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Abstract

Pyrethroid insecticides have been used for more than 40 years and account for 25% of the global insecticide market. With increasing use of pyrethroids over recent years, human exposure to these chemicals is more likely. Because of the assumed harmless effects of their use, pyrethroids have grown to become the major class of pesticides for household, agricultural and public health applications worldwide. The acute toxicity of pyrethroids to adults has been well-documented by the U.S. Environmental Protection Agency as moderately toxic to mammals. However, there is little evidence regarding the potential toxicological effects of the chemicals on mammalian neurodevelopment. Recent publications suggest that pyrethroids inhibit neurite outgrowth in PC12 cells; however there is limited information available that has examined this phenomena on mammalian neurons.

In the current study, we aim to develop a neurodevelopment assay that could be utilized to investigate the effects of pyrethroids on the development of neuronal cells from undifferentiated neocortical cells cultured from the embryonic *Mus musculus* brain. Throughout the 14 day culture, the differentiation of the mouse neocortical cells into neurons were monitored with fluorescent microscopy and measured via percent differentiated calculations. The results of this study revealed that neurite outgrowth begins at day eight of culture, and maximum differentiation of the cells into neurons occurred at day 14 of the culture, with a percent differentiation of 64.8%. It is predicted that future pyrethroid exposure of these differentiated cells will inhibit neuronal differentiation in correlation to this timeline, with maximum inhibition of differentiation occurring at the fourteenth day of growth for this particular culture timeline.

This evidence would suggest that these compounds may act as developmental neurotoxicants in mammals.

Introduction

Pyrethroid insecticides have been used for more than 40 years and account for 25% of the global insecticide market (Shafer et al., 2005). Both the naturally occurring pyrethrins isolated from the plant genus *Chrysanthemum* (Tran et al., 2006) and their synthetic equivalents the pyrethroids are potent and effective insecticides to which significant amount of human exposure is attributed. However, with few reports of human fatalities in regard to these chemicals, mammals have been found to be approximately three times less sensitive than insects to the toxic effects of the pyrethroids due to a faster rate of metabolic disposal of the chemicals from the body, and a much higher body temperature (Ray and Fry, 2006). Because of the assumed harmless effects of their use, pyrethroids have grown to become the major class of pesticides used for household, agricultural and public health applications worldwide.

Pyrethroids are one of the most popular pesticides used across the globe today, and their popularity is continuing to grow. To date, there are 16 pyrethroids registered for legal use in the United States alone in both agricultural and everyday consumer products (Bryant and Bite, 2003). These increasing opportunities for chemical exposure have raised doubt as to whether or not the pyrethroids are actually safe to man. As a number of recent publications have suggested that potential effects of these synthetic pesticides not considered in their original investigations may be pivotal for human health, it is necessary that new studies be conducted in a more accurate evaluation of these chemicals.

The acute toxicity of pyrethroids to adults has been well-documented, and the U.S. Environmental Protection Agency has documented them as moderately toxic (EPA Category II) to mammals (Soderlund et al., 2002). Despite the abundance of this information, there is little to no evidence regarding the potential effects of the chemicals on the neurodevelopment of

mammals. Under the Food Quality Protection Act of 1996, the U.S. Environmental Protection Agency (EPA) has been required to assess all potential risks that might be associated with any mammal that might be subjected to experiencing contact with the chemicals from either agricultural or consumer applications of the product (Shafer et al., 2005). Following a thorough assessment, the EPA is required to include a safety factor in evaluating the potential risk against developing individuals to the toxicity of the pesticides (Shafer et al., 2005).

The general unanimity of the few studies that are available that have aimed to investigate the potential detrimental effects of the pesticides on developing individuals have found that the extent of the age-related toxicity by the pyrethroids appears to be much larger than other classes of pesticides, regardless of use of type I or type II pyrethroid compounds. In a 1994 study with weanling and adult rats, 11 day-old weanling rats were observed to be 16 times more sensitive than adults to acute lethality to the type II pyrethroid deltamethrin, and 21 day-old rats were 7 times more sensitive than adults to acute lethality to deltamethrin (Sheets et al., 1994). The results of this study demonstrated that there is a large age dependence on the acute toxicity of pyrethroids to developing rats, which have lower metabolic capabilities than adult rats.

Though this general conclusion has been made, a safety factor can be included in the EPA evaluation only if significant scientific data exists regarding which possible effects the synthetic insecticides may have on developing mammals in regard to the developmental neurotoxicity (Shafer et al., 2005). Since there are very minimal amounts of studies that have been conducted to evaluate the effects of the pyrethroids on the developmental neurotoxicity of neonates, the current study aims is one small step toward this goal.

In the current study, a neurodevelopment assay was evaluated for the development of neuronal cells from undifferentiated neocortical cells cultured from the embryonic *Mus musculus*

brain. The neocortical cells were maintained in a fourteen-day culture, and the differentiation of the cells into neurons were monitored with fluorescent microscopy and measured via percent differentiated calculations. We hypothesize that cell differentiation into neurons will begin at day ten of the culture, and maximum differentiation will be at day fourteen of the culture. In future study, this neurodevelopment assay will be used to evaluate the potentially detrimental effects of the pyrethroids on developing neurons to investigate whether or not these compounds may also act as developmental neurotoxicants in mammals.

Materials and Methods

Embryonic mouse cortical tissue was purchased from BrainBits, LLC (Springfield, IL, USA). Prolong Gold antifade reagent with DAPI was purchased from Invitrogen (Grand Island, NY, USA). Anti-Neuron specific beta III Tubulin antibody [2G10] - Neuronal Marker was purchased from Abcam, PLC (Cambridge, MA, USA). Anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 was purchased from Invitrogen (Grand Island, NY, USA). All other reagents and chemicals were purchased from Sigma-Aldrich, Co., LLC (St. Louis, MO, USA).

