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Studies of Norspermidine Uptake in Drosophila Suggest the Existence of Multiple Polyamine Transport Pathways

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STUDIES OF NORSPERMIDINE UPTAKE IN *DROSOPHILA* SUGGEST THE EXISTENCE OF MULTIPLE POLYAMINE TRANSPORT PATHWAYS

by

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ABSTRACT

Polyamines are a class of essential nutrients involved in many basic cellular processes such as gene expression, cell proliferation, and apoptosis. Without polyamines, cell growth is delayed or halted. Cancerous cells require an abundance of polyamines through a combination of synthesis and transport from the extracellular environment. An FDA-approved drug, D,L-α-difluoromethylornithine (DFMO), blocks polyamine synthesis but is ineffective at inhibiting cell growth due to polyamine transport. Thus, there is a need to develop drugs that inhibit polyamine transport to use in combination with DFMO. Surprisingly, little is known about the polyamine transport system in humans and other eukaryotes. Understanding the transport system would allow us to identify compounds that inhibit polyamine transport, which could then be used in tandem with DFMO to treat cancer. Our laboratory has identified one gene in *Drosophila*, called CG32000, as a component of this transport system, and numerous other candidate genes remain to be tested.

To better characterize this system, this project investigated the ability of the *Drosophila* transport system to take up a toxic polyamine analogue called norspermidine, with the initial goal of developing a new screening method to find polyamine transport genes. My experiments have demonstrated significant differences in norspermidine uptake and toxicity between *C. elegans* and *Drosophila* which may imply a secondary polyamine transport system in higher eukaryotes. In the long term, it is hoped that this thesis will facilitate the development of more effective cancer medications by providing new information about the polyamine transport system.

DEDICATION

To my incredibly supportive and patient wife, Arletta. Thank you for your constant encouragement and for putting up with many long hours and late nights.

ACKNOWLEDGMENTS

I would like to thank the many people who made this thesis possible: Dr. Laurence von Kalm for his guidance and mentorship throughout the process; Dr. Ken Teter and Dr. Claudia Andl for their patience and helpful comments as members of my thesis committee; Michael Haney, Corey Seavey, and David Brown for their training, ideas, suggestions, and assistance with experiments; and my friends and family for their support and encouragement. I've learned so much during my time in the lab, and it's all thanks to all of you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Physiological Roles of Polyamines:

Polyamines are a class of crucial nutrients that are involved in many cell processes such as gene expression and cell growth and cell death (Childs, 2003). Common polyamines include putrescine, spermine, and spermidine. In mammals, spermine and spermidine are present in much higher levels than putrescine, from which they are synthesized (Pegg, 2009). At physiological pH, polyamines are polycationic and therefore able to interact with negatively charged regions of macromolecules such as DNA and RNA. Because of these interactions, polyamines have been implicated in the synthesis, stability, and transcription of DNA, as well as ion channel regulation and phosphorylation of proteins (Hochman, 1978; Tabib, 1994; Panagiotidis, 1995; Childs, 2003). On a broader level, studies suggest that polyamines play a role in longevity and stress response (Eisenberg, 2009; Rhee, 2007; Minois, 2011). Polyamines are present in all cells and levels of polyamines are carefully regulated, indicating their universal importance (Russell, 1983). Regulation of polyamine levels is accomplished through synthesis, transport, and catabolism.

Regulation of Polyamine Levels:

The polyamine synthesis pathway begins with arginine and methionine (Figure 1). Arginase converts arginine to ornithine, which is then converted to putrescine by ornithine decarboxylase (ODC) (Hochman, 1978; Tabib, 1994; Panagiotidis, 1995; Childs, 2003). Methionine is converted to S-adenosyl-L-methionine, decarboxylated, and then added to putrescine to produce spermidine or to spermidine to produce spermine. The pathway can also be reversed to produce putrescine

from spermine or spermidine. The conversion of ornithine to putrescine by ODC is a crucial step in this pathway. Transcription of the ODC gene is tightly regulated and the ODC protein is post-translationally regulated. The ODC antizyme inhibits ODC and facilitates its degradation (Minois, 2011). These mechanisms ensure that cells maintain proper levels of polyamines.

