Examining the Neuroprotective Effects of 7-hydroxy-2-(2-phenylethyl)chromone in Glutamate Excitotoxicity Model of C. elegans

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Abstract

There are 600 neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS) that are affecting about 50 million Americans each year. Glutamate excitotoxicity is a pathway that plays a role in neuronal death and degeneration across several neurodegenerative diseases. In the Williams’ lab, 5-HPEC has proven to have neuroprotective activity. Because 5-HPEC and 7-HPEC are a subclass of 2-(phenylethyl)chromone, this study examines if 7-Hydroxy-2-(2-phenylethyl)chromone (7-HPEC) will have the same properties and neuroprotective effects in C. elegans as their family compounds. Developed in the Driscoll lab, a glutamate excitotoxicity model of C. elegans was used to prove the effects of 7-HPEC. The results indicated that although 7-HPEC does not have a significant impact on neuroprotective effects in a C. elegans glutamate excitotoxicity model, there is a decrease due to its effects but is not comparable to its 5-HPEC. 7-HPEC had limited effects when quantifying the number of necrotic neurons.
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<th>Description</th>
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<tbody>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CGC</td>
<td>Caenorhabditis Genetics Center</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>GLT-3</td>
<td>Glutamate Transporter 3</td>
</tr>
<tr>
<td>iGluRs</td>
<td>Ionotopic Glutamate Receptors</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>5-HPEC</td>
<td>5-hydroxy-2-(2-phenylethyl)chromone</td>
</tr>
<tr>
<td>6-HPEC</td>
<td>6-hydroxy-2-(2-phenylethyl)chromone</td>
</tr>
<tr>
<td>7-HPEC</td>
<td>7-hydroxy-2-(2-phenylethyl)chromone</td>
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**Introduction**

Neurodegenerative disorders like Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS) are affecting about 50 million Americans each year.\(^1\) As one age, the likelihood of developing a neurodegenerative disease increase due to the loss and degradation of neurons. The symptoms can include but are not limited to cognitive functions, loss of muscle function, and a decrease in locomotor movement.\(^2\) Neurons are unable to grow back into a healthy neuron and function properly, thus causing the neurons to die. Some common mechanisms include of neuron degeneration are mitochondrial loss, oxidative stress, and excitotoxicity.\(^3\) Research has shown that glutamate excitotoxicity, in particular, can play a major role in neurodegenerative disorders.

Glutamate excitotoxicity is a pathway that plays role in neuronal death across several neurodegenerative diseases. This is due to the neurotoxic effects of glutamate being displayed in both glutamate receptors and non-glutamate receptors, ion channels, and transporters. Glutamate is a free amino acid in the central nervous system (CNS) that acts as an excitatory neurotransmitter and contributes to learning and memory.\(^3\) In both the CNS and peripheral nervous system (PNS), glutamate gets released into the synapse and binds to glutamate receptors. By binding to the post-synaptic receptor, a cation channel opens up in order to depolarize the neuron. The post-synaptic neuron will then become activated until the excitatory neurotransmitter detaches from the receptor. Of importance to this study, ionotropic glutamate receptors (iGluRs) play a major role with it being a ligand-gated ion channel for fast synaptic responses.\(^4\) Ionotopic receptors are divided into N-methyl-D-aspartate (NMDA) receptors and non-N
MDA. The ligand-gated receptors allows ions such as sodium ($\text{Na}^+$), potassium ($\text{K}^+$), and calcium ($\text{Ca}^+$) from entering or leaving the cell membrane (Figure 1).

Another subtype is $\alpha$-amino-3-hydroxy-5methyl-4-isoxazolepropionic (AMPA) which causes an influx of sodium and ultimately changes the charge on the neuron due to the binding of glutamate.\textsuperscript{5} Both NMDA and AMPA receptors are activated by glutamate. However, glutamate receptors can be toxic to neurons when there is an excessive amount of it that causes a toxic influx of calcium.\textsuperscript{6} Because there is a continuous binding of the excitatory neurotransmitter that is unable to detach from the receptor, this leads to
excitotoxicity and cell death (Figure 2). In an effort to design novel drugs to treat neurodegenerative diseases, suitable models that display glutamate excitotoxicity are needed.

