Advances in a *C. elegans* model of Alzheimer's disease for drug screening against neurodegeneration

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Abstract –

Alzheimer's disease (AD) is the sixth leading cause of death in the United States, yet no treatment effectively prevents, halts or reverses the disease. Progress in developing treatments is hampered by the extensive time required for traditional mouse models of AD to age before displaying histological hallmarks of AD. We set out to test whether a novel transgenic model of AD using the nematode *Caenorhabditis elegans* may be used to more rapidly determine efficacy of candidate treatments through high throughput screening of behaviors. Our lab previously showed that antagonists for the conserved Sigma-2 receptor (Sig2R) are protective against age-dependent degeneration of cholinergic neurons caused by the human plaque protein, amyloid precursor protein (APP), in this model. To investigate how inhibition of Sig2R protects neurons, we tested whether knockdown of Sig2R via RNA interference prevented decline of two behaviors that depend on these cholinergic neurons. We found that RNAi treatment normalized one of the two behaviors in this AD model suggesting that the antagonists act against the Sig2R *in vivo* for neuroprotection. The success of this study suggests that this behavioral readout might be used to screen for additional pharmaceutical and genetic modifiers of Sig2R on neurodegeneration. In addition to developing this high throughput behavioral screening, we also built transgene components to generate an improved second generation *C. elegans* model of AD that conveniently tags the APP protein with mCherry for *in vivo* fluorescent visualization. Our results set the stage for further drug discovery using our *C. elegans* models of AD.

Background –

Alzheimer's disease (AD) has been a devastating challenge to the research, medical, and public community for more than a century. In 1906, AD was described as a neurodegenerative disorder associated with progressive cognitive and memory impairment. Nearly a century later, the etiology of AD remains unknown and there is no treatment to effectively prevent, halt, or reverse the disease.[1] Mitochondrial and endoplasmic reticulum (ER) driven apoptosis has been implicated in disease-specific protein accumulation during the early-stages of AD pathogenesis.[1] Research now focuses on identifying both the molecular mechanism of the disease and potential biological targets for therapeutic drugs that will inhibit abnormal protein accumulation in the brain and improve cognitive function.

Protein misfolding and ER stress contribute to AD-related degeneration in a subset of cholinergic neurons important for memory. In particular, β-amyloid (Aβ) peptide accumulates in senile plaques within the brain of AD patients due to abnormal processing of the amyloid precursor protein (APP).[2] Identical plaques also form in the brains of those with Down

Syndrome because these individuals carry an extra copy of the human *APP* gene on chromosome 21. More than 75 percent of individuals in this population develop early-onset AD.[3] Mice with three copies of chromosome 16, orthologous to the critical region of human chromosome 21, display degeneration of cholinergic neurons in the basal forebrain similar to those with AD and Down syndrome.[4] Therefore, overexpression of APP due to duplication may be one way to generate a predictable model of AD.

The laboratory developed a novel transgenic *C. elegans* model of AD that displays age-related and APP-related neurodegeneration. This model pan-neuronally expresses a single human *APP* gene (SC_APP) in addition to endogenous expression of the worm ortholog, *apl-1* (amyloid precursor-like protein-1). We used the *C. elegans* model system because of its short life span, transparent body, highly conserved genes, and rapid genetic manipulation. The simplified nervous system of the worm, containing only 302 neurons, has been exquisitely described in terms of its anatomy, lineage, and intrinsic molecular profile. The transparent body of the worm has made it possible to use *in vivo* fluorescent markers to track changes in morphology of cell bodies and axonal projections in live animals over the nematode life cycle from larval stage to death by senescence. We showed that our model displays progressive, age-dependent degeneration of specific cholinergic neurons, ventral cord neuron (VC) 4 and 5.

Researchers found that the six VC neurons, particularly VC4 and VC5 proximal to the vulval, are important for initiating successful egg-laying events.[5] The VC4 and VC5 neurons have also been implicated in swim initiation.[6] Therefore, egg-laying and swimming behaviors represent a sensitive readout of VC neuron activity in the *C. elegans* model system. Using our model, we have previously shown that Day 3 adult worms retain significantly more eggs and exhibit defective swimming with respect to both AD control and wild-type animals. These results suggest that neurodegeneration observed in our model might correlate with functional behavioral deficits in egg laying and swimming.

Together with trackable neurodegeneration and easily measured behavior, our *C. elegans* model has proven to be useful in target identification and validation. We identified a novel target, Sig2R, based on a loss-of-function model that showed reduced neurodegeneration relative to non-AD control animals. While the relationship between Sig2Rs and AD has not been studied, it was well established that Sig2R ligands can act as agonists at the receptor to induce apoptosis in cancer cells.[7] A preliminary screen of 5 compounds identified a Sig2R-specific ligand with neuroprotective benefits. This compound has since been shown to reduce cognitive deficits in a mouse model of AD. Specifically, we have shown that this Sig2R antagonist can also improve cognition in double transgenic mice homozygous for the London (V717I)[8] and Swedish (KM670/671NL)[9] mutant APP forms. Identification and validation of a lead compound was an important outcome for establishing our model in future drug discovery efforts.

Current gaps in our understanding include the mechanism through which this Sig2R-antagonist is providing neuroprotective influence and how expression of human APP is causing age-dependent degeneration specifically in the VC4 and VC5 neurons. Our hypothesis states that the cellular pattern of neurodegeneration seen in our *C. elegans* model of AD is correlated with accumulation of mCherry-tagged APP and that Sig2R antagonism provides protection by reducing the level of APP in the VC4 and VC5 neurons. In order to test this, we first need to correct several imperfections in our model, namely three point mutations in the human APP gene and an insertion that caused the mCherry sequence to be out-of-frame. I addressed these problems by 1) fixing the *hAPP* construct using site-directed mutagenesis and 2) inserting this new construct using Mos1-mediated single-copy insertion (MosSCI). Next, I wanted to develop

a reverse genetic screen to find genes important for Sig2R's functional influence on neurodegeneration. I set out to test 1) whether egg laying and/or swimming behavior could provide a measurable phenotype necessary for rapid screening of genes and 2) if RNAi knockdown of the Sig2R could improve behavior in our AD model. These goals are necessary to understand the mechanism by which our hit compound, the Sig2R-antagonist, provides neuroprotective influence in the face of APP dysregulation.