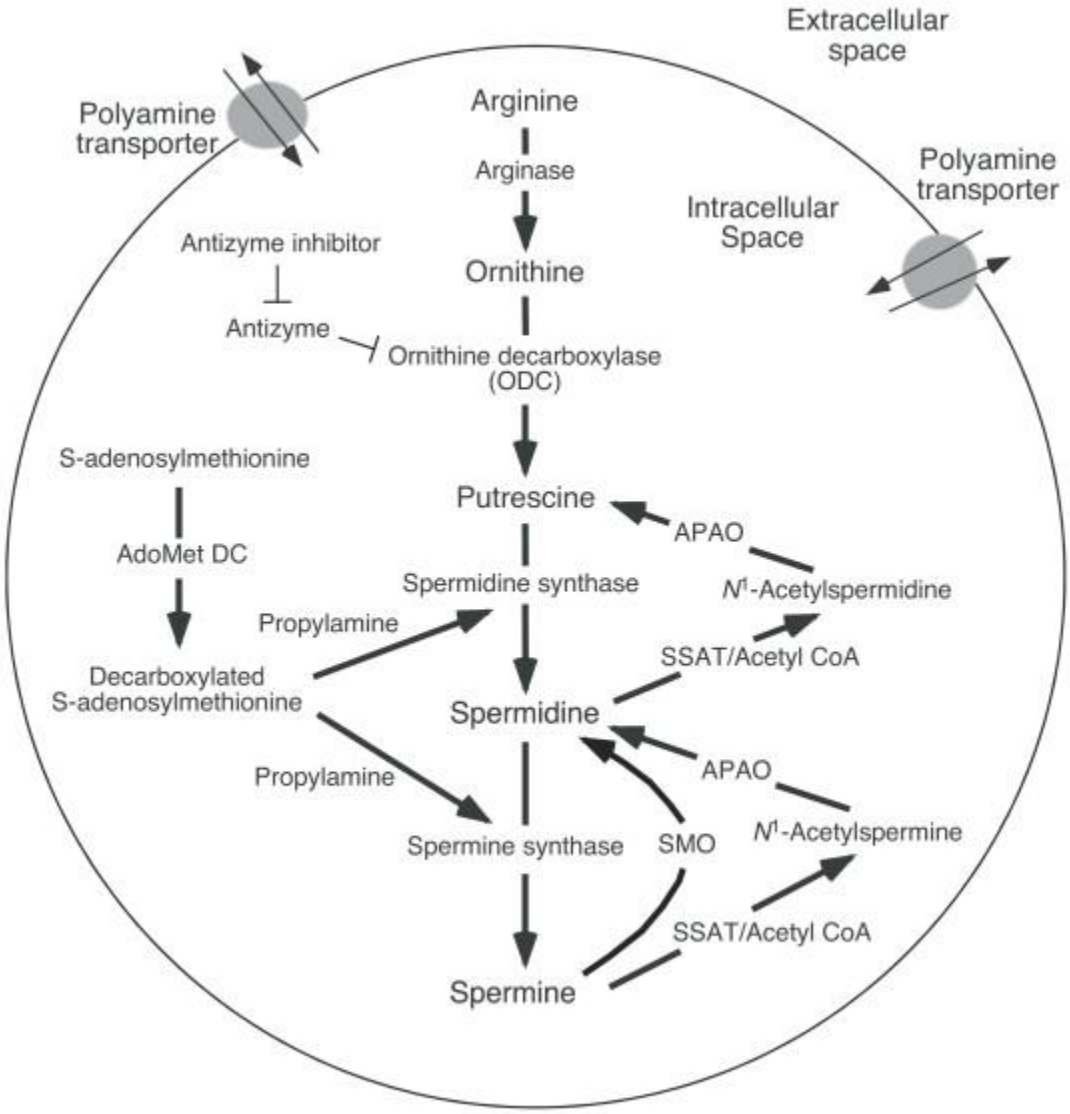

Figure 1: Regulation of cellular polyamine levels. Cells maintain the necessary levels of polyamines through synthesis from arginine and S-adenosylmethionine and through polyamine transport. (Soda, 2011).

Polyamines and Disease:

Polyamine metabolism is important in several human pathologies. Leishmaniasis is a neglected tropical disease caused by the *Leishmania* parasite, and an estimated 2 million cases occur annually, often resulting in lasting disability (Desjeux, 2001). Levels of putrescine, spermidine, and spermine fluctuate during the cell cycle of the *Leishmania* parasite and peak during logarithmic growth, indicating the importance of polyamines in their development (Bachrach, 1979). The malarial parasite also depends on high levels of polyamine biosynthesis for development and synthesis of proteins and amino acids, because its human erythrocyte host cells lack the enzymes needed to synthesize polyamines (Assaraf, 1984). The protozoan *T. cruzi* is the causative agent for Chagas' disease, a neglected tropical disease that affects 8 million people worldwide and for which few effective drug treatments are available (Bern, 2011). Unlike other protozoans, *T. cruzi* depends solely on polyamine transport to obtain polyamines and will die if its supply is lost, making the polyamine transport system in *T. cruzi* a promising target for new drugs (Reigada, 2017). Multi-drug resistant Mycobacterium tuberculosis is yet another emerging world health concern, and a 2013 study suggests that polyamines may be a contributing factor (Sarathy, 2013). Beyond pathogenic diseases, polyamines have been linked to inflammatory diseases, liver disease, stroke, Alzheimer and Huntington's disease (Hussain, 2017; Minois, 2011).

Polyamines and Cancer:

Polyamines play a significant role in cancer. Because of their high rate of growth, cancer cells require high levels of polyamines (Luk, 1987). This is reflected by increased levels of blood and urine polyamines in cancer patients. Blood and urine polyamine levels return to normal levels once the cancer has been completely eradicated (Loser, 1990; Chatel, 1987; Kubota, 1985; Uehara, 1980). It has further been noted that higher polyamine levels in the blood and urine correspond with more severe prognoses (Durie, 1977). An increased capacity to synthesize polyamines correlates with increased production of proteinases to degrade tissues around a tumor resulting in higher levels of extracellular polyamines. Immune cells in the high polyamine environment of the tumor show reduced anti-tumor function which may be related to high levels of polyamines (Soda, 2011). Thus, blocking the supply of polyamines to cancer cells presents a promising therapeutic strategy.

An existing polyamine synthesis-blocking, FDA-approved drug called difluoromethylornithine (DFMO) has shown promise in treating cancer, especially pancreatic cancer (Mohammed, 2014). This is noteworthy because pancreatic cancer was the third leading cause of cancer deaths in the United States in 2016 (Siegel, 2016). Pancreatic cancer is especially dangerous because physical symptoms – such as weight loss and fatigue – are not specific to the disease, which complicates diagnosis and delays treatment. When the cancer is finally diagnosed, few effective treatments are available.

