Reducing Excessive Microglia Activation to Reduce Neurodegeneration

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Introduction

The detriment of Alzheimer's disease (AD) can be accurately summarised in a single quote from Auguste Deter, the first person in history to be officially diagnosed with the neurodegenerative disease. "I have so to speak, lost myself," were the words she said to her doctor, Alois Alzheimer, in 1901(Lawton, 2024). More than 100 years later, this sentiment is still apparent amongst Alzheimer's patients, as the struggle for a cure persists. The hallmark symptoms of AD are neuroinflammation, oxidative stress and injury of cholinergic neurons, with the number of AD patients projected to increase to 150 million by 2050 (Gou, 2024), the staggering social and economic implications are apparent, as it is currently the world's most expensive disease (Cohen, 2015), with the World Health Organisation estimating the global cost of dementia could reach $2.8 trillion by 2030 (Brunier et al., 2021). Though possible cures have been deemed rooted in our cells, preventative measures such as lifestyle choices can reduce the risk of the disease developing: cardiovascular diseases, lack of sleep and exercise are all linked to perpetuating the chances of Alzheimer's disease (Genova, 2017). In my research, I sought to investigate the methods of reducing excessive microglial cell inflammation, such as possible epigenetic treatments for AD, commenting on the complexity of the problem at hand, whilst elucidating the future climate for treatments of AD. In this essay, I reflect on my shortcomings and my skills developments after undertaking this research project, whilst providing insight into how this will inform my later career prospects.

Section 1: Research Context and Summary

In 1986, the nuclear factor in B lymphocytes binding to the kappa enhancer of the gene encoding the k light-chain of immunoglobulin was discovered. The genesis of the name of NF-kB was derived from the fact that it was a nuclear factor (transcription factor) that bound near the kappa light chain gene in B cells or NF-kB. David Baltimore, one of the key discoverers of the transcription factor, further uncovered the significance of the kappa enhancer protein binding site kB in promoting transcription activity in B cells. Through additional research and evidence, it can no longer be rebuked that the most important role performed by NF-kB is its contribution to inflammation and immunity responses.

The mammalian NF-kB factor family consists of 5 members: NF-kB1 (p105/p50), NF-kB2 (p100/p52), p65 (RelA), Rel B and c-Rel. Any two members of the family can form homo or heterodimers that bind to the IkappaB protein (inhibitor of NF-kB) and sequester in the cytoplasm in an inactive form, then migrate to the nucleus when the cell is stimulated. Overactivation of NF-κB signalling not only promotes tumour cell survival, invasion, metastasis, genomic instability, and metabolic abnormalities, but also reshapes the immune-suppressive microenvironment, promoting immune escape and resistance to immunotherapy.

Acetylation is an important mode of post-translational protein modification in eukaryotic cells. Both histone and non-histone proteins can be reversibly acetylated so as to alter gene expression and the function of proteins. Depending on the necessity of specific gene expression, a dynamic equilibrium between acetylation by histone acetyltransferases and deacetylation by histone deacetylases continuously undergoes alterations to maintain the acetylation of proteins in the cell. Post-translational modification can affect protein-protein interactions, enzyme activity and the regulation of many cellular processes. Inflammation of a cell can be incurred when alterations in the equilibrium result in a pathological state, affecting protein-protein interactions, enzyme activity and the regulation of many cellular processes. In the nucleus, DNA is wrapped around small proteins (histones). Together, these form the 'nucleosome' which is the fundamental packing unit of DNA. Upon the modification of histones, their interaction with DNA changes and alters the chromatic structure to expose promoter regions where transcription factors, such as NF-kB, can bind to initiate numerous cellular processes, such as inflammation.

Important post-translational methods of modification, namely acetylation and methylation, have been identified as important regulatory methods for determining the nuclear activity of NF-kB and how it impacts the transcription of genes. Other examples of post-translational methods of modification include phosphorylation and ubiquitination. The RelA subunit of the NF-kB family is the most studied for its regulation by lysine acetylation and methylation. Subsequently, DNA binding, transcription activity and protein stability are distinct NF-kB functions that can be investigated using specific lysine residues of RelA (Chen et al., 2015).

