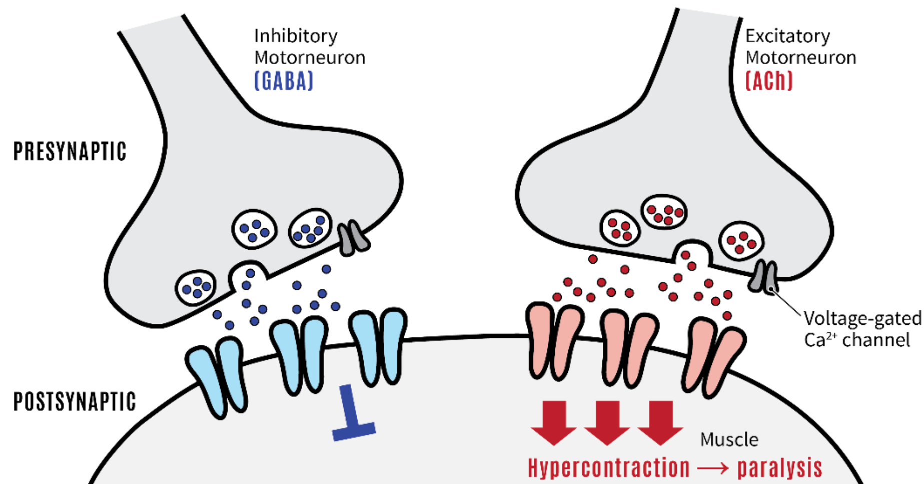

Figure 1. Structure of the *C. elegans* NMJ. UNC-2 is a subunit of VCGGs (modified from Kowalski 2015 based on Droual 2011).

We set out to better define the relationship between the egg-laying rates of wild-type and *unc-2* mutant *C. elegans* over its four-day fertile period (Altun & Hall 2009). Our hypotheses were as follows:

H_{01}: Time from incubation has no effect on the egg-laying rate of *C. elegans*.

H_{a1}: Time from incubation has an effect on the egg-laying rate of *C. elegans*.

H_{02}: The *unc-2* mutation has no effect on the egg-laying rate of *C. elegans*.

H_{a2}: The *unc-2* mutation has an effect on the egg-laying of *C. elegans*.

H_{03}: The effect of time from incubation on the egg-laying rate in *C. elegans* is the same in wild-type and mutant replicates.

H_{a3}: The effect of time from incubation on the egg-laying rate in *C. elegans* is not the same in wild-type and mutant replicates.

We predicted that egg-laying rates would not remain constant through our observation period. *C. elegans* lays eggs in a biphasic manner, alternating between periods of activity and inactivity (Waggoner et al. 1998). Additionally, Muschiol, Schroeder & Traunspurger (2009) observed that the rate of egg-laying in wild-type *C. elegans* followed a bell-shaped curve over age (Figure 2). We also predicted there would not be a difference in egg-laying rates between

wild-type and mutants. The *egl-19* and *cca-1* genes encode for similar α1 subunits in VGCCs (Mathews et al. 2003), and overlap in their pathways could compensate for any deficiencies in defective UNC-2 proteins. As *unc-2* does not participate in the regulation of timing of egg-laying, but rather works to propel the signals initiating or inhibiting egg-laying, the effect of time from incubation should be the same in both wild-type and mutant.