Polyamine Transport:

While DFMO is a promising treatment option, it is limited as a chemotherapeutic agent because malignant cells are also able to transport polyamines from their environment. The polyamine transport system (PTS) is understood in *E.coli* (Kashiwagi, 1995), yeast (Uemura, 2005), and some unicellular protozoans (Marie-Pierre, 2005; Machius, 2007). In these organisms, polyamine transport is controlled by a complex of proteins: for example, in E. coli the proteins potB and -C form a channel while potA and -D coordinate with them to control spermidine uptake (Kashiwagi, 1995). However, only a few scattered components of the polyamine transport system in multicellular animals have been identified (Belting, 2003; Uemura, 2008; Heinick, 2010; Hiasa, 2014), and the overall mechanism is not yet understood. Currently, there are three principle proposed models of polyamine transport, as discussed in a review by Poulin et al (Poulin, 2012): glypican-1-dependent endocytosis (Belting, 2003), caveolin-mediated endocytosis utilizing an unidentified receptor (Uemura, 2010), and entry via an energy-dependent channel or transporter followed by packaging into endosomes for storage (Soulet, 2004). The three models are not necessarily mutually exclusive, and polyamine transport may occur via combinations of these models. Therefore, elucidating the polyamine transport system in eukaryotes would facilitate the development of a transport inhibitor to be used in conjunction with DFMO.

As part of our effort to understand the PTS in *Drosophila*, our laboratory has identified a gene, CG32000, that is required for polyamine transport and which has a human orthologue, ATP13A3. CG32000 encodes a type $P5_B$-ATPase, a subfamily of proteins whose function was previously unknown. CG32000 encodes multiple proteins via alternative splicing and therefore may encode

a family of polyamine transporters. A closely related $P5_A$-ATPase encoded by CG6320 was not found to play a role in transport, indicating a high level of specificity and providing a useful control. Based on the complexes observed in *E. coli* and yeast, polyamine transport in higher organisms is also likely to involve a complex of proteins and therefore it is important to identify additional genes required for polyamine transport. Additionally, as our laboratory continues to investigate the role of CG32000, identifying its partners will facilitate our understanding.

The DFMO Assay:

Previous studies have indicated that the polyamine transport system in *Drosophila* is highly comparable to that of vertebrates, making *Drosophila* a good model for understanding the PTS (Tsen, 2008; Wang et al 2017). Our laboratory currently uses a DFMO based assay for screening candidate genes which involves knocking out the gene of interest in *Drosophila* using RNAi. To achieve this, fly strains are engineered so that one strain expresses the GAL4 transcription factor, a protein found originally in yeast, linked to a ubiquitously expressed promoter such as that for actin (Figure 2). This strain is crossed with another strain that contains an upstream activating sequence (UAS) that binds GAL4 upstream of a sequence that will produce double stranded RNA (dsRNA) complimentary to the gene of interest. When the GAL4 and the UAS sequences are present in the same animal, Gal4 activates transcription of the dsRNA, which is processed by Dicer and used to degrade RNA transcribed from the gene of interest. In this way, the functions of genes that may be otherwise lethal can be tested without maintaining a stock of mutant animals (Duffy, 2002). Once flies with the desired gene knockout have been bred, the offspring larvae are grown in the presence of DFMO and polyamines. DFMO disables their ability to synthesize polyamines,

forcing them to transport to survive. Because polyamines are added to the fly media, animals with a functional transport system can transport polyamines and survive in the presence of DFMO. If a crucial transport gene has been knocked out, the animals will be unable to transport and will die.

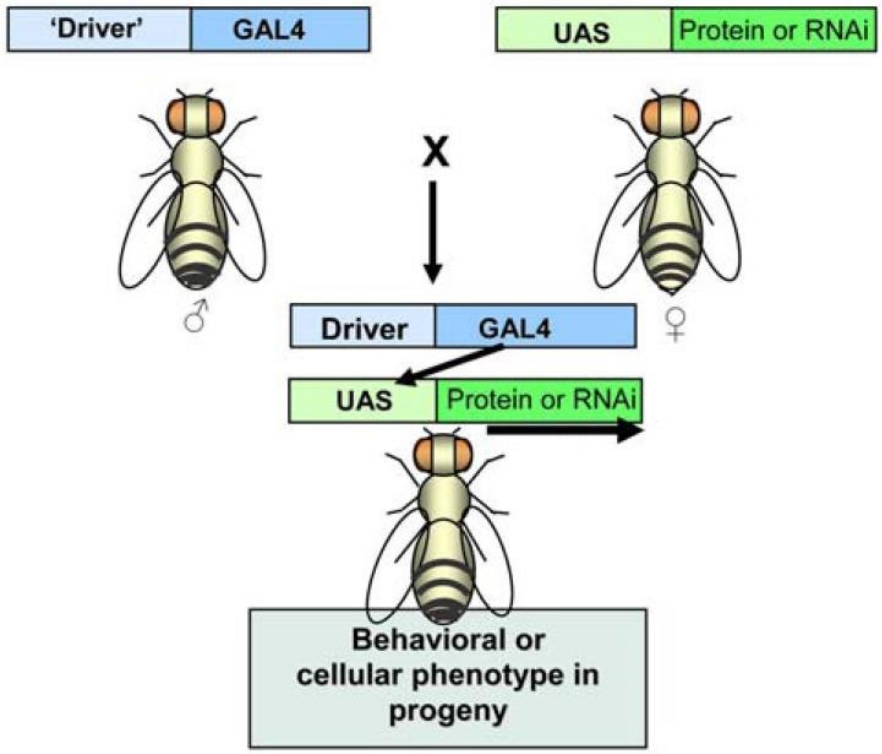

Figure 2: GAL4/UAS system in Drosophila. Flies expressing the GAL4 transcription factor are crossed with flies expressing an Upstream Activating Sequence (UAS) that binds GAL4 to allow transcription of the desired gene or dsRNA for RNAi. (Caldwell, 2013).

