“The Impact of Superimposed Acute Systemic Inflammation on Brain Immune Responses in Alzheimer’s Disease Mouse Models”

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Introduction

Alzheimer's Disease is a devastating neurodegenerative disorder characterised by the accumulation of neurofibrillary tangles and amyloid plaques. Alzheimer's Disease causes damage to brain cells and the leading cause of dementia. It has emerged in

recent years that inflammation occurring outside the brain (i.e. systemic inflammation) can potentially have negative impacts on brain tissue and that the Alzheimer's Diseased brain is particularly vulnerable to this.

I set out with ambitious goals for my research project. My research project was initially aimed at investigating how imposed acute systemic inflammation disrupts brain barriers and cognitive function in the APP/PS1 mouse model of Alzheimer's Disease. In essence, I intended to assess how infection occurring in the body periphery had an effect on the brains of normal versus Alzheimer Disease mouse models in the hours following infection.

I was to look at not only the physiological effects of this infection, but also the psychological implications through the analysis of cognitive data. I would also be looking at different stages of the immune response in these brain tissues, tracking the infection from 2 to 36 hours.

Early in my research project I began to experience roadblocks that made these goals that I had set out to achieve seem impossible. At first, the gaps in my knowledge and my inability to complete every single thing that I wanted to do in the timeframe that I had created for myself was frustrating. I had to continuously revise my plan again, and again as self-imposed deadlines would pass, or if I wasn't happy with my results. This led to some feelings of frustration. I soon learned, however, that *research is not linear*. The whole essence of research is figuring things out — if the answers were already clear there would be no need for research/exploration in the first place. My perspective shifted, and project management and personal development sessions provided to me from the Laidlaw Leadership Development Programme helped me to clarify what I wanted to get out of this experience. I wanted to obtain reliable results, even if they were not the results, I went into the project

expecting, I wanted to broaden my knowledge in this field and I wanted to gain confidence and conviction in my ability to do these things.

I redefined my goals, and chose to explore the differences in immune responses in the brain of the mice after 2 hours. My aim was to uncover the mechanisms underlying the relationship between systemic (body) inflammation and neuroinflammation in an Alzheimer Disease mice. I quickly learned that prior knowledge and making new discoveries truly go hand in hand, so I invested a great deal of time into reading papers to broaden my knowledge of inflammatory pathways and Alzheimer's Disease pathology, which allowed me to notice things in my results that I had previously overlooked and gain a deeper understanding of various aspects related to my research project.

Methods

4 groups of mice were obtained, two groups were transgenic Alzheimer's Disease mice (APP/PS1). The other two were wild-type (i.e. non-transgenic/'normal') mice. All of these mice were aged to 14-16 months, since Alzheimer's disease is typically a disease of older age. Within these groups half of the mice were treated with Lipopolysaccharide (LPS), a component of gram-negative bacteria that elicits an immune response, and half with sterile saline.

The major part of my research involved immunohistochemical staining. This is a method of staining tissues that exploits the components of the immune system. It works through use of antibodies which are specific Y-shaped proteins naturally produced by immune cells in response to antigens (foreign bodies). They bind to specific target antigen during the immune response.
This can be exploited by creating antibodies in different animals that bind to a plethora of different biomolecules/targets including normal cellular proteins from another species. This allows us to examine the levels and location of specific proteins of interest in a disease condition. Afterwards this so-called 'primary antibody' can be recognised and bounded by secondary antibodies which contain some form of indicator that allows us to visualise the location of the target. Thus antibodies can be used to 'label' targets of interest.

During the course of my research project I labelled various different molecules in the mouse brains that are indicators of inflammation. I began by labelling a molecule known as 'p65'. This movement of this molecule into the nucleus of a cell is an indicator of the beginnings of the immune response. Following three failed attempts of this, I finally obtained successful staining of p65, and could visualise the molecule in the nuclei of cells. Understanding where I went wrong with these initial stains, I went on to stain for other inflammatory molecules (cytokines) CXCL1, CCL3 and IL-1β. I also attained stains of microglial cells in the brains which are a type of immune cell in the brain that carry out important immune functions. I achieved this by labelling a molecule called IBA1 present in microglia.

Additionally I applied quantitative PCR, a technique used to examine gene expression, in order to quantify some of the same targets as above.

Results

I observed differences in the localisation and the distribution of microglia in the brains of the mice. Microglia also appear in various forms in the brain.

