CYCLIC GMP: A SATIETY SIGNAL IN C. ELEGANS

Ji S. Park
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CYCLIC GMP: A SATIETY SIGNAL IN C. ELEGANS

A thesis submitted in partial fulfillment of the requirements for the degree of Master at Virginia Commonwealth University.

by

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Richmond, Virginia
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I have grown and learned many things in my relatively short course of study here and all past educational experiences leading up to my time here at VCU, and I owe thanks to all of those individuals who have been of tremendous support throughout this journey.

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Abstract

CYCLIC GMP: A SATIETY SIGNAL IN C. ELEGANS

By Ji Su Park, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master at Virginia Commonwealth University.

Appetite control and satiety mechanisms help animals maintain energy homeostasis; however, these mechanisms can be misregulated, leading to overweight and obesity. 

Caenorhabditis elegans is an excellent model system to study appetite and satiety because of its conserved behavioral aspects of satiety and conserved molecular mechanisms. ASI senses nutrition and its activity is required for the behavioral state of satiety quiescence. The purpose of this thesis project was to elucidate the function of cGMP signaling in ASI by looking at behavioral effects from the pharmacological use of sildenafil (Viagra), a PDE inhibitor, and the effects on ASI activation from mutating guanylyl cyclase DAF-11. Sildenafil treatment increases satiety quiescence and decreases fat storage in a PDE-dependent manner. The daf-11 mutation decreased overall
fluorescence intensity of ASI activation and the frequency at which ASI activated by about 50% compared to wild-type worms, suggesting that DAF-11 plays an important role in ASI to promote satiety.
Chapter 1 Introduction

1.1 Importance of understanding appetite control in the context of the obesity epidemic

In 2008, more than 1.4 million adults were overweight and more than half a billion were obese worldwide. Approximately 2.8 million people die year globally from a complication of being overweight or obese\(^1\), such as heart disease, stroke, type 2 diabetes, and certain types of cancer, which are some of the leading causes of preventable death. In the United States alone, $147 billion were spent in estimated medical costs in 2008, and individuals who are obese spent an average of $1,429 more in medical care than those who are not obese\(^2\).

The factors contributing to body weight are complex and involve a combination of an individual’s genetics and environment, but body weight is ultimately a result of the body’s balance of energy and caloric intake. Animals naturally have appetite control and satiety mechanisms in place in order to maintain energy homeostasis. However, these mechanisms can be misregulated, which can lead to eating over the daily caloric requirement, energy imbalance, and subsequent weight gain. The importance of understanding appetite control and satiety continues to be more pressing with the steady rise of the global obesity epidemic.
1.2 What is satiety?

Satiety is the feeling of fullness that results in the cessation of feeding, and is a complex, yet widely conserved behavior in many animals. A series of satiety behaviors have been reported and repeatedly observed in many animals, including mice, rhesus monkeys, human infants; upon feeling full, the animal stops eating, grooms and explores for some time, and then rests. These behaviors are a result of many integrated signaling molecules and neuronal networks in order for an animal to maintain proper energy homeostasis.

In mammals, satiety results from various signals from the gut and adipose tissue relaying and converging at multiple levels of the central nervous system. Neuropeptides, such as cholecystokinin (CCK) released from the gut in response to dietary nutrients, enter the brain directly from circulation, or its signal is input at vagal afferents innervating the viscera and gastrointestinal tract, which then connect to higher brain centers. Mechanoreceptors are also present on vagal afferents that are located on branches lining the gastrointestinal tract, which are sensitive to stomach and intestinal volume and pressure. Signaling from all levels is then integrated in the hypothalamus and brainstem, which then interact with higher brain centers to terminate meals, including reward centers such as the ventral tegmental area and nucleus accumbens, and areas involved in executive decision making such as the prefrontal cortex.
1.3 *C. elegans* as a powerful model for studying satiety

Although knowledge about satiety signals has increased dramatically within the past 50 years, many details of the molecular and neuronal mechanisms by which satiety is signaled remain unclear. The largest obstacle in studying appetite and satiety in mammals is the degree of complexity that is encountered and a vast number of technical limitations. With trillions of connections among billions of neurons, it is an especially difficult endeavor to study detailed neuromolecular mechanisms underlying appetite control in mammals.

