Biology Honors Thesis

Investigating the Relationship between Environmental Lead Exposures and the Onset of Alzheimer's Disease Pathologies

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A thesis submitted to the Department of Biology, East Carolina University, in partial fulfillment of the requirements for Biology Honors Thesis

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I hereby declare I am the sole author of this thesis. Dr. Jamie DeWitt provided guidance on some it's content and one image was provided by Annalise vonderEmbse. This project was but one small piece of a very large project, but I alone was responsible for that piece. This thesis has not been submitted elsewhere as coursework for this or another degree.

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ABSTRACT – It is been estimated that nearly 5 million Americans suffer from Alzheimer's Disease (AD), making finding its cause one of medicine's top priorities. It has long been known that genetics plays a major role in the development of Alzheimer's Disease, but this does not fully explain why or when the disease manifests itself. Alzheimer's is likely not a single-origin disease, but rather a disease that arises from a combination of both genetic and environmental factors that occur in the "right mix" at the "right time" to produce a phenotype indicative of AD. A major component of Alzheimer's is the plaques of amyloid-β that form in the brains of its victims. One of the cells in the brain that has been shown to control the levels of amyloid-β is the microglia, though scientists debate over exactly how microglia does this. Studies show that these microglia experience a "critical window" of heightened susceptibility to environmental contaminants early in development from post-natal day (PND) 5-15. In this study, 3x-Tg-AD mice were dosed during this critical window with a proven neurotoxicant, lead acetate, in the form of drinking water. The levels and states of microglia were observed and compared to the levels of control mice. Taking this a step further, the levels of amyloid-β were tested using enzyme-linked immunosorbent assay (ELISA). This study hopes to uncover the relationship between microglia and amyloid-β

levels as well as determine whether introduction of an environmental toxicant (i.e. lead) during a period of heightened susceptibility could exacerbate the onset of AD pathologies later in life. In the end, no major correlation could be detected. The ELISA showed inconclusive evidence to provide a correlation between the environmental contaminants and amyloid-β levels. Complementary evidence, however, suggests that there may have been a flaw in the ELISA methodology. We believe this because confocal images show accumulated amyloid-β in the brain, but each ELISA result yielded that the levels were below the limits of detection. The DeWitt lab plans to revamp its methods and replicate this project because it represents a novel undertaking in science and the results have the potential to make waves to drastically change how Alzheimer's Disease is analyzed and how it can be prevented.

Acknowledgments

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Introduction

Alzheimer's Disease and Amyloid-β

Alzheimer's Disease (AD) is a late-onset neural disease that affects basic brain functions like memory and thinking skills. Those afflicted with Alzheimer's Disease have neurofibrillary tangles and plaques made up of amyloid-β protein throughout the brain (USDHHS, 2012). This can be seen in Figure 1. AD pathogenesis is widely believed to be driven by the production and deposition of these amyloid-beta peptides, which hinder neuron to neuron signaling at the synapse and eventually leads to cell death (Murphy & Levine, 2010).