Figure 2. Overstimulation of glutamate causes a toxic influx of calcium

In a past study by Mano and Driscoll, glutamate excitotoxicity was previously explored in Caenorhabditis elegans (C. elegans) and was found to be similar to neurodegeneration in humans which was proven useful for the Williams lab. C. elegans is the animal model of this research because of its low cost and the ability to provide data from a whole animal perspective with the sensory and neuromuscular system by readily
visualizing the neurons. They developed a model, ZB1102, that contains the glutamate transporter 3 knockout (GLT-3 KO) to study glutamate excitotoxicity. By having the GLT-3 KO, glutamate is unable to be removed, thus causing overstimulation of neurotransmitters. Despite the lack of evolutionary complexity, *C. elegans* share many conservative molecular pathways as well as cellular mechanisms with mammals, thus allowing for comparative studies. In other words, *C. elegans* contain a genome that shares homology with mammals. As a result, the considerable attributes *C. elegans* can be utilized are found in a multitude of different models of research for neurodegenerative diseases.

![Chemical structure of 5-Hydroxy-2-(2-phenylethyl)chromone](image)

*Figure 3. The chemical structure of 5-Hydroxy-2-(2-phenylethyl)chromone*

In a study by Yoon *et al.*, 5-hydroxy-2-(2-phenylethyl)chromone (5-HPEC) (Figure 3) showcased neuroprotective activity against glutamate-induced excitotoxicity in primary cultures of rat cortical cells. The Williams’ lab sought to investigate the work of 5-HPEC in *C. elegans* and was established to be a suitable model for testing suitable drug candidates. As stated in Madeline Harding’s Senior Individualized Project, 39.23 mM 5-HPEC significantly reduced the quantity of necrotic neurons in the glutamate excitotoxicity models. 5-HPEC is part of the family compound, 2-(2-phenylethyl)chromone which is part of the class of chromones (Figure 4). They contain pharmacological properties and derivatives that can lead to new therapeutic agents.
As a subclass of 2-(2-phenylethyl)chromone, 7-hydroxy-2-(2-phenylethyl)chromone (7-HPEC) (Figure 5) has the potential to have neuroprotective effects against glutamate excitotoxicity. In this study, however, 7-HPEC will be used to compare 5-HPEC. The difference in the compound is the hydroxyl group at the 7 position instead of the 5 position and the goal is to investigate the difference it makes.

By using *C. elegans* as the model animal, we will be able to investigate the link between glutamate excitotoxicity and 7-HPEC. The two assays will be a quantification of necrotic neurons in the heads of the model animal and investigation of the lifespan. Previous work done in the Williams’ lab done by Harding proved 5-HPEC has significant neuroprotective effects as the p-value is 0.0279. Many of neurodegenerative diseases are terminal and can greatly diminish the life expectancy once diagnosed. We sought to determine if an analogous decrease in life expectancy would be seen in *C. elegans*. If there is a link, it could be possible that 2-(phenylethyl)chromone as a whole has
neuroprotective activity. With the results, there are potential treatments for neurodegenerative diseases that will affect millions of people worldwide.
Materials and Methods

C. elegans Strain Types

With C. elegans being used as the model animal, the different strains were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (CGC, St. Paul, MN, USA). The two different types of strains that were used are N2 Ancestral and ZB1102. N2 Ancestral is used as the wild-type and ZB1102 has the knockout gene of glutamate transporter 3.

Table 1. C. elegans strain types used

<table>
<thead>
<tr>
<th>STRAIN TYPE</th>
<th>PHENOTYPE</th>
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<tbody>
<tr>
<td>N2</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZB1102</td>
<td>Glt-3 KO</td>
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Maintenance of Nematodes

Once the C. elegans were obtained from the CGC, they were first moved to a 60 mm petri dish of agar with a layer of *Escherichia coli* (OP50) by being chunked. With the original petri dish, a sterilized spatula was used to cut a square with at least 1 C. elegans and placed it upside down on the new petri dish. The OP50 solution was made by streaking OP50 bacteria, also acquired from the CGC, onto an agar petri dish. Then, a colony of bacteria was added to LB broth, a tablet of LB dissolved in 50 mL of Milli-Q water was autoclaved.

Preparation of Agar Petri Plates

The materials used for the agar solution were 3 g sodium chloride, 2.5 g peptone, 17 g of agar, and 975 mL of 18.2 MΩ Milli-Q water that was autoclaved for an hour in a 2000 mL Erlenmeyer flask. Once the solution was autoclaved and cooled down for 20
minutes, 25 mL of 1 M potassium phosphate at pH 6, 1 mL of 1 M calcium chloride, 1 mL of cholesterol, and 1 mL of 1 M magnesium sulfate were added. The agar solution was poured into 60 mm petri dishes. The plates were left at room temperature overnight in a laminar flow hood and then placed in a 4°C freezer until needed.