While the DFMO assay is useful, it has several limitations. Genes must be tested individually, and several treatment conditions must be set up for each gene because of the various controls used. Because the majority of the larvae will survive and must be counted at least daily over the course of 2-3 weeks, tracking the results of the assay is similarly labor-intensive.

An Alternative Approach:

Norspermidine, a toxic polyamine, presented a promising alternative screening method. Norspermidine is an analog of spermidine, differing by only one carbon atom. In *C. elegans*, it was used in an assay to find the polyamine transport gene CATP-5, an ortholog of *Drosophila* CG32000 (Heinick, 2010). In an assay similar to the DFMO assay described above, nematodes were exposed to norspermidine, and worms with a functional transport system did not survive. Mutants with a nonfunctional transport system, however, were able to survive when grown in the presence of lethal levels of norspermidine. The work demonstrated that CATP-5 was necessary for norspermidine uptake. As this discovery led to the identification of CG32000 as a polyamine transport system component in *Drosophila*, a norspermidine assay appeared to be a promising approach to identifying additional components. If a norspermidine assay could be replicated in *Drosophila*, it would only require two treatment conditions to test a gene (the lethal concentration of norspermidine and the control), and vials could be rapidly checked for survivors that would indicate a positive result. Additionally, multiple fly strains with different gene knockdowns could be tested in the same vials and either eliminated together or tested individually if survivors were observed. All other advantages aside, a norspermidine assay could simply provide a second line of evidence to confirm results from the DFMO assay.

In this study, I investigated the effect of norspermidine on *Drosophila* lines with functional and nonfunctional transport systems and determined the feasibility of developing a norspermidine assay in *Drosophila* to screen for genes associated with the polyamine transport system.

METHODS

Determining the lethal dose of norspermidine for *Drosophila*:

To investigate whether disrupting CG32000 conferred norspermidine resistance, the lethal concentration of norspermidine for wild-type OreR flies was first identified to provide a point of comparison.

200 female OreR flies and 50 males were allowed to breed and lay their eggs on an agar plate containing grape juice, which provided an ideal environment for larval growth and subsequent collection. The following protocol was used to produce these grape plates: 376 mL ddH2O and 126 mL grape juice were added to a 2 L flask along with a magnetic stir rod. The mixture was stirred until homogenous and 15 g agar and 6 g sucrose were then added. The flask was covered with aluminum foil and heated at 450 degrees C until boiling occurred, after which it was removed from the hot plate for 5 minutes. The flask was then placed again on the hot plate and stirred until the contents reached 60 degrees C. 10 mL of 100% ethanol and 5 mL glacial acetic acid were added, and the flask was stirred for an additional 3 minutes. The mixture was poured into Petri dishes and left overnight to set before being stored at 4 degrees C until used.

A small amount of yeast paste (made from instant yeast and water) was applied to each grape plate to provide food for the adult flies. Grape plates were replaced regularly to ensure that sufficient numbers of eggs would be obtained and cleared of larvae and yeast paste 4 hours before collection so that larvae would emerge synchronously. Larvae were collected at the first instar stage and placed into vials containing jazz mix medium (a standard fly food containing yeast, agar, cornmeal,

and sugar) obtained from Fisher Scientific and varying concentrations of norspermidine, with 50 larvae per vial.

Vials were prepared as follows:

$$\frac{226.8\ g\ jazz\ mix}{1050\ mL\ total\ food} \times (\#\ of\ vials) \times 6.0\ mL\ food\ per\ vial = amount\ of\ jazz\ mix\ needed$$

$$\frac{1200\ mL\ H2O}{1050\ mL\ total\ food} \times (\#\ of\ vials) \times 5.4\ mL\ food\ per\ vial = amount\ of\ water\ needed$$

Jazz mix and water were combined in a 500 mL Erlenmeyer flask along with a magnetic stir rod, covered with aluminum foil, and heated at 415 degrees C until boiling. The temperature was then reduced to 225 degrees C for 10 minutes before being pipetted into vials, with 5.4 mL per vial. Vials were then placed in a 55-degree C water bath for 10 minutes. 98% norspermidine (obtained from Sigma-Aldrich and stored at 20 degrees C) was then added in the appropriate concentrations and each vial was mixed thoroughly. Vials were allowed to cool at room temperature for at least 30 minutes and any condensation was removed before larvae were added.

<u>Strains used in norspermidine sensitivity experiments:</u>

OreR: This strain is considered to be wild-type.

CG6230: CG6230 encodes a highly similar protein to CG32000 that is not transport-related. RNAi, together with the Gal4 system, was used to produce a ubiquitous targeted gene knockdown.

CG32000 B exon: This strain has a 2 kilobase mobile element insertion in the B exon of CG32000. To verify that the insertion disrupted CG32000 activity and prevented polyamine transport, larvae

were exposed to DFMO and polyamines in the following combinations and concentrations (Table 1):

Table 1: Drug concentrations for DFMO assay.