Interestingly, many aspects of the appetite-controlling behavior and of the molecular pathways are conserved\(^7\)–\(^9\) in a simpler organism, the *Caenorhabditis elegans*, a small, free-living soil nematode. Worm behavior after feeding mimics behavioral aspects of satiety and postprandial sleep in mammals. Additionally, satiety signaling in *C. elegans* is partly regulated through a TGF-β and cGMP (cyclic guanosine monophosphate) pathway that is also conserved in mammals\(^10\).

In addition to conserved behavior and mechanisms, the simplicity of the nervous system of *C. elegans* makes it a great model to use in order to study neuronal mechanisms of appetite control. *C. elegans* only has 302 neurons and is the only organism whose entire neural network is mapped by EM reconstruction.

Furthermore, using *C. elegans* as a model presents numerous other conveniences. *C. elegans* are self-fertilizing hermaphrodites (with about 0.02% existing as males through the non-disjunction of the sex chromosome during meiosis). The life cycle period
from egg to adult is about 3 days, with each worm laying about 300 eggs, which allow large numbers of worms to be bred cheaply, easily, and quickly. The worm is transparent under the microscope, which allows for many accessible techniques and assays. *C. elegans* is also a powerful genetic model because they are amenable to genetic manipulation. The haploid genome size is only 100 megabase pairs (Mb)$^{11}$, compared to about 3,200 Mb in humans$^{12,13}$. DNA can be injected directly into the gonad of the worm under a microscope to create transgenic worms and RNA interference knockdown of gene expression to assess the role of genes can be accomplished simply by feeding them bacteria that expresses the RNAi of choice. Through the study of a much simpler model organism such as *C. elegans*, the core molecular basis of the appetite-controlling behavior can still be unraveled without the complexity that come with mammalian models.

1.4 Feeding and satiety behaviors in *C. elegans*

*C. elegans* primarily feeds on bacteria. It pumps food through a large pharynx, and the food is crushed as it passes into the intestine. *C. elegans* actively seek out food depending on the food quality, which is defined as its ability to support growth, and visibly prefer high quality food. Worms switch between two modes of movement when seeking for good food—dwelling and roaming. Dwelling is low-speed movement with frequent stops and backing up in the reverse direction in a restricted area. Roaming is rapid straight movement, which is common on bad quality food as the worm continues to search for better food$^{14}$. 
A third behavioral state in worms, called satiety quiescence, is the cessation of all feeding and movement. The development of quiescence seems to resemble the sequence of mammalian satiety behaviors and is a sleep-like state that results from being satiated. It depends on food quality, requires nutritional signals from the intestine, and is affected by previous feeding history; for example, fasting before refeeding enhances quiescence.

1.5 Neuronal and molecular regulation of satiety in C. elegans

Although not all details are known, it is clear that a simple neuronal circuit consisting of a cGMP (cyclic guanylyl monophosphate) molecular pathway and a TGF-β molecular pathway is involved to signal postprandial satiety. ASI, a pair of amphid neurons in the head of C. elegans, responds to nutritional cues in the environment and is part of the neural circuit that ultimately signals postprandial satiety. Worms lacking ASI do not show satiety quiescence and spend more time roaming in search of food. ASI also regulates the worm’s developmental choices depending on the worm’s nutritional environment; under an adverse condition such as starvation, C. elegans larvae enter a dormant developmental stage called the dauer larva to prolong its life span. Without ASI, the worm frequently enters dauer formation even in a food-rich environment, suggesting that the ASI is important for sensing nutrients.

Satiety quiescence requires intact cGMP signaling in ASI. cGMP is an important second messenger in a variety of mammalian cell signaling processes, such as blood pressure, reproduction, phototransduction in the eyes, circadian rhythms.
and intestinal homeostasis\textsuperscript{7}, and body size regulation and exploratory behavior in invertebrates\textsuperscript{23}. This cGMP pathway is also conserved in mammalian appetite control systems\textsuperscript{24}. The \textit{C. elegans} genome contains 34 guanylate cyclases (GCY), including 27 membrane-bound and 7 soluble GCYs. The soluble GCYs are expressed in sensory neurons and not required for satiety quiescence (personal communication, Young-Jai You). Of the 27 membrane-bound GCYs, 3 of them are known to be important for satiety quiescence: DAF-11, GCY-18, and GCY-28. Previous data show that a worm goes into satiety quiescence, possibly by ASI activation when food is replaced with 8-Br-cGMP (a membrane-permeant form of cGMP), even in the absence of food. Furthermore, the addition of 8-Br-cGMP brings a worm out of its dauer phase and rescues satiety quiescence in animals deficient in certain GCYs. This suggests that a GCY may be directly involved in receiving nutrient signals from the worm’s environment, or at least act downstream of the nutrient signal. Further investigation is required in order to validate the role of these GCYs and cGMP in the activation of ASI.