Methods –

Strains.
The strains generated and used for this study include JPS67 and LX959. The LX959 strain integrated GFP expressed specifically in the ventral cord neurons 1-6 by a ventral cord neuron-specific promoter. This strain also contained a lin-15B genetic background so that its genotype was vsIs13 IV; lin-15b(n765) X. A single-copy of the *hAPP* gene on chromosome II was integrated into a background LX959 strain with *unc-119* (ed3) rescue marker. The genotype for the JPS67 strain can be described by vsIs38 II; unc-119(ed3) III; vsIs13 IV; lin-15B(n765) X. All strains were maintained at room temperature and fed *E. Coli* OP50 on NGM plates.

Site-directed mutagenesis to repair model
We used the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) to make specific changes to the DNA sequence of our *hAPP* gene cloned in the Gateway pDONR221 vector. We repaired the three point mutations at position 585, 926, and 1082. Primers 5' CTGAAGAAAGTGACAATGTGGATTCTGCTGATG 3' and 5' CATCAGCAGAATCCACATTGTCACTTTCTTCAG 3' contained the desired substitution C→T at position 585. At position 926, primers 5' CTCGAGACACCTGGGGATGAGAATGAACATGC 3' and 5' GCATGTTCATTCTCATCCCCAGGTGTCTCGAG 3' were designed with the substitution G→A. The G→A substitution at position 1082 was fixed using primers 5' GTTATCCAGCATTTCCAGGAGAAAGTGGAATC 3' and 5' GATTCCACTTTCTCCTGGAAATGCTGGATAAC 3'. To repair the frameshift in the mCherry sequence, primers 5'GCAGATGCAGAACTACCCAGCTTTCTTGTAC 3' and 5' GTACAAGAAAGCTGGGTAGTTCTGCATCTGC 3' were used to delete AG at position 2082-83. These primers were used to synthesize mutant molecules by PCR (95°C for 50 seconds, 60°C for 50 seconds, 68 for eight minutes) and transform them into XL 10-Gold Ultracompetent cells. Colonies containing kanamycin-resistant pDONR221 vector plasmid were isolated after plating on kanamycin overnight (≥16 hours). Minipreparation (Qiagen) of plasmid DNA was used to confirm the correct sequence by next generation sequencing in the Institute for Cellular and Molecular Biology (ICMB) Core Facilities.

Assembly of 3' entry clone.
Following the Gibson Assembly protocol (E5510) developed by New England BioLabs (NEB), mCherry was inserted into the pDONR P2-P3 vector with *unc-54* UTR. DNA segments for the insert and vector were generated by PCR (95°C for 30 seconds, 55 for 30 seconds, 55-65°C gradient for 5:30 minutes (vector) or one minute (insert)) using primers 5' GTGGCATGGATGAATTGTATAAGACTAGTTAGCATTCGTAGAATTCC 3' and 5' GTTATCTTCTTCACCCTTTGAGACTGCCCGGGCGGATCCCTCCACTTTGTA 3', and 5'

TACAAAGTGGAGGGATCCGCCCGGGCAGTCTCAAAGGGTGAAGAAGATAAC 3' and 5' CTTATACAATTCATCCATGCCACCTG 3', respectively. To confirm the size and yield of our product, we ran each PCR product on an 8% agarose gel. GenElute® PCR Clean-Up Kit was used to purify our PCR amplification product. This product was used in a Gibson Assembly Reaction (50°C for 15 minutes) to combine the DNA segments. XL 10-Gold Ultracompetent cells were transformed with the assembly reaction and the plasma DNA extracted and purified by miniprep. Sequence analysis by the ICMB Core Facility confirmed our final plasmid.

Assembly of a transformation-ready transgene.
We used Gateway® Technology through the Multisite LR reaction to recombine the *Prab3* pan-neuronal promoter in the 5' entry clone, *hAPP* gene in the middle entry clone, and mCherry;*unc-54* UTR in the 3' entry clone into the MosSCI destination vector pCFJ150. The promoter had been cloned into the pDONR P4-P1 vector. The LR recombination reaction was incubated overnight at room temperature and transformed into One Shot®TOP10 Chemically Competent cells. Colonies were isolated on ampicillin plates, incubated in 3 mL of LB with 1 mM kanamycin overnight and prepared using QIAprep Spin Miniprep Kit for microinjection into the *ttTi5605 C. elegans* strain with a MosSCI background.

Construction of Transgenic Animal
Single-copy transgenes constructed in the PCFJ150 vector using Gateway cloning were introduced into *ttTi5605 C. elegans* following the Mos1-mediated single-copy insertion (MosSCI) method. Our final construct *Prab3::hAPP::unc-54UTR* was microinjected into the distal arm of the gonad of young adults from the *ttTi5605* strain (EG4450) containing the *Drosophila* Mos1 element. The target construct contained a 2.1 kb *C. briggsae unc-119* rescue marker and the transformation-ready transgene flanked by Mos1 site sequences for insertion at the Mos1 site on chromosome II by homologous recombination. Following injection, we selected for animals that were moving and lacked red fluorescence in the pharynx. The presence of a small percentage of animals with red pharynx suggested that the transposase worked.

RNAi Preparation
The *vem-1* RNAi and empty vector plasmid pL4440 colony were selected from the Ahringer library (Genesequence) and grown overnight in LB with 50 μg/mL of ampicillin. At least 24 hours before the experiment, the *vem-1* RNAi culture was seeded onto ampicillin-containing NGM-agar plates with 1 mM isoprophylthiogalactoside (IPTG). Plates without IPTG were seeded with the L4440 empty backbone and these were considered control RNAi plates.