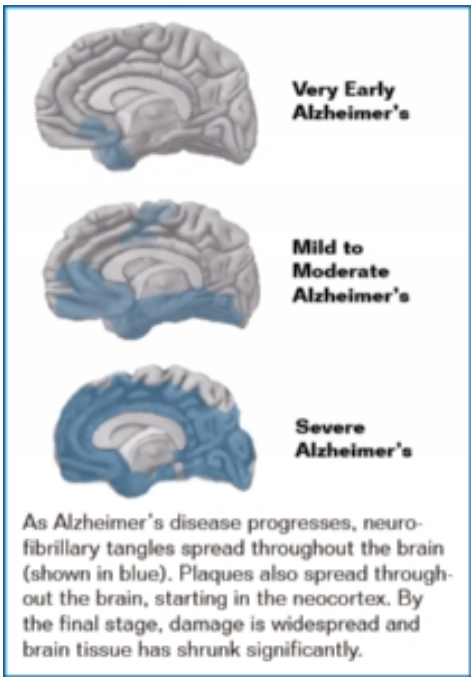

Figure 1: Visual depiction of AD progression

Microglial Effects on Amyloid-β

One cell that is associated with amyloid-β plaques in the AD brain is the microglia, the innate immune cells of the brain. Microglia act as microphages in the central nervous system, clearing cellular debris and dead neurons through the process of phagocytosis. It is very controversial whether microglia have a beneficial or detrimental function (Dheen et al. 2007). Some studies show that microglia actually reduce AD pathology by phagocytosing amyloid-β, while others suggest that, when activated, microglia increases the deposition of amyloid proteins (amyloidosis) (Weitz & Town, 2012). A visual of activated vs. ramified microglia can be seen in Figure 2. In a recent review of microglia in health and disease, Saijo and Glass (2011) emphasized that existing data is insufficient to rule microglia as exclusively

neuroprotective or neurodegenerative in the AD brain. Microglia may protect the brain by phagocytosing amyloid-β and clearing plaques in AD pathology but alternatively, amyloid-β may activate microglia, induce pro-inflammatory mediators and lead to subsequent neuron elimination. This brings up another plausible explanation that microglia may additionally increase neurodegeneration through the secretion of proinflammatory signals. One study suggests that in response to accumulating amyloid-β proteins, microglia generate a toxic inflammatory response that accelerates synaptic and neuronal injury. It is a persistent and nonresolving inflammatory response and is a key feature in the development of Alzheimer's disease (Woodling et al. 2014). Many proinflammatory signaling pathways are linked to the development of neurodegeneration. This process also plays a major role in other neurodegenerative diseases like Parkinson's disease, prion diseases, and multiple sclerosis (Dheen et al. 2007). Further studies support this claim by saying that the presence of acute phase proteins and oxidative damage proves that amyloid-β plaques are the foci of local inflammatory responses. This same study shows that fibrillar forms of amyloid-β have been shown to activate tyrosine kinase-dependent signal transduction cascades, resulting in inflammatory responses in microglia (McDonald et al. 1998). Despite the controversy, it is widely agreed upon that the actions of microglia in AD, whether detrimental or beneficial,

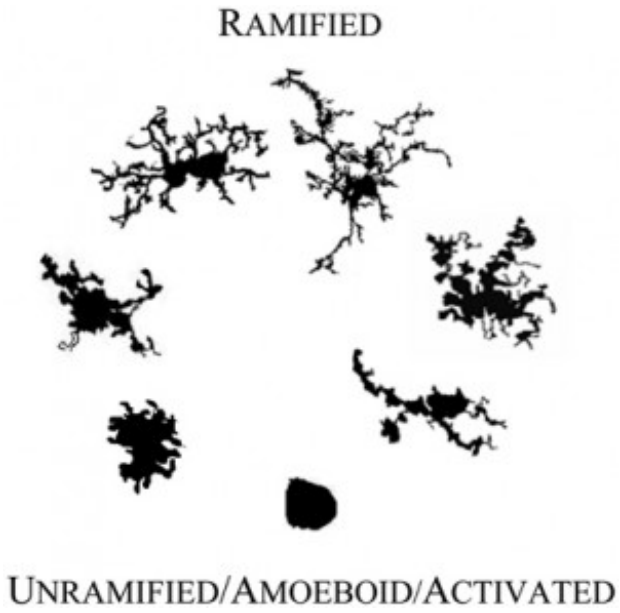

Figure 2: Activated vs. Ramified Microglia

depend on the conditions under which the microglia encounter AD pathologies (Weitz & Town, 2012).

Critical Windows

Despite being a late-onset disease, usually occurring after the age of 60, the origins of Alzheimer's may arise very early in development, long before the brain has reached full maturity (Basha et al. 2005). Microglia experience a "critical window" during development, or a period of heightened susceptibility to disturbances such as aberrant protein expression associated with congenital genetic mutations or exposures to exogenous agents such as environmental contaminants. These disturbances can potentially reprogram the organism and increase the risk of disease later in life. Moreover, mounting evidence suggests that developmental exposure to environmental agents leads to latent neurotoxicity in adult organisms (Bash et al, 2005). Alzheimer's is likely not a single-origin disease, but rather a disease that arises from a combination of both genetic and environmental factors that occur in the "right mix" at the "right time" to produce a phenotype indicative of AD.