Suspected to act downstream of cGMP is a Transforming Growth Factor-β (TGF-β) superfamily member called DAF-7. TGF-β signaling has recently been implicated in mammalian weight\textsuperscript{25}, appetite\textsuperscript{26}, and fat mobilization\textsuperscript{27}, and has been extensively studied in other systems such as cell proliferation, differentiation, and tumor formation in mammals\textsuperscript{28}. Once ASI is activated, DAF-7 is expressed in ASI and is released\textsuperscript{10,29}. It was also shown that \textit{daf-7} expression in ASI is dependent on DAF-11\textsuperscript{30}, suggesting cGMP acts upstream of DAF-7 expression. Worms lacking DAF-7 show a decrease in satiety quiescence, similar to ASI-ablated worms. Additionally, upon treatment with 8-Br-cGMP
rather than food, *daf-7* expression is enhanced, suggesting further that cGMP release follows ASI receiving the nutrition signal which is then followed by *daf-7* expression. The mechanistic details between the formation of cGMP and *daf-7* expression are not entirely known, but it is suspected that cGMP signals through a combination of cyclic nucleotide gated channels (CNGs), such as that formed by TAX-2 and TAX-4 subunits, and cGMP-dependent protein kinases (PKGs), namely EGL-4, to upregulate the expression of *daf-7* 

Intracellular cGMP would be degraded by phosphodiesterases (PDEs) to turn off the signal after cGMP action is completed. The *C. elegans* genome encodes six different PDEs, PDE-1 through 6, and each is known to have a closely related human homolog. PDE-1, 2, 3, and 5 cleave cGMP, while PDE-4 and PDE-6 are specific for cAMP. It is unclear whether and how the inhibition of PDEs and therefore an increase in intracellular cGMP levels would affect satiety.

RIM is a pair of motor neurons and RIC is a pair of interneurons also in the head of the worm that have been shown to be involved in hunger. Following expression, DAF-7 is released from ASI and binds with TGF-β receptor DAF-1 on RIM and RIC to inhibit the action of RIM and RIC neurons. This inhibition of RIM and RIC then may subsequently allow the worm to go into satiety quiescence. When RIM and RIC were ablated in *daf-7* mutants, the quiescence defect of *daf-7* mutants in satiety was rescued, suggesting that RIM and RIC act downstream of DAF-7 to antagonize DAF-7 function.
1.6 Experimental approach and hypothesis:

The molecular mechanisms by which cGMP signaling regulates appetite are still unclear. In this project, we focused on the cGMP signaling pathway and its role in ASI activation that eventually leads to satiety quiescence. In order to examine the function and importance of cGMP signaling in ASI in controlling feeding and satiety behaviors, we used sildenafil (Viagra), a commercially available PDE inhibitor, to increase intracellular level of cGMP. Sildenafil occupies the same Q pocket that cGMP binds to human PDE-5. There is also high sequence homology between the human PDE-5 Q pocket and that of C. elegans PDEs that bind cGMP (personal communication, Young-Jai You), suggesting that sildenafil will also competitively inhibit C. elegans PDEs. We predicted that through the competitive inhibition of PDEs by sildenafil and blocking the degradation of cGMP, levels of cGMP in ASI would potentially be increased and ASI would be activated, subsequently increasing satiety quiescence, decreasing food intake, and consequently decreasing worm fat stores.

DAF-11 is one of the transmembrane GCYs previously shown to be important for satiety quiescence and is known to be expressed in ASI, along with at least four other amphid neurons. ASI specific expression of DAF-11 in daf-11 mutants rescued the satiety quiescence defect of daf-11, showing that ASI is the action site of DAF-11 in satiety quiescence. In order to investigate whether a GCY is required for ASI activation, DAF-11 was chosen as the first candidate to test. It was hypothesized that ASI in daf-11 mutants would not activate even with the presentation of nutrient-rich stimulus such as
Luria broth (LB), because we predicted that DAF-11 acts downstream of the nutrient signal.
Figure 1. Proposed model of ASI activation with cGMP signaling and TGF-β signaling pathways