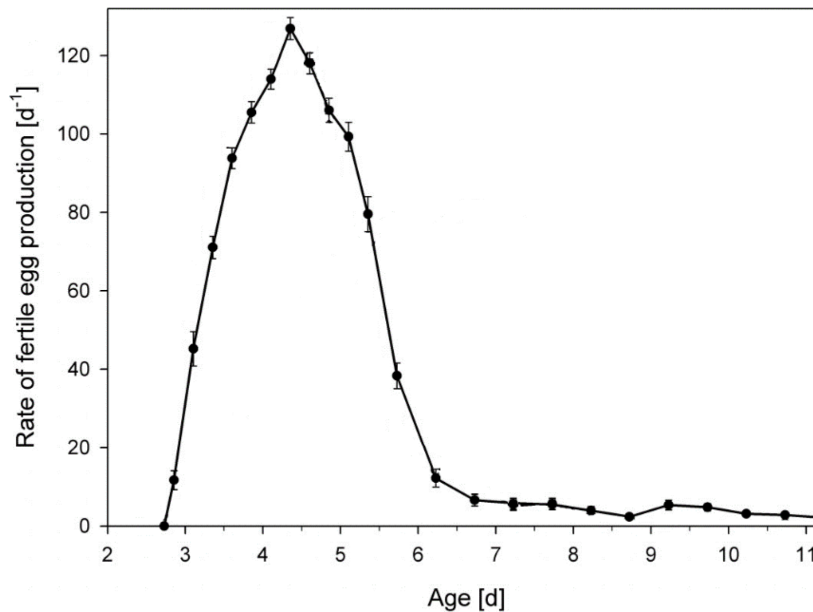

Figure 2. Egg-laying rate in N2 *C. elegans* over age. $n = 69$ until age 5.7 days, $n = 36$ after. Bars represent standard error (modified from Muschiol, Schroeder & Traunspurger 2009).

Defective VGCCs cause several human diseases, such as hypokalemic periodic paralysis and Timothy syndrome (Lainé et al. 2014). Gaining a better understanding of the functions of VGCC components of *C. elegans* could provide invaluable insight to the pathology of these diseases, as well as pharmacological uses (Catterall 2011).

Methods

For this experiment we obtained two stocks of *C. elegans*, one of the wild-type N2 strain and the *unc-2* mutant VC854 strain. The stock provided contained only hermaphrodite nematodes in varying stages of their lifecycle, from unhatched eggs to mature adult worms. We used the wild-type replicates ($n = 5$) as our control group and the mutants ($n = 5$) as our experimental

group. Over a period of four days, we observed and quantified their egg-laying behaviour. This four-day period ensured that we would be able to observe the egg-laying rates through the entire reproductive cycle of mature hermaphrodites (Altun & Hall 2009).

In preparation for our experiment, we used a dissecting microscope to identify and isolate five L4-stage worms from each strain using a sterile platinum-tipped worm pick. We identified L4 worms through a characteristic white semi-circle at the vulval region (Figure 3) (Corsi, Wightman & Chalfie 2015). This distinguishing feature is present only in L4 worms (M. Chow, 2016, Biology Lab Technician, pers. comm). The vulvae of worms in other stages were identified as a black spot approximately halfway down the length of the body (Altun & Hall 2009).

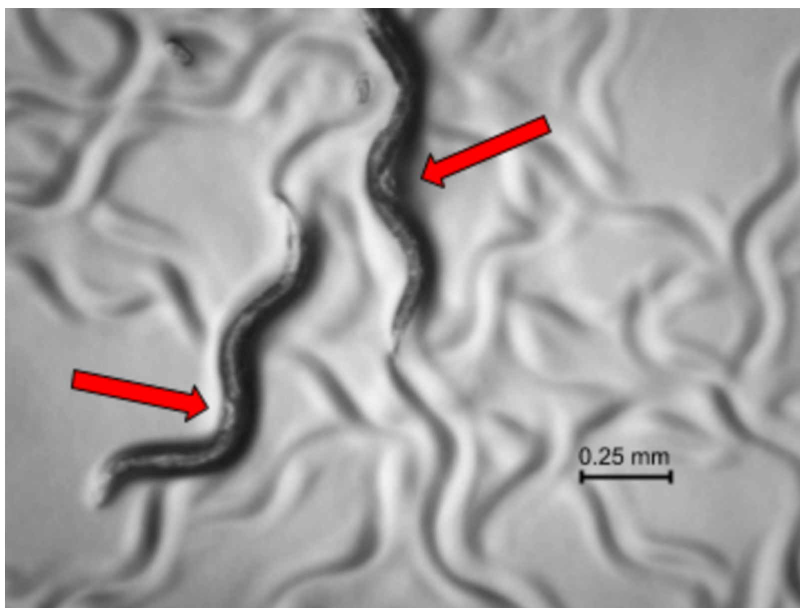

Figure 3. Arrows indicate the white semi-circle at the vulva region, the distinguishing feature of L4 worms, located approximately midway through the length of the organism (photo credit Mindy Chow, University of British Columbia).