Preparation of Poly-D-Lysine Coverslips

Small, round glass coverslips were obtained and placed in a 24-well cell culture plate. Aliquots of 100 µl of Poly-D-Lysine solution were added to each coverslip. The Poly-D-Lysine solution was allowed to incubate and dry to coverslips for 2 hours at 25°C under a hood. Once incubation time had elapsed, any remaining Poly-D-Lysine which had not dried to coverslips was removed and discarded. Each coverslip was then washed with 1000 µl of sterile H_2O and were air dried.

Neocortical Cell Culturing

Papain buffer (100 µl papain enzyme: 400 µl buffer) was incubated in a 30°C water bath for 30 minutes. Two hundred and fifty µl of the papain buffer was transferred to a centrifuge tube containing 400 µl Hibernate-A-$CaCl_2$™. The neocortical tissue medium was removed from its container and moved to a separate centrifuge tube for temporary storage. The mixture of papain buffer and Hibernate-A-$CaCl_2$™ solution was then transferred into the container containing the neocortical tissue. The cortex tissue was incubated in this solution at 30°C for 30 minutes. When tissue pieces were settled at the bottom of the container, all solution was removed from the tissue and considered waste. The cortex tissue was then resuspended in 1 ml of its original medium and gently titrated via use of a 200 µl pipette. The remaining medium was then added to the titrated tissue. The container containing the titrated tissue was then set aside for a brief time as to allow any tissue pieces to settle to the bottom of the container. The titrated solution was then transferred into a 15 ml conical vial. The solution was centrifuged at 200 x g for 4 minutes. The resulting supernatant was removed and discarded. The remaining pellet was resuspended in 1 ml Hibernate EB™ solution (2% B27 and 0.5 mM Glutamax).

A 20 µl aliquot of the cells was transferred into a microcentrifuge tube containing a 20 µl aliquot of 0.4% typan blue and were mixed and counted via use of a hemocytometer. The cells were then diluted in preparation for plating ~1500 – 2000 cells/well. The cells were then seeded for culture on previously prepared Poly-D-Lysine coverslips. The cells were incubated for a total of 14 days in a humid 37°C CO_2 incubator. Neurobasal medium (Hibernate EB solution™) and cytosine β-D-arabinofuranoside was added to each cultured coverslip every 2 days.

Permeabilization and Blocking

Coverslips of neuronal cell culture were chosen to be permeabilized at a schedule of two every two days in an effort to observe and analyze cell cultures arrested at different points of time during growth. Each coverslip was washed with PBS buffer three times, with each wash lasting approximately 5 minutes. An aliquot of dissolved 0.3 % Triton X-100 in PBS buffer solution was then added to each coverslip. The cells were then permeabilized in this solution for 5 minutes. The 5 minute PBS buffer wash was then performed three more times following permeabilization. Cells were finally blocked in PBS containing 1% for 1 hour at 25°C.

Primary Antibody Solution

Anti-Beta-III-Tubulin 1° mouse antibody was diluted by a 1:200 dilution in PBS containing 1% BSA according to the following calculation: 1 µl: 200 µl 1% BSA/PBS. This 1° antibody solution was stored on ice. A small glass slab was obtained and covered in Parafilm secured with tape. Two 50 µl aliquots of 1° antibody were aliquoted on isolated areas of the Parafilm-covered glass slab. Follwing the 1 hour blocking period, coverslips were carefully removed from the solution via forceps and gently inverted (cell side down) over each 1° antibody aliquot (carefully, as to not create any air bubbles under the coverslip. The whole glass slab was then covered with another sheet of Parafilm, and coverslips were incubated in 1° antibody at 4°C overnight.

Secondary Antibody Solution

Following incubation of cells in diluted primary antibody solution, the Parafilm was removed from the glass slab setup and coverslips were gently placed into a clean cell culture plate via forceps. The coverslips were washed in PBS buffer three times, with each wash lasting approximately 5 minutes. A 1:10 dilution of anti-mouse 2° antibody (anti-mouse IgG conjugated

to Alexa Fluor 488) was further diluted according to the following: 7 µl 2° antibody: 4 ml PBS containing 1% BSA. Two ml of the diluted 2° antibody was applied to each coverslip in separate wells for 2 hours at 25°C. The cell-culture plate was covered in aluminum foil so as to allow incubation to occur in the dark. Following the 2 hour incubation in diluted 2° antibody, the solution was removed and added to waste. The coverslips were then washed in PBS buffer three times, with each wash lasting approximately 5 minutes. Washes were performed under the aluminum foil to prevent 2° antibody light overexposure.

Nuclear Staining

Coverslips were then incubated in 3 µl 4',6-diamidino-2-phenylindole nuclear counterstain (DAPI) for 1 minute. Each coverslip was again washed with PBS buffer for 5 minutes.

Mounting for Visualization

Glass microscope slides were obtained upon time for fluorescent microscopy analysis. Coverslips were inverted over the glass microscope slide with use of forceps, carefully as to reduce the appearance of any air bubbles under the coverslip. The coverslips were adhered to the glass slide via a small amount of clear nail lacquer. Microscope slides that were fixed were allowed to dry overnight at 4 °C in the dark before being analyzed via microscopy.

Slides were observed and analyzed for neurite outgrowth via fluorescent microscopy on a Nikon Eclipse 80/X-cite Series 120 microscope equipped with a digital camera. For each microscope slide, digital photos were taken of ten randomly chosen microscope frames. Three sets of photos were taken for each randomly chosen frame- one under bright white light, one under blue fluorescent light for the illumination of anti-Neuron specific beta III Tubulin, and one under fluorescent green light for the illumination of DAPI-stained cell nuclei.