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Contents:	ddH2O	5 Mm DFMO	0.4 mM Putrescine	0.4 mM Putrescine 5 mM DFMO	0.2 mM Spermidine	0.2 mM Spermidine 5 mM DFMO

CG32000 RNAi: A strain expressing RNAi against CG32000 was also tested. The experimental design was the same as for the CG32000 B exon.

Determination of Developmental Profiles:

OreR larvae were exposed to 0, 5, 10, 15, 20, 25, and 30 mM norspermidine and adult flies were counted daily to determine the wild-type developmental profile under each condition. B exon larvae were then exposed to the same concentration and their developmental profile was similarly observed.

RESULTS

Determination of a Lethal Concentration of Norspermidine to *Drosophila:*

In *C. elegans* the lethal concentration of norspermidine is 3 mM (Heinick, 2010). I therefore grew wild-type *Drosophila* OreR strain larvae in media containing 0, 1, 2, 3, 4, and 5 mM norspermidine. Survival rates at these concentrations of norspermidine are shown in Figure 3. Surprisingly, statistical analysis (1-way ANOVA) revealed no differences in larval survival within this range of concentrations with 100 percent viability observed in each case.

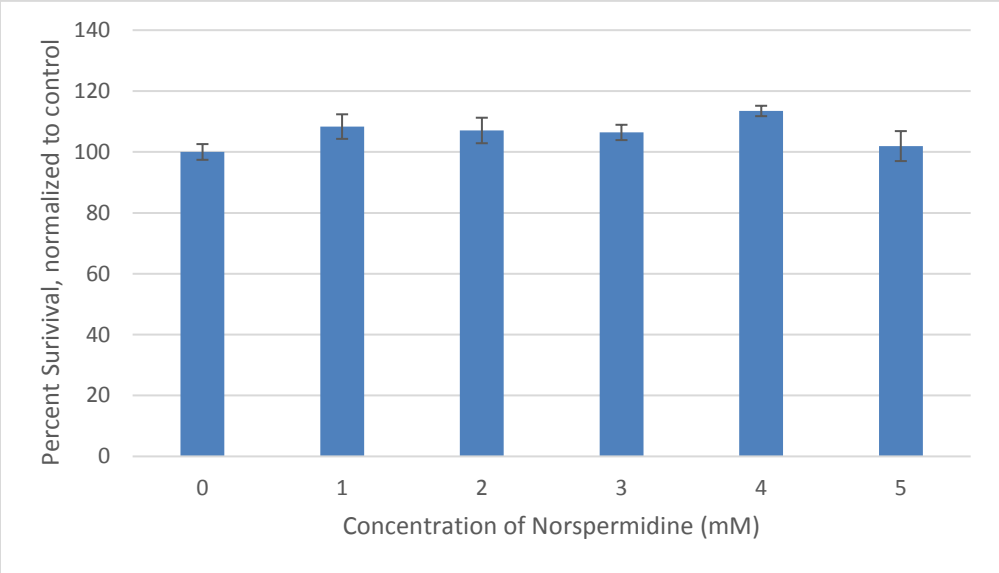

Figure 3:Percent survival of OreR larvae in 0-5 mM norspermidine. Each concentration was tested in triplicate. Error bars reflect standard error. Data were normalized with the 0 mM control set as 100%.

Based on these results I tested norspermidine at higher concentrations up to 30mM (Figure 4). Statistical analysis (1-way ANOVA) revealed significantly reduced viability compared to the 0

mM control at concentrations around 10 mM and higher. Complete lethality was observed at 30 mM.

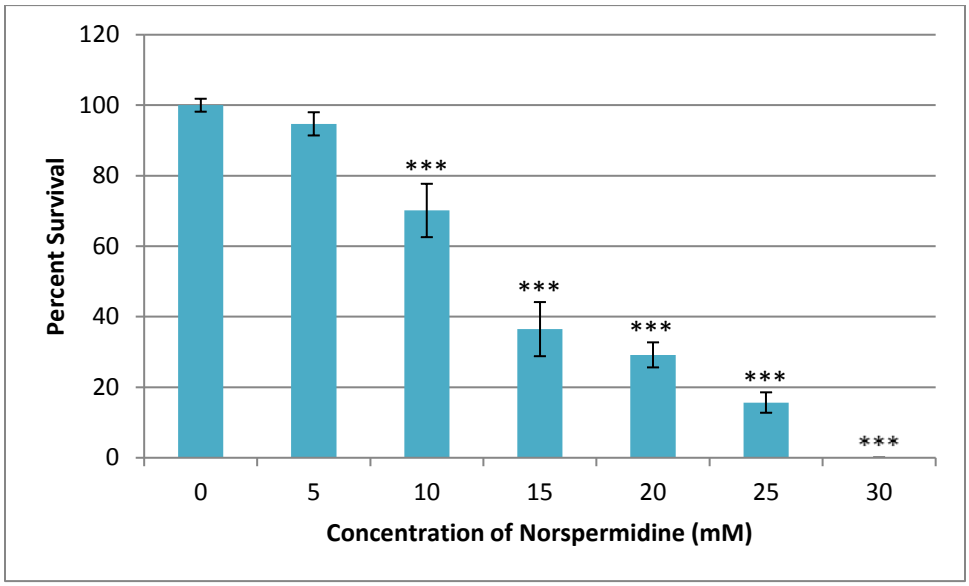

Figure 4:Percent survival of OreR larvae in 0-30 mM norspermidine. All concentrations were tested in triplicate. Error bars reflect standard error. *Data were normalized with the 0 mM control set as 100%. P<0.001 for all concentrations above 5 mM.*

Knockdown of the Polyamine Transport Gene CG32000 Does Not Prevent Uptake of Norspermidine.