ASI senses a nutrient signal, possibly by a GCY. cGMP is formed which subsequently interacts with members of the TGF-β pathway, which inhibit RIM/RIC to stop the hunger signals and the worm can go into satiety quiescence. Once produced by a GCY, cGMP is degraded by PDEs.
Chapter 2 Materials and Methods

2.1 General methods and worm strains

Worms were grown and handled as described previously\textsuperscript{34}, except worms were maintained on NGMSR plates\textsuperscript{35}. All worms were maintained at 20°C on \textit{Escherichia coli} strain HB101 unless indicated otherwise. The wild-type strain was \textit{C. elegans} variant Bristol, strain N2. Mutant strains used were TQ1828 \textit{pde-1(nj57) pde-5(nj49)} \textit{I; pde-3(nj59)} \textit{II; pde-2(tm3098)} \textit{III}, RB2279 \textit{pde-5(ok3102)} \textit{I}, and \textit{daf-11(jt195)}.

For synchronizing L1 larvae, worms on stock plates were washed and bleached as described previously\textsuperscript{36}, then harvested eggs were rocked in M9 buffer overnight at 20°C. Newly hatched L1 larvae were transferred onto plates seeded with \textit{Escherichia coli} strain HB101.

2.2 Food intake assay

Food intake was measured as described previously\textsuperscript{10} but including steps to treat plates with sildenafil. One colony of \textit{E. coli} strain HB101 and one colony of \textit{E. coli} HB101 expressing fluorescent mCherry protein were separately inoculated in 50 ml LB each and grown overnight at 37°C, and then seeded on 60 mm NGMSR plates, which were then incubated overnight at 37°C. Plates were stored at room temperature for at least one night. The seeded 60 mm NGMSR plates were treated with 100 \textmu{l} of dimethyl sulfoxide.
(DMSO) for control worms or 100 μl of 400 μM sildenafil for experimental worms, so that DMSO was no more than 1% of agar volume and the final concentration of sildenafil is 20 μM. Treated plates are left to dry overnight at room temperature. L4 worms were picked to treated plates seeded with HB101, placed in a dark chamber that stays undisturbed on the countertop at 25°C and given 24 hours to develop into adults. At 24 hours, the worms are transferred to treated plates seeded with mCherry HB101 and are left undisturbed for 1 hour, then treated with 100 μl of 1M sodium azide to fix their feeding status. Worms were mounted on 3% agar pads on glass slides and observed with a Zeiss Axio A2 Imager microscope with a 10X objective lens. Images were acquired using Zeiss Axiovision software and fluorescence was quantified using ImageJ software. For food intake assays done in conjunction with locomotion assays, each worm was treated with 5 μl of 1 M sodium azide to fix its feeding status at the end of the assay and imaged.

2.3 Locomotion monitoring assay

Locomotion was monitored and recorded in non-fasted conditions as described previously, but including steps to treat plates with sildenafil and changing the volume of the mCherry bacteria suspension. 35 mm NGMSR plates were treated with 25 μl of DMSO and 25 μl of 400 μM sildenafil, and left to dry overnight at room temperature. The mCherry bacteria suspension was prepared as described previously. On the day of the locomotion assays, 3 μl of prepared mCherry bacteria suspension was pipetted onto a
treated 35 mm NGMSR plate and allowed to dry completely. Adult worms that were transferred to treated HB101 plates 24 hours prior are transferred singly to individual plates prepared for locomotion assays.

2.4 Oil-Red-O Staining

60 mm NGMSR plates were seeded with *E. coli* strain HB101 and treated with DMSO and sildenafil as described above for food intake assays. L4 worms were picked and transferred to seeded plates, placed in a dark chamber that stays undisturbed on the countertop at 25°C, and given 24 hours to develop into adults. Then the worms were washed and stained with Oil-Red-O stain as previously reported, except that worms were grown on the drug-treated plates and the freeze-thaw cycle steps were omitted. Worms were mounted on 3% agar pads on glass slides and observed with a Zeiss Axio A2 Imager microscope with a 10X objective lens. Images were acquired using Zeiss Axiovision software and the intensity of the red stain was quantified using ImageJ software.