**Treatment of C. elegans with Vehicle and 7-HPEC**

The vehicle used for the *C. elegans* was a 1% acetone solution in OP50. The total volume in the 1.5 Eppendorf tube is 1000 μL, 10 μL is acetone and 990 μL is OP50. To 35 mm Petri plates, 50 μL of the 1% acetone solution was added to each for the worms to be grown on. For 7-HPEC drug treatment, 5 mg of 7-HPEC was measured into a 1.5 mL Eppendorf tube. Sterile-filtered acetone was then added in 10 μL increments, vortexing after each addition until 7-HPEC completely dissolved. The solution turned into a clear purple color. Then, 3.2 μL of the 7-HPEC and acetone solution, which is 1% of the total volume, was added to 316.8 μL of OP50. 50 μL of the 19.81 mM 7-HPEC solution was then added to 35 mm Petri plates.

**M9 Solution**

A mixture of 6 g of sodium phosphate, 3 g of potassium phosphate, 5 g of sodium chloride, and 1000 μL of magnesium sulfate was added to a one-liter volumetric flask. Enough Milli-Q water was added to the neck of the flask and the mixture was stirred on a hot plate until the solution dissolved. The stir bar was then removed, and Milli-Q water was added to the 1000 mL line in the flask. The solution was distributed to 500 mL bottles and autoclaved for 1 hour.

**Age-Synchronizing C. elegans**
For the lifespan assay, the worms were age-synchronized to grow until L4 stage and for neuron counting, the worms were age synchronized until L3 (Figure 6). At L4 stage, nematodes began their adult phase and can be differentiated by their white kidney-like shape (Figure 7). At L3, necrotic neurons were shown to be more prevalent than at any other stage as shown by the Driscoll lab.

Figure 6. The nematode is shown at the L3 stage.15

Figure 7. The nematode is shown to be at the L4 stage.15

Plates with a great number of adults that have eggs were washed multiple times with 700 mL of M9 and then placed into an Eppendorf vial. The tube was centrifuged for 1 minute at 3000 rpm and then the supernatant was disposed of without disrupting the pellet of worms. This process was repeated two more times. To the existing 700 mL of M9 solution and pellets, 1 mL sodium hydroxide and 1 mL of 4% Clorox bleach were added and then vortex for 30 seconds intervals for about 3 minutes until the worms were completely dissolved. M9 was added to fill the vial and was then centrifuged. The supernatant was discarded and added 700 mL of Milli-Q water which was then vortexed
for 3 seconds. After centrifuging the vial, it was repeated two more times. Lastly, the supernatant was discarded and 500 mL of M9 was added to be mixed in with the pellet and then transferred 60 μL of the solution onto an agar plate after removing 60 μL of the solution from the vial. The plate was then looked at under the microscope to view the eggs.

**Lifespan Assay**

For both the control and 1% acetone solution, 20 L4 worms were added to 35 mm plates. Each day, the worms moved to another plate if they were alive. The alive were seen moving and the dead worms were shown to not move or did not have a response to the platinum pick. The data was recorded until all the worms were dead.

**Prepare Slides for Neuron Counting**

With one microscope slide, a drop of 4% Agarose was added to the center of the slide using a disposable pipette. Another slide was placed on top and pressed so that the agar could spread to about the size of a nickel. The slide with the tape was carefully removed without disturbing the agar and about 10 μL of Milli-Q water was added to the center. About 50-60 worms were placed in the water at the L3 stage using a platinum pick. A slide cover was carefully placed on top of the water with little air bubbles.

**Differential Interference Contrast Microscopy**

To determine the number of necrotic neurons per worm in the head, a differential interference contrast (DIC) microscope (Zeiss Axiophot 2) (Carl Zeiss Inc., Thornwood, NY, USA) was used at 63x focus. A necrotic neuron was identified in the head, between the mouth and pharynx, as an empty vacuole (**Figure 8**), and at least 200 worms were quantified. Each strain was blinded before collecting data to restrain experimenter bias.
Statistical Analysis

Results were analyzed using Graphpad Prism 9 software (Graphpad Software, Inc., La Jolla, CA, USA). One-way ANOVA with Tukey’s Post Hoc multiple comparison was used to compare the statistical difference between the different groups.
Results

The objective of the study is to investigate the neuroprotective effects of 7-HPEC in *C. elegans*. The two assays used were lifespan assay and using DIC microscopy to quantify the number of necrotic neurons present in two different strains: N2A (wildtype) and ZB1102 (GLT-3 KO) in the head of the model animals.