Having established a lethal concentration of norspermidine I next asked if norspermidine utilizes the PTS to gain entry into cells. To test this hypothesis, I asked if norspermidine uptake was inhibited by RNAi knockdown or mutation of CG32000. Our laboratory has shown that RNAi against CG32000 blocks polyamine transport (Barnett and von Kalm; unpublished). The CG32000 mutation tested is a mobile element (Piggy Bac) insertion in the 5'UTR of the B exon, and in

homozygous condition this mutation also blocks polyamine transport. As controls I also tested strain OreR and RNAi against CG6230 which encodes a related P type-5A ATPase which is not required for transport (Barnett and von Kalm; unpublished). Results from these experiments are shown in figure 5. A 1-way ANOVA revealed no statistically significant differences between the genotypes tested. No survivors were observed in OreR, CG6230 and CG32000 B exon mutants grown in 30 mM norspermidine. A few animals (5/200) survived after RNAi knockdown of CG32000. These data suggest that norspermidine uses an alternative transport system from CG32000 to gain entry to cells.

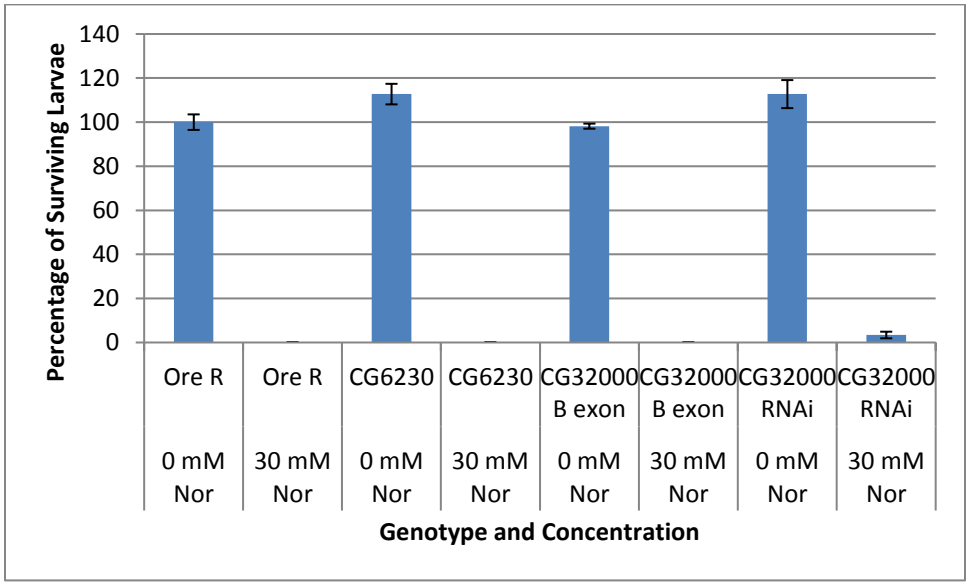

Figure 5:RNAi knockdown and mutation of CG32000 does not block uptake of norspermidine. Percent survival of OreR, CG6230 RNAi, CG32000 B exon mutants, and CG32000 RNAi larvae in 0 and 30 mM norspermidine. All experiments were performed in triplicate and error bars reflect standard error. Data was normalized with the 0 mM OreR control set as 100%.

To ensure that the B exon and RNAi CG32000 strains were genetically intact I asked if these strains were unable to transport polyamines in the presence of a lethal concentration of DFMO.

DFMO blocks polyamine synthesis and forces larvae to rely on transport of exogenous polyamines to survive. If the larvae have a functional transport system, the presence of polyamines (putrescine and spermidine) will provide rescue. Otherwise, larvae will not survive. Data for the B exon mutant are shown in figure 6 (see Methods for experimental details). Lethality caused by 5 mM DFMO could not be rescued by exogenous putrescine or spermidine indicating that the PTS was non-functional in these animals. Similar results were observed for RNAi against CG32000 (data not shown).

