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" Effects of common perturbing agents on neurons and glial cells in culture"

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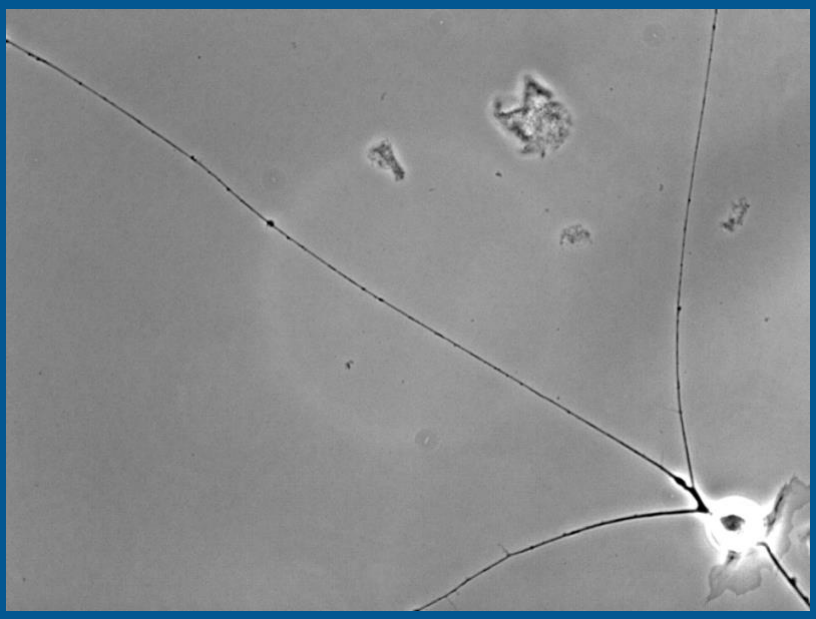

Preliminary study on the effects of an autophagy agonist on axonal transport in *Gallus gallus* peripheral sympathetic neurons

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Introduction:

Proteostasis is an integrated set of biological pathways associated with biogenesis, protein folding, trafficking, and degradation of proteins intracellularly and extracellularly. Disruption in proteostasis can lead to neurodegenerative diseases, like Alzheimer's. Underlying proteostasis is autophagy, a major mechanism for removing protein aggregates (Novack, 2017). Autophagy is a temporary survival mechanism that maintains intracellular homeostasis, remodeling development, and regulates metabolism (Wang et.al, 2015). In neurons, autophagy is essential for cell growth, synaptic formation/plasticity, and transporting essential molecules in autophagic vacuoles across axons (Lee, 2012). Previous studies examining the relationship between autophagy and neurodegeneration have displayed a negative correlation (Nilsson et.al, 2013). These findings insinuate that there may also be a negative correlation between autophagic vacuole velocity and neurodegenerative diseases, such as Alzheimer's.

Additional studies have examined the relationship between proteostasis and Alzheimer's. Studies have shown that the disruption of proteostasis and autophagy results in a cascading effect of Beta-amyloid aggregates collecting externally on the myelin sheath of axons, ultimately inhibiting neuron communication and function (Genereux, 2015). These findings suggest that autophagic vacuole transport down axons are adversely affected. This study aims to examine autophagy in *Gallus gallus* Peripheral Sympathetic Neurons (PSNs). Specifically, to investigate whether acute exposure of an autophagy agonist can increase the velocity of autophagic vacuoles. Physiologically, if autophagy becomes inefficient this can result in neurodegenerative disorders causing dementia. Autophagy agonists may be an avenue for remedying neurodegenerative diseases (Novack, 2017).

Prior studies have examined the efficacy of autophagy agonists on reducing the effects dementia. Tetrandrine is an autophagy agonist that has displayed neuroprotective effects, when administered intraperitoneally (IP injection), to rats with dementia (Yan-ling et.al, 2016). This drug is a member of the bisbenzylisoquinoline alkaloids from *Stephaniae tetrandrae*, a traditional Chinese medical herb. Tetrandrine has also been shown to have an underlying mechanism associated with the production of intracellular reactive oxygen species after a 24-hour exposure in liver cells with cancer (Wang et.al, 2015). Thus, tetrandrine poses as a potential therapeutic remedy for neurodegenerative diseases like Alzheimer's. The suggested mechanism of tetrandrine interacting with neurons, is by upregulating autophagic transport of autophagic vacuoles. It is hypothesized that acute exposure to tetrandrine will increase the velocity of autophagic vacuoles along the axons in 1-day old PSNs.