One such form of microglia seen were ramified microglia, characterised by small cell bodies and long, highly branched (ramified) processes. These microglia are in a 'resting state' constantly monitoring the brain environment for signs of damage or infection. I also observed microglia in an 'activated state' with short and thickened processes. When microglia are in an active state, it means they have transitioned from their resting state to a more responsive and dynamic state triggered by stimuli such as damage, infection, or disease. In this activated state, microglia begin to produce proinflammatory molecules (cytokines) such as those mentioned previously. These active/hypertrophic microglia were present in all groups around a cavity in the brain known as the ventral 3rd ventricle.

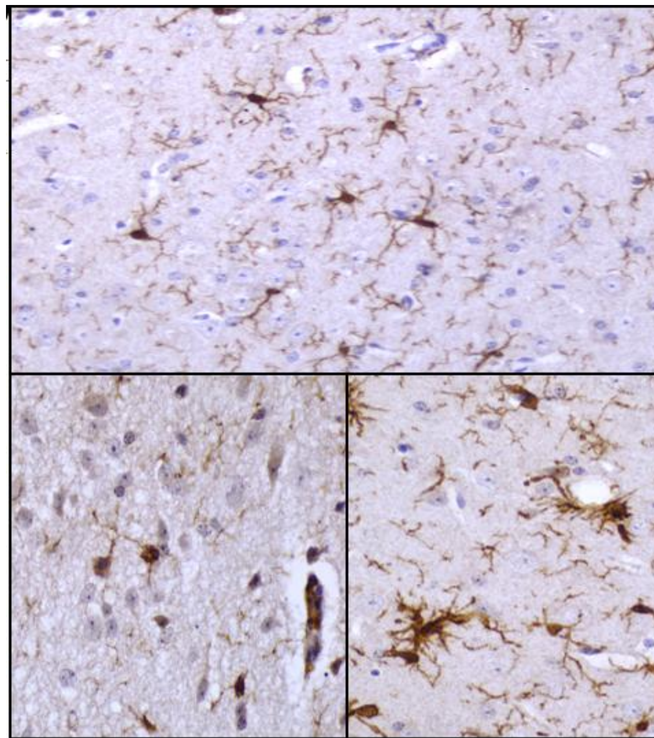


Figure 1: The different forms of microglia observed in the brain following labelling with IBA1. Top image, ramified microglia. Bottom left, ameboid (highly active) microglia. Bottom right, activated microglia.

In the transgenic Alzheimer Disease mice, even in the absence of bacterial LPS, I observed groups of these activated microglia around amyloid plaques - these are abnormal aggregates of amyloid beta protein that are thought to be one of the primary causes of Alzheimer's disease as they disrupt normal cell functioning and trigger inflammation.
The hippocampus was one area of the brain where this feature was abundant. This is a part of the brain that plays crucial roles in memory, cognition and emotion. The activated microglia as a result of the plaques suggests that the microglia here are more sensitive to stimuli as they are already in a reactive state. This heightened state of responsiveness and increased likelihood to contribute to neuroinflammation is termed 'Microglial Priming' which was evidenced in my observation.

The transgenes can therefore more readily initiate inflammatory responses, potentially worsening their condition.