Fluorescent Microscopy Analysis

Digital photos were analyzed via NIS-Elements BR 3.2 computer programming, and two sets of cell counts were taken for each microscope slide: a count of the total number of cells, and a count of the total number of differentiated cells. Cells were considered differentiated if they exhibited evidence of a developing soma at least twice the nucleus diameter, and/or visible neurite outgrowth. For each slide, a percentage of cells differentiated was calculated.

Results

The mouse neocortical cells were treated with Hibernate EB™ solution and cytosine β-D-arabinofuranoside, and were cultured for a total of 14 days. For every two days of culture, cells were arrested at different periods of growth and fixed to microscopes slides for visualization and cell counting. Neocortical cells which displayed a small, standard round shape, within which a cell nucleus was visible, were considered undifferentiated. Those neocortical cells that were fixed and observed at the beginning of the cell culture timeline remained significantly undifferentiated, and were observed to display nothing more than cell nuclei (Figure 1). Neocortical cells that were observed to have differentiated into neuronal cells demonstrated a cell body with a minimum diameter of double the cell's nucleus, observable neurite outgrowth of axonal cytoskeletons, and/or the formation of filapodia-like projections (Figure 2).

In observation of the morphology of the undifferentiated mouse neocortical cells into neuronal structures, a clear development of the undifferentiated neocortical cells into the formation of neurons can be observed (Figure 3). At day two of the cell culture, cells remained significantly undifferentiated, and only cell nuclei were present (Figure 3A). During day four of culture, the appearance of cell bodies were evident, and most of those observed were at least

double the cell nucleus in size (Figure 3B). Day six of culture yielded cells that demonstrated cell bodies with a slight increase in size, demonstrating an increase in cell growth with the progression of time (Figure 3C). At eight days of culture, the beginnings of neuronal class III β-tubulin microtubule elements were observed as illuminated by the fluorescein-labeled 2° anti-mouse antibodies tagging the β-III tubulin of the developing neurons (Figure 3D). By 10 days of culture, the differentiating cells displayed evidence of developing neurite outgrowth of axonal cytoskeletons (Figure 3E). The observation of the microtubule elements at 12 days of differentiation revealed a display of a more wispy appearance of its developing neuronal cytoskeleton; these wispy elements resembled the formation of filopodia-like projections (Figure 3F). At 14 days of differentiation in cell culture, a large majority of the cells were observed to have formed into semi-mature neurons, with observable somas, axons, and the beginnings of dendritic elements (Figure 3G).

Neocortical cells that were observed to have an absolute bare minimum of differentiated were those that had been cultured for the least amount of time in the 14- day cell culture time frame; these cells were arrested at two days of growth. Out of the 202 total cells that were counted by their cell nuclei, only eight cells were considered to display cell bodies that were at least twice the diameter of their cell nuclei. These eight cells were thus considered to demonstrate the beginnings of cell differentiation, making up a mere 4% percent differentiation at two days of culture (Table 1). Neocortical cells that were observed to demonstrate a maximum percentage of differentiation during the 14-day cell culture were those that had been cultured for 14 days. There were 236 total cells that had been counted from the randomly-selected 10 frames of reference from this fixed microscope slide. Of this total cell count, 153 cells were determined to have demonstrated differentiation into neuronal cells. For the end of the current study's 14-

day cell culture, the maximum percent differentiation of cells into neurons for this culture was represented by a percentage of 64.8% (Table 1).

In observation of the morphology of the neocortical cells from the current study's 14-day culture into neurons, the percent differentiation of cells into neurons was observed to increase over time (Figure 4).