Egg-retention Assay
Strains LX959 and JPS67 were used. To prepare a homogenous population of age-matched eggs, non-starved adult worms were treated with bleach (5M NaOH) and the eggs remaining in the liquid were transferred to clean NGM plates seeded with an *E. coli* OP50 lawn. Eggs were allowed to develop for 3 days (~100 hours) so that worms reached a late larval stage 4. About 100 animals at this stage were transferred to RNAi and control RNAi plates and allowed to develop to day 2 adults (40-50 hours) at room temperature. After 60 animals were individually dissolved in 60 μL of 5% bleach and eggs retained within the dissolved adult animal were counted.

Multi-Fluid Chamber Assay

Standard soft lithography techniques were used to fabricate devices for use as a microfluidic system.[10] A master template was first generated with patterned features in a double layer of 50 μM thick photoresist (100 μM total thickness)(Riston® Dry Film Photoresists, Dupont, Wilmington, DE) using a photomask (Cad Art Services, Brandon, OR). All devices were molded from this master template in polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Corning, NY). The first layer of PDMS prepolymer was cast against the master template and cured in an oven for 20 min at 70°C. An inlet/outlet was created at one end of the device using a nanoport. Following this, a second layer was cast and cured for 30 min at 70°C. PDMS was removed from the template and a blunt epidural needle (0.070 mm) was punched multiple times into the device to form fluid inlet and outlet holes. We exposed the devices to oxygen plasma (120 W, 2 s) in order to irreversibly bond the devices to the coverslip. Prior to adding animals, the devices were flushed a few times with ethanol, deionized water, and NGM buffer, respectively. Day 2 animals were transferred from a seeded plate to an unseeded plate to clean off the bacteria food. About 20-25 animals were loaded into the inlet of the device and 10μL of diacetyl (Sigma-Aldrich, St. Louis, MO) was immediately added to the nanoport opposite to the inlet where the worm were loaded. The time started when the diacetyl was added to the device. At 5-minute increments for 15 minutes, the number of animals in each zone (3 total zones) was counted. The strains used (LX959 and JPS67) were prepared as described above (*Egg-retention Assay).*

Results –

Specific changes to our AD model is necessary to visualize APP accumulation

The facile genetics of the *C. elegans* model allowed us to easily and specifically edit the DNA sequence in our existing AD model so that in future studies we can visualize the human APP protein (*hAPP*) using fluorescent markers and provide information about the developmental timing and spatial location of gene activity relative to neurodegeneration. We used a series of molecular biology techniques to repair our *C. elegans* model of AD with pan-neuronal expression of a single copy of human APP tagged with mCherry at the C-terminal. Three point mutations and an insertion mutation were detected in our PCFJ150 vector recombined to contain *Prab-3*::*hAPP*::*mCherry*::*unc-54-UTR*. The large 17 kb size of this construct impaired our success with mutagenesis. Thus, we set about to fix these problems in the smaller 8,000 kb pDONR 221 middle entry clone with the *hAPP* gene inserted. To repair the point mutations, we used specifically designed primers (see *Methods*) to carry out three site-directed mutagenesis reactions to substitute C→T at position 585, G→A at position 926, and G→A at position 1082. A two-nucleotide insertion of AG at the end of the APP sequence caused the mCherry;*unc54*-UTR construct to be out of the reading frame. Using primers 5'GCAGATGCAGAACTACCCAGCTTTCTTGTAC 3' and 5' GTACAAGAAAGCTGGGTAGTTCTGCATCTGC 3', targeted deletion of AG at position 2082 and 2083 by site-directed mutagenesis corrected for this problem. After completion of each round of mutagenesis, Sanger sequencing performed in the ICMB Core Facilities confirmed sequences. All four mutagenesis reactions successfully achieved the desired mutation.

Next we constructed a *hAPP* transgene with C-terminal mCherry tag that we plan to introduce in our *C. elegans* model. We took advantage of modern modular cloning methods in order to assemble a fluorescent reporter construct within the 3'-untranslated region (UTR) and tag the *hAPP* gene without disrupting the original sequence context or appropriate expression patterns. We recombined the corrected APP entry construct with 5' and 3' entry clones in a single LR recombination reaction with the PCFJ150 destination vector. We used a *Prab-3* promoter in the pDONRP4-P1R vector and *mCherry::unc-54-UTR* in the pDONRP2-P3 vector for the 5' position and 3' position entry clones, respectively. We used the *Prab-3* promoter in order to achieve pan-neuronal expression of APP. The *unc-54*-UTR is a common UTR used in transgenes to enhance expression. The Gateway Multisite vector PCFJ150 was selected as the destination vector in preparation for transformation into the *ttTi5605 C. elegans* containing the *Drosophila* Mos1 element at the ttTi5605 site on chromosome II. We chose this strain based on previous work in the laboratory and elsewhere demonstrating successful transgene integration following the published Mos1-mediated single-copy insertion technique.[11] The PCFJ150 vector contains the Multisite Gateway® cassette with attR4-attR3 sequences flanked by targeting and selection Mos1sequences. These Mos1 sequences are necessary for homologous recombination and insertion at this *ttTi5605* Mos1 site. The PCFJ150 vector also contains a 2.1 kb *C. briggsae unc-119* fragment that rescues the *unc-119(ed3)* mutation in the *ttTi5605* strain. This mutation normally causes partial paralysis, small body and brood size, and an inability to form dauer larvae in response to starvation conditions. In preparation for the LR reaction, we needed to construct a 5' entry clone encoding *mCherry::unc*-54-UTR. We decided to use the Gibson Assembly protocol to insert the mCherry gene sequence in front of the *unc54*-UTR sequence on the pDONR P2-P3 vector. In a single reaction, we achieved a fully ligated, double-stranded plasmid vector ready for use. Using this UTR vector in an LR reaction, we successfully produced a transformation-ready transgene as shown in *Supplemental Figure S1*.