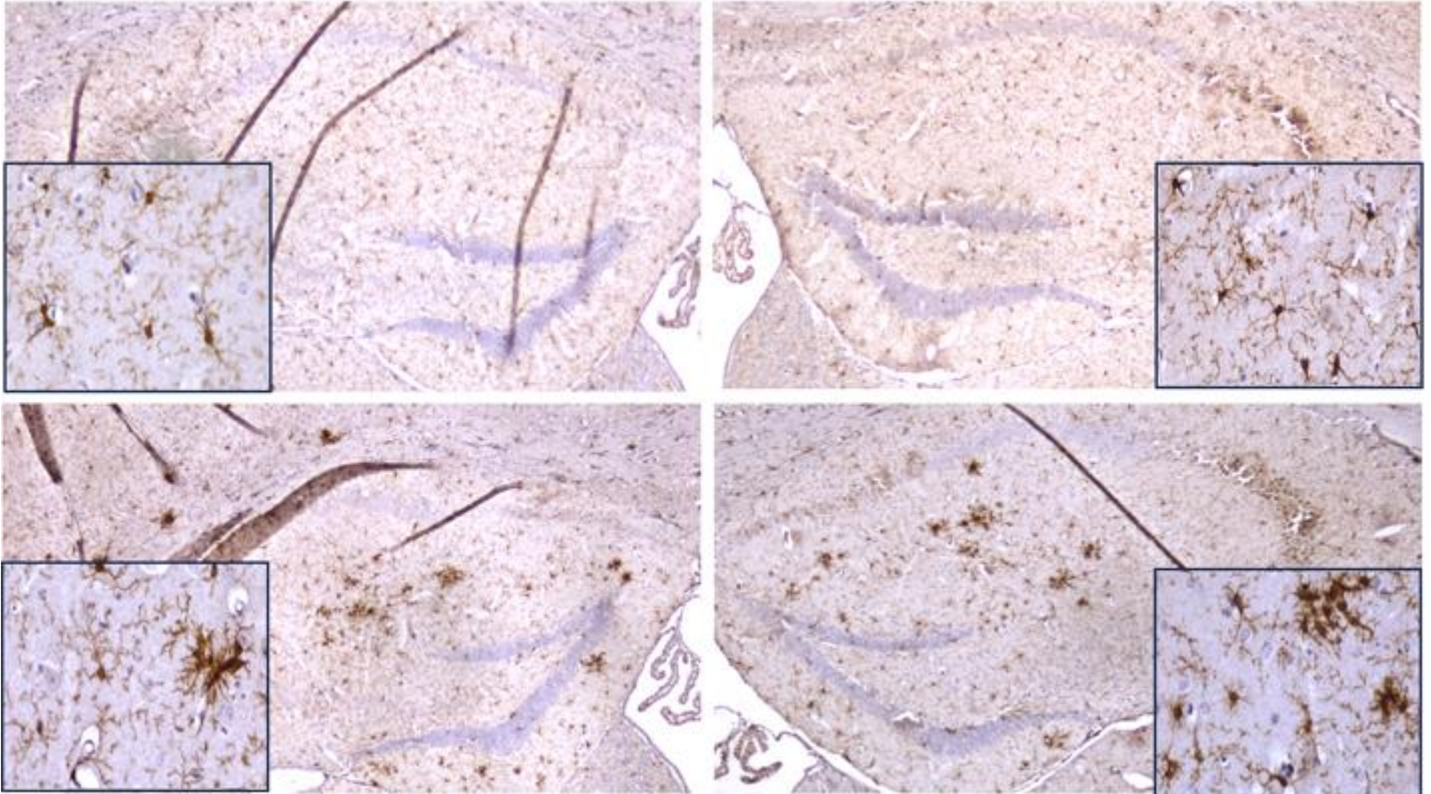


Figure 2: Microglia in the hippocampus of each of the four groups.
Top left- Wild type Saline, Top right- Wild type LPS, Bottom left- Transgene Saline, Bottom right- Transgene LPS. Activated microglia are observed around the plaques of the Transgenic (bottom two images) animals.