**Necrotic Neurons using DIC microscopy**

Using the DIC microscopy, necrotic neurons viewed as vacuoles were counted in the head of the animal (Figure 8).

![Figure 8](image)

*Figure 8. Necrotic neuron identification. A) Healthy wild-type N2A with no necrotic neurons present. B) ZB1102 strain with one necrotic neuron in the blue circle. These are representative images viewed at 63x magnification on a Zeiss Axiophot 2.*

**Necrotic Neurons in Controlled *C. elegans***

![Graph](image)

*Figure 9. There were significantly more necrotic neurons in ZB1102 animals than N2. Error bars are standard error of the mean (p<0.0001). N2 n=249, ZB1102 n=255.*
As predicted, ZB1102 model had significantly more necrotic neurons than N2A (p<0.0001) (Figure 9). On average, the untreated wild-type worms had about .623 necrotic neurons and the untreated GLT-3 KO worms had about 1.69 necrotic neurons.

**Necrotic Neurons in *C. elegans* with the Vehicle**

![Graph showing necrotic neurons in N2 and ZB1102 with and without acetone treatment.](image)

*Figure 10. There were significantly more necrotic neurons in ZB1102 animals than N2. Error bars are standard error of the mean (p<0.0001). N2 and ZB1102 n=189.*

As with the untreated model, ZB1102 animals had significantly more necrotic neurons than N2A (p<0.0001) (Figure 10). On average, the untreated wild-type worms had about .53 necrotic neurons and the untreated GLT-3 KO worms had about 1.35 necrotic neurons. Unexpectedly, treatment with 1% acetone increased the average number of necrotic neurons compared to the untreated models.

**Necrotic Neurons in *C. elegans* with 7-HPEC**

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Figure 11. Treatment of 19.81 mM 7-HPEC has decreased the quantity of necrotic neurons found in each model animal. Error bars are standard error of the mean (p<0.0001). N2 and ZB1102 treated with 1% acetone and 19.81 mM 7-HPEC n=216.

Treatment of 19.81 mM 7-HPEC had an impact in decreasing the number of neurons for the wild-type strain as well as the GLT-3 KO strain (Figure 11). The average number of necrotic neurons N2 with 1% acetone and 19.81 mM 7-HPEC was about .47 compared to 1.21 necrotic neurons ZB1102 with 1% acetone and 19.81 mM 7-HPEC had.

Comparison of Necrotic Neurons between 5-HPEC and 7-HPEC
Figure 12. Comparison between 58.67 mM 5-HPEC and 19.81 mM 7-HPEC in both N2A and ZB1102 animals. Error bars are standard error of the mean (p<0.0001). ZB1102 treated with 1% acetone and 58.67 mM 5-HPEC n=39 and ZB1102 treated with 1% acetone 19.81 mM 7-HPEC n=216. Data for 5-HPEC was acquired from Skyler Rogers.

Between the two treatments, 58.67 mM 5-HPEC and 19.81mM 7-HPEC, there was a significant difference since the p<0.05. The average necrotic neurons found in ZB1102 with 5-HPEC was about .67 and the average number of neurons found in ZB1102 treated with 7-HPEC was 1.21.
**Lifespan Assay**

Without the drug or vehicle, the two strains had the same rate after day 10. Although N2A started to die off quicker, it was not apparent until day 7 when 13 N2A worms died compared to 9 ZB1102 worms died the same day. The 1% Acetone treatment altered the rate of death in both. All the *C. elegans* animals were dead by day 26 in both the control and acetone lifespan assay.