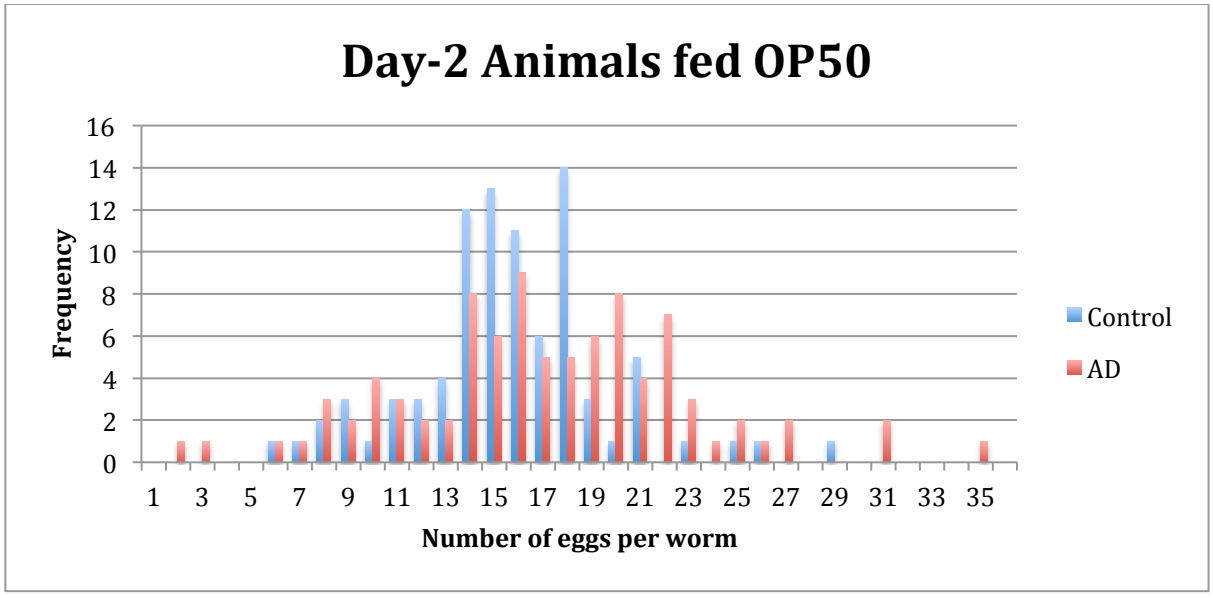
We made use of the well-characterized *ttTi5605* strain with positive and negative markers to select for an insertion event in which the transgene and selection markers are incorporated into the genome by gene conversion. Animals that successfully incorporated the final construct with the *unc-119*(+) gene, a positive selection marker, appeared to move and survive starvation conditions. To differentiate from animals that integrated the transgene from animals that only carry the extrachromosmal array, we coinjected the PCFJ90 vector, which contains *mCherry* under a pharynx-specific promoter, to provide a negative selection marker. Under fluorescence microscopy, we observed animals with red pharynx in our population of ttTi5605 strains. This suggested that the transposase excised the Mos1 transposon and caused a double-stranded DNA break, which could be repaired with the transgene or the red pharynx insert. Only animals that both moved and did not fluoresce red were selected.

Loss-of-function Sig2R mutation normalizes behavior in AD model animals

One approach to high-throughput screening (HTS) involves a chemical-to-gene approach where 'hit' compounds are identified based on their ability to rescue adverse phenotypes to wild-type phenotypes. Based on our previous studies showing a relationship between neurodegeneration and behavior, we set out to test whether functional deficits in egg laying behavior also reflect Sig2R's functional influence on neurodegeneration. As a proof of principle, we showed that 14.77% of day-2, "middle-aged" adult control, strain LX959, animals without the extra copy of

hAPP retained over 17 eggs. Control animals show a relatively clear bell-shaped curve (average of 14.69 eggs), whereas our AD model with a single extra copy of *hAPP* (SC_APP), strain JPS67, have a slight bimodal distribution. As a result, there is not a large difference between averages in the AD (average of 16.07 eggs) and control animals, but a larger percentage of age-matched day-2 AD animals (41.11%) that retained over 17 eggs. Because these AD animals also show significant neurodegeneration at middle age, these results support our initial hypothesis that neurodegeneration in VC4 and VC5 neurons correlates with functional deficits in egg-laying behavior. Therefore, egg laying represents both a screenable behavior and a rapid behavioral readout of neurodegeneration in our control and AD animals.