After L4 identification, we transferred each organism to a separate petri dish. The dishes had been previously prepared with an agar base and swabbed with *E. coli* to serve as a food source. We transferred the worms using sterile technique and sealed the petri dishes with Parafilm M® to ensure that the agar would not dry out. We stacked the dishes in two groups of five replicates, one for each strain, and stored them in a clear plastic box that was incubated at

20°C for approximately 24 hours. We chose this temperature as *C. elegans* is optimally maintained at 20°C in laboratory settings (Stiernagle 2006).

For each day of the observation period, we first transferred the hermaphrodites to new petri dishes treated with *E. coli*. We recorded all progeny, defined as eggs and hatched worms, using a dissecting microscope with the aid of a grid. At the end of each observation period, we sealed all new and previously used petri dishes with Parafilm M®, placed them back inside the box, and returned the box to the 20°C incubator for another 24 hours. This process was repeated for each observational day (Figure 4). During handling and observations, the abiotic factors remained consistent for all replicates as they were exposed to the same temperature and light intensity variations. To minimize the effects, we limited our observational period to three hours.

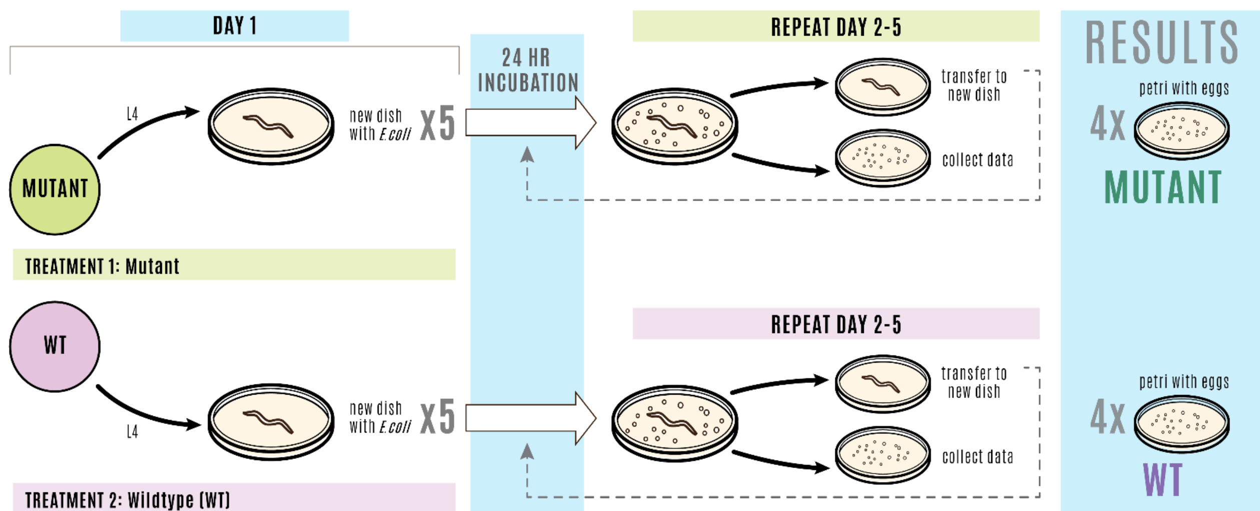

Figure 4. Outline of the experimental setup.

We conducted a two-way ANOVA test for statistical significance of our data using the software StatPlus to obtain the *p*-values for our three hypotheses. This statistical test was chosen as we had two independent factors, time from incubation of the replicates in days and the type of strain. Additionally, we calculated the mean rate of eggs laid per day per nematode strain and the 95% confidence intervals (C.I.), which allowed us to note any potentially significant differences between wild-type and mutant egg-laying rates for each day.