Goal and Overview

In this project, we hypothesize that early-life exposure to a neurotoxic chemical (i.e. lead) would alter the microglia, making them over or under sensitive to aberrant protein expression in the brain, and lead to the early onset of Alzheimer's Disease pathologies.

Therefore, the goal of this project is to evaluate a critical window of microglial susceptibility to an environmental agent in a vulnerable genetic model, the 3x-Tg-AD mouse, and correlate changes in microglial number and activity with early-onset AD pathologies, such as amyloid-β levels. Detrimental interactions of a developing organism with environmental factors during susceptible windows of development are putative pathways to myriad diseases later in life. For example, early life exposure to tobacco smoke increases the risk of childhood asthma, which subsequently increases the risk of additional diseases in adulthood, including obesity, behavioral disorders, lung cancer, and allergies. Insults during development, whether from altered genetic mutation or from signaling potentially reprogram an organism's life-long trajectory for disease later in life. This idea can be seen in Figure 3. Diseases of aging such as AD are thought to arise from developmental reprogramming, and evidence suggests that microglia, the resident macrophages of the brain, are key players in AD pathology.

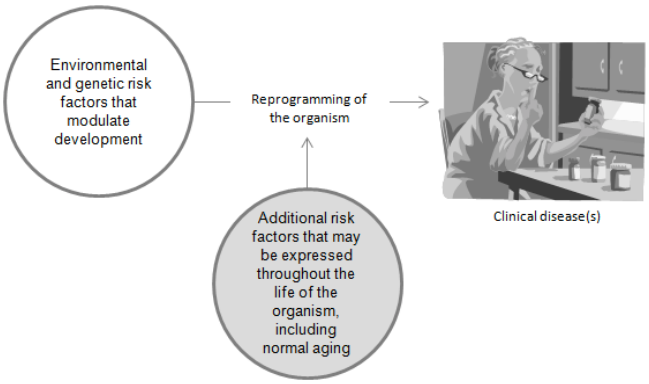

Figure 3: A general schematic of how environmental and genetic risk factors may contribute to clinical diseases in the aged, including additional lifetime risks. This proposal will consider *both* environmental and genetic risk factors as well as normal aging in an innovative model that evaluates both cellular and molecular markers of Alzheimer's disease pathology.

Importance

This project is important because it further adds to our knowledge and understanding of the cellular mechanisms of AD and related neurodegenerative diseases as they relate to

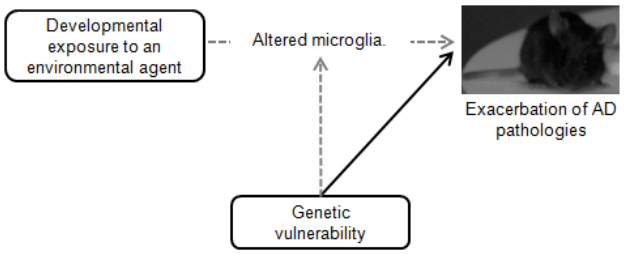

Figure 4: Schematic depicting potential mechanistic pathways to be explored. Solid lines indicate known pathways whereas dashed lines indicate potential pathways.

microglia. It also represents a very novel undertaking, as it has been the first project to explore the impacts of both environmental agents *and* critical windows of developmental susceptibility in AD.

As we are specifically focused on the etiologic synergism between environmental agent exposure and genetic vulnerability as a double-hit model that more closely mimics realistic exposures than does a single-hit model, we hope to provide a robust model of AD. This double-hit model is outlined in Figure 4. Additionally, as AD is likely a disease of developmental reprogramming, this study will be particularly useful in designing early-intervention treatment strategies that focus on developmental processes. While many published studies report on the effects of aging microglia in the Alzheimer's brain, few studies have evaluated developmental impacts to microglia and subsequent AD pathologies.