After the discovery of NF-kB almost 40 years ago, the knowledge about its applications have greatly developed; the exploration of its role in numerous cancers, autoimmune diseases and cardiovascular diseases has implicated an overarching conclusion: it is an important protein (Gou, 2024). The most prevalent and best studied of the NF-kB family, p50-p65 heterodimer (Rel A), was the subunit that we focused on during the project. In resting cells, the inactive form of p50-p65 is present in the cytoplasm, bound to inhibitory proteins known as IkBs. The presence of specific inducers such as inflammatory cytokines (TNF), bacterial products (LPS) or oxidative stressors (hydrogen peroxide), can induce phosphorylation and ubiquitination of IkBs so they degrade and release the p50-p65 dimer, translocating it into the cell nucleus. As seen in **Figure 1.**, the transcription factor, NF-kB, can upregulate specific genes, stimulating the whole cell through inducing cellular cascades and subsequent processes (Ghizzoni et al. 2011).

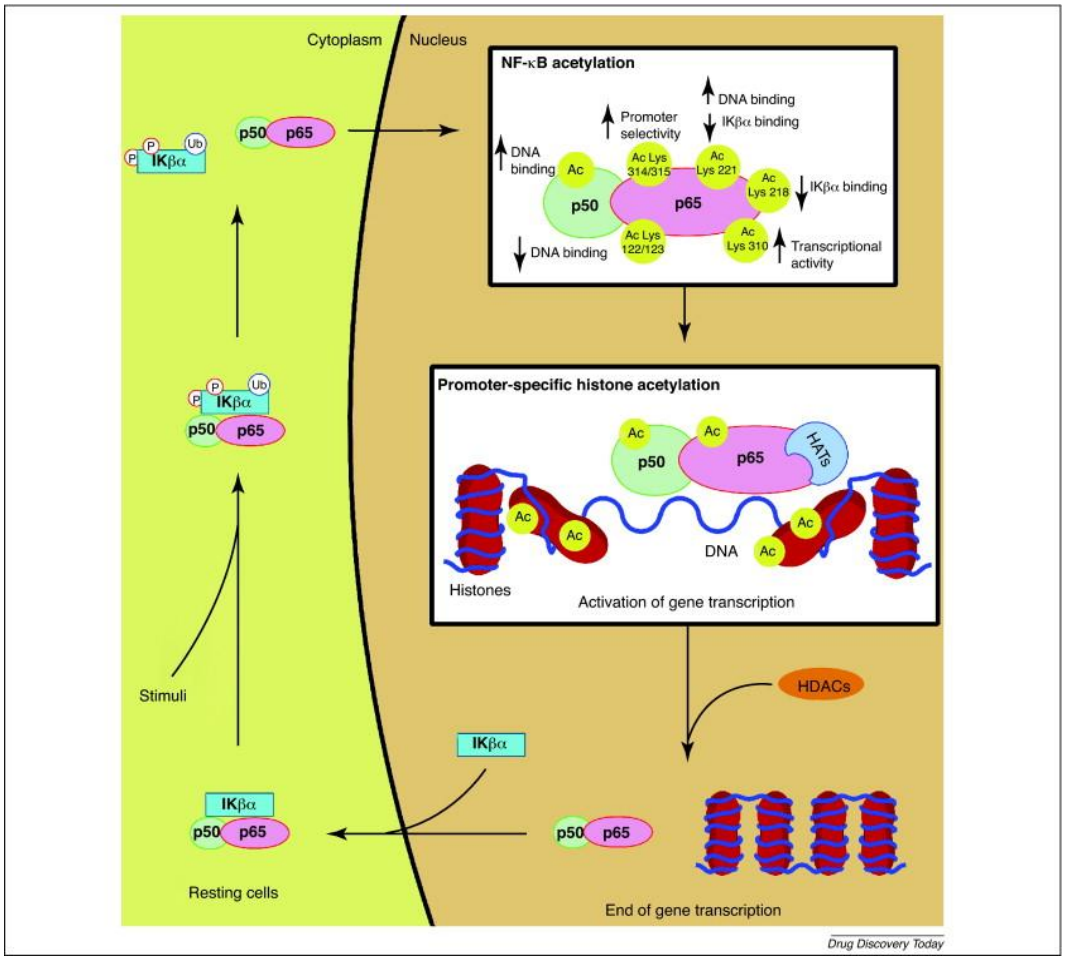

Figure 1. The degradation of IkB in cell cytoplasm, causing the translocation of NF-kB (p50-p65) into the cell nucleus where it can bind to DNA promoters and carry out promoter-specific histone acetylation to upregulate gene expression (Ghizzoni et al., 2011).

Seven lysines have been identified within RelA. These residues include lysines (K) at positions 122, 123, 218, 221, 310, 314 and 315. Acetylation of K122 and K123 by p300/CBP seems to negatively regulate NF-κB mediated transcription by reducing RelA binding to the κB enhancer. Whereas acetylation of K314 and K315 by p300 affects neither NF-κB shuttling, DNA binding, nor the induction of anti-apoptotic genes, but differentially regulates the expression of specific sets of NF-κB target genes.
In the duration of the research project, we used mutants of the Rel A subunit, namely K314 and K122, to test how altering specific residues on these subunits affects their location in the cell and, subsequently, activation of the cell through debilitating their ability to alter gene expression.