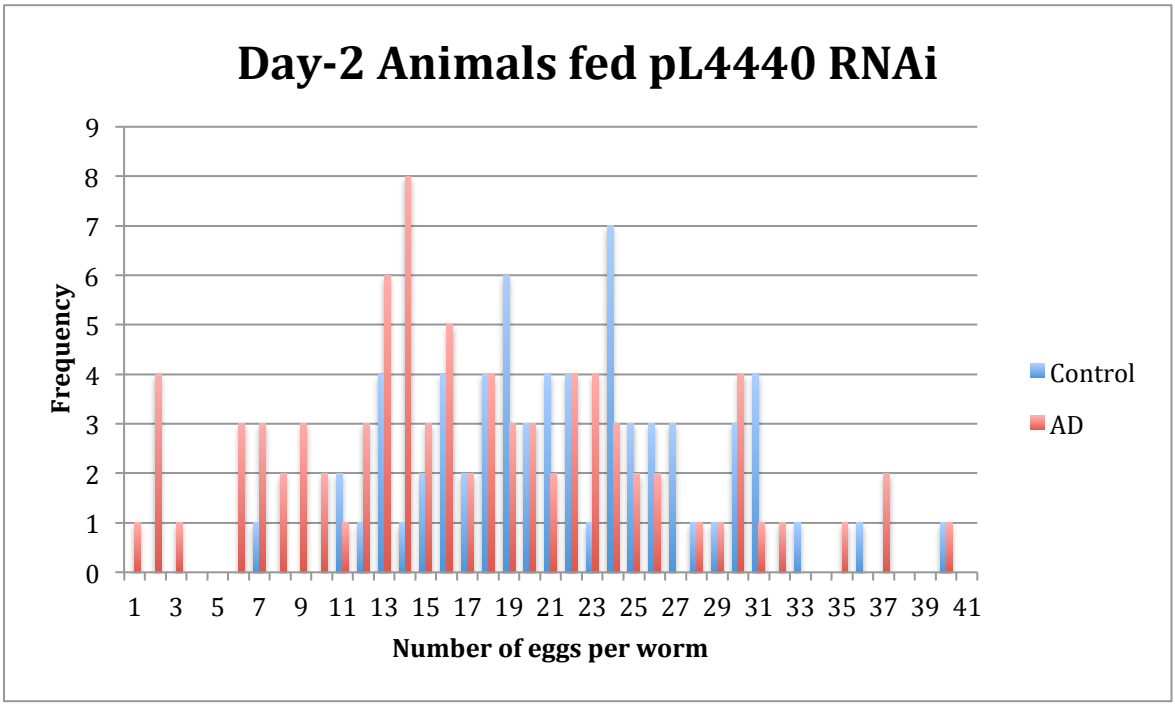
Figure 1. **Neurodegeneration is correlated temporally with defects in egg laying behavior.** A histogram of day-2 control (blue) and AD animals (red) displays the frequency or number of individual animals (y-axis) that retained a discrete number of eggs (x-axis). We show that a greater number of AD animals retained more than 17 eggs. 14.77% of 88 total control animals vs. 41.11% of 90 total AD animals retained over 17 eggs. This is clearly observed in the distribution where the results from control animals and AD animals are unimodal and bimodal, respectively.



This HTS approach is especially useful to identify genes and gene pathways when combined with RNA-mediated interference (RNAi). To better understand if the neurodegeneration seen in our AD animals is influenced by Sig2R activity, we tested whether Sig2R knockdown by RNAi would normalize egg laying behavior in our AD animals. This experiment serves as a positive control for our behavioral assay since we have previously shown that elimination of Sig2R is neuroprotective. The Sig2R was recently identified as the progesterone receptor membrane component 1.[12] The *vem-1* gene is ortholous to the mammalian VEMA, a membrane-associated progesterone receptor component, and has a well-known role in axonal guidance in *C. elegans.*[13] Animals were fed *vem-1* RNAi or pL4440 empty vector at larvae stage 4 and scored for functional deficits in egg-laying as day 2 adults. We showed that RNAi treated animals were able to retain a similar number of eggs (25% retained over 17 eggs) as non-AD control animals

fed *E. coli* OP50 (*Supp. Figure S2*). However, animals fed pL4440 empty vector RNAi bacteria showed different egg laying behavior than when fed OP50 bacteria. The relationship between egg laying behavior and overexpression of APP was reversed so that more control animals (68.66%) retained over 17 eggs than AD animals (38.04%) (*Figure 2*). These results suggest that RNAi bacteria, not knockdown of Sig2R, affect egg laying behavior. Consequently, our positive result with RNAi treatment in AD animals is unclear and egg laying is not a useful behavior for RNAi screens.

Figure 2. **Differences in bacterial strains affect the relationship between egg laying behavior and APP overexpression.** A histogram of control and AD animals fed pL4440 empty vector RNAi shows the number of animals that fall into each 'bin' that represents a discrete number of eggs. **A)** Animals fed pL4440 empty vector RNAi showed different distributions in egg laying behavior than when fed *E. coli* OP50. 68.66% of 67 total control animals and 38.04% of 92 total AD animals retained over 17 eggs. The control distribution (average of 20.76 eggs) was shifted to the right whereas the **B)** AD distribution (average of 15.64 eggs) was shifted to the left. These results are opposite of what we would have expected.



We next investigated whether locomotion may represent an alternative behavioral readout of VC neuron function in our RNAi screen. First, we tested whether swimming and crawling are affected in our AD model. We developed a microfluidic chamber assay where worms alternatively swim in open areas and crawl through a field of pillars. Animals are injected at the start 6.3 mm from the first pillar field as shown in the experimental set up in *Supp. Figure S3*. The goal of the design was to have animals race to an attractant at the other end so that defects in motility and transitioning between swimming and crawling will differentiate AD animals from non-AD control animals. An attractant gradient of 10 µL 1:1000 diacetyl was sufficient to encourage day-2 wild-type animals to maneuver through all three pillar fields within 15 minutes.

Significantly less ($p < 0.05$) day-2 AD animals were able to make it into the third zone nearest the 'goal' after the same time period (*Figure 3*). For each trial, the percentage of animals in each zone was averaged at each 5-minute time point and a t-test for significance was performed. The inability of AD animals to perform basic locomotor tasks suggests that the ventral cord neurons are necessary for proper swimming, crawling, and/or gait transitions. Additionally, animals treated with *vem-1* RNAi were capable of reaching the goal as frequently as non-AD control animals (*Figure 4*). It is important to note that although we did not calculate statistical significance ($p>0.1$) between the untreated AD animals and AD animals treated with *vem-1* RNAi, we do notice a clear difference between these groups in zone 3 after 15 minutes from the start time. This clear trend in our AD animals toward control levels suggests that swimming is a useful behavior in RNAi screening. We plan to perform additional trials with all control groups (non-AD animals with *vem-1* RNAi or pL4440 RNAi) and larger sample sizes in order to statistically prove that knockdown of Sig2R can reverse abnormal swimming behavior to control phenotypes. We expect to see a similar trend in our AD animals treated with *vem-1* RNAi when compared to non-AD control animals treated with pL4440 RNAi. Overall, these results support our hypothesis that loss of Sig2R function is neuroprotective and thereby sustains normal swimming, crawling, and gait transitions.