Furthermore, this study encompasses a broader public health issue, which is that Alzheimer's disease and many other neurological disorders are related to aging. Alzheimer's alone affects 5.4 million Americans annually, costing $385 billion per year (Swerdlow, 2011). The mechanism of Alzheimer's disease is complex and remains unclear. With the baby boomer era aging, the number of those who develop Alzheimer's disease is likely to increase precipitously. It is incumbent for biomedicine to resolve this conundrum.

Materials and Methods:

Primary Culture and Dissection of embryonic peripheral chick neurons

Embryonic peripheral chick neurons were prepared and dissected according to Morris's method (Morris, 2017a-b). Chick neurons and glial cells were cultured in F+ medium, which consisted of 100ml Leibovitz L-15 medium, 2mM glutamine, 0.6% glucose, 100 U,μg/ml pen/strep, 10% fetal bovine serum, and 50 ng/ml neuron growth factor.

Coverslip Preparation

Published methods were used for coverslip treatment and preparation (Morris, 2017b).

Stock Solution and Cell Culture Exposure

Preparing Stock Solution

50mg of tetrandrine was purchased from abcam, catalog No. (ab142464). 11 mg of tetrandrine was vortexed in 11 ml of Dimethyl sulfide (DMSO) to produce a 1mg/1ml stock solution. DMSO was used as the solvent, because it most effectively dissolves tetrandrine, which as both polar and non-polar groups (abcam).

Control condition

The control condition was prepared by mixing 12.5ul of DMSO with 2ml of F+ medium. First, the original media in wells, containing a high-density volume of 1-day old PSNs, was aspirated. Subsequently, the control media was administered to the wells with PSNs and incubated for 2 hours at 37^{o}C.

Experimental conditions

Two different concentrations of tetrandrine were administered to separate wells containing peripheral sympathetic neurons. 10uM concentrations of tetrandrine were prepared by mixing 12.5ul of the stock solution with 2ml of F+ medium. And 5uM concentrations of tetrandrine were prepared by mixing 6.25ul of stock solution with 2ml of F+ medium. First, the original media in wells, containing a high-density volume of 1-day old PSN's, was aspirated. Subsequently, either 2ml of 10uM or 5uM tetrandrine was administered to the wells with PSNs and incubated in their respective medias for 2 hours at 37^{o}C.

Chip Chamber Protocol

After the 2-hour incubation for the control and experimental conditions, published methods were used to prepare Chip Chambers in order to examine the neurons microscopic observation (Morris, 2017c).

Data Collection

Images were capture using a Nikon Eclipse E200 microscope at 40x and 100x magnification, Apple iMac Computer with Operating System: MacOS X 10.13.6, and SPOT software: SPOT Basic version 5.4.3, and an Idea 3.0 MP Color Mosaic camera. Once a neuron with axons was located, autophagic vacuoles were imaged using video-enhanced phase contrast microscopy. Axons containing autophagic vacuoles were focused at 40x and 100x magnification. 30-second or 1-minute time lapses captured at 1/5 frame per second (FPS) were collected. Time lapses were discarded if no vacuole movement could be observed with the naked eye. Phase dense ovoid structures that were stationary were excluded from analysis, because motion is a key characteristic of autophagic vacuoles. According to previous studies, a stationary phase dense ovoid structure cannot be confidently determined as an autophagic vacuole (Morris and Hollenbeck, 1993). Thus, only moving phase dense ovoid structures were analyzed. Furthermore, vacuoles that could not be observed for the entire time lapse were excluded from data analysis. The control condition, experimental conditions, and all images were collected in collaboration with Hannah Woloschuk. Furthermore, James Marcucella's, 2018, results related to ROS and tetrandrine exposure in PSNs were incorporated into this study. Collaborating with Marcucella afforded the opportunity to potentially determine if ROS produced by tetrandrine is also increased in PSNs, as seen in prior studies analyzing cancer cells and tetrandrine exposure (Wang et.al, 2015). Marcucella captured fluorescent images using Nikon Eclipse E200 microscope with a SPOT Firewire 2 camera and the same computer operating system.