2.5 Calcium imaging

60 mm NGMSR plates seeded with *E. coli* strain HB101 were treated with 600 µl of 20 mM 8-Br-cGMP (Sigma-Alrich) so that the final concentration of 8-Br-cGMP is 1 mM. Treated plates are left to dry to 1 hour. *daf-11* dauer worms with the GCaMP
transgene expressed in ASI were picked under fluorescence to sort transgenic worms, transferred to a 8-Br-cGMP treated plate (~20-30 worms per plate), and grown for 48 hours at 15°C. Calcium imaging experiments were done as previously reported\textsuperscript{15} with 1-day old adult \textit{daf-11} worms, using Luria broth (LB) as the stimulus.
Chapter 3 Results and Discussion

3.1 Sildenafil increases satiety quiescence

To examine how an increase of intracellular cGMP levels would affect satiety quiescence, a locomotion monitoring assay was done on wild-type worms on plates treated with DMSO and 20 µM sildenafil as described in Materials and Methods. The assay showed that sildenafil increased the time each worm spent in satiety quiescence, to almost double the time that worms spent in satiety quiescence in control conditions (Figure 2).

3.2 Sildenafil decreases fat storage

The increase of satiety quiescence by sildenafil treatment suggests that sildenafil reduces food intake, and subsequently fat storage. To test this idea, Oil-Red O staining was done on wild-type worms treated with DMSO and sildenafil for 24 hours as described in Materials and Methods. Stain intensity was analyzed with ImageJ. The average stain intensity of sildenafil-treated worms was significantly lower (p = 0.00007) than the DMSO-treated worms, indicating that sildenafil-treated worms had lower fat stores (Figure 3B).

In order to confirm sildenafil’s action to increase intracellular cGMP levels via the competitive inhibition of PDEs, Oil-Red O staining was also done using PDE
mutants. First, worms containing mutations in all PDEs specific for cGMP were used\textsuperscript{20}. Quadruple mutants of \textit{pde-5 pde-1; pde-3; pde-2} were treated under the same conditions as wild-type worms and the stain intensity was compared. There was no significant difference between the average stain intensities of DMSO- and sildenafil-treated mutants (Figure 4B). The experiment was repeated with \textit{pde-5} single mutant worms and again, there was no significant difference between the two groups (Figure 5B). These results show that sildenafil decreases fat storage by increasing intracellular cGMP levels in a PDE-dependent manner.

3.3 Sildenafil effect in food intake

Sildenafil decreases fat storage, which suggests that sildenafil reduces food intake. To test how an increase of intracellular cGMP levels would affect a worm’s food intake, the food intake of wild-type worms was measured after being treated on DMSO and sildenafil for 24 hours as described in Materials and Methods. The fluorescence intensity from \textit{E. coli} strain HB101 expressing mCherry protein was analyzed with ImageJ. The average fluorescence intensity between DMSO and sildenafil-treated worms was not significantly different (Figure 6B). However, this may be explained by various technical reasons and limitations of the food intake assay, as will be discussed in further detail in the Discussion section.
3.4 Guanylate cyclase DAF-11 is important for ASI activation

DAF-11 expressed in ASI is important for satiety quiescence\textsuperscript{10}. To directly test whether DAF-11 is necessary for ASI activation, ASI activity of \textit{daf-11} mutant worms was recorded via calcium imaging as described in Materials and Methods. The individual traces were normalized (before the presentation of stimulus) so that the mean for the first 15 s is 1, and then averaged. ASI in \textit{daf-11} mutant worms did show activation, but at a much lower overall fluorescence intensity compared to wild-type worms (Figures 7A and 7B) and with ASI activation at about a 45\% frequency compared to 100\% frequency in wild-type worms (Figure 7C). This suggests that though DAF-11 is clearly important for full ASI activation, its functional role in activation is still unknown.
Figure 2. Sildenafil increases satiety quiescence

Wild-type worms were treated with DMSO and sildenafil starting from L4 stage. After 24 hours of treatment, their locomotion was recorded using the motion tracking system as described in Materials and Methods. Treatment with sildenafil significantly increased satiety quiescence. Control: n = 15, sildenafil: n = 28.
The diagram compares the percentage of time spent in different activities between Control and Sildenafil conditions. The activities are categorized as Roaming, Dwelling, and Quiescence. The chart illustrates that Sildenafil condition has a higher percentage of time spent in Dwelling compared to the Control condition.
Figure 3. Sildenafil decreases fat storage in wild-type worms

A) Wild-type worms were treated with DMSO and sildenafil starting from L4 stage.
After 24 hours of treatment, worms were washed from the plate, stained with Oil-red O stain, and imaged. B) The stain intensities of the images were measured and averaged.