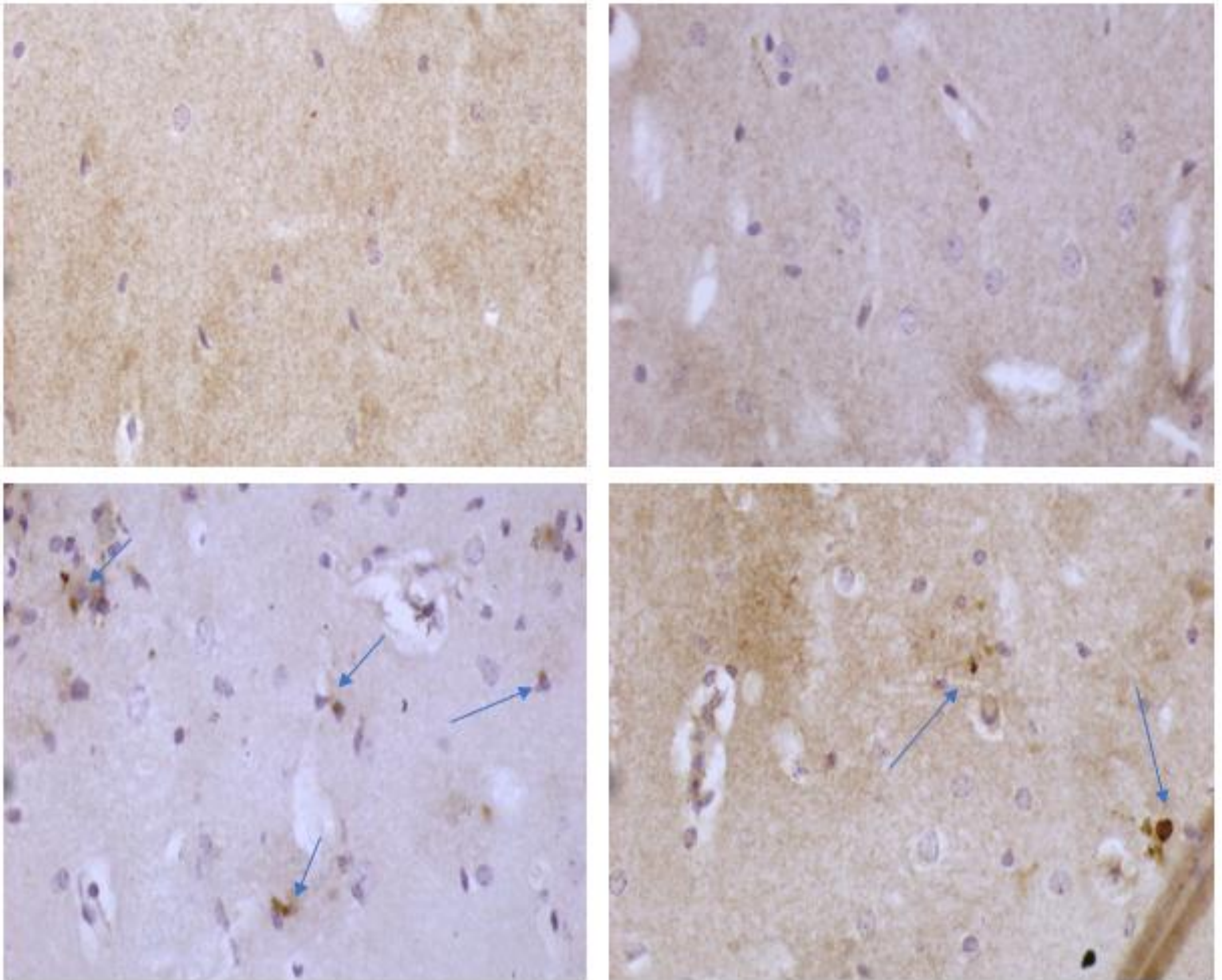


Figure 3: The hippocampus of each of the four groups, Wild type Saline, Wild type LPS, Transgene Saline, Transgene LPS. CCL3 expression observed in the hippocampus of Transgenic animals. Arrows indicating location of CCL3.

Looking at CCL3 labelling, this theory is further corroborated. Despite being treated with LPS, the Wild Type 'normal' mice didn't express notable CCL3 in the hippocampus. CCL3 is an important molecule (cytokine) that upregulates the immune response. It attracts white blood cells to the sites of damage/infection. Notable expression of cytokine was only found in the hippocampi of Alzheimer's disease mice, even without administration of any bacterial LPS.

This is evidence that these active microglia surrounding plaques in Alzheimer Disease are contributing to an ongoing state of chronic inflammation in the brain, independent of any contribution from peripheral inflammation.

The enhanced sensitivity of 'primed' microglia to subsequent stimuli is demonstrated by the Alzheimer's disease mice treated with bacterial LPS. In these mice, the extent of microglial activation appeared to be more extensive in the hippocampus, often reaching out further from the plaques. In contrast, Alzheimer's disease mice treated with saline displayed activated microglia limited to regions immediately surrounding the plaques.