Through conducting literature reviews, I sought to contextualise and learn more about the functions of NF-kB as well as the uses of acetylation in epigenetic treatments, not just AD (Ghizzoni et al., 2011). It has been shown that the p65 NF-kB subunit (Rel A) is acetylated at specific sites by different HATs. Acetylating at different lysine residues can do different things. Recent work by Yang *et al.* showed that acetylation at Lysine 310 enhances the transcriptional activity of p65 by impairing the methylation of lysine residues 314 and 315, which is important for the ubiquitination and degradation of chromatin-associated p65. By contrast, acetylation at other positions decreases the NF-κB transcriptional activity.

Acetylation on the lysine residues 122 and 123 by p300 and PCAF reduce binding of NF-κB to the DNA κB enhancer and facilitate binding to IκBα and subsequent export from the cytoplasm. Acetylation at lysine residues 314 and 315 do not affect the general transcriptional activity of NF-kB but they modulate the expression of specific sets of genes. This helps to indicate that site specific acetylation of P65 regulates the specificity of NF-kB-dependent gene expression.

Upon analysing the accumulation of this data, it can be concluded that direct acetylation and deacetylation of specific lysine residues in the p50/p65 subunits of NF-kB play an important regulatory role in the functions of the transcription factor. This indicates that the resulting effect of both HAT and HDAC inhibition depends on the selectivity for specific NF-κB acetylation sites. The crucial role of NF-κB acetylation and deacetylation in the regulation of NF-κB-mediated gene expression raises the idea to modulate inflammatory responses by modulating NF-κB acetylation levels with HAT and HDAC inhibitors (Durham et al., 2017). NF-kB has been shown to interact with both HATS (co-activators for gene transcription) and HDACs (repressors of gene transcription by deacetylating histones at promoters of NF-kB target genes) (Ghizzoni et al., 2011).

When undertaking the practical work of the project, we transfected BV-2 microglia cells with a plasmid expressing an NF-kB-GFP fusion protein. We also had some mutated versions where some of the lysine residues that can be acetylated had been replaced with arginine (has a similar structure to lysine but cannot be acetylated). The other RelA mutants we used to have residues that were replaced by glutamine which has a structure that mimics an acetylated lysine residue. As depicted in **Figure 2. And Figure 3.,** once we transfected the cells with the fusion protein, we activated some of the microglia cells and analysed them under a fluorescence microscope to see if the mutations had affected the localisation of the NF-kB subunit.