![Graph showing lifespan comparison between N2 Control and ZB1102 Control](image)

**Figure 13.** The wild-type animals had a faster death rate compared to the ZB1102 strain with the GLT-3 KO. Average lifespan per strain represented as mean ± SE. N2A: 92.42 ± 16.01, ZB1102: 145.38 ± 14.22. p-value (compared to N2A) < 0.05 for ZB1102. n=105 for N2A and n=153 for ZB1102.
Figure 14. Treatment with 1% Acetone led to a decrease in lifespan for the wild-type animals. With 1% acetone treatment, ZB1102 animals had about the same death rate. They also had a faster death rate compared to the control and wild type with acetone. Average lifespan per strain is represented as mean ± SE. N2A: 35.14±6.99, ZB1102: 44.99 ± 9.31. p-value (compared to N2A) < 0.05 for ZB1102. n=125 for N2A and n=120 for ZB1102.
Discussion

The objective of this study was to investigate the neuroprotective effect of 7-HPEC on glutamate excitotoxicity using *C. elegans* as the model animal. 7-HPEC was chosen due to its compound structure being similar to 5-HPEC, a compound having neuroprotective activity in cell culture. The structural difference between the two is that 7-HPEC has a hydroxyl group at the 7th position rather than the 5th position. In this experiment, it was implicated that 7-HPEC had a slight effect in reducing the number of necrotic neurons in the GLT-3 knockout animals. To measure neurodegeneration, a lifespan assay with the control and vehicle was done as well as using DIC microscopy to quantify the number of necrotic neurons of each strain.

**DIC Microscopy**

The glutamate knockout strain ZB1102 had significantly more necrotic neurons than the wild-type N2A strain. The result from the study is consistent with past studies which support the idea that the GLT-3 KO gene directly causes an increase in necrotic neurons. ZB1102 having fewer neurons with the treatment of 7-HPEC can indicate that there are neuroprotective effects against glutamate excitotoxicity in the GLT-3 KO model (Figure 11). Compared to the 5-HPEC treatment, nematodes that were treated with 7-HPEC had on average, more neurons per worm which can conclude that the drug may not be a possible treatment for neurodegenerative diseases (Figure 12). 7-HPEC may not have as much neuroprotective activity when compared to 5-HPEC considering it is not a serotonin ligand.

The concentration of 7-HPEC was much lower than 5-HPEC with it being 19.81 mM and 5-HPEC being 58.67 mM. This can give an explanation as to why the drug did
not work as well as expected. However, if the concentration of 7-HPEC was much higher than 5-HPEC and there were no significant difference, this could mean that the concentration was passed it’s threshold.

**Lifespan Assay**

The results from the lifespan assay were unexpected as there was little change in the difference between the wild type and GLT-3 KO strains. This does not accurately express the neuroprotective effects of glutamate excitotoxicity as it was expected for the GLT-3 KO strain to have a quicker death rate and shorter lifespan. If there is an influx of calcium that leads to cell death, the conclusion is that there would be a shorter lifespan. However, it could be possible that neurons do not accurately measure the cause of a shorter lifespan. Life expectancy among the United States population is about 76 years old.\(^1\)\(^6\) Although the severity of the disease, treatment, and symptoms differ, the life expectancy can vary for those who are suffering from neurogenerative diseases which can range from 56 to 80 years old.\(^1\)\(^7\) A novel drug can potentially decrease the amount of necrotic neurons and quality of life but may not increase or neutrally affect life expectancy.

**Limitations and Further Direction**

A limitation was that there could have been human error in handling the *C. elegans* when transferring them from plate to plate, especially because two people were handling them and had different methods. When comparing the two drugs, it might not have been accurate considering that there was a significant difference in the number of worms quantified, 39 worms were counted for 5-HPEC and 216 worms were counted for 7-HPEC. Future possible studies are changing the concentration of the 7-HPEC such as
decreasing or increasing the amount to accurately understand the effect of the drug. This can ensure that there is maximum concentration when dissolved in acetone. Instead of focusing on the lifespan assay and neuron counting, future studies can focus on neuron counting and a locomotor assay. A locomotor assay can improve upon whether glutamate excitotoxicity impacts locomotor activity. Another possible future direction is adding the JCB169 and CB6193 strains. JCB169 has GLT-3 KO as well as BUS-8 mutation whereas CB6193 only has the BUS-8 mutation. The BUS-8 mutation could allow for better absorption of 7-HPEC, therefore, increasing the neuroprotective effects.

In conclusion, this study showed that 7-HPEC does not have a significant neuroprotective effect against glutamate excitotoxicity in both the wild-type and GLT-3 KO strains. There were a decreased amount of neurons in both strains. However, there will need to be further research on the impacts of 7-HPEC to hopefully treat neurodegenerative diseases. Work from the Williams’ lab as suggested a potential drug, 5-HPEC, being interactions with Serotonin receptors, however, it does not seem that 7-HPEC has the same interactions and that may explain the lack of neuroprotective activity.
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