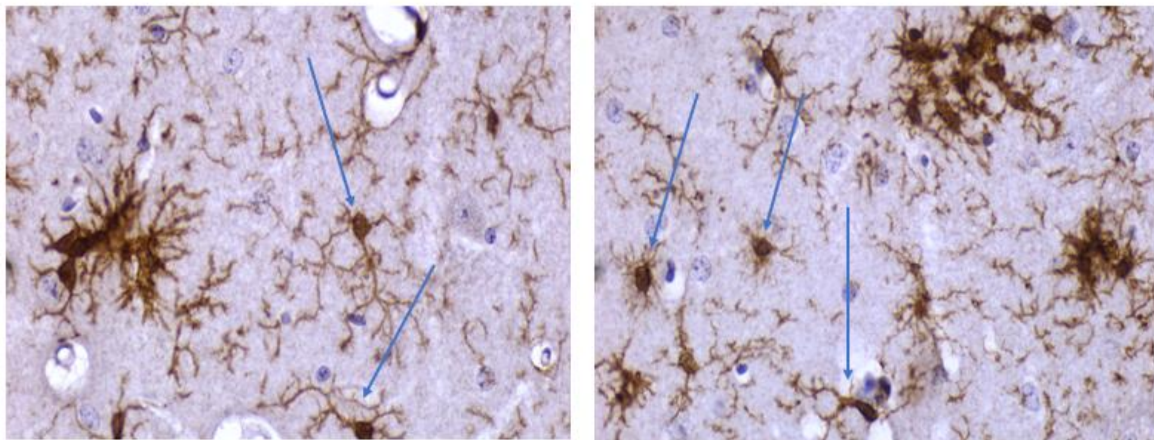


Figure 4: Left image displays the proximity of ramified (resting) microglia to the plaques in Alzheimer's disease saline treated mouse models. Right image displays activated microglia close to the plaques in Alzheimer's disease LPS treated mouse models. Arrows are pointing to the microglia

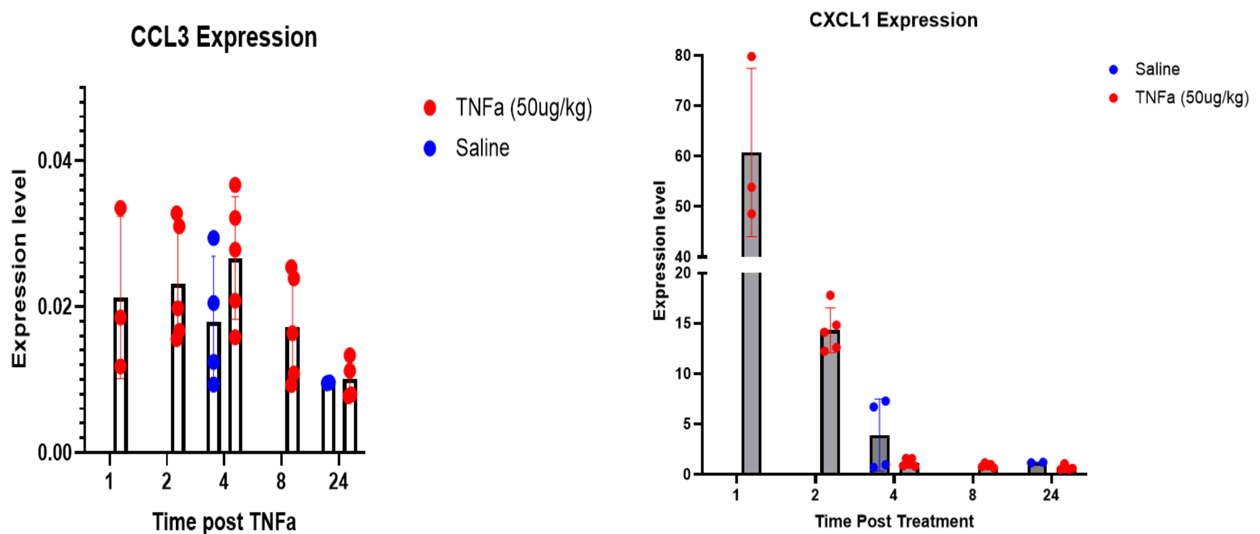
qPCR (quantitative polymerase chain reaction), is a laboratory technique used to measure the amount of a specific DNA sequence in a sample. I made use of a version of this technique called RT qPCR measures the amount of RNA in samples. RNA is the molecule that encodes proteins. By measuring the RNA in a sample, we can quantify how much of a particular transcript is present in cells, which may be predictive of how much of that protein will be made. For this I used brain tissue from a new group of all normal mice that had been

treated with either saline solution or TNFα (a key mediator of inflammation that can be activated by LPS). This was also administered in the periphery in normal mice.

I found that the pro-inflammatory molecule CCL3 is stimulated by TNFα. I created a time course to track the differences in CCL3 expression level over time. I found that the expression of this cytokine is highest 1-4 hours post TNFα administration.