Figure 3. **Animals with an extra copy of *hAPP* show functional deficits in a multi-fluid chamber assay.** The percent number of animals in each zone (x-axis) at 15 minutes after starting the race was averaged across three trials and recorded on the y-axis. From several different trials, a total of 87 and 84 animals of AD and control, respectively, were scored at 5-minute increments after diacetyl was added (time = 0). A 15-minute time period was sufficient for significantly more wild-type animals to reach zone 3 relative to AD animals as measured by t-test ($p<0.05$). There was no significant difference in animals that reached zone 1 in the same time period.

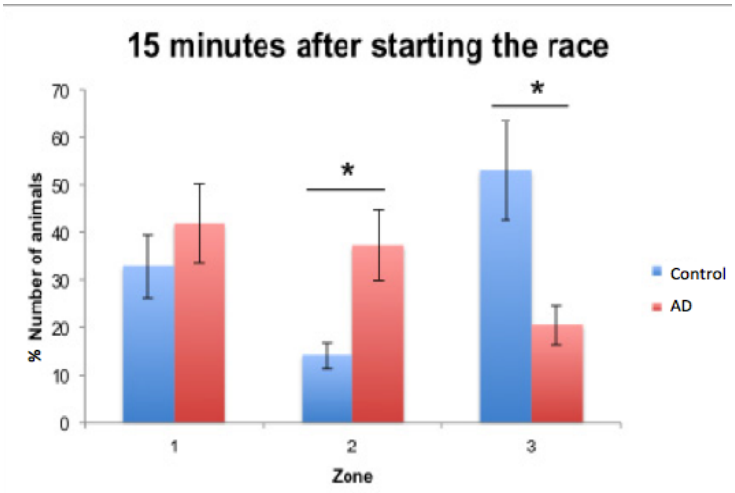
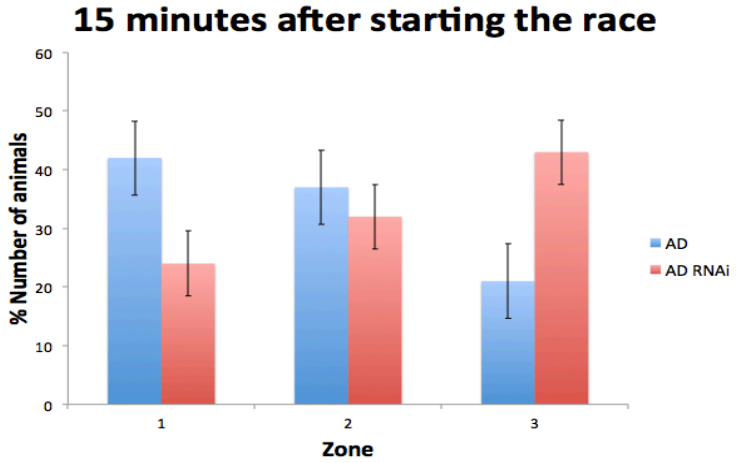


Figure 4. **RNAi knockdown of Sig2R normalizes multi-fluid chamber behavior in AD animals.** Untreated AD animals and AD animals treated with *vem-1* RNAi (total of 57 animals) at larval stage 4 were scored as day-2 adults. The percent number of untreated and treated AD animals in each zone (x-axis) at 15 minutes after starting the race were averaged across three trials and recorded on the y-axis. 43% of *vem-1* RNAi treated AD animals were able to reach the

third zone after 15 minutes. Similar results were recorded with non-AD control animals (53% reached zone 3), suggesting that knockdown of Sig2R modulates motility and/or transition between swimming and crawling. No statistical significance using t-test ($p>0.1$) was found between the two groups in each zone; this is most likely due to the low sample size of each group.



Discussion –

There is no existing model powerful enough to answer all the questions surrounding the century old debate of AD. In this study, we set out to establish the *C. elegans* model system for future drug discovery efforts in AD. Despite only having 959 somatic cells, the *C. elegans* system is a sophisticated and complex multicellular organism with highly conserved protein functional and structural properties. We confirmed and expanded upon previous work in the laboratory demonstrating the use of a novel transgenic *C. elegans* model of AD for large-scale target validation and high throughput compound screens that would otherwise be impossible in higher order mammalian models.

We have shown that overexpression of a key, highly conserved genes can result in easily measured phenotypes. Both locomotion and egg-laying behavior were altered in our *in vivo* model upon pan-neuronal expression of an extra copy of human APP. The goal of our behavioral studies was to identify a screenable behavior that could be used in a large-scale RNAi screen for important genes mediating Sig2R influence on neurodegeneration. It is important to understand the mechanism of drug action in order to avoid unanticipated side effects in later clinical trials. We found that although egg laying behavior can be rapidly scored, cheap to prepare, and easy to replicate, it is adversely altered by bacterial strains necessary for RNAi studies. Alternately, we demonstrated that locomotion efficiency could be a rapid and sensitive behavioral readout of neurodegeneration that is needed to facilitate high quality and high-throughput screens.

Our AD model showed impaired motor skills necessary for efficient gait transition and swimming. These results were measurable in animals known to display significant neurodegeneration in VC4 and VC5 neurons at day 2. AD animals were not able to navigate our multi fluid chamber at the same efficiency level of control animals that could quickly travel through alternating zones of open areas and fields of pillars within 15 minutes. The ability to differentiate between control and AD animals was an important outcome of this behavioral assay. We propose that the inability of our AD animals to produce efficient movement is due to

defective production and control of locomotor gaits by central nervous system neurons, specifically the VC neurons. Coincidental neurodegeneration of VC4 and VC5 in our model, therefore, seems to be sufficient to cause functional defects in motor skills that can be quickly and easily scored.