Data Quantification

Autophagic vacuole criteria

FIJI Version 1.0 was used to measure the velocity of autophagic vacuoles moving anterograde and retrograde across axons. Anterograde movement is towards a synapse, and retrograde movement is towards a cell soma. The criteria for identifying autophagic vacuoles is as follows. The size, phase density, and movement were the necessary criteria. Phase dense ovoid structures that could be visually observed moving either in an anterograde or retrograde direction along an axon, between consecutive image frames, and have an area between 7-11 um^2 were classified as autophagic vacuoles (Hollenbeck, 1993). The shortest movement observed visually in this study was 0.036 um/sec. The edge of a vacuole was determined by enlarging an image by 20-30 times and examining where the pixels of the phase-dense molecule faded into the lighter colored axon. The darker opaque pixels were considered the vacuole and the lighter pixels were considered the axon. See Figure 1, for a visual on how the edge of an autophagic vacuole was distinguished from an axon.

Calculating Velocity

In the control condition 5 autophagic vacuoles were analyzed, in the 5uM tetrandrine treatment 5 vacuoles were analyzed, and in the 10uM tetrandrine treatment 5 vacuoles were analyzed. A total of 138 instantaneous velocities, and 3 average velocities were calculated for a total of 11.5 minutes. In the control and experimental conditions, the instantaneous and average velocity of autophagic vacuoles were quantified. The velocity of an autophagic vacuole was determined by measuring the distance one of its edge's travels between subsequent images in units of pixels. Once a vacuole was located for analysis, the images were expanded by 20-30 times. The end of a vacuole was determined and tracked along its trajectory frame by frame. For each frame collected in a time lapse, the coordinates of the end of an autophagic vacuole were recorded. The distance, in pixels, traveled between each image was calculated using Pythagoreans theorem. Subsequently, this pixel distance was converted to micrometers using one of the following conversion formulas. At 40x there are 6.21 pixels / 1um. And at 100x there are 15.5 pixels / 1um. Since the times lapses were collected at 1/5 FPS, there is 5 seconds between each image. Thus, the instantaneous velocity of an autophagic vacuole between each image in a time lapse was calculated by dividing distance traveled over time. The average velocity was calculated by averaging all the instantaneous velocities within each condition. See Figure 1, for how the distance vacuoles travel, in pixels, are converted to um/s.