However, statistical evaluation found this effect of treatment (saline vs. TNFα) is not significant. This could further substantiate why despite being treated with bacterial LPS, there was no CCL3 expression observed in the hippocampus of the LPS-treated Wild Type mice following staining. Microglial activation in response to the amyloid plaques had a greater influence on CCL3 expression than whether or not an inflammatory response had been induced in mice.

I did the same with another pro-inflammatory cytokine called CXCL1. Similarly statistical analysis by means of a two-way ANOVA revealed that there was not a significant difference due to treatment. However there were significant differences in CXCL1 expression at different timepoints, with the greatest expression levels seen at 1 hour post TNFα



treatment.

Figure 5. Graph of CCL3 and CXCL1 qPCR results.

As previously mentioned, CXCL1 was another cytokine that I visualised using immunohistochemistry. It was observed at the choroid plexus of the lateral and 3rd ventricle. The Choroid plexus is a specialised structure in the brain that produces and regulates the composition of Cerebrospinal Fluid (CSF) - the fluid that surrounds and protects the brain and spinal cord. The choroid plexus also acts as a vital barrier between the blood in the body and the Cerebrospinal fluid in the brain.

Although I did not observe significant CXCL1 in the brain tissue, the outer 'epithelial' cells of the choroid plexus were stained dark brown. This may indicate that the Choroid plexus is actively responding to the inflammatory signal triggered by LPS.
IL-1β is another key inflammatory molecule that I stained for. In LPS treated animals (both wild type and Alzheimer's disease mouse models), I saw white blood cells in the blood vessels of the choroid plexus.

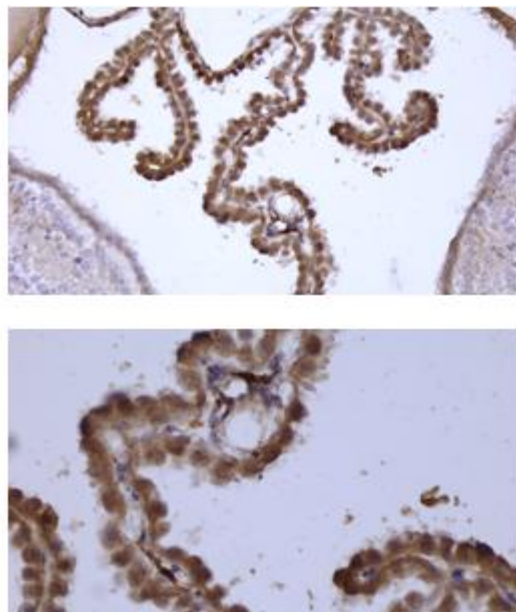


Figure 7: Image of white blood cells (dark brown staining) inside of the choroid plexus of an LPS treated mouse

During the course of my investigations there was one area in particular that peaked my interest. This was the bottom (ventral) area of the aforementioned 3rd ventricle (also referred to as the infundibular recess). This ventral area of the brain is heavily involved in hormone regulation and involuntary, or autonomic, bodily functions. This ventricle again acts as a barrier between the blood coming from the body and the brain. Interestingly, I observed highly active 'ameboid' microglia (see figure 1) surrounding the ventral 3rd ventricle in almost every animal across all the groups. These microglia are characterised by their dark appearance and lack of cell bodies. They were especially prevalent closer to the top and the bottom of this ventricle.

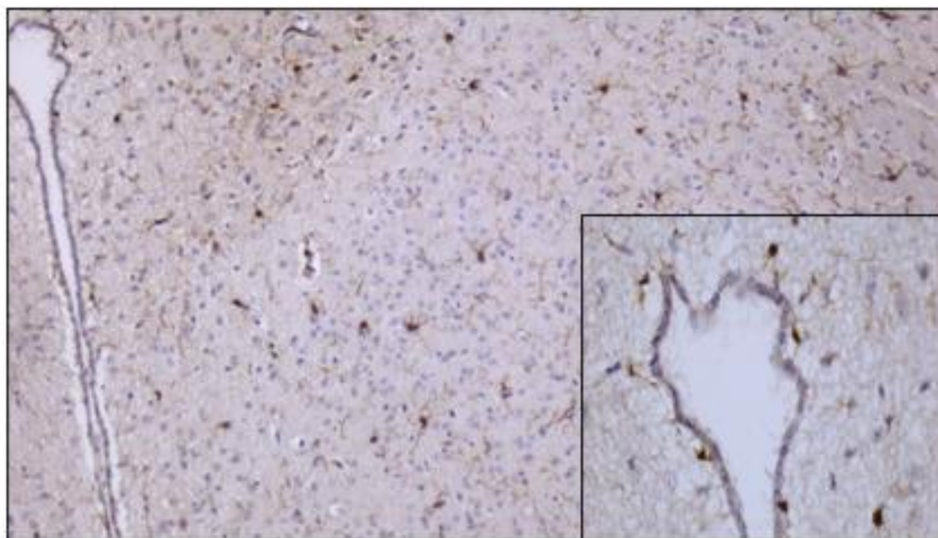


Figure 8: Image of the ventral 3rd ventricle. Close up Image of ameboid microglia around the top area of the ventricle

The literature concerning the ventral 3rd ventricle has shown that this ventricle is surrounded by tanycytes. Tanycytes are long, thin specialised cells that play important roles in various physiological processes, especially transducing changes in CSF glucose concentration to the brain cells that control energy status. They essentially send information about the CSF to cells inside the brain tissue. Interestingly tanycytes have been shown to have various functional chemokine receptors, including IL-6, TNFa. Proving that they do

have the potential to receive messages from pro-inflammatory molecules. They also have a close interaction with microglia.

Interestingly, the same occurrence was observed around the other brain ventricles. Due to the nearly-identical arrangement of microglia and various chemokines (CCL3,CXCL1) around this area of the brain in mice across treatment groups, I have reason to believe that the microglia here have a 'sensing function'. It is my hypothesis that the microglia surrounding this ventricle in particular constantly receive stimulation by the CSF causing them to remain in this activated state similar to the fashion observed in the hippocampus of Alzheimer Disease mouse models.

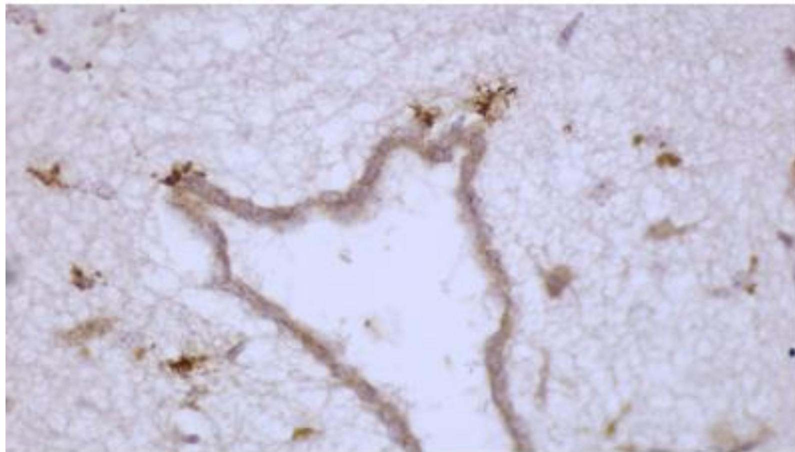


Figure 9: Close up Image of CCL3 expression in the same location (same animal)

I believe that the tanycytes present here not only work in tandem with these resident microglia, but also send out their long processes to interact with cells much further away from the site of inflammation (in areas such as the ventromedial Hypothalamic Nucleus) to upregulate the immune response. Of course this hypothesis would require further validation through additional experiments to confirm these findings and explore potential mechanisms in greater detail. Nonetheless this a start for understanding the role of the ventral 3rd ventricle and the interactions between tanycytes and microglia in the inflammatory response.

Conclusion

Despite only observing slight differences between the brain's response to systemic inflammation in wild-type versus Alzheimer's disease mice brains, a main takeaway was that this study elucidated the role of Alzheimer's disease pathology (in the form of plaques) on a state of ongoing inflammation in the brain. A similar influence of possible 'resident' active microglia surrounding the brain ventricles in all mice brains also may give insight into brain inflammatory processes. Perhaps a greater dosage of LPS to the mice would have made clearer the differences existing between treatment groups.

Overall I learned that despite challenges and adversity, in research it is important to be able to critically assess what has gone wrong experimentally and use that knowledge to inform your next steps. My leadership development experience helped to tie this all together, helping me build confidence in myself and my abilities. There is much still to be done in this area of research. This experience lit a fire in me to expand my knowledge in this field and grow to be able to help find the answers to these burning questions.