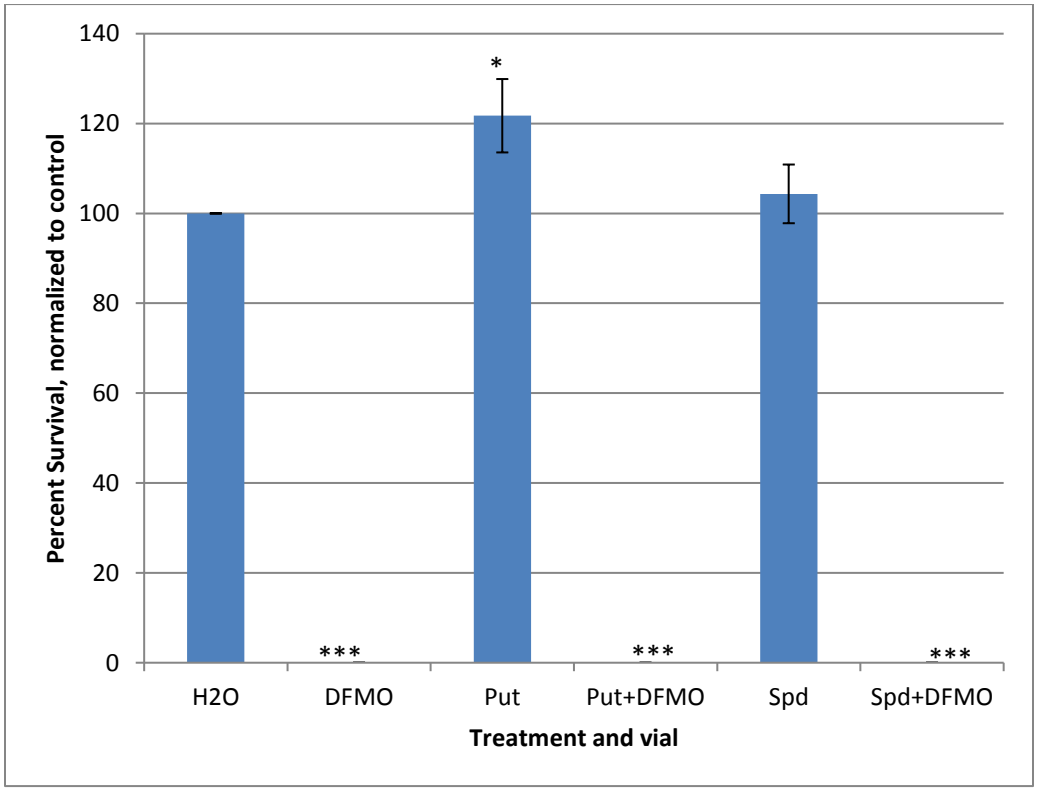

Figure 6:Testing of B exon stocks. Exogeneous polyamines fail to rescue B exon CG32000 larvae grown in a lethal concentration of DFMO. A 1-way ANOVA revealed that changes in survival between the control and polyamine + DFMO conditions is statistically significant (P<0.001). All experiments were performed in triplicate and error bars reflect standard error. Data were normalized with the 0 mM OreR control set as 100%.

Developmental Profiles of OreR and B exon CG32000 mutants in the presence of norspermidine.

The surprising finding that norspermidine did not utilize the PTS involving CG32000 led me to ask if animals exhibited abnormal developmental profiles. If animals grown on norspermidine were severely developmentally delayed, then the absence of survivors for B exon and RNAi CG32000 in Figure 5 might reflect this rather than the use of an alternative transport system by norspermidine. Developmental data for OreR and the B exon mutant are shown in Figures 7 and 8 respectively.

For OreR, animals grown on all concentrations of norspermidine except 30 mM, which was lethal, began to emerge within 2 days of control 0 mM norspermidine animals. In addition, peak eclosion numbers (the point at which no more flies emerged) occurred at approximately the same time for all concentrations of norspermidine tested. For B exon mutants concentrations above 20 mM were lethal, however initial eclosion and peak eclosion times were similar for all concentrations tested. Thus, severe developmental delay does not seem to be a viable alternative explanation for my observations.

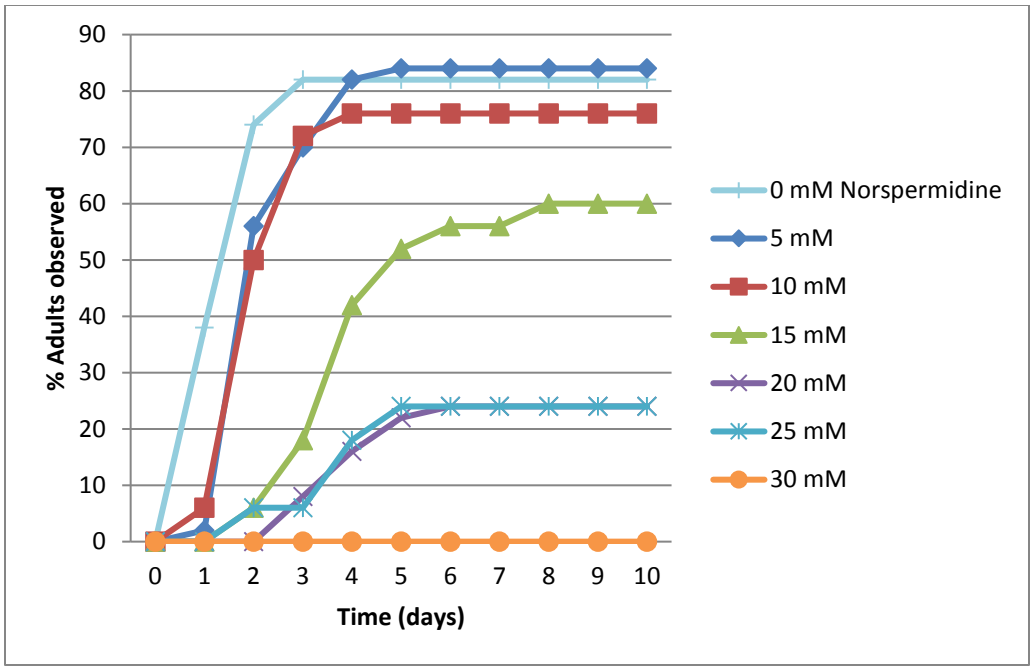

Figure 7:Developmental profile of OreR during norspermidine exposure. Cumulative average percent of adult OreR flies observed after each day for a range of norspermidine concentrations. The first day that adult flies were observed in the control is counted as Day 1.