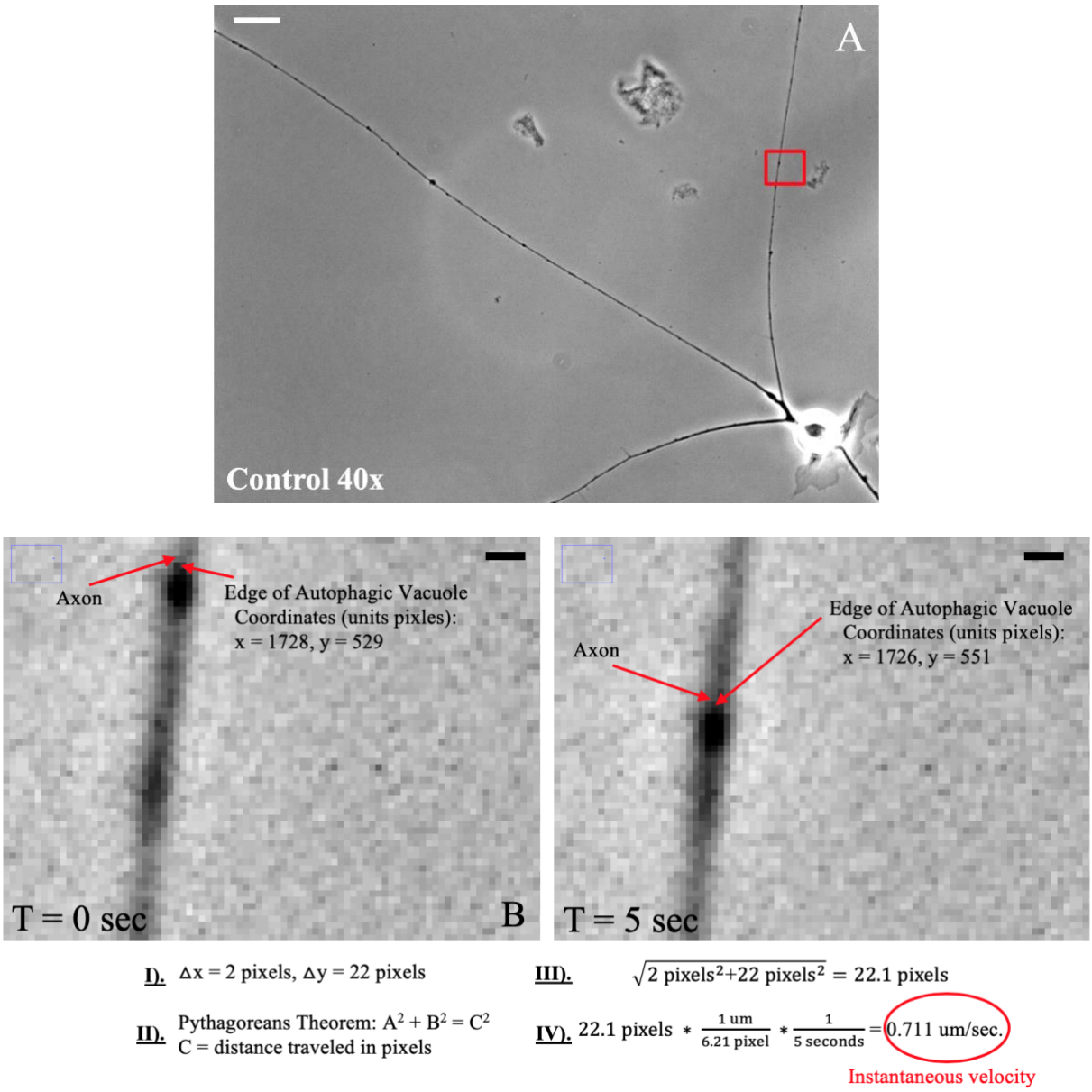

Figure 1: Determining the edge of an autophagic vacuole and calculating velocity protocol
Figure 1A displays a transmitted light image of a 1-day old PSN at 40x after a 2hr exposure to 12.5ul DMSO (control condition). Both retrograde and anterograde movement of autophagic vacuoles were observed in the three axons shown in the top image. The red box is one area where vacuole movement was seen. The bottom two images, in Figure 1B, display the area of the red box in the top image. The sequence of bottom images shows how the edge of an autophagic vacuole was distinguished from the axon, using the protocol described in the *autophagic vacuole criteria section* of the methods and materials. The contrast and brightness were increased to observe a more precise difference between the edge of a vacuole and an axon. There is a five second difference between the two images in Figure 1B. Pythagoreans theorem was used to determine the distance a vacuole traveled, and a conversion formula was used to convert pixels into meters. The white scale bar in the top left corner Figure 1A is 20um. The black scale bar in the top right of the images in Figure 1B are 1um.

Results:

Collaborating with James Marcucella afforded the opportunity to understand how tetrandrine affects intracellular ROS levels in PSNs. As shown in Figure 2, Marcucella displayed that tetrandrine significantly increased ROS levels in the cell bodies of PSNs (Marcucella, 2018). This is significant, because these data support that tetrandrine was interacting intracellularly similar to previous studies (Wang et.al, 2015).

Time lapse images collected for the control and acute 5uM and 10uM tetrandrine treatments displayed autophagic vacuole movement. Both anterograde and retrograde movement were observed amongst the 15 vacuoles analyzed. Figure 3 represents a time lapse of a control sample at 40x magnification. The red box representing the area magnified in Figure 3, shows the retrograde movement of one autophagic vacuole examined. The other 14 vacuoles analyzed between the control and tetrandrine treatments displayed the same retrograde movement observed in Figure 3, as well as anterograde movement. No vacuoles analyzed displayed both retrograde and anterograde movement. Additionally, the movements captured at 100x magnification looked analogous to the vacuole's motion represented in Figure 3.

The *Calculating velocity* protocol, described in the *Data Quantification* section of the Materials and Methods, was used to calculate the instantaneous velocity of vacuoles analyzed in each time lapse collected. The average velocity of vacuole movement in each condition was calculated by combining all the instantaneous velocities in a single condition. Figure 4 represents the results of the average velocity calculated in the control and acute tetrandrine treatments. A Students-T test determined that there is no significant difference in vacuole velocity between the control and both experimental conditions at a level of $P = 0.05$.