Results

We obtained three p-values from our two-way ANOVA test: $p_1 = 0.0006$, $p_2 = 0.3$ and $p_3 = 0.06$. Out of the three hypotheses, only the p value for H_1 was statistically significant. There was no overlap between wild type Day 2 and wild type Days 1 and 4; the significant difference on this day likely contributed to the statistical significance of the p value for H_1. We found both strains had a greater rate of eggs laid on the two middle observation days and decreased rates on Days 1 and 4 (Figure 5). The overlap of 95% C.I.s between mean wild-type and mutant egg-laying rates on Days 1, 3, and 4 indicated there was no significant difference on these days (Figure 5).

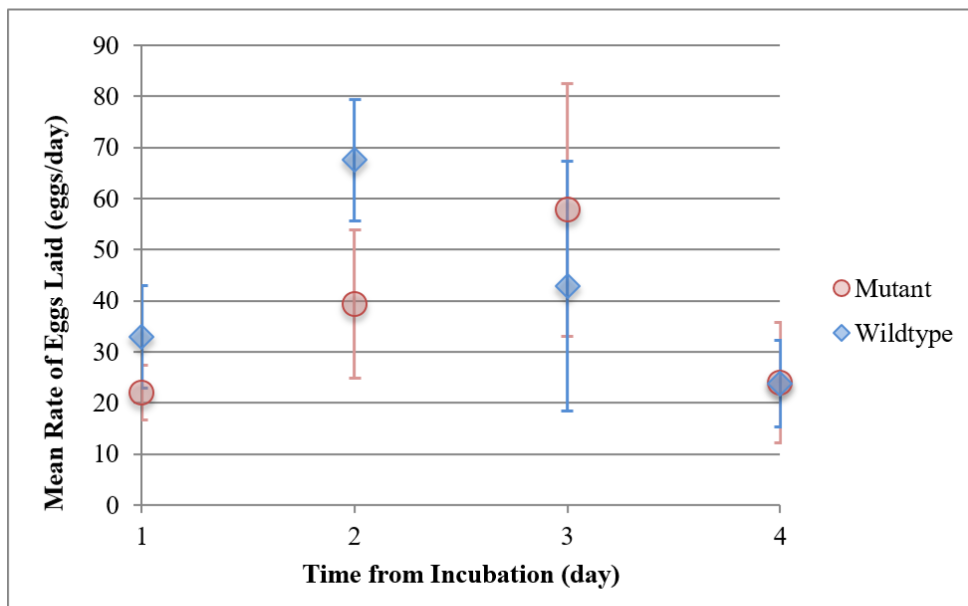

Figure 5. The mean rate of eggs laid by wild-type and *unc-2* mutant *C. elegans* each experimental day. Vertical bars represent 95% confidence intervals. $n = 5$, $p_1 = 0.0006$, $p_2 = 0.3$, $p_3 = 0.06$.

Throughout the experiment, we observed that the wild-type nematodes were more active than the mutant nematodes. Tracks on the *E. coli* lawn caused by the organism's movement were more abundant and covered larger areas in wild-type replicates than in mutant replicates. This observation was also consistent in newly hatched larva. Eggs were laid singularly along these tracks. Occasionally we also found pairs of eggs, but clusters of eggs greater than two were not observed.

Discussion

We rejected the null hypothesis for H_1 and provided support for the alternate, indicating that time from incubation has an effect on the rate of eggs laid by *C. elegans*. However, we failed to reject the null hypothesis for both H_2 and H_3. Thus, the presence of the mutation in the *unc-2* gene has no effect on the rate of eggs laid and the effect of time from incubation on the egg-laying rate is the same in both the wild-type and mutant strain. The statistical results were consistent with our predictions that the mutant *unc-2* gene may not affect the rate of eggs laid. Despite being expressed in the HSN and VC motor neurons that control egg laying (Mathews et al. 2003), it appears there is no direct relationship between the *unc-2* mutation and the rate of eggs laid. We found similar egg-laying rates between wild-type and mutant replicates, which both followed a bell-shaped distribution over time (Figure 5). These observations suggest neurotransmitter release is still occurring even with compromised presynaptic activity. Zhang et al. (2008) observed HSN activity without external presynaptic excitation, suggesting HSNs have an internal modulation mechanism controlling egg-laying events. Thus, HSNs could still exhibit normal activity even with decreased UNC-2 function in presynaptic motor neurons. Instead, the HSNs could be responding to residual neurotransmitter release, spontaneous neurotransmitter release, or other neuropeptides. While primarily expressed in muscles, *egl-19* is also found in some neurons, and thus can be responsible for at least some neurotransmission (Lee et al. 1997). Spontaneous postsynaptic excitation in the absence of calcium has also been previously observed (Richmond and Jorgensen 1999), demonstrating that HSNs can still be excited without functional UNC-2 proteins. In addition to the neurotransmitters serotonin and acetylcholine, HSNs also contain at least one neuropeptide (Waggoner et al. 1998), which may act directly on vulval