Treatment with sildenafil significantly decreased fat storage (p = 0.000072).
Control: n = 20, sildenafil: n = 20.
A. Control

B. Sildenafil

**ORO (arbitrary units)**

![Graph showing ORO (arbitrary units) for Control and Sildenafil]
**Figure 4. Sildenafil has no effect on fat storage in pde-5 pde-1; pde-3; pde-2 mutants**

A) Quadruple pde mutant worms (pde-5 pde-1; pde-3; pde-2) were treated with DMSO and sildenafil starting from L4 stage. After 24 hours of treatment, worms were washed from the plate, stained with Oil-red O stain, and imaged. B) The stain intensities of the images were measured and averaged. Treatment with sildenafil had no effect on fat storage (p = 0.298783). Control: n = 40, sildenafil: n = 40.
A. Control Sildenafil

B. ORO (arbitrary units)

Control Sildenafil
Figure 5. Sildenafil has no effect on fat storage in *pde-5* mutants

A) *pde-5* mutant worms were treated with DMSO and sildenafil starting from L4 stage. After 24 hours of treatment, worms were washed from the plate, stained with Oil-red O stain, and imaged. B) The stain intensities of the images were measured and averaged. Treatment with sildenafil had no effect on fat storage (*p* = 0.323411). Control: *n* = 25, sildenafil: *n* = 25.
A.

Control

Sildenafil

B.

<table>
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<table>
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**Figure 6. Sildenafil effect on food intake**

A) Wild-type worms were treated with DMSO and sildenafil starting from L4 stage. After 24 hours of treatment, their food intake was fixed with sodium azide and the worms were imaged. B) The fluorescence intensities of the images were measured and averaged. There was no significant difference between the control and sildenafil groups.

Control: n = 25, sildenafil: n = 25.
A. Control  

Sildenafil

B. Fluorescence (arbitrary units)
Figure 7. Decreased intensity and frequency of ASI activation in *daf-11* mutants

*daf-11* dauers were treated with 8-Br-cGMP for 48 hours to bring them out of the dauer stage and transferred off 8-Br-cGMP to be grown to adults. Worms were loaded into microfluidic devices and ASI activation was recorded via calcium imaging as described in Materials and Methods, using LB as the stimulus. A) ASI activation in control worms (n= 9), B) ASI activation in *daf-11* mutants (n = 9), and C) the frequency of ASI activation in control worms and *daf-11* mutants (control n = 9, *daf-11* n = 9).
A. 

B. 

C. 

Frequency of ASI Activation

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<th>Percentage</th>
<th>wild-type</th>
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3.5 Discussion

Appetite control and satiety mechanisms are crucial for an animal to maintain healthy energy homeostasis. cGMP signaling has been previously shown to be involved in *C. elegans* satiety and its role was further confirmed with the progression of this project. In support of the hypothesis, treatment with sildenafil did increase satiety quiescence and decreased fat storage, possibly by increasing intracellular cGMP levels through its inhibition of PDEs. However, it is not known whether sildenafil acts solely in ASI, which can be addressed by targeting the expression of PDE-5 only in ASI and testing whether this will rescue the sildenafil effect in fat storage. Also, if sildenafil is increasing satiety by its inhibition of PDEs, then it is expected for the fat content of the PDE mutants to look similar to the lower fat content shown in sildenafil-treated wild-type worms. However, the fat content of the PDE mutants resembled the higher fat content of the control wild-type worms. The reasons for this discrepancy are unclear, but one possible explanation could be the differences in the length of time that the worms do not have PDE, as the PDE mutants do not have PDEs their entire lifetime and could have adapted to this condition, whereas the PDEs of sildenafil-treated wild-type worms are inhibited for only 24 hours. Another explanation could be variations in staining between the control and the PDE mutant experiments. Possible future experiments to resolve this discrepancy are described below in the Future Directions section.