Furthermore, data received from Hannah Woloschuk demonstrated that 5uM and 10uM tetrandrine did not have a significant effect on the number of autophagic vacuoles present per 1um in 1-day old PSNs. These findings suggest that the highest concentration of tetrandrine tested, 10uM, did not significantly increase autophagy in PSNs. (Woloschuk, 2018).

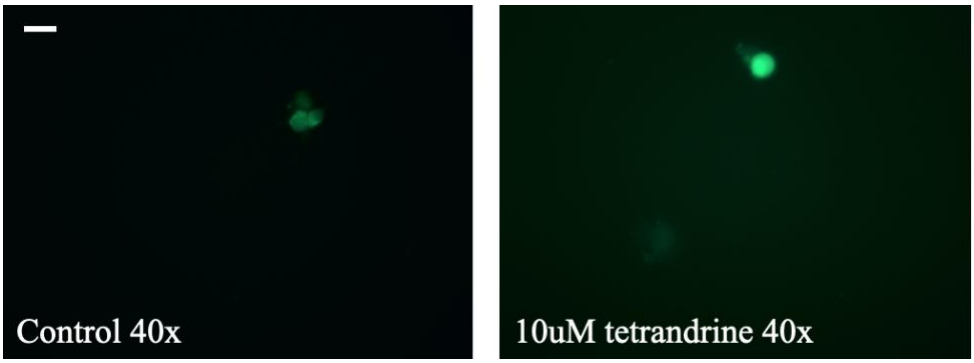

Figure 2: Tetrandrine produces reactive oxygen species (ROS) in PSNs
Data collected from James Marcucella displays ROS production in a PSN without tetrandrine administered and in a PSN exposed to 10uM tetrandrine for 2hrs. Using the fluorescent probe 2',7', dichlorofluorescin diacetate (DCF), ROS levels were measured using published methods (LeBel, Ischiropoulos, & Bondy, 1992). The green dots labeled are the cell bodies of PSNs. Both images were captured at 40x magnification. The image on the left is a control condition (DMSO). And the right image represents ROS production after a 2hr exposure of 10uM tetrandrine. The scale bar in the top left corner of Figure 2 is 20um.