muscles (Zhang et al. 2008). As a result, the egg-laying rates of mutant worms were unaffected by the *unc-2* mutation and were subjected to the same temporal effects as wild-type organisms.

For H_1, we did not expect a consistent rate of egg-laying over time due to its temporal egg-laying pattern, which consists of short episodes of egg-laying separated by longer periods of inactivity (Waggoner et al. 1998), the duration of which is randomly distributed (Zhou, Schafer & Schafer 1998). This temporal pattern is controlled by the HSNs, which further provides support for our alternate hypothesis where time from incubation affects the rate of eggs laid (Branicky et al. 2014). In our results, we note a peak in egg-laying 48-72 hours from the first incubation, suggesting that all replicates, both wild-type and *unc-2* mutants, follow a bell-shaped distribution with regards to time from incubation and the rate of eggs laid (Figure 5). This is consistent with the model found by Muschiol, Schroeder & Traunspurger (2009), who noted that the rate of egg-laying in N2 *C. elegans* sharply increased following reproductive maturity until its maximum at 108.2 hours post-hatch (Figure 2). This is roughly two days following the L4-to-adult molt, which occurs 45-50 hours post-hatch (Altun & Hall 2009). Our wild-type replicates (Figure 5) closely followed the left-skewed bell-curve model (Figure 2).

Adult hermaphrodite worms lay 4-10 eggs/hour (Lints & Hall 2009b; Waggoner et al. 1998). We expected to see a rate of 96-240 eggs/day for each worm in our experiment, but these numbers fell short, ranging from 10-85 eggs/day in our wild-type replicates. A possible reason for this decreased rate was the handling of the worms during our observational period. Mechanical stimulation, such as vibration, is known to inhibit egg-laying in worms (Sawin 1996). However, this was unavoidable when we transferred the worms from the previous day's plate to new plates, a process that took roughly three hours per day. Light stimulus and the duration of light exposure also affect HSN excitability, which in turn decreases the rate of eggs laid (Branicky et al. 2014). During handling, all replicates were subject to same unregulated abiotic

factors, such as ambient room temperatures and fluorescent lighting, for the same period of time, so any outside conditions would affect all replicates equally. In addition, we selected for L4 worms at the beginning of the experiment, which were still maturing into adults on Day 1. As hermaphrodites only produce oocytes after the L4-to-adult molt when sperm production has stopped (Altun & Hall 2009), fertilized eggs are accumulated and laid after worms have fully matured into adults. Thus, worms were not capable of egg-laying for a portion of the observation period in Day 1.

Conclusion

From our results, we rejected H_{01} and provided support for H_{a1}, showing that time from incubation has a statistically significant impact (p_1=0.0006) on the rate of eggs laid by *C. elegans*. This agrees with our prediction that rate of egg-laying in *C. elegans* fluctuates through its reproductive period. We failed to reject both H_{02} (p_2 = 0.3) and H_{03} (p_3 =0.06), which indicates the *unc-2* mutation does not have an effect on the egg-laying rate, and that the effect of time from incubation is the same in both wild-type and mutant organisms. We found no statistically significant difference in egg-laying rate between wild-type and mutant replicates. These results suggest *unc-2* does not play a critical role in egg-laying and other pathways present may compensate for the effects of the *unc-2* mutation.

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