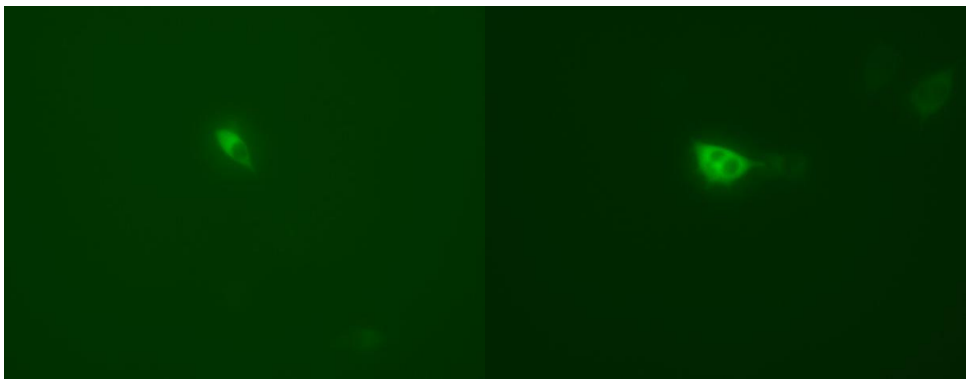

Figure 2. K314R mutants presenting localisation of NF-kB-GFP fusion protein before activation with LPS.

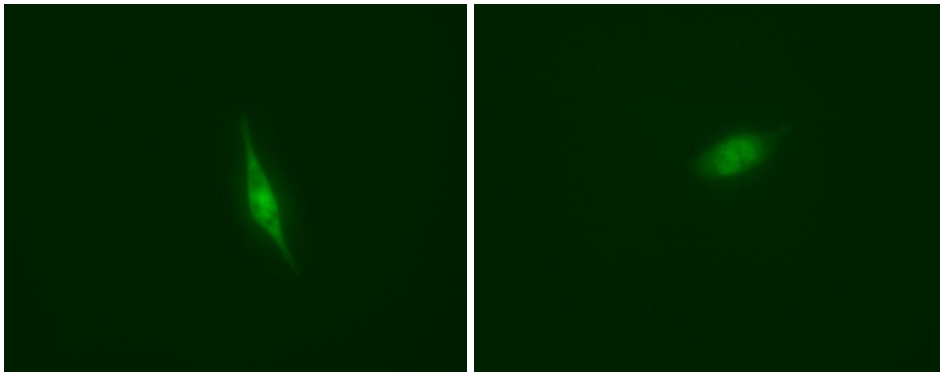

Figure 3. K314R mutants containing NF-kB-GFP fusion protein after being activated with LPS.

Section 2: Results and Analysis

As can be seen in **Figure 2.** and **Figure 3.**, the location of the NF-kB-GFP fusion protein changes when comparing cells activated by LPS and the resting cells. As the expected impact of K314 residue mutations is a change in what specific genes are expressed, it is expected that the NF-kB should still move into the nucleus after activation of the cell via a stimulus. After collecting the images of the successful transfected cells, I used ImageJ to quantify how the intensity of the fluorescence, or the Gray Value, differs in different areas of the cell. As I found some difficulty with culturing the cells, the transfection and translocation in some of the mutants was not successful, therefore, I only gained viable results from the K314R mutants. Though this was very much a downside in my project as I lost the ability to compare how different mutants interacted within an activated cell, the experience and results I gained were formative and I believe representative of previous research data.

After quantifying and plotting the data in **Figure 4.**, I used R studio to measure the total area under the curve then the area under the curve, which I approximated, was the nucleus. As the plot represented the intensity across the whole cell, I needed a method to compare the localisation of the fluorescence, and therefore, the RelA mutants. I had only just familiarised myself with R studio this year and it took me a while to figure out the code but once I did through lengthy research and countless YouTube videos, I simply substituted what data was necessary to create the plot and calculate the area under the curve (AUC). At first, I tried to come up with the code that could select the desired vectors from the total dataset so as to measure the specific area that would be the cell nucleus, but after a while I realised, I could just copy this data and create a separate data set to simplify the process.