Methods

Background

In a 2005 study, Basha et al. exposed Long-Evans rats to lead (as lead acetate) in drinking water during early development from postnatal day (PND) one through PND20 and examined several endpoints relevant to AD, including levels of amyloid precursor protein (APP) and amyloid-β in brains of aged rats. They also exposed a different group of rats to lead during senescence (18-20 months of age) to determine if later life exposure to an environmental agent increased markers of AD. In developmentally exposed rats, markers of AD were transiently expressed in young animals (i.e., PND50) and were overexpressed in these animals as they aged. In rats exposed during senescence, no changes in markers were noted. Therefore, Basha concluded that developmental exposure to lead produced latent effects on genes associated with neurodegenerative processes during old age and that developmental events influenced later life neurodegenerative changes. Due to these findings, we assessed our animals at ages relevant to the 3x-Tg-AD mouse: PND50, three months of age (PND90), and six months of age (PND 180).

Experimental Approach

Our experimental approach included an exposure period encompassing a critical window of susceptibility for microglia. During prenatal development, microglial colonization of the brain occurs and their numbers increase dramatically between PND5 and PND15 (Harry and Kraft, 2012). This postnatal period represents a critical window of susceptibility for microglia, where their numbers increase and their phenotypes mature (Harry and Kraft, 2012). It is this window that we are interested in disrupting with lead exposure.

The study began with 24 triple-transgenic dams that were predisposed to develop

Alzheimer's disease. Therefore, our model can be seen as a push model. Of these 24 dams, 12 were labeled as control and the remaining 12 labeled as treated. Once each dam had her litter, they were culled to no more than six animals per dam, balanced by sex, if possible. A low dose of lead as lead acetate (100 parts per million) was administered to the mice via gavage dosing. The concentration of 100 parts per million has been shown to be well within the range of human consumption and gavage dosing is a reliable and safe way to dose the animal. Animals were given 0.1 mL of dosing solution per 10 g of daily body weight. Offspring were examined daily from PND5-PND21 and any deviations from general health, growth, and behavior were noted. At PND21, all offspring were weaned and placed in a same sex sibling group. At PND50, PND 90, and PND 180, one offspring of each sex per litter (if possible) will be evaluated for microglia and AD endpoints.

The AD endpoint that I was responsible for during my time in the DeWitt lab was analysis of the amyloid-β levels. After euthanasia, brains of both the control and treated offspring were taken out and subsequently had the hippocampus removed. Amyloid-β levels were then determined by an enzyme-linked immunosorbent assay (ELISA). A BetaMark Beta Amyloid x-42 ELISA kit, manufactured by Covance, was used.

Results

A two-sample t-test was performed on each age group to determine if the data was significantly different and if the changes in the data could have happened by chance. In the end, no significant difference was detected between the absorbance values of the control and treated populations in any age group. A standard curve was created by plotting the mean absorbance for each standard concentration (x axis) against amyloid-β concentration (Y axis) in pg/mL. We then drew a best fit curve through the points in the graph and found that each value obtained in both the control and treated groups were below the limits of detection for the ELISA.

In the PND 50 age group, control groups showed a mean ELISA absorbance value of 0.1425 while the treated group showed a mean of 0.1516. The p-value associated with this age group was 0.37. Table 1 shows this data.

In the PND 90 age group, control groups showed a mean ELISA absorbance value of 0.1739 while the treated group showed a mean of 0.1310. The p-value associated with this age group was 0.108. Table 2 shows this data.

In the PND 180 age group, control groups showed a mean ELISA absorbance value of 0.0512 while the treated group showed a mean of .0498. The p-value associated with this age group was 0.505. Table 3 shows this data.

Rather than reject the null hypothesis that early-life exposure to a neurotoxic chemical (i.e. lead) would alter microglia and lead to the early onset of Alzheimer's Disease pathologies (i.e. increased amyloid-β deposition), the DeWitt lab feels that further investigation should be completed to see if these values are actually indicative of the true values or if a flaw in methodology could be the problem.

t-Test: Two-Sample Assuming Equal Variances

Age: 50; Control versus Treated

	Control	*Treated*
Mean	0.1425	0.1516
Variance	0.000112833	0.0002683
Observations	4	5
Pooled Variance	0.000201671	
Hypothesized Mean Difference	0	
df	7	
t Stat	-0.955240986	
P(T<=t) one-tail	0.185636145	
t Critical one-tail	1.894578604	
P(T<=t) two-tail	0.371272289	
t Critical two-tail	2.364624251	

Table 1: PND 50 Statistics

t-Test: Two-Sample Assuming Equal Variances

Age: 90; Control versus Treated

	Control	*Treated*
Mean	0.173857143	0.1308
Variance	0.00265681	0.0025909
Observations	7	10
Pooled Variance	0.002617264	
Hypothesized Mean Difference	0	
df	15	
t Stat	1.707835423	
P(T<=t) one-tail	0.054136656	
t Critical one-tail	1.753050325	
P(T<=t) two-tail	0.108273312	
t Critical two-tail	2.131449536	

Table 2: PND 90 Statistics

t-Test: Two-Sample Assuming Equal Variances

Age: 180; Control versus Treated

	Control	*Treated*
Mean	0.051222222	0.0498
Variance	1.66319E-05	0.000008075
Observations	9	5
Pooled Variance	1.37796E-05	
Hypothesized Mean Difference	0	
df	12	
t Stat	0.686895904	
P(T<=t) one-tail	0.252603188	
t Critical one-tail	1.782287548	
P(T<=t) two-tail	0.505206375	
t Critical two-tail	2.178812827	

Table 3: PND 180 Statistics

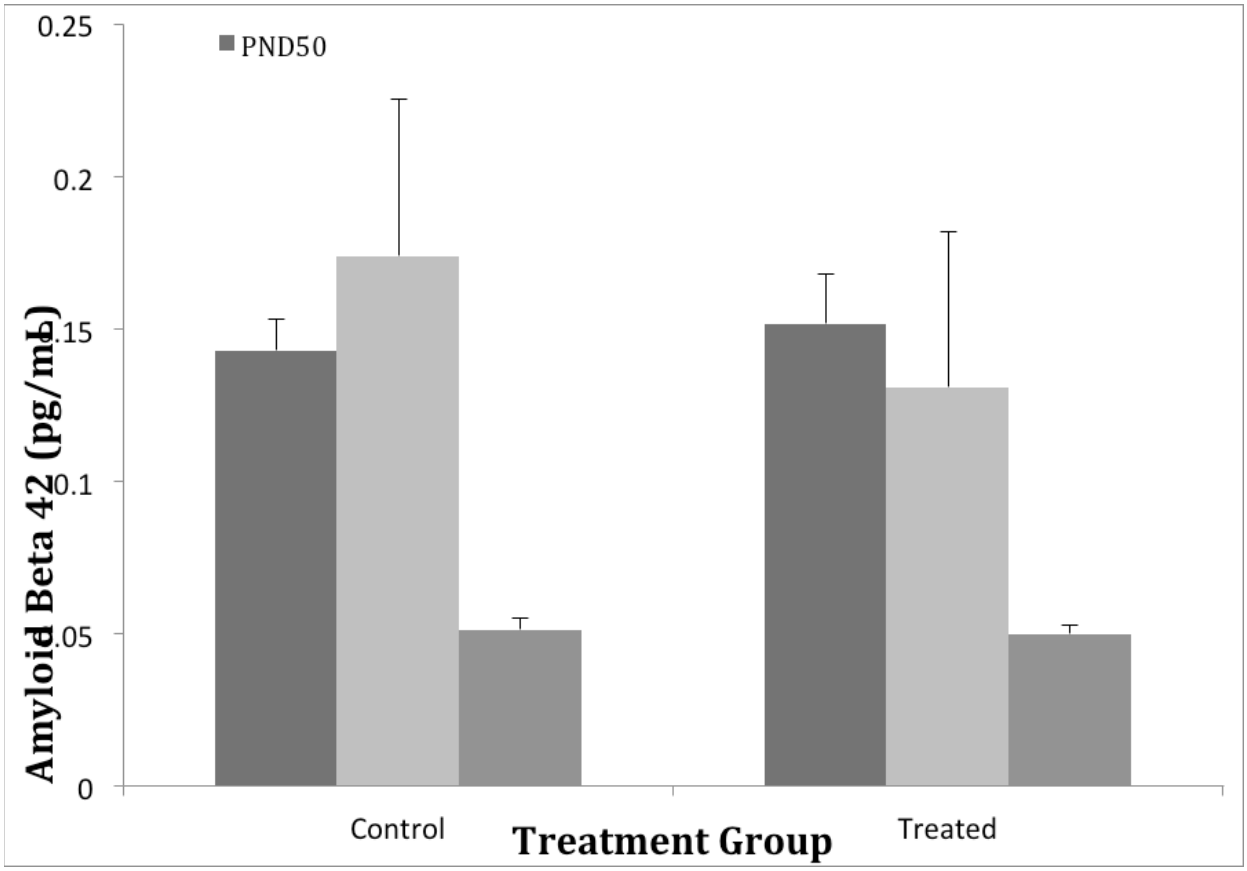

Figure 5: Graph of Mean ELISA Absorbance Values (control vs. treated)

Discussion

Innovation

This research project was truly a novel one. We know that in some individuals, AD arises from genetic mutations. We also suspect that environmental influences contribute to the risk of AD in individuals regardless of genetic makeup and that microglia play a role in AD pathologies. We do not know, however, how genetic vulnerability *and* environmental insults interact to result in AD pathologies. Our project takes an innovative turn away from traditional, single risk factor approaches to develop a robust model of AD that incorporates the dual risk factors of genetic vulnerability and an environmental insult. Thus, our model of detrimental exogenous interactions with a genetic vulnerability more closely approximates developmental scenarios experienced in a real world scenario. Additionally, by evaluating things like microglia, *APP* gene expression, or in this case, levels of amyloid-β, we can start to tease apart the cellular and molecular mechanisms that lead to AD pathologies under a system of gene x environment interactions. The risk of AD attributable to genetics is about 70% (Vilatela et al., 2012). However, "genetics" does not denote just heritable mutations, it includes mutations induced by, for example, exposure to exogenous agents that disrupt protein expression and ultimately, a functional endpoint. Our project is particularly innovative because it combines a heritable mutation with exposure to a well-known neurotoxic agent, lead. By examining AD endpoints like levels of amyloid-β, we can determine if developmental exposure to lead impacts traditional indicators of AD known to be exacerbated in the 3x-Tg-AD mouse model. Layering on microglia number and morphology with co-localization of amyloid-β will provide additional novel data about the early-life role of a cell known to be associated with AD pathologies and will provide insight

into whether microglia play a neuroprotective or neurodegenerative role in AD.

Analysis of Results

Being that this project was so innovative, it came with a large number of unknowns. It is a natural occurrence that novel research must sometimes undergo a series of "growing pains" before truly significant and meaningful data collection can occur. Everything from the homogenization procedure to which ELISA kit to use was a result of literature reviews and making educated guesses, but in the end, the DeWitt lab feels we may need to repeat this study and make a few adjustments to our methodology. We remain confident that our hypothesis is a valid one. The major reason we feel this way is because the lab took a confocal image of a PND 90 male (Figure 6) brain that showed significant amounts of amyloid-B expression, as well as colocalization with microglia. This tells us that amyloid-β IS present in the brains of the animals, yet every animal came back well below the ELISA's limit of detection. Therefore, we can assume that a major problem we face deals with the homogenization procedure. One problem could stem from the amyloid-β becoming denatured for some reason during homogenization. We will need to take a look at the chemicals and mixtures and how they may possibly have had an adverse affect of the protein structures or concentrations. Another explanation could simply be we did not use enough brain tissue to get an accurate reading. Though we are not as concerned with absolute concentration as we are relative concentrations, we thought that approximately ½ of the hippocampus should have yielded enough amyloid-β to be detected, but that may not be the case. In the future, we plan to experiment with this to find out exactly how much of the brain needs to be used. It is possible that the entire hippocampus or even the entire brain may need to be homogenized and used in the ELISA in order to yield proper results.

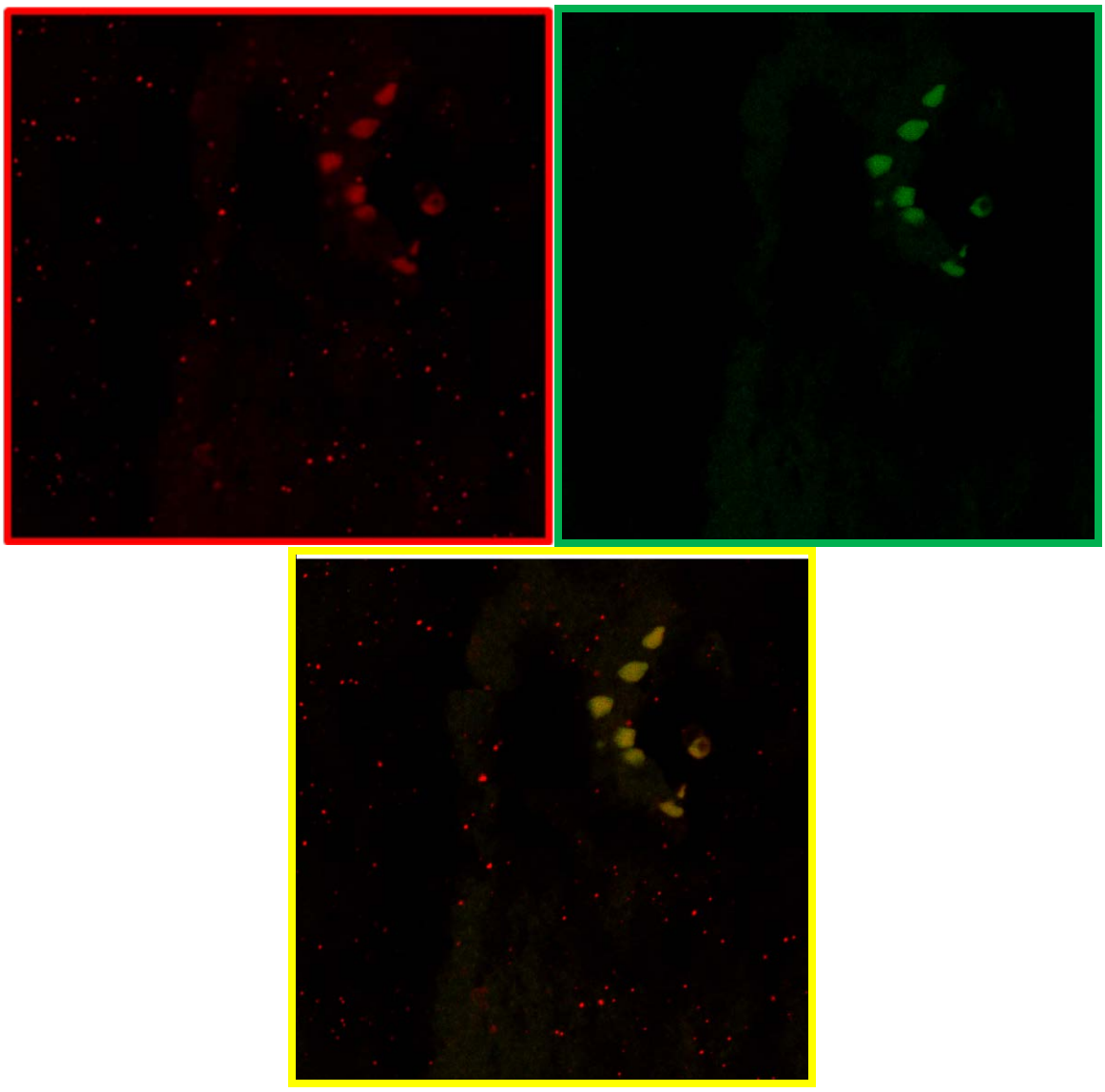

Figure 6: Picture from a male control PND90 with amyloid-beta in red (Cy5 fluorophore), microglia in green (FITC fluorophore) and the overlap in yellow.

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