Evoking a Reaction: Alzheimer's Disease, Sex and Astrocytes

Discovering Patterns in Astrocyte Reactivity in the APP/PS1 Hippocampus

Internship Report (2020-2021)
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Layman's Summary

Alzheimer's disease is a neurodegenerative disorder with no current cure. Even though women are significantly more affected, the disease has global reach, as 55 million people are suffering from it worldwide. Alzheimer's disease is still not entirely understood but an important factor is the accumulation of a small protein (amyloid-beta) which grows into larger plaques throughout the brain. Astrocytes, a cell-type involved in maintaining the central nervous system, are significantly affected by this accumulation. As a result, astrocytes go into an altered, reactive state. The implication of this state is not entirely clear, but changes in gene expression point towards a less neuroprotective profile. Like in Alzheimer's diseases, sex differences are observed within astrocytes as well. The relationship between reactive astrocytes, sex and Alzheimer's disease might thus be an important link in understanding the disease. Therefore, this study performed a visual, exploratory analysis on male and female mouse brains, comparing astrocytes between healthy brains and brains expressing amyloid-beta plaques. Significant spatial relationships between amyloid-beta plaques and reactive astrocytes were observed. Astrocyte reactivity, as well as increases in astrocyte amounts, was somewhat concentrated to a particular brain region (the dentate gyrus). Finally, no significant sex differences were found but further steps have been suggested. This study therefore confirmed a relationship between astrocytes and amyloid-beta plaques and indicated several follow-up studies. Overall, we provided relevant information regarding a relationship that is still not entirely understood which might be important in, not only understanding Alzheimer's Disease, but also finding a cure.

Abstract

Alzheimer's Disease (AD) affects 55 million people worldwide and women are at a significantly higher risk of diagnosis than men. Although the exact mechanisms are not completely understood yet, amyloid-beta (Aβ) plaques are thought to play an important role. Other pathological markers, such as reactive astrocytes, have been observed. Astrocytes are a type of glial cell that play a large role in neuronal upkeep and signalling. When located close to Aβ plaques, they become reactive by undergoing hypertrophy and overexpressing intermediate filaments such as glial fibrillary acidic protein (GFAP). We have used APPswe/PSEN1dE9 mice, hereafter called APP/PS1, to investigate the relationship between astrocyte reactivity, Aβ plaques and sex. Using immunohistochemistry, we compared healthy and AD astrocyte reactivity by measuring GFAP-positive cell count and fluorescence in the hippocampus and its subregions (CA1, CA3 and DG). We validated the presence of reactive astrocyte clusters in APP/PS1 mice across hippocampal regions and found that reactivity and cluster formation was significantly higher in the DG of APP/PS1 mice. We also found that astrocyte count increased significantly in the DG in a somewhat concentrated manner. Finally, a spatial relationship between reactive astrocyte clusters and Aβ plaques was established, as plaques were rarely present without astrocytic clusters and vice versa. No significant sex differences were found, though a more detailed plaque analysis is recommended. Future studies might therefore include performing a more in-depth analysis of plaque and cluster colocalisation, so that quantitative differences can be established. Additionally, a more in-depth analysis of the DG might an interesting topic for future research as well.

Prefix

Though this project took up a substantial amount of time, I also worked on a behavioural study regarding the subarachnoid haemorrhage (SAH). We hypothesised that SAH recovery is limited because of an overactive immune response. Therefore, we did behavioural tests on mice that received an operational SAH, and mice that received a SHAM operation. Several mice from either group were also given an intraperitoneal injection of a neutralizing monoclonal antibody directed against murine C5, thereby suppressing immune response, whereas the others were given an IgG isotype. My role in this project was to handle, inject and perform all behavioural experiments (including an open field test, a Barnes Maze, and a novel object recognition test). I also performed numerous neurological tests to confirm post-operative animal wellbeing and helped with perfusions after test completion. I will continue this project in the coming year, by analysing the data, performing more immunohistochemistry, and writing another in-depth report.

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Introduction

Alzheimer's Disease and the Amyloid Cascade Hypothesis

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder and accounts for 60-70% of all dementia cases (1). Currently, 55 million people are suffering from AD worldwide, which is expected to increase to over 100 million by the year 2050, likely becoming strenuous for health-care systems globally (2,3). Though several pathological changes are observed, the underlying cause of AD is yet to be established (4). Consequently, there is no cure for the disease but only treatments for its symptoms (5). A well-known pathological feature is the accumulation of amyloid-beta peptides (Aβ) leading to Aβ plaques (4). Due to a cleavage of amyloid precursor protein (APP) into several amino acid fragments, either Aβ40 or Aβ42 is formed. These accumulate into large, insoluble amyloid fibrils eventually resulting in plaques across the brain (4). The strong correlation between the occurrence of AD and Aβ plaque formation prompted the amyloid cascade hypothesis, suggesting that Aβ plaques cause other pathological AD markers and hence, AD itself (6). However, an increasing number of researchers are refuting this hypothesis as it underestimates the disease's pathological complexity, and drugs successfully limiting plaque formation have not resulted in clinical benefits (7–9). Research delving deeper into the exact pathology of AD is therefore crucial.

Astrocyte Reactivity

Because neurodegenerative diseases were mostly associated with neuronal death, the role of glial cells gained interest relatively recently. Earlier, astrocytes were only considered to play a minor, supportive role or studied as a reliable marker for diseased tissue (10,11). It is now known that astrocytes have numerous functions, such as controlling blood flow, regulating the blood-brain barrier together with pericytes, and controlling formation, maintenance, function, and removal of neuronal synapses (12). Their ability to so is, in part, contingent on their expression of intermediate filaments (IF), the most important being glial fibrillary acidic protein (GFAP) (12). In the presence of Aβ plaques, astrocytes go into a reactive state and, though this response is still not entirely understood, a key feature of astrocyte reactivity is an upregulation of GFAP (12,13). It is likely that this reactive response initially allows for astrocytes to regulate and internalise Aβ, thereby sequestering plaques. However, these benefits appear to coincide with adverse effects such as abnormal Ca^{2+} signalling, excitotoxicity, abnormal energy metabolism, neurovascular dysregulation and neuroinflammation (14,15). The possibility therefore arises that reactive astrocytes play a larger part in the exacerbation than prevention of AD. Perhaps further research into the relationship between reactive astrocytes and Aβ would aid in the understanding of the mechanisms at play.

Sex Differences

Research into sex differences remains limited, especially considering the disproportion in AD cases, as two-thirds are female (16,17). Astrocytes might provide insights to this disproportion as they express enzymes involved in steroid synthesis, metabolism and release neuroprotective steroids, thereby possibly having a sex-dependent response to AD (18,19). Observations in this realm have been made before. Astrocyte resistance to oxygen-glucose deprivation appears sex dependent, likely due to higher activity in aromatase, which is an enzyme converting testosterone to estradiol, in females than in males (20). Other studies have found that perinatal testosterone, oestrogen and progesterone affect astrocytic inflammatory response, the two latter also affecting mitochondrial function (18,21,22). Because astrocyte response appears to vary between sex in numerous ways, it is important to consider how astrocyte response to Aβ plaques might differ between sexes as well. This may, not only provide information about astrocytic sex-based responses, but also AD pathology itself.

General Aim and Research Question

In both APPswePS1dE9 (APP/PS1) mice and healthy mice, we measured GFAP-positive cells and Aβ plaques in the hippocampus, the Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3) and Dentate Gyrus (DG). In doing so, we examined the level of astrocyte reactivity, the number of GFAP-positive astrocytes and the spatial relationships between reactive astrocytes and Aβ plaques across the hippocampus. Finally, this analysis was also completed per sex, allowing for a detailed sex-based investigation. Considering the complicated relationship between Aβ plaques, reactive astrocytes, and the observed sexual differences, our research aims to answer the following question: is there a pattern in hippocampal astrocyte reactivity and count in APP/PS1 mice? More specifically: do astrocytes become reactive in concentrated clusters? Do these clusters coincide with amyloid beta plaques? Are there differences between hippocampal regions? Are there any sex-related differences in astrocyte reactivity?

Methods

Animals

The procedures outlined below were compliant with the protocols and guidelines approved by the Animal Ethics Committee of the Central Authority for Scientific Experiments on Animals of the Netherlands (CCD), and the standards set by EU Directive 2010/63/EU. Housing occurred in standard conditions with ad libitum access to food, water, and continuous exposure to background music. Mice were handled using the palming method. 51 mice (26 females and 25 males) with a C57BL/6 background were housed in group sizes of 3-5 mice and separated by sex. To compare AD mice to WT mice, heterozygous double transgenic APPswePS1dE9 (APP/PS1) mice were compared to their wildtype (WT) littermates (26 APP and 24 WT). PCR tests were performed on all animals with primers targeting to the two transgenes expressed by APP/PS1 mice (human/mouse chimeric APP/PS1 with the K595N/M596L Swedish mutation and human PS1 carrying the Exon 9 deletion).

Tissue preparation

At an age of approximately 9 months, mice were anesthetised with 0.1 ml Euthanimal 20% (Alfasan 10020 UDD) and killed by intracardiac perfusion-fixation with 4% PFA in PBS. Brains were removed and post-fixed for 48 hours in 4% PFA in PBS, pH7.4 at 4°C. After approximately 48 hours, the brains were transferred to 30% glucose with 0.05% sodium azide. Using a cryostat, the brains were sliced coronally at a thickness of 30 µm and stored in cryopreservation medium (19% glucose, 0.031% sodium azide, 37.5% ethylene glycol in 0.2M PB).

Immunohistochemistry

Hippocampal slices were collected free floating in PBS in a 12 well plate and transferred between different well plates by netted cups. They were rinsed and washed in PBS (3x 10 min). Blocking medium, consisting of 10% NDS and 0.4% Triton-X in 1x PBS was added to each well and transferred to a shaker for at least an hour. 200µl of primary antibody medium consisting of 0.1% Triton-X, primary GFAP antibody (rabbit polyclonal, 1:1 diluted in glycerol, primary 1:1000, Agilent Dako Z0334) and primary 6E10 antibody (mouse, 1:1 diluted in glycerol, 1:1000, BioLegend Sig-39300) in 1x PBS was added. All slices were incubated on a rocker overnight at 4°C. Samples were washed with 1x PBS (3x 10 min). 200 µl secondary antibody medium consisting of donkey-anti-rabbit 594 (1:1000) and donkey-anti-mouse 647 (1:1000) was added with 0.13% Triton-X in 1x PBS, and then placed on a shaker at room temperature for 2 hours. The slices were rinsed with MilliQ, collected, and transferred to glasses. Mowiol and cover glasses were used to mount the slides.

Imaging

In total, 150 hippocampal 8-bit images were recorded using an AxioScope A1 microscope. One sample, Female APP/PS1 8, was not imaged as that animal was blind in one eye, possibly affecting the outcome of the analysis. 3 images at 5, 10 and 20x magnification were taken per animal with the following exposure times: Ch1-GFAP 148ms; Ch2-6E10 1472ms; Ch4-Hoechst 30ms) (Fig 1).

Only 5x magnified images were processed and analysed using ImageJ. The hippocampus and its subregions were manually selected per slice from the Hoechst channel. White matter around the hippocampus, cuts and tears were excluded from analysis. All images were processed on a scale of 0.4885 pixels/micron APP/PS1 slices were randomly matched with same sex WT slices for further image analysis. As there were an uneven number of APP/PS1 to WT females, one female WT sample was excluded. Among the males, the image quality of an APP/PS1 slice was also deemed unusable, hence two WT samples were also excluded to create an even ratio of WT to APP/PS1 samples. Therefore, 46 samples were included in the final analysis (n = 23 per group).

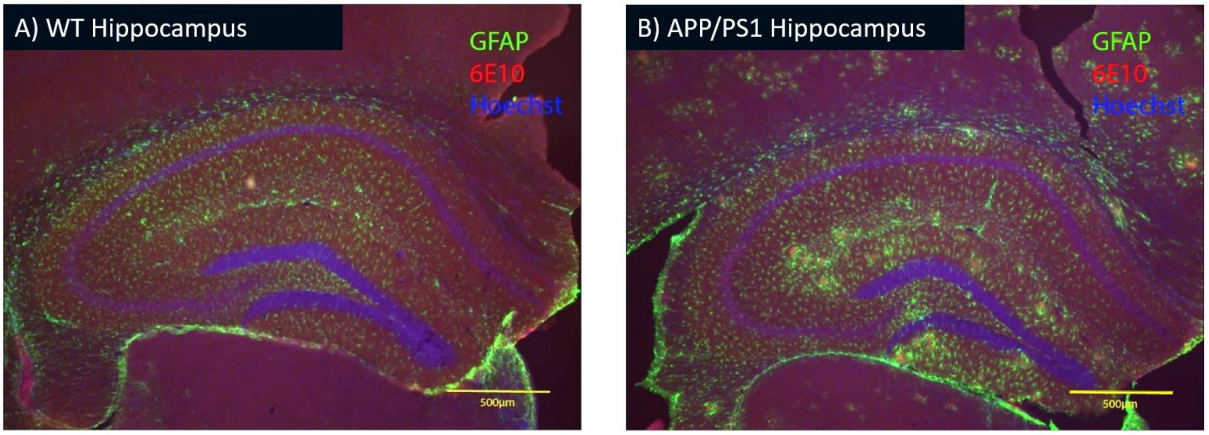

Figure 1: Images of APP/PS1 and WT Hippocampus. Microscopy images at 5x magnification of the hippocampus of A) a WT slice and B) an APP/PS1 slice. Plaques (6E10) are shown in red, astrocytes (GFAP) in green, and cell nuclei (Hoechst) in blue. Scale is 500 µm.

In APP/PS1 slices, astrocyte clusters (clusters) were selected from the GFAP channel and marked as ROIs with a circular selection of 60 pixels (122.82 microns) in diameter. In the Hoechst channel, an identical 'near' and 'far' selection was made in the same hippocampal region (Fig 2). Near selections were made directly adjacent to the cluster and far selections were made no less than 120 pixels (245.64 microns) away from the original cluster. This was repeated in WT slices, except clusters were also selected in the Hoechst channel. In total, 46 clusters were selected in the DG, 32 clusters in CA3, and 23 clusters in CA1. No more than 3 clusters were selected within the same slice and region.

Image background was subtracted using a rolling ball radius of 50.0 pixels, after which fluorescence was measured. In the binary options, the image was set to have a black background before it was converted to binary using the threshold tool. In our images, the Moments algorithm provided the most accurate results and was used for all slices (Fig 2). The tool 'analyze particles' with a threshold size of 10-200pixels^2 and sphericity of 0.00-1.00 was used. This way, data on the number of GFAP-positive astrocytes (cell count) was obtained. Plaques were visually determined from the 6E10 images.

Co-localisation of clusters with Aβ plaques was detected by overlapping GFAP and 6E10 images. In this analysis, regions in which clusters appeared present were manually selected. From this, selections were only determined to be clusters if they had a fluorescence intensity higher than 100 000, a value determined from the cluster analysis.

Statistical Analysis

GraphPad Prism 9 was used for all statistical analyses. When comparing three or more groups, a 2-way ANOVA with Tukey's or Sidak's multiple comparison was used ($\alpha = 0.05$). When comparing two groups, and hence pooling genotype or sex, an unpaired two-tailed T-test was used. ROUT ($Q = 1$) was used for outlier detection, but no outliers were found. In total, 5 samples were excluded from analysis as explained earlier. Therefore, 46 brain slices (1 per mouse) were used for analysis of which 24 were female and 22 were male (the ratio of APP/PS1 to WT was kept equal at 23 per group).

Results

Hippocampal and regional selections, along with (reactive astrocyte) cluster selections were analysed in 9-month-old APP/PS1 and WT mice. The analysis included a specific comparison between hippocampal GFAP-fluorescence intensity (Fig 3A) and the number of GFAP-positive astrocytes (count) (Fig 3D). These were further subdivided and analysed per hippocampal region (Fig 3B/C/E/F). As there was no significant sex-based difference, sexes were pooled in the results indicated in Fig 3 are pooled.

There were no significant hippocampus-wide differences but there was a significant difference within the DG

No significant differences were found between models in the hippocampus-wide fluorescence and count comparison though a trend was observed in count, as WT slices had a lower count than APP/PS1 slices ($p < 0.09$) (Fig 3A/B). A two-way ANOVA-analysis between region and model revealed a significant difference in the DG, as APP/PS1 slices had a significantly higher fluorescence ($p < 0.01$) and count ($p < 0.01$) than their WT counterparts (Fig 3C/D). There were no significant differences in the CA1 and the CA3 and the DG had a significantly higher fluorescence than CA1 ($p < 0.01$) and CA3 ($0 < 0.01$) in the APP/PS1 model, which was not reflected in the count analysis (Fig 3E/F). No significant sex differences were found (supplementary Fig 1).

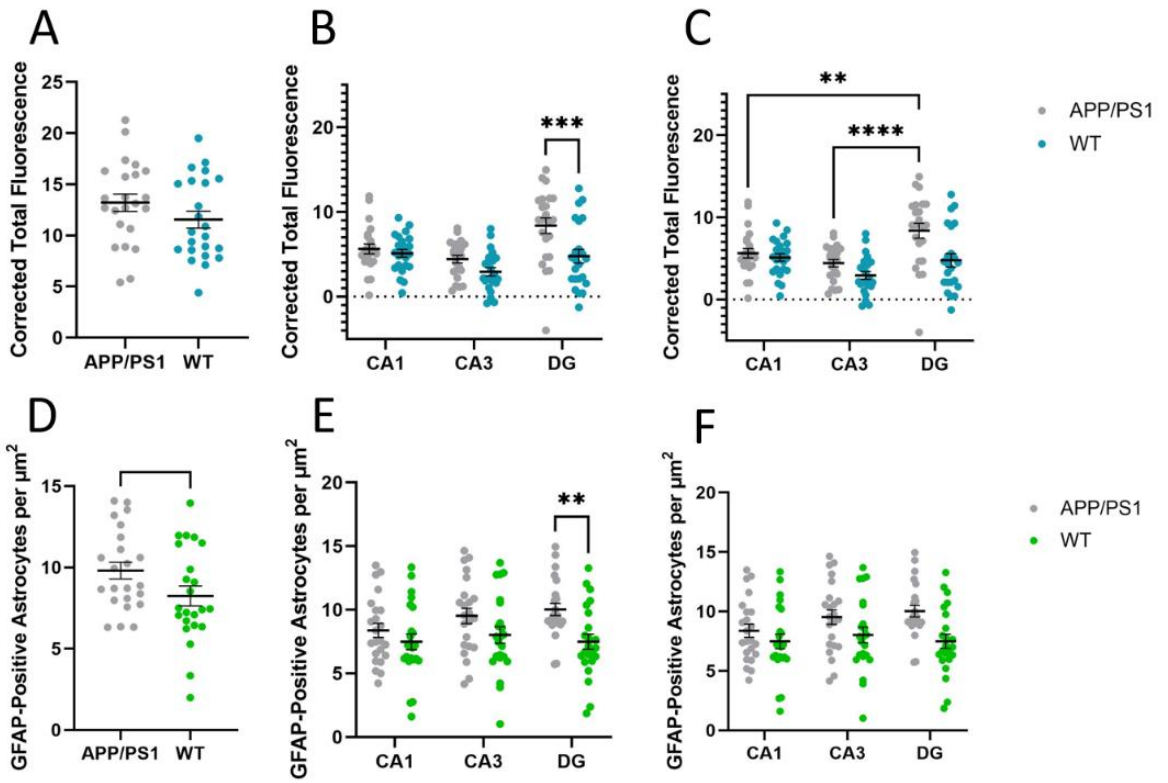

Figure 3: hippocampal fluorescence and count analysis. Corrected total fluorescence comparing WT and APP/PS1 slices in A) the entire hippocampus and B) hippocampal subregions, as well as C) differences between hippocampal subregions within WT and APP/PS1 slices. The same analysis was repeated using corrected GFAP-positive astrocyte count, comparing WT and APP/PS1 slices in D) the entire hippocampus and E) hippocampal subregions, as well as F) comparing subregions within APP/PS1 and WT slices. A/B/C) APP/PS1 is indicated in grey; WT is indicated in blue. D/E/F) APP/PS1 is indicated in grey; WT is indicated in green. * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$. $p > 0.05$. Mean and SEM are indicated. WT: N = 23; APP/PS1: N = 23.

Astrocytes had a significantly higher fluorescence and GFAP-positive cell count in cluster regions

There was no significant difference between selected WT clusters (supplementary Fig 3). Hence, all selections (meaning the cluster itself, the near and far selection), were collapsed. In the fluorescence analysis, there was a significant difference between cluster and near selections ($p < 0.01$) but no difference between far and WT selections. In the CA1 and CA3 there was also no significant difference between near and far selections (Fig 4A). The count analysis had similar results, except for the fact that no significant difference was found between the cluster and near selections and, in the DG significant was only found between cluster and WT, as well asl near and WT ($p < 0.01$) (Fig 4C). There was a regional difference between clusters in fluorescence, as DG had a significantly higher fluorescence than CA3 ($p < 0.05$) (Fig 4B). No significant regional differences were found in the count analysis (Fig 4D). In both analyses, no sex differences were found (supplementary Fig 2)

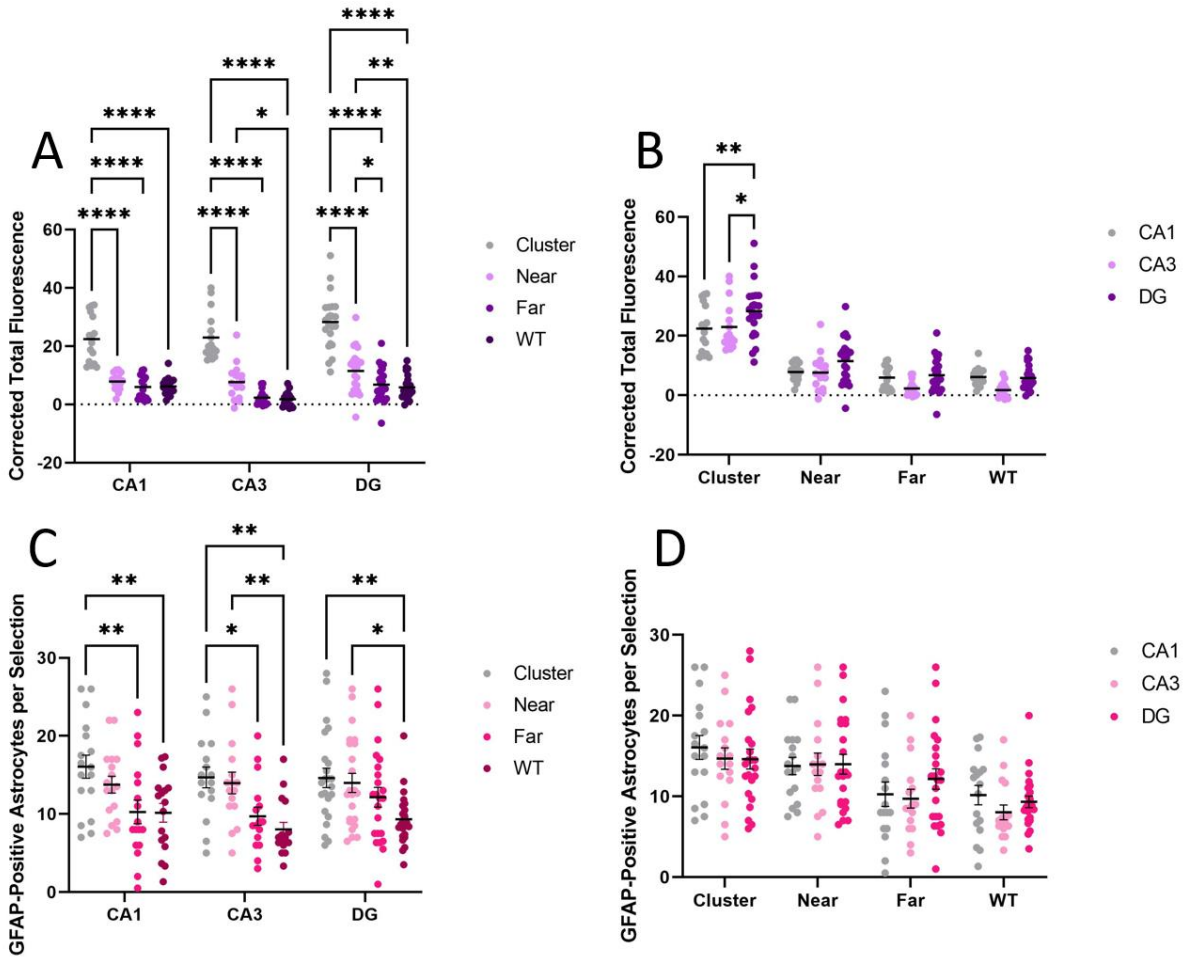

Figure 4: GFAP cluster analysis in GFAP-positive cell count and fluorescence. Corrected total cell fluorescence comparing differences between A) cluster, near, far and WT selections and B) selections within hippocampal regions. The same analysis was done using GFAP-positive astrocyte count comparing C) cluster, near, far and WT selections and D) selections within hippocampal regions. A) Clusters are indicated in grey, near selections in light purple, far selections in purple and WT selections in dark purple. B) Clusters are indicated in grey; near selections in light pink, far selections in bright pink and WT selections in darker pink. C) CA1 is indicated in grey, CA3 in light purple and DG in purple. D) CA1 is indicated in grey, CA4 in light pink and DG in bright pink. * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$; ns indicates $p > 0.05$. CA1: N = 16; CA3: N = 16; DG: N = 23.

Clusters and plaques colocalised significantly more frequently than occurring alone throughout the hippocampus, except in the CA1

In the comparison between the proportion of colocalised to non-colocalised plaques and clusters, several observations can be made. Approximately 75% of clusters colocalised to plaques so approximately 25%, which is significantly less, did not ($p < 0.01$) (Fig 5A). In the reverse analysis, the same was found, as approximately 75% of plaques colocalised to clusters whereas approximately 25% did not which is, again, significantly different ($p < 0.01$). This indicates that plaques and clusters colocalise significantly more frequently (Fig 5A). Dividing this analysis into hippocampal subregions showed similar results within the DG and CA3 (supplementary Fig 4), but not in the CA1. Though plaques still showed a significant colocalization to clusters, the colocalization from clusters to plaques was approximately 50% and therefore insignificant (Fig 5C).