Though the food intake assays did not show any significant differences in food intake between control and sildenafil-treated worms, sildenafil-treated worms showed
decreased fat storage. This may be due to unknown side effects of the drug or it could possibly be from decreasing food intake, which may not have been captured properly due to technical limitations of the assay. The assay is only a snapshot of fluorescence from the ground bacteria in the worm gut at the time of fixation with sodium azide, which is not a direct reflection of cumulative food intake or frequency of pharynx pumping (=feeding rate). It also does not account for any defecation cycles that may have changed, so fluorescent protein may be retained in the gut longer. This is why the staining of the worm's fat stores can be considered to be a better reflection of the long-term effects of sildenafil treatment on energy balance.

cGMP signaling seems to be downstream of ASI receiving a nutrient signal because exogenous 8-Br-cGMP mimics the effect of food. Also, 3 GCYs were found to be important for satiety quiescence, including DAF-11. For this reason, it was hypothesized that a GCY is involved in detecting the nutritional signal in the ASI, and mutating DAF-11 would abolish ASI response to LB. Although the daf-11 mutation did not fully eliminate ASI activation, it did decrease the overall fluorescence intensity and the frequency at which ASI activated by about 50% compared to wild-type worms. This indicates that DAF-11 does have a significant role in ASI activation, but the degree of its importance is not yet clear. It is possible that some biological redundancy exists for daf-11 as there are other GCYs that are important for satiety quiescence. There could also be redundancy at the neural circuit level; ASI is connected to the neuron AIA by gap junctions, which could also receive nutritional signals from other neurons and be
involved in activation of ASI. It is possible that in *daf-11* mutants, AIA can be activated by nutrients, and that signal indirectly activates ASI less reliably and to a lesser degree.
Chapter 4 Conclusions and Future Directions

4.1 Conclusions

From the results of this project, it can be concluded that a cGMP signaling pathway is involved in ASI activation and satiety quiescence. Sildenafil increases satiety quiescence and decreases worm fat stores in a PDE-dependent manner, potentially by increasing intracellular cGMP levels through its competitive inhibition of PDEs. Finally, DAF-11 seems to have an important role in mediating ASI activation, though the exact function or the extent of its role in ASI is still unclear. It will be interesting to continue to illuminate more details about the cGMP signaling pathway in ASI in the endeavor to construct a more holistic understanding of appetite control and satiety.

4.2 Future Directions

In order to eliminate questions about whether the actions of sildenafil actually increase intracellular levels of cGMP or when it might increase in the course of ASI activation, it would be extremely valuable to be able to have a better reflection of intracellular cGMP levels of ASI. cGi500, a fluorescence-resonance-energy-transfer (FRET) based cGMP indicator will be introduced as a transgene into worm ASI using a gpa-4 promoter in various worm mutants and cGMP levels will be measured in different given conditions. Furthermore, rescue of the sildenafil phenotype of pde-5 mutants by
expressing pde-5 only in ASI using the gpa-4 promoter would confirm that the sildenafil effect we found is through ASI.

To provide a better comparison between the ORO staining of wild-type worms and PDE mutants, wild-type worms and all PDE mutants will be treated with sildenafil and stained in one experiment to eliminate the possibility of variation in experimental conditions or staining. Furthermore, to more accurately mimic the intracellular conditions of PDE mutants, which do not have PDEs their entire lifetime, worms will be treated with sildenafil for their entire lifetime rather than only for 24 hours.

To further elucidate the role of DAF-11 in ASI activation, there are several additional experiments that will be conducted. daf-11 mutants will be crossed with an integrated line of wild-type worms carrying the transgene to express GCaMP in ASI then homozygosed to reduce any variations coming from the copy number variation from the additional GCaMP transgene. In addition, to confirm that the 50% of reduction in ASI activation is due to the daf-11 mutation failing to produce cGMP, daf-11 mutants will be treated with 8-Br-cGMP and the ASI activation will be measured. If 8-Br-cGMP could activate ASI of daf-11 mutants more reliably than LB, it would show that the 50% of reduction in ASI activation in daf-11 mutants by LB is indeed due to the daf-11 function in nutrient sensing. Short-term treatment with RNAi for DAF-11 in wild-type worms will also be used to eliminate the possibility of overall worm adaptation to the daf-11 mutation. Also it is unclear whether the daf-11 mutation is a complete knockout in its function, so treatment with RNAi for DAF-11 of daf-11 mutants will be useful to do in order to completely block the formation of DAF-11. Finally, the origin of the intracellular
calcium will be traced in the ASI and neighboring neuron AIA, which connects to the ASI via gap junctions. If calcium originates from the neuron processes, then a different neuron may be relaying signals to ASI in order for it to activate. If calcium is originated from the cell body rather than the cell processes, ASI might be activated by action of AIA. It would be enlightening to see whether AIA has a role in ASI activation and may contribute to the biological redundancy of DAF-11, which could be why only mutating DAF-11 might not completely abolish ASI activation.
Literature Cited


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