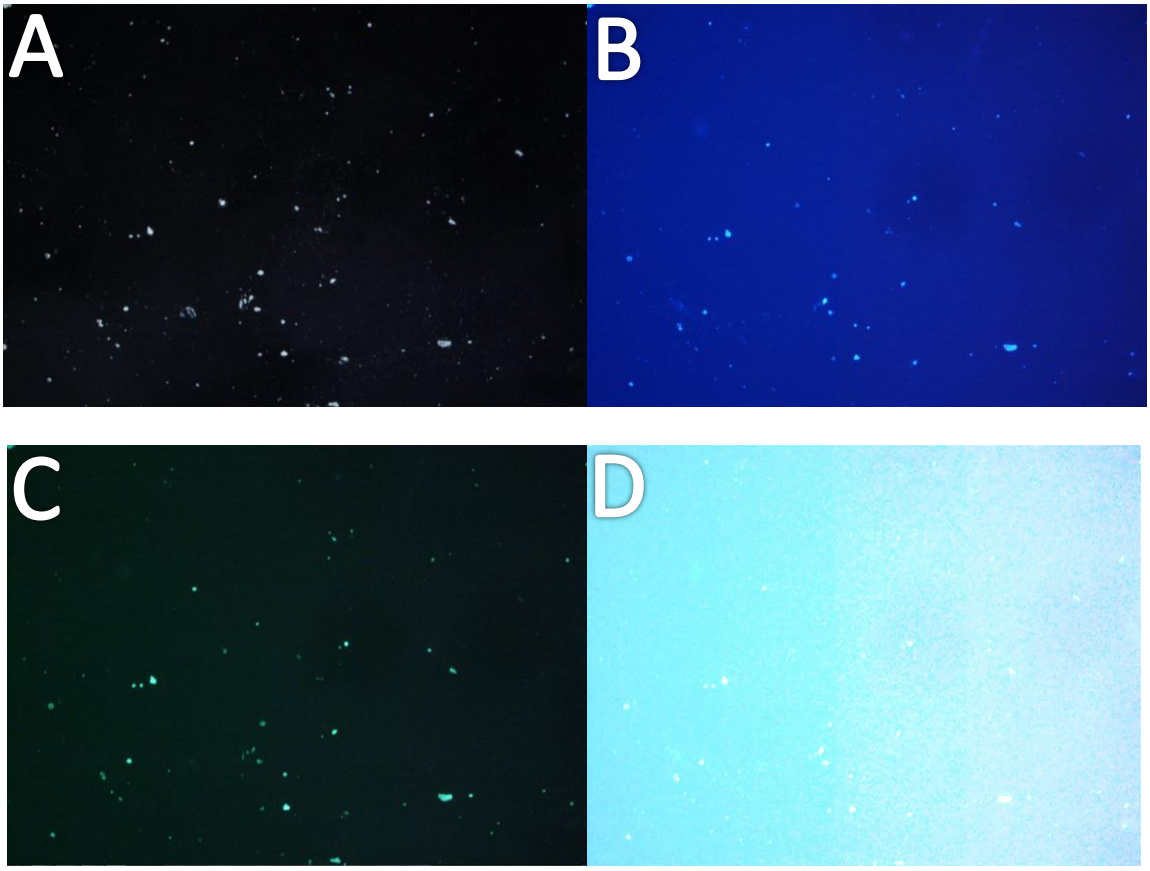

Figure 1: Structure of Undifferentiated Mouse Neocortical Cells. Photos were taken at 40x magnifications following the first two days of cell culture. At the time of photography, cells were visualized under **(A)** white light, **(B)** blue fluorescent light, illuminating fluorescein-labeled 2° antibody, tagging beta III Tubulin, and **(C)** green fluorescent light, illuminating cell nuclei stained with DAPI nuclear counterstain. Photo **(D)** is a representation of the 2 day-old undifferentiated cells composed via superimposing photos **(B)** and **(C)**.

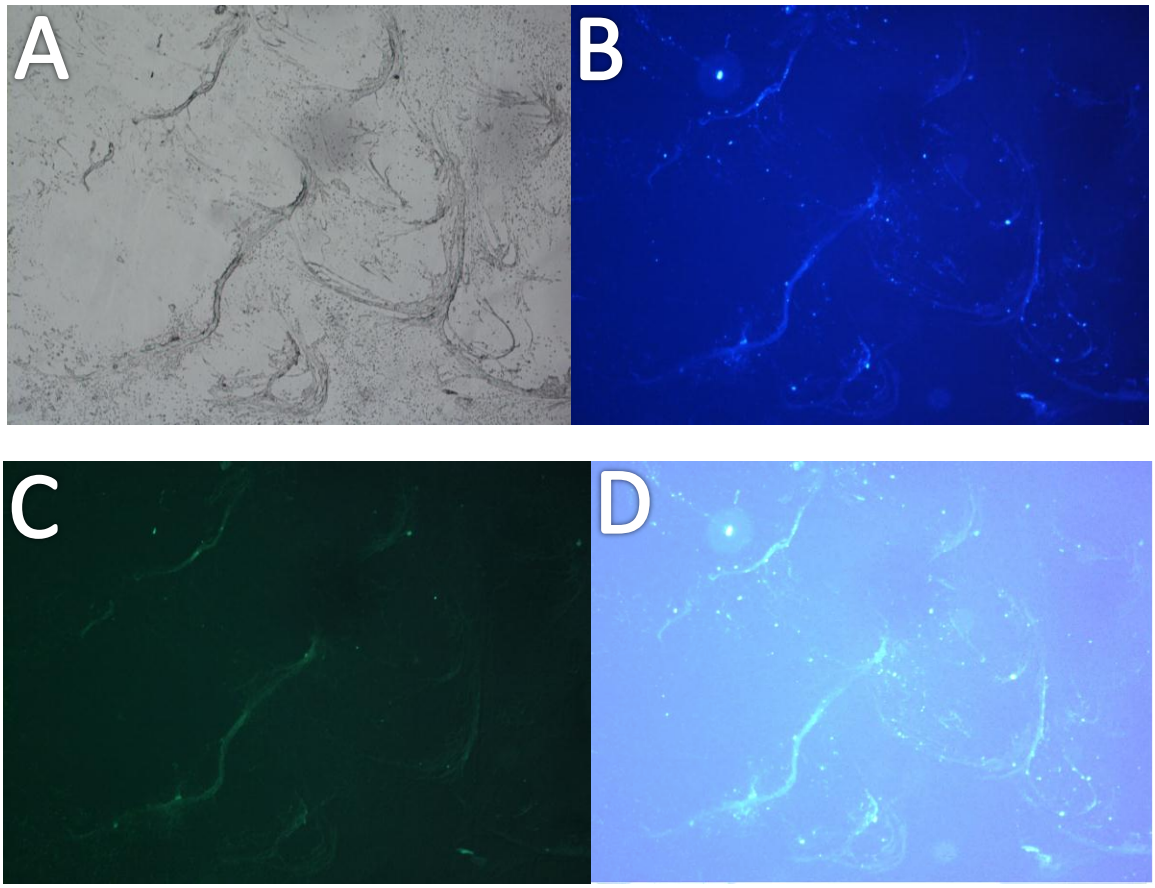

Figure 2: Structure of Differentiated Mouse Neocortical Cells. Photos were taken at 40x magnifications following 14 days of cell culture. At the time of photography, cells were visualized under **(A)** white light, **(B)** blue fluorescent light, illuminating fluorescein-labeled 2° antibody, tagging beta III Tubulin, and **(C)** green fluorescent light, illuminating cell nuclei stained with DAPI nuclear counterstain. Photo **(D)** is a representation of the 14 day-old differentiated cells composed via superimposing photos **(B)** and **(C)**.