The code I used to plot and calculate the data:

```
ggplot(Data_Values, aes(x = `Distance_(Inches)`, y = Gray_Value)) + geom_line(colour = "black") + labs(x = "Distance (Inches)", y = "Gray Value") + theme_minimal()

LPS.40F.Nuc.Dis <- Values_40f $ 'Distance_(Inches)'
LPS.40F.Nuc.GV <- Values_40f $ Gray_Value
auc <- trapz(LPS.40F.Nuc.Dis, LPS.40F.Nuc.Dis)
print(paste("Area under the curve:", auc))
```

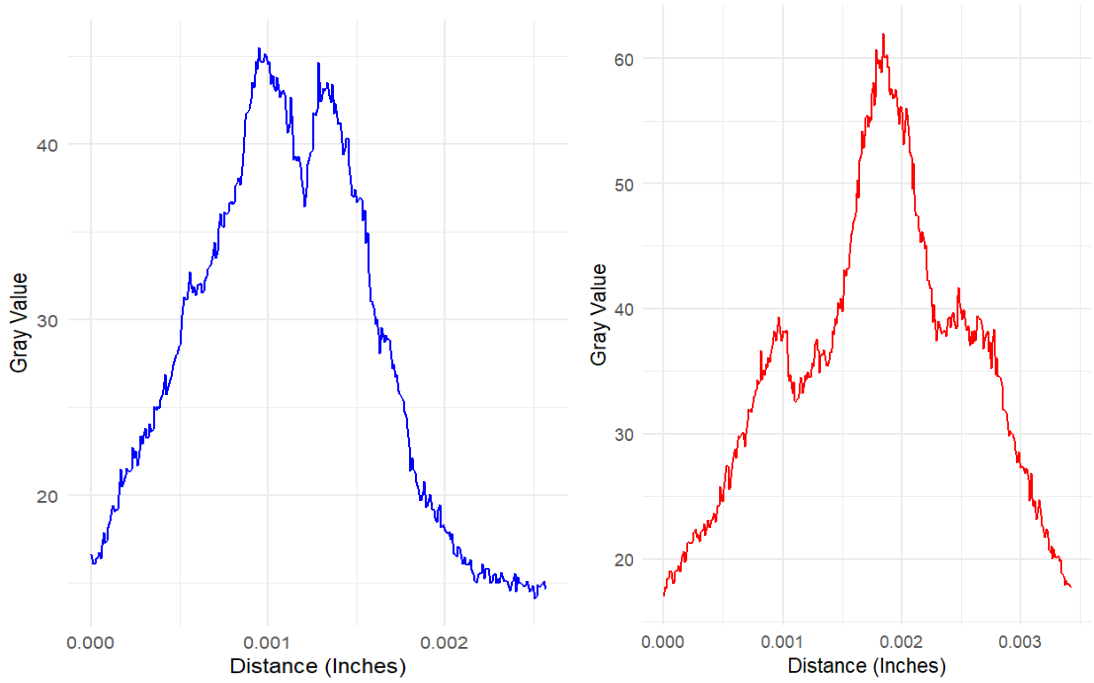

Figure 4. Resulting plot of Gray Values after creating a cross section of the cell images to quantify the fluorescence and to compare how it varies in a cell. **Left**: Variation of Fluorescence (emitted from NF-kB-GFP fusion protein) across a resting cell. **Right:** Variation of Fluorescence across an activated cell.

When comparing the plots in **Figure 4.,** the fluorescence varies greatly when comparing the cytoplasm and nucleus. In the left plot, there is a visible decline in the Gray Value (fluorescence) at the area that represents the cell nucleus. In comparison to the right plot, there is a sizable increase of fluorescence in the cell nucleus.