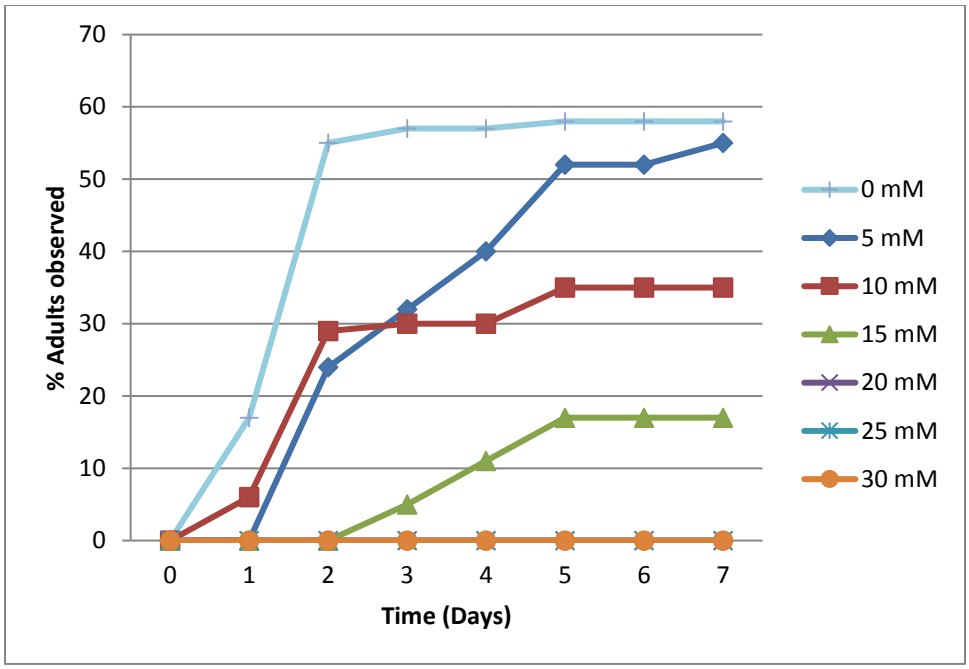

Figure 8:Developmental profile of CG32000 B exon mutant flies during norspermidine exposure. Cumulative average percent of adult CG32000 B exon mutant flies observed after each day for a range of norspermidine concentrations. The first day that adult flies were observed in the control *is counted as Day 1.*

DISCUSSION

The initial goal of this project was to investigate the feasibility of using norspermidine to screen for genes involved in polyamine transport. A major advantage of this approach over the current DFMO based approach used in the laboratory is that animals can only survive if they are unable to transport polyamines. Thus, batches of genes could be tested simultaneously and if a few survivors are observed the batch could be broken down to individual genes for further testing. In contrast in the DFMO screen animals must transport to survive and genes are tested individually in this survival based assay. Unfortunately, my data suggest that a norspermidine based screen will not be feasible in Drosophila.

There were three major findings from my work. First, the lethal concentration of norspermidine in *Drosophila* (30 mM) is ten times higher than the lethal concentration in *C. elegans* (Heinick et al 2010). Second, my data show that norspermidine gains entry to cells via an alternative mechanism to that used by CG32000, a gene required for polyamine transport. Third, I have shown that my data cannot be explained by severe developmental delay associated with uptake of norspermidine.

The lethal concentration of norspermidine for wild-type *C. elegans* was observed to be 3 mM (Heinick, 2010). *Drosophila* required ten times this amount. This may indicate an increased ability to export or metabolize norspermidine in *Drosophila,* thereby mitigating its toxicity. Alternatively, a separate transport system with a decreased affinity for norspermidine (compared to *C. elegans*) may also explain these results. Support for multiple transport pathways in *Drosophila* comes from recent work using a chemical approach showing that polyamine transport inhibitors differ in the relative abilities to block the uptake of the native polyamines (Wang et al., 2017). This study also

showed that a cocktail of polyamine transport inhibitors is more effective than individual inhibitors alone to blocking transport. One limitation of this study is that the norspermidine was not tested on *C. elegans* to confirm a similar level of toxicity as observed by Heinick et al.

While mutations disrupting the *C. elegans* gene CATP-5 enabled worms to survive even in 5 mM norspermidine, flies with a nonfunctional CG32000 gene (the orthologue of CATP-5) showed no decrease in sensitivity to norspermidine compared to wild-type flies, either in terms of increased survival or decreased developmental delay. Exposing the same strain of flies to DFMO and polyamines confirmed that the flies were unable to transport native polyamines, as the presence of exogenous polyamines was unable to provide rescue from DFMO. Knocking down CG32000 using RNAi yielded comparable results. One explanation may be that at sufficiently high concentrations, norspermidine uptake occurs via a secondary transport system. To further address this question it will be important to test the ability of the native polyamines to competitively inhibit norspermidine uptake. If an alternative transport system is used the native polyamines may not be able to competitively inhibit norspermidine transport.

Finally, while the lethal concentration of norspermidine is 30mM for OreR, it is 20mM for the B exon mutant of CG32000 (cf. Figures 7 and 8). Overall viability is compromised in the B exon mutant (Barnett and von Kalm unpublished) presumably due to inability to transport polyamines. This coupled with exposure to norspermidine may increase the sensitivity to this compound.

Future studies should be directed at better understanding the mechanism of norspermidine uptake and how it relates to polyamine transport in general. If, as recent evidence suggests (Wang et al, 2017 and this study), there are multiple transport systems, it will be important to understand them

to facilitate the design of effective polyamine transport inhibitors to be used in conjunction with DFMO in cancer therapy.

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