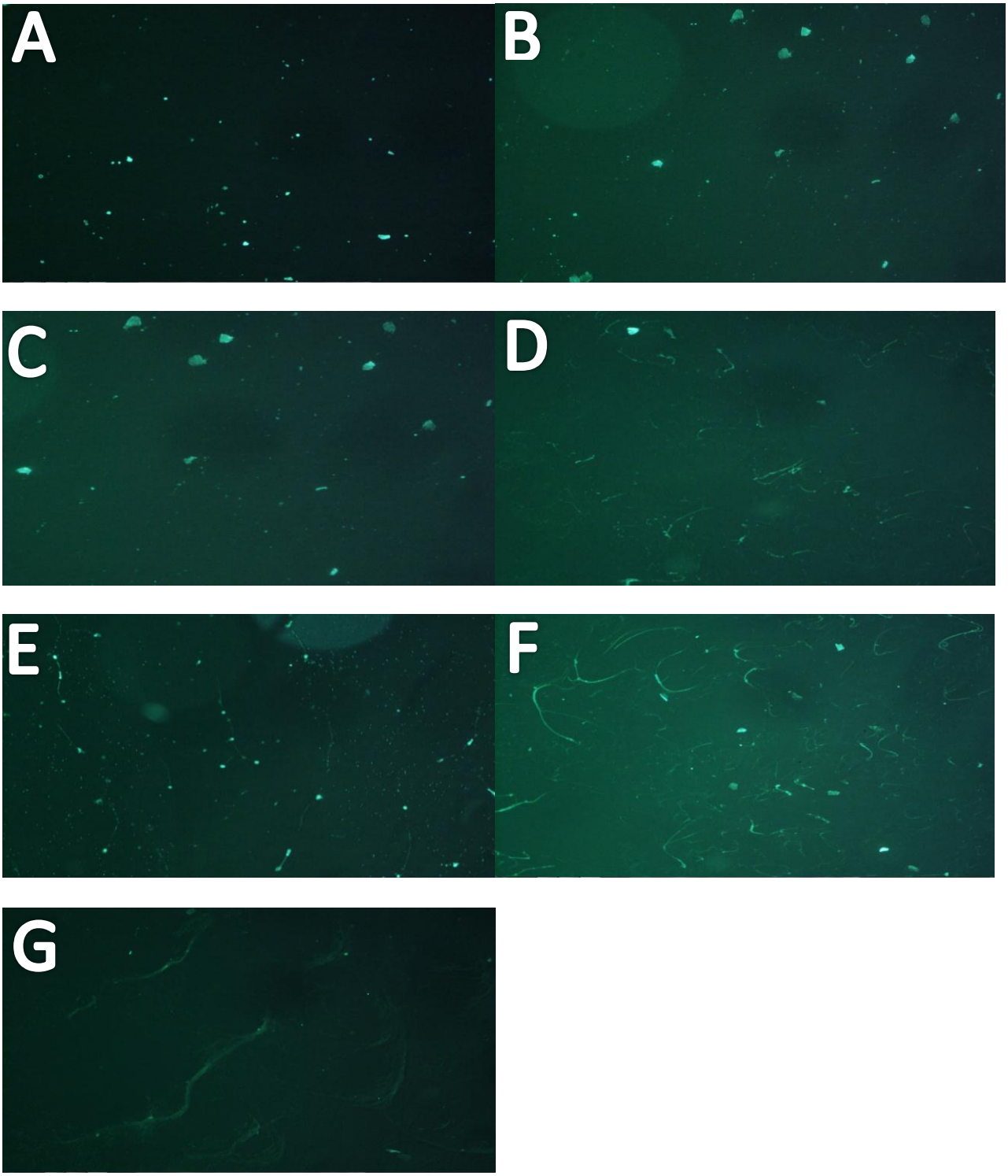

Figure 3: Morphology of Mouse Neocortical Cells Treated with Hibernate EB™ solution and Cytosine β-D-Arabinofuranoside over Time. Photos were taken at 40x magnifications under blue fluorescent light, illuminating fluorescein-labeled 2° antibody, tagging beta III Tubulin in differentiating neurons. At the time of visualization and photography, cells had been cultured for **(A)** 2 days, **(B)** 4 days, **(C)** 6 days, **(D)** 8 days, **(E)** 10 days, **(F)** 12 days, and **(G)** 14 days.

Table 1: Percent Differentiation of Mouse Neocortical Cells into Neurons over a 14-Day Timeline.

Day of Culture	Total Number of Cells	Number of Differentiated Cells	Percent Differentiation
2	202	8	4.0%
4	203	40	19.7%
6	161	32	19.9%
8	221	71	32.1%
10	225	75	33.3%
12	234	117	50.0%
14	236	153	64.8%

Cells were removed from culture and fixed for visualization via fluorescent microscopy at two-day intervals over a 14-day timeline. A total cell count via illumination of cell nuclei, and a count of the total number of differentiated cells were taken from 10 frames of each microscope slide magnified at 100x. A percentage of differentiated cells was then calculated for each slide. Percent differentiation of cells for every two days of culture is displayed in the table above.

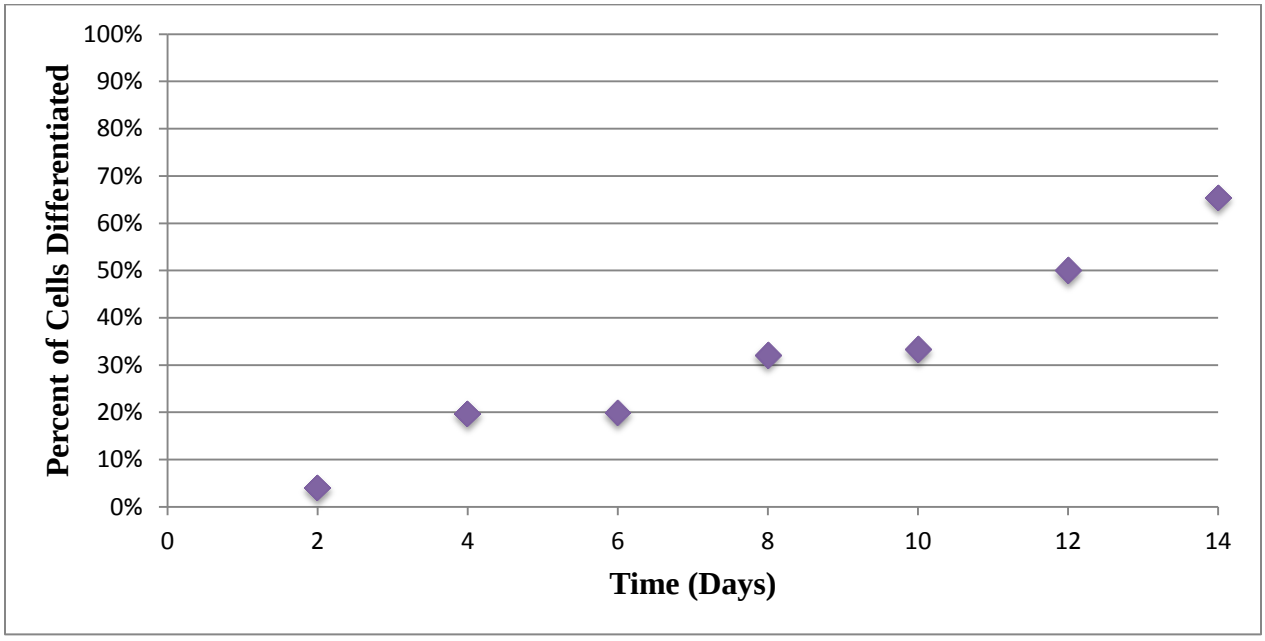

Figure 4: Percent Differentiation of Mouse Neocortical Cells over Time. Displays a plot of the percent of neocortical cells differentiated into neurons over time (days). The percent differentiation was considered over a culture timeline of 14 days.

Discussion

Pyrethroids are synthetic pesticides that can be found almost everywhere today. What may be most surprising to the general public worldwide is that these chemicals can be found in schools and restaurants, in mosquito-abatement programs, on livestock, and in the majority of households amongst products that many people utilize daily: roach, ant, and flying insect spray; flea bombs; roach traps and baits; pet tick-and-flea collars and shampoos; and lice and scabies shampoos for humans (Agency for Toxic Substances and Disease Registry, 2003). It is also possible for people to ingest pyrethroids through the eating of conventionally-grown fruits and vegetables (Agency for Toxic Substances and Disease Registry, 2003). With the potential for people and mammalian animals alike to encounter these chemicals from a variety of sources of daily lifestyle, mammals may become exposed to pyrethroids through a variety of sources in daily lifestyle, via: the spraying of a product that can be used to kill flies or roaches, the accidental ingestion of the chemicals after getting them on skin or food, or the absorption of the chemicals through the skin while bathing a pet or from lice shampoos.

With pyrethroid chemicals being encountered by most people from day to day, the acute toxicity of pyrethroids to adults has been investigated, and the U.S. Environmental Protection Agency has documented them as moderately toxic (EPA Category II) to mammals (Soderlund et al., 2002). However, despite the abundance of this information on acute neurotoxicity to adult mammals, there is little to no evidence regarding the potential neurotoxicity effects of the chemicals on the development of mammals. In fact, the "Toxicological Profile for Pyrethrins and Pyrethroids" published by the Center for Disease Control and Prevention has a specific heading for "Developmental Effects" on human health caused by exposure to pyrethroids. This document is blank under the heading of "Developmental Effects", indicating that there is not yet any

evidence on whether or not pyrethroid exposure has the potential to cause harmful neurodevelopmental effects (Centers for Disease Control and Prevention, 2003).

Further, the official document, "Safety of Pyrethroids for Public Health Use" published by the World Health Organization (WHO) has a specific heading which attempts to address the "Neurodevelopmental Effects" of pyrethroid exposure; however, the evidence of very few studies is presented under this heading, indicative that very few studies that aim to address this concern have been conducted at all (World Health Organization, 2005). The result of a 1991 study conducted in Sweden was amongst one of the few posted by the WHO under the heading of "Neurodevelopmental Effects". This study that was conducted on neonatal mice concluded that oral administration of deltamethrin (0.7 mg/kg bw/day by gavage in a sonicated mixture of egg lecithin, peanut oil and water on seven consecutive days) during rapid brain growth (postnatal days 10–16) induced changes in the density of muscarinic cholinergic receptors in the brain shortly after exposure, (this effect was also observed in adult animals treated in the neonatal period (Eriksson and Fredricksson, 1990). In addition, the animals were observed to demonstrate choreoatetotic symptoms at only a slightly higher dose (1.2 mg/kg bw) (Eriksson and Fredricksson, 1990). The World Health Organization utilized the evidence of this study to demonstrate that there can be possible detrimental neurodevelopmental effects on developing young. However, in further reflection of these results, the WHO considers the quantitative interpretation of these findings to human health unclear due to the particular administration procedure used in the study.

Another study that may highlight the potential for developmental neurotoxicity due to pyrethroid exposure concerned Mexican children whom had been exposed to a mixture of agricultural pyrethroids that had been used for agricultural purposes during their development.

The observations of this study revealed that these children had adverse effects on motor skills, memory, attention, and learning (Guillette, et al., 1998). There is potential that the pyrethroids may have been the causal link between the children and the development of their adverse effects; however, this study proved to be a very limited one, and further investigation is necessary (Schettler, 2001).

Two additional studies concerning the pyrethroid deltamethrin demonstrated that: exposure to this pesticide has the potential to induce apoptosis in cultured mouse cerebral cortical neurons (Wu et al., 2003), and that low-level in utero exposure of embryonic rats to deltamethrin has to potential to cause increased hippocampal acetycholinesterase activity and decreased learning and memory between the six to twelve weeks of the postnatal period (Aziz et al., 2001).

The studies such as those mentioned above are only some of the few which have attempted to investigate into the dangerous developmental neurotoxicity effects of pyrethroids. With only a very limited amount of studies available, with some of which that have since been considered “unclear” or “limited” in regards to human health, there is a necessity for further studies to be conducted. The European Union (EU) has begun to raise concern regarding potential neurodevelopmental effects of pyrethroids (Lautraite and Sargent, 2009). It is time to begin to provide the regulatory agencies in North America and the EU with conclusive research on this topic (Lautraite and Sargent, 2009).

In a 2006 study conducted by the Department of Biology at Adelphi University, the potential deleterious effects on neurite outgrowth of cultured PC12 cells were investigated in an attempt to address the possible toxicological effects of bifenthrin, one of the newest additions to the synthetic pyrethroid insecticides that has exhibited a wider range of insecticidal properties

than previous pyrethroids (Tran et al., 2006), on neuronal development. With this assessment, the scientists hoped to address the potential consequences of maternal exposure to pyrethroids on the developing nervous system of mammalian young.

The study utilized rat PC12 cells of the neuronal cell line to assess whether bifenthrin affected viability of the PC12 cells and the neurite outgrowth of the cells when seeded in nerve growth factor. The viability of the PC12 cells given the control treatment was 100%. Technical grade bifenthrin was not found to be toxic to PC12 cells at a concentration of 10^{-3} M, which was the highest soluble concentration of bifenthrin tested; cell viability was determined to fluctuate around 110% for cultures of 72 and 96 hours, and be greater than 120% for 24 hour cultures (Tran et al., 2006). In terms of neurite outgrowth, the percentage of cells with neurites observed in the control cells after 48 hours of treatment was considered to be 110%. The concentrations of technical grade bifenthrin observed to inhibit neurite outgrowth by approximately 35% and 75% after 48 hours of treatment were 10^{-6} M and 10^{-3} M (Tran et al., 2006).

In analyzing these findings, it was determined that the technical grade bifenthrin had detrimental effects on neurite formation at concentrations of bifenthrin which prove to have no effect on actual cell viability. Since bifenthrin has previously been classified as a moderate hazard by the WHO and therefore, has been considered safe for household use, it is no surprise that cell viability was not affected. However, in considering the effects of bifenthrin on neurite outgrowth of developing cells in the absence of cell death, the perceived advantageous use of this pesticide may not be as ideal as previously determined, even at levels of low toxicity.

The results of this study suggest that bifenthrin and other pyrethroids may have a significant and detrimental effect on the developing nervous system, in the absence of determined toxicity. Exposure of a mammalian maternal system to these pyrethroids may not

elicit immediate lethal effects on the adult, but may induce gradual tissue degeneration of the reproductive system (Tran, et al., 2006). Exposure to levels of pyrethroids that have previously been determined "safe" for a mammalian adult may actually lead to eventual neurodevelopmental abnormalities and defects of prenatal young exposed to degenerated utero tissues.

The abovementioned study asserts crucial theories on the potentially detrimental effects of pyrethroids on developing mammalian young; however, further investigation with additional study is necessary to support these assumptions and conclusions. Just as the abovementioned study aimed to develop a neurodevelopment assay of cultured PC12 cells to assess the neurodevelopmental effects of pyrethroids, the purpose of the current study is develop an assay of cultured neuronal cells, which might be used in the future to similarly assess the neurodevelopmental effects of pyrethroids. With the development of an assay that utilizes neurons, the detrimental effects of pyrethroids on developing neurons can provide more accurate conclusions on the neurodevelopmental effects of these pesticides. This study is essentially the first step in working toward establishing such an assay.

The current study aims to demonstrate the necessity of a neurodevelopmental assay in order for further investigation into the potential of pyrethroids to cause developmental effects on developing mammalian neurons. This assay allows for the monitoring of the development of neurons in cell culture, in preparation for the evaluation of the effects of pyrethroids on developing neurons. The benefits of using a cell culture and neurodevelopmental assay to prepare for the evaluation of the pyrethroids proves to be a cheaper, more time-efficient method in collecting experimental results (National Research Council of the National Academies, 2003), and it is an experimental method that can be considered highly ethical, as well. The use of an

efficient, reliable neuronal culture can spare the lives of over laboratory rodents, in addition to sparing the expenses of scientists. For these reasons, the neurodevelopmental assay developed in the current study as opposed to the more traditional method of utilizing whole animals will prove most efficient and effective in the next step of this project- evaluating the neurodevelopmental effects of pyrethroids.

The assay that had been developed in this study established a timeline for maximum cell differentiation of the mouse neocortical cells into neurons during a 14-day cell culture. In addition, parameters for defining whether or not a cell was considered differentiated were also determined. Mouse neocortical cells that were fixed and observed at the beginning of the cell culture timeline remained significantly undifferentiated, and were observed to display nothing more than cell nuclei (Figure 1). Neocortical cells that were observed to have differentiated into neuronal cells demonstrated a cell body with a minimum diameter of double the cell's nucleus, observable neurite outgrowth of axonal cytoskeletons, and/or the formation of filopodia-like projections (Figure 2). Although the appearance of cell bodies were evident, and most were at least double the cell nucleus in size (Figure 3B) by day four of culture, the beginnings of neuronal class III β-tubulin microtubule elements were not observed until day eight of culture (Figure 3D). By 10 days of culture, the differentiating cells displayed evidence of developing neurite outgrowth of axonal cytoskeletons (Figure 3E) and by day 12 of culture, the formation of filopodia of the developing neurons were observed (Figure 3F).

At 14 days of differentiation in cell culture, a large majority of the cells were observed to have formed into semi-mature neurons, with observable somas, axons, and the beginnings of dendritic elements (Figure 3G). These neocortical cells that had been cultured for 14 days also displayed a maximum amount of differentiation into neurons for this particular 14-day cell

culture. For the end of the current study's 14-day cell culture, the maximum percent differentiation of cells into neurons for this culture was represented by a percentage of 64.8% (Table 1).

The fact that ~65% of the neocortical cells had demonstrated differentiation into neurons at day 14 of the culture does support the preliminary hypothesis that maximum differentiation will be at day 14 of the culture; however, since only ~65% of cells had differentiated at day 14, it may be that a culture with a timeline longer than 14 days should be conducted in order to establish an assay with 100% differentiation, and to determine a new timeline for this culture. In observation of the morphology of the neocortical cells from the current study's 14-day culture into neurons, the percent differentiation of cells into neurons was observed to increase over time (Figure 4). With this observation with a direct relationship between differentiation and time, it can be hypothesized that the cells would continue to differentiate in greater calculated percentages with an increase in time for a culture to progress, beyond the 14 days of the present study.

In reflecting upon the results of the current study, it can be concluded that the second part of the preliminary hypothesis, which predicted that cell differentiation into neurons would begin at day 10 of the culture, was not very far off. The current study defined differentiation of a cell into a neuron as a cell body with a minimum diameter of double the cell's nucleus, observable neurite outgrowth of axonal cytoskeletons, and/or the formation of filapodia-like projections (Figure 2). During day four of culture, the appearance of cell bodies were evident, and most of those observed were at least double the cell nucleus in size (Figure 3B); therefore, the current study considered these cells as being differentiated. However, in defining cell differentiation by visible evidence of developing neurite outgrowth of axonal cytoskeletons, as the studies in the

primary literature do (Tran et al., 2006), then this observation was seen by day 10 of culture, (Figure 3E), and these results do support the preliminary hypothesis.

In the 2006 study conducted by the Department of Biology at Adelphi University, where PC12 cells were stimulated with Nerve Growth Factor, a 100% maximum differentiation of the cells into pseudo-neuronal cells was observed after 48 hours of culture. In analyzing these results with the results of the current study, where only a 4% differentiation rate was observed, questions arise concerning such a large inconsistency in results. These concerns might be addressed with the following considerations: Tran et al., 2006 utilized rat PC12 cells which were stimulating to grow neurites to resemble neurons, while the current study cultured mouse neonatal neuronal cells; Tran et al., 2006 utilized Nerve Growth Factor to stimulate the PC12 cells to grow neurites, while the current study utilized Hibernate EB™ solution (2% B27 and 0.5 mM Glutamax).

The 2006 study conducted at Adelphi University found that bifenthrin does inhibit neurite formation in the absence of cell death on PC12 cells differentiating into pseudo-neuronal cells (Tran et al., 2006). In reflection of the results of this study, the following conclusions and considerations have been made: that bifenthrin exposure in utero has the potential to lead to neurodevelopmental defects, and that chronic exposure to bifenthrin may lead to neurodegenerative disease (Tran et al., 2006). Therefore, with the wide use of bifenthrin as a pesticide with a great selective toxicity to insects, the determined “safe use” of this particular pesticide should be scrutinized. In addition, these results received from a study which utilized bifenthrin might cause one to scrutinize the use of all other pyrethroids as well, until their effects on developmental neurotoxicity are proved harmless.

In contrast to the 2006 study conducted at Adelphi University, which aimed to investigate the potential developmental neurotoxicity effects of the pyrethroid bifenthrin (Tran et al., 2006), the current study aims to establish the beginnings of a reliable assay where the potential developmental effects of pyrethroids could be assessed on actual developing neurons.

In preparation for future studies, a first step would be to either evaluate a neuronal culture with a longer timeline, or to culture cells according to the 14-day timeline of the current study, but to utilize a fresher, or different neuron nutrient medium in an attempt to encourage a greater maximum differentiation at an earlier point in the culture. In developing the next cell culture, consideration should be taken in ensuring more accurate results by: performing maximum differentiation calculations in duplicate for each day of culture being evaluated, and having a negative experimental control for DAPI nuclear counterstain of cells only.

A next step might be to perform the actual assay by adding a pyrethroid to the developing cells: first, at a time of little to no cell differentiation at the earliest days of the culture, and then at the day of culture determined to demonstrate maximum cell differentiation (for the current study, at day 14 [Table 1]). The chosen pyrethroid should then be applied in differing concentrations of strength to determine if the treatments inhibit or discourage cell differentiation, at all; and if so, at which concentrations, so that a dose-responses curve might be created. A long-term future goal would be to repeat the experiment with multiple pyrethroids first in one class, and then moving on to another.

As mentioned earlier, the official document entitled “Safety of Pyrethroids for Public Health Use” published by the World Health Organization has little information on the topic of “Neurodevelopmental Effects” of pyrethroids (World Health Organization, 2005). In addition, the document entitled “Toxicological Profile for Pyrethrins and Pyrethroids” published by the

Center for Disease Control and Prevention has absolutely no information under the heading of "Developmental Effects" (Centers for Disease Control and Prevention, 2003). This study is one small step that attempts to prepare to address one potentially very large, yet silent issue.

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