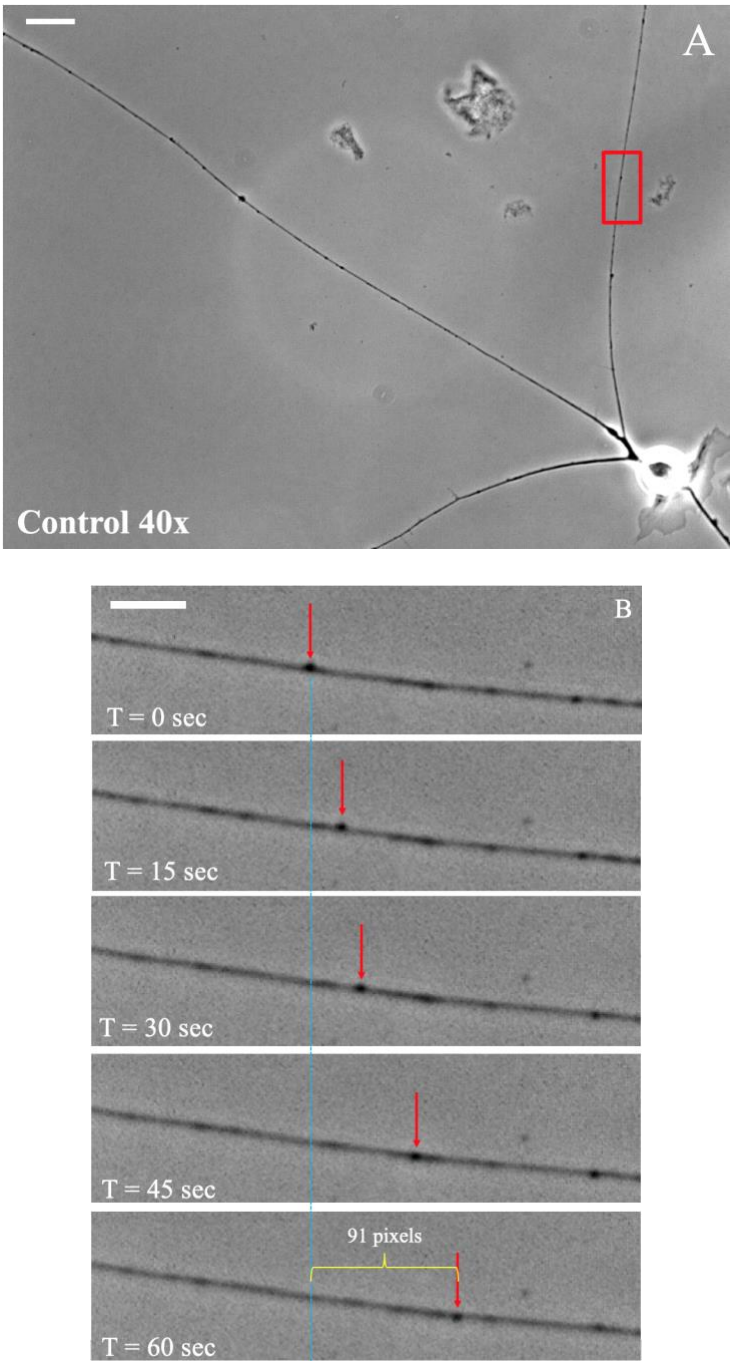

Figure 3: Autophagic vacuole movement in a 1-day old control peripheral sympathetic neuron. Figure 3A is a transmitted light image of a 1-day old PSN at 40x after a 2hr exposure to 12.5ul DMSO (control condition). The series of images below (Figure 3B), represent a time lapse of the area in the red box. The red arrows indicate the location of an autophagic vacuole displaying retrograde movement. There is a 15 second difference between subsequent images in Figure 3B. The blue dashed line is the initial location of the vacuole being analyzed. And the yellow bracket on the bottom image of Figure 3B, is the total distance the vacuole moved during the time lapse. The scale bar in the top left corner of Figure 3A is 20um. The scale bar in the top left corner of Figure 3B is 5um.

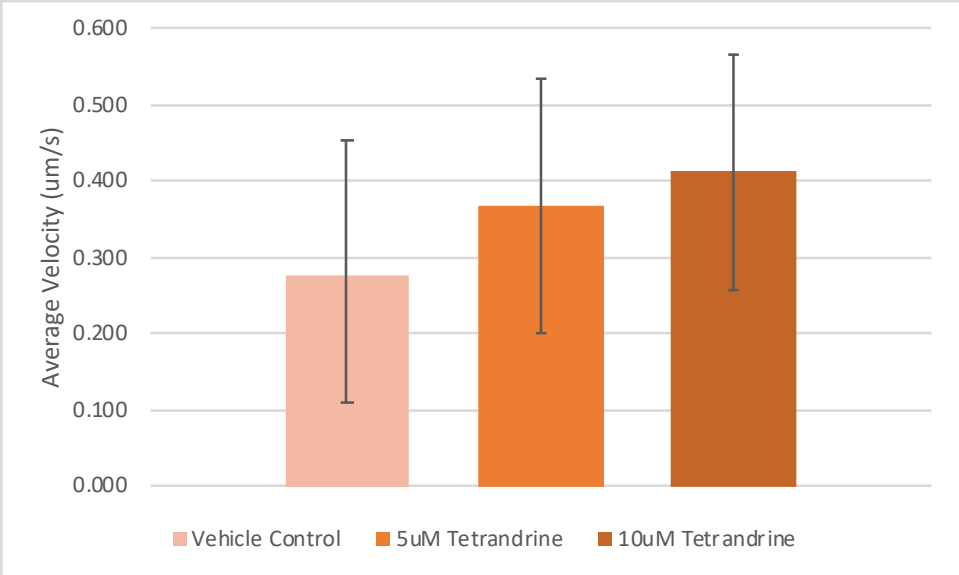

Figure 4: Average velocity of autophagic vacuoles in 1-day old PSNs following 2-hour exposure to tetrandrine

Figure 4 represents the average velocity in um/s, of autophagic vacuoles in the control and experimental conditions. The different conditions were a vehicle control (12.5ul DMSO), 5uM tetrandrine, and 10uM tetrandrine. Chip chambers containing 1-day old PSNs were administered their respective condition for 2 hours. A Students T-test demonstrated no significant statistical difference between the experimental conditions and the control at P = 0.05 (92 instantaneous velocities were compared to each other).