Without LPS table

Mutant Type	AUC	Nucleus AUC
K314R (a)	0.04231	0.01591
K314R (d)	0.02818	0.01546
K314R (f)	0.07552	0.03245

With LPS

Mutant Type	AUC	Nucleus AUC
K314R (d)	0.02539	0.04307
K314R (e)	0.0673	0.04571
K314R (h)	0.04779	0.03222

Figure 5. The table above presents the individual cell images that were measured to quantify the fluorescence. In the first table, the cell mutants that had not been activated by LPS were measured. The total area under the curve (AUC) presents how much of the fluorescence is present in the cell. The higher the number, the higher the average fluorescence across the cell. As we were attempting to discern how the fluorescence, and therefore the mutants, changed after activation of the cell, I also compared the approximated area of the curve that represents the nucleus. When comparing the average AUC of the cell and nucleus between activated and inactivated mutants, on average the fluorescence in the nucleus increases as the K314R mutant does not necessarily impact localisation of the protein, but it impacts what genes are expressed. Subsequently, though the cells still seem to be successfully activated, further research would be required to investigate whether gene expression still remains the same.

Section 3: The Future of the Field

David Selkoe at Harvard Medical has described AD as filled with “roiling scientific controversies” (Lawton, 2024). Through collecting numerous publications, the future of finding a cure for the disease seems hopeful. In addition, recent evidence has suggested that lifestyle has just as much of an impact as genetics. Sleep, diet, and suffering from other cardiovascular diseases has been shown to increase the risk of developing AD. Therefore, it can be argued that a cure is not only rooted in epigenetic modifications and inflammatory suppressants, but also early in our lives where we still have a chance to make a difference. It is challenging to tackle the issue of how to promote the rapid and consistent application of cellular and animal studies to human applications. The diversity of the disease spectrum and inter-species variability are significant obstacles. More advanced and closer to the real human environment research methodologies are required, encompassing spatial multi-omics, single-cell sequencing, organoids, genetically engineered animal models, 3D bioprinting, and other cutting-edge techniques.

Summary of possible epigenetic modifications to combat AD:

DNA Methylation*:*

- Alterations in DNA methylation is linked to AD. Increased methylation is seen in genes such as *PSEN1*, *APOE*, and *MTHFR*.
- DNA methylation is managed by DNA methyltransferase (DNMT) enzymes, but targeting these for treatment is challenging due to the lack of specific inhibitors.

Histone Modifications*:*

- Histone deacetylase (HDAC) enzymes play a role in AD pathology, with increased expression of HDAC2 and HDAC3 negatively impacting cognitive function.
- HDAC inhibitors have shown promise in improving cognition in AD models, though they do not prevent neuronal degeneration.

Non-coding RNAs (ncRNAs)*:*

- Altered expression of ncRNAs, especially microRNAs, is linked to AD. For example, miR-29 and miR-16 regulate the expression of genes involved in amyloid pathology.
- Targeting ncRNAs offers therapeutic potential, but delivery challenges remain a barrier.

The NF-κB signalling pathway plays a pivotal role in numerous human diseases by regulating various immune responses and contributing to inflammatory lesions. Therefore, it is essential to develop targeted therapies that address specific effectors involved in these diseases. However, the complexity of the NF-κB pathway, with its involvement in multiple physiological responses, presents a challenge in avoiding side effects associated with its inhibitors. Future research must focus on creating more selective NF-κB inhibitors to treat inflammatory disorders effectively. While the therapeutic potential of targeting NF-κB signalling is significant, it is equally important to address the concerns related to adverse effects due to its broad biological functions. As highlighted by this article, therapies targeting NF-κB will inevitably provoke side effects (Wood, 2018). Thus, precise drug design, synthesis, and delivery methods are crucial, with nanomaterials playing a key role in advancing these approaches. Future efforts should aim to enhance the efficiency of NF-κB-targeting therapies while minimising adverse outcomes, paving the way for breakthroughs in immunotherapy.