This sensitive behavioral correlate of neurodegeneration demonstrated by our AD model in our multi-fluid chamber suggests that we could use this assay to find genes important for Sig2R-mediated modulation of neurodegeneration. To do so, we plan to perform an RNAi knockdown screen of potentially relevant genes. The ability use RNAi knockdown at later developmental stages or even beyond development in the worm greatly facilitates functional genomic studies of genes important in aging. One way to manipulate Sig2R function is also with RNAi. However, it has been shown that the efficiency of knockdown by RNAi in worms is reduced when targeting multiple genes.[14] Instead, we will need to manipulate Sig2R function pharmacologically. The multi-fluid chamber assay can support this need as animals are accessible to simultaneous treatment with RNAi and a known compound. Thus, we can treat animals with a Sig2R antagonist or agonist by soaking to influence neurodegeneration in either direction, while simultaneously using RNAi to knockdown a single gene in our AD model. Employing our multi-chamber behavioral readout, we then can determine whether knockdown of the single gene could either "break" or rescue the influence of Sig2R modulation on the AD phenotype. In this way, we will be able to develop an understanding of the mechanism of drug action.

To achieve our second goal of investigating the causal relationship between APP accumulation and neurodegeneration in VC4 and VC5 neurons, we fixed the *hAPP* construct in our AD model. Since the Sig2R antagonist has been shown to be protective against neurodegeneration, we reasoned that this protection might come about by reduction of APP levels in VC neurons. This can only be tested in our new model strain, in which the *mCherry* gene is in reading frame with *hAPP*. We will use this model to reconfirm our neurodegeneration and behavior results and provide more information about the role of APP in neurodegeneration and cell protection by Sig2R antagonism.

The present study is important for guiding future high-throughput screening studies investigating novel therapeutic targets for the treatment of AD. Instead of screening for AD-specific functional activity in a cell-based assay, we demonstrated that our *in vivo* model of AD could be used to select for genes important for Sig2R's influence on neurodegeneration. From a reductionist approach, our *in vivo* model system represents an enormous advantage to the pharmaceutical industry. It is easily maintained in the laboratory and can rapidly develop from egg to adult within 3 days, producing about 300 progeny each. In this short period of time, millions of animals can be produced and analyzed in microtitre plates or in a single well of a 96-well plate with large sample sizes. The sensitivity of the worm to RNAi treatment further accelerates the target identification and validation process that is necessary for pharmaceutical development. We plan to use an RNAi screening technique to probe the mechanism of action of our novel Sig2R ligands in our AD worm. Towards this end, we have produced a behavioral assay, locomotor performance in a multi-fluid chamber, to screen for the effects of single gene knockdown on Sig2R ligand efficacy. More broadly, this behavioral correlate of neurodegeneration holds the potential to accelerate target validation and drug discovery for neurodegenerative diseases into higher order mammalian systems and humans. Continued work in our new and improved *in vivo* model will be needed to establish its use in further drug discovery efforts.

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Supplemental Information.
Supplemental information includes three figures providing results from the final sequencing analysis of our transformation-ready transgene and from the Egg-retention Assay using *vem-1* RNAi treatment. In addition, we have provided a diagram of the device used in our Multi-fluid Chamber Assay.

Supplemental Figure S1. The final sequence for the transformation ready-transgene contained the 3' *Prab3* promoter, *hAPP* gene, C-terminal tag with mCherry sequence (yellow), and 5' *unc-54*-UTR (green). The APP construct contained the desired mutations that repaired the three point mutations (at position 585, 926, and 1082; highlighted in blue) and the insertion at position 2082-83 (pink) that had caused the mCherry sequence to be out-of-frame.

ATGCTGCCCGGTTTGGCACTGCTCCTGCTGGCCGCCTGGACGGCTCGGGCGCTGGAG
GTACCCACTGATGGTAATGCTGGCCTGCTGGCTGAACCCCAGATTGCCATGTTCTGT
GGCAGACTGAACATGCACATGAATGTCCAGAATGGGAAGTGGGATTCAGATCCATC
AGGGACCAAAACCTGCATTGATACCAAGGAAGGCATCCTGCAGTATTGCCAAGAAG
TCTACCCTGAACTGCAGATCACCAATGTGGTAGAAGCCAACCAACCAGTGACCATC
CAGAACTGGTGCAAGCGGGGCCGCAAGCAGTGCAAGACCCATCCCCACTTTGTGAT
TCCCTACCGCTGCTTAGTTGGTGAGTTTGTAAGTGATGCCCTTCTCGTTCCTGACAAG
TGCAAATTCTTACACCAGGAGAGGATGGATGTTTGCGAAACTCATCTTCACTGGCAC
ACCGTCGCCAAAGAGACATGCAGTGAGAAGAGTACCAACTTGCATGACTACGGCAT
GTTGCTGCCCTGCGGAATTGACAAGTTCCGAGGGGTAGAGTTTGTGTGTTGCCCACT
GGCTGAAGAAAGTGACAATGTGGATTCTGCTGATGCGGAGGAGGATGACTCGGATG
TCTGGTGGGGCGGAGCAGACACAGACTATGCAGATGGGAGTGAAGACAAAGTAGTA
GAAGTAGCAGAGGAGGAAGAAGTGGCTGAGGTGGAAGAAGAAGAAGCCGATGATG
ACGAGGACGATGAGGATGGTGATGAGGTAGAGGAAGAGGCTGAGGAACCCTACGA
AGAAGCCACAGAGAGAACCACCAGCATTGCCACCACCACCACCACCACCACAGAGT
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TATCCAGCATTTCCAGGAGAAAGTGGAATCTTTGGAACAGGAAGCAGCCAACGAGA
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CGCCGCCTGGCCCTGGAGAACTACATCACCGCTCTGCAGGCTGTTCCTCCTCGGCCT
CGTCACGTGTTCAATATGCTAAAGAAGTATGTCCGCGCAGAACAGAAGGACAGACA
GCACACCCTAAAGCATTTCGAGCATGTGCGCATGGTGGATCCCAAGAAAGCCGCTC
AGATCCGGTCCCAGGTTATGACACACCTCCGTGTGATTTATGAGCGCATGAATCAGT
CTCTCTCCCTGCTCT
ACAACGTGCCTGCAGTGGCCGAGGAGATTCAGGATGAAGTTGATGAGCTGCTTCAG
AAAGAGCAAAACTATTCAGATGACGTCTTGGCCAACATGATTAGTGAACCAAGGAT
CAGTTACGGAAACGATGCTCTCATGCCATCTTTGACCGAAACGAAAACCACCGTGG
AGCTCCTTCCCGTGAATGGAGAGTTCAGCCTGGACGATCTCCAGCCGTGGCATTCTT
TTGGGGCTGACTCTGTGCCAGCCAACACAGAAAACGAAGTTGAGCCTGTTGATGCCC
GCCCTGCTGCCGACCGAGGACTGACCACTCGACCAGGTTCTGGGTTGACAAATATCA
AGACGGAGGAGATCTCTGAAGTGAAGATGGATGCAGAATTCCGACATGACTCAGGA
TATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCAGAAGATGTGGGTTCAAACAAA
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ACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGA
GGTTGACGCCGCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACG
GCTACGAAAATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAGACCCAGCTTT
CTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACA
GGTCA
CTATCAGTCAAAATAAAATCATTATNNGCCATCCAGCNGATNNCCC
…
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ATTGATATTTATATCATTTTACGTTTCTCGTTCAACTTTCTTGTACAAAGTGGAGGGA
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GACGCAAGACTCAAGGTAAGTTTAAACAGTTCGGTACTAACTAACCATACATATTTA
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AATTGTATAAGACTAGTTAGCATCGTAGAATTCCAACTGAGCGCCGGTCGCTACCAT
TACCAACTTGTCTGGTGTCAAAAATAATAGGGGCCGCTGTCATCAGAGTAAGTTTAA
ACTGAGTTCTACTAACTAACGAGTAATATTTAAATTNTCAGCATCTCGCGCCCGTGC
CTCTGACTTCTAAGTCCAATTACTCTTCAACATCCCTACATGCTCTTTCTCCCTGTGCT
CCCACCCCCTATTTTTGTTATTATCAAAAAACTTCTCTTAATTTCTTTGTTTTTTAGCT
TCTTTTAAGTCACCTCTAACAATGAAATTGTGTAGATTCAAAAATAGAATTAATTCG
TAATAAAAAGTCGAAAAAAATTGTGCTCCCTCCCCCCATTAATAATAATTCTATCCC