Discussion and Conclusion:

It was hypothesized that the autophagy agonist, tetrandrine, would significantly increase the velocity of autophagic vacuoles across axons in 1-day old peripheral sympathetic neurons (PSNs). However, the results collected in the current study appear to contradict the hypothesis. Although acute exposure of increasing concentrations of tetrandrine suggests to have a positive trend with vacuole velocity, this trend was not statistically significant. As shown in Figure 4, administering 5uM and 10uM tetrandrine for 2hrs to PSNs, the average vacuole velocity seems to increase. However, the velocity of only 5 autophagic vacuoles in the control and 10 vacuoles in the experimental conditions were quantified. Thus, a final conclusion on tetrandrines effectiveness as an autophagy agonist, in PSNs, cannot be conclusively determined at the moment. Consequently, this positive trend is intriguing and future studies should analyze more vacuoles to obtain more conclusive results.

Previous studies examining cancer cells and ROS established that 30uM tetrandrine induces apoptosis. And 5uM tetrandrine administered to liver cells with cancer induced autophagy (Wang et.al, 2015). These results insinuate that PSNs may be more resistant to tetrandrine compared to liver cells with cancer. But, PSNs cannot be assumed to express similar apoptotic characteristics as cancer cells.

Determining how tetrandrine interacted intracellularly with PSNs was important to understand how applicable this experimental design was to prior studies. Marcucella's, 2018, results displayed that tetrandrine increases ROS levels in PSNs compared to control groups

(Marcucella, 2018). Observing an increase in ROS using tetrandrine is similar to previous studies (Wang et.al, 2015). Ultimately, Marcucella shows that tetrandrine affects PSNs in a relatively predictable fashion, implying this experimental design is a practical model for study.

Furthermore, Woloschuk's, 2018, results supplement the current study by providing additional insight into tetrandrines affects in 1-day old PSNs. It was determined that even at the highest concentration tested (10uM tetrandrine) the amount of autophagic vacuoles per 1um was not significantly different than the control. Future studies should administer concentrations greater than 10uM tetrandrine to PSNs, to determine exactly at what concentration PSNs are apoptotic. This would grant the ability to test if the highest concentration of tetrandrine, that is not cytotoxic to PSNs, could elicit a significant increase in autophagy. Nevertheless, these preliminary results suggest that acute exposure to 10uM tetrandrine is not affecting autophagy in PSNs.

In relation to neurological diseases, the results of Woloschuk's and the current study imply, that tetrandrine's ameliorating effects observed in rodents, diagnosed with vascular dementia, are not the result of increasing autophagic vacuole velocity or autophagy in neurons (Yan-ling et.al, 2016). Compared to the results in the current study, Yan-ling's findings suggest that tetrandrine was affecting some other aspect of the rodent's physiology, rather than the velocity of autophagic vacuole transport across axons. However, it is important to mention, that previous studies used different methods of tetrandrine administration, an *in vivo* model, and examined cognitive abilities. Contrastingly, this current study used an *in vitro* model and analyzed tetrandrines effect in specific cells.

A limitation of this study, was that Beta-amyloid plaques were not used in experimentation. Thus, the effectiveness of tetrandrine on axonal transport with neurons that have a similar phenotype to a neurological disorder is only speculation at the moment. Another limitation was that only 1-day old PSNs, with autophagic vacuoles demonstrating movement were examined. Future experiments should administer concentrations of tetrandrine higher than 10uM, as well as other autophagy agonists, to PSNs of varying ages, with and without Beta-amyloid plaques. The purpose would be to gain more insight into how autophagy agonists effectiveness is related to aging and neurodegenerative disease.

Despite, acute exposure of 5uM and 10uM tetrandrine not being an effective autophagy agonist in 1-day old Peripheral Sympathetic Neurons (PSNs), this study enhances our understanding of proteostasis. It has been shown that hindering proteostasis can cause neurodegenerative diseases, such as Alzheimer's (Novack, 2017). Considering that autophagy underlies proteostatsis, and is a major mechanism for removing protein aggregates, like Beta-amyloid, perhaps the mechanism by which tetrandrine alleviated symptoms of dementia in effected rodents, was not by increasing autophagy compared to control groups; but, rather by increasing autophagy in rodents with dementia to a level that is commensurate to rodents without dementia (Novack, 2017, Yan-ling et.al, 2016)? It is pertinent to note, this proposed mechanism is speculation, from coupling the current preliminary results and previous studies. Ultimately, further exploring and comprehending the mechanism of neurodegenerative diseases will help enable bio-medicine to discover and develop an effective remedy.

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