Moving forward, several strategies for targeting NF-κB signalling should be explored: (1) developing inhibitors that can simultaneously target multiple key nodes within the pathway; (2) improving the bioavailability, safety, and stability of small molecule drugs; (3) increasing drug specificity to reduce interference with other cellular functions; and (4) investigating natural products and traditional medicine for potential therapeutic applications.

Future Prospects

According to a recent New Scientist article, a vaccine is currently being developed as a possible method of utilising the immune system to target amyloid beta plaques (one of the main symptoms of AD). Florida based biotechnology company Vaxxinity, are planning to begin phase three clinical trials on their most advanced vaccine, UB-311. By 2030, the vaccine could be administered to people diagnosed with mild Alzheimer's, revolutionising how we combat AD through 'reducing cognitive decline by around 50 per cent', says the Chief Executive of Vaxxinity.

In contrast to this, Biogen (another biotechnology company) based in Cambridge, Massachusetts has recently abandoned Aducanumab, a monoclonal antibody and the first FDA approved AD drug since 2003. Biogen has now expressed favour in a similar monoclonal antibody, Lecanemab, which has been developed alongside Japanese firm, Eisai. In clinical trials, this drug produced significant reductions in cognitive decline. In addition, it increased the participants' ability to live independently in most, but not all cases. When comparing current vaccines to monoclonal antibodies, the latter appear to be more successful as they specifically target the amyloid plaques rather than the naturally existing soluble-protein form of amyloid in the brain. Yet, these antibodies remain a work in progress

as they disintegrate relatively quickly, requiring continuous intravenous administration, at a substantial sum of $26,500 per patient per year. The significance of the UB-311 vaccine is a promising prospect as it is not only cheaper and easier to administer compared to monoclonal antibodies, but it is a form of active immunity where the body can continuously utilise the immune system to combat the development of AD.

The discovery and formulation of cutting-edge drugs is not the only method on the horizon for reducing the expansion of AD. It has been increasingly made apparent that our cardiovascular health, and how we maintain it, can influence our chances of developing AD, with various autopsies signifying that 80% of people with AD also suffer from cardiovascular diseases (Genova, 2017). This includes high blood pressure, diabetes, obesity, high cholesterol, all possible contributors to dementia. Studies have shown that it is the maintenance we do when we are young that can truly impact our health as we age. Not only is it our physical health that can influence this likelihood, but also maintaining our neural plasticity and cognitive reserve.

Neural plasticity is the ability to constantly remake new synapse connections, through learning new skills and seeking out new experiences. In maintaining this habit, even as you age, it can prove to be a sufficient buffer as ageing depletes your synapses as well as making it more difficult to form new synapses. Cognitive reserve is the amount of excess functional synapses an individual has, which can also act as a buffer for those that develop dementia. People who have higher levels of education and a high degree of literacy who also regularly engage in mentally stimulating activities all have more cognitive reserves (Genova, 2017). Ideally, learning new things and building more cognitive reserve is not done through simply learning and retrieving information; ideally, they should be as deep as possible, engaging your emotions and senses consistently.

Conclusion

Throughout this reflective research essay, I have sought to examine how the climate for AD treatment is rapidly evolving. From conducting literature reviews, to carrying out my own lab work, my comprehension of what it means to carry out research has distinctly evolved. In diversifying my skill set and having the opportunity to work outside my comfort zone, I have learnt so much about the life of an academic.

I have sought to highlight how epigenetic modifications can equally contend with cutting edge drug development. I have also exemplified how more holistic and early preventative measures can do a lot to sustain our ability to keep forging new neuronal connections and strengthen us against the symptoms of AD, even after we develop it.
The dynamic and homeostatic features of the body mean that diseases and health are interlinked; our lifestyle choices have many, seemingly undetectable effects on our body that we may not be aware of until it is too late. It is important to keep in mind that how we prioritise our health now, can have even more long-lasting effects than any treatment or drug; the importance of advocating for responsibility over our health is just as much a priority as it is pioneering new medical treatments.

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