AAAATCTACACAATGTTCTGTGTACACTTCTTATGTTTTTTACTTCTGATAAATTTTTT
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ATCGGAAGAAGGTTTGGGTTTGAGGCTCAGTGGANNNGAGTAGAAGTTGATAATTT
GAAAGTGGAGTAGTGTCTATGGGGTTTTTGCCTTAAATGACAGAATACATTCCCAAT
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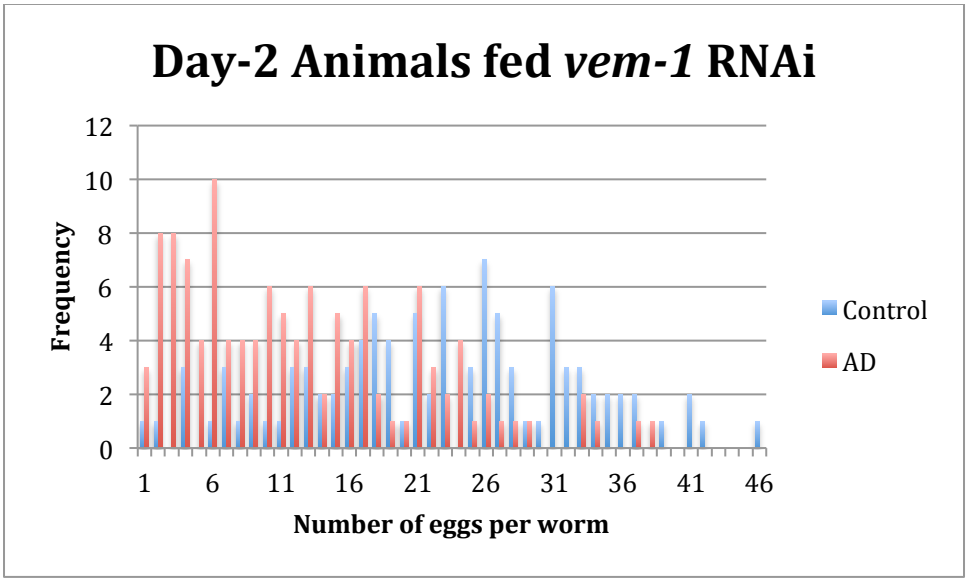
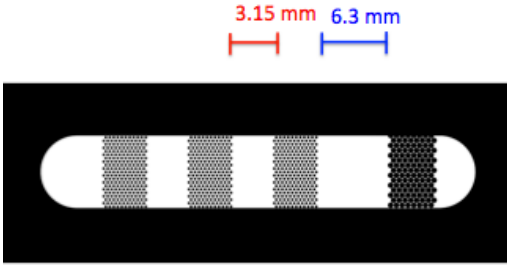
TGGANCTCNNGGNAGCGTTNACGCNGCCNCNATATCCCCNNTANTGAGTCG

Supplemental Figure S2. RNAi knockdown of *Sig2R/vem-1* normalizes swimming behavior in AD animals. Cumulative results from three separate trials show that animals fed **A)** *vem-1* RNAi at larval stage 4 (120 total animals) retained less eggs than animals fed **B)** pL4440 empty vector (left; 122 total animals). A left-shifted change toward non-AD control egg laying behavior (average of 11.525) suggests that knockdown of Sig2R normalizes behavior in our AD animals.



Supplemental Figure S3. Experimental design for the multi-fluid chamber assay. The first area (black) has pillars with a larger diameter than the pillars in the other 3 areas (grey). The space between the pillars in the first area is too small for adult worms to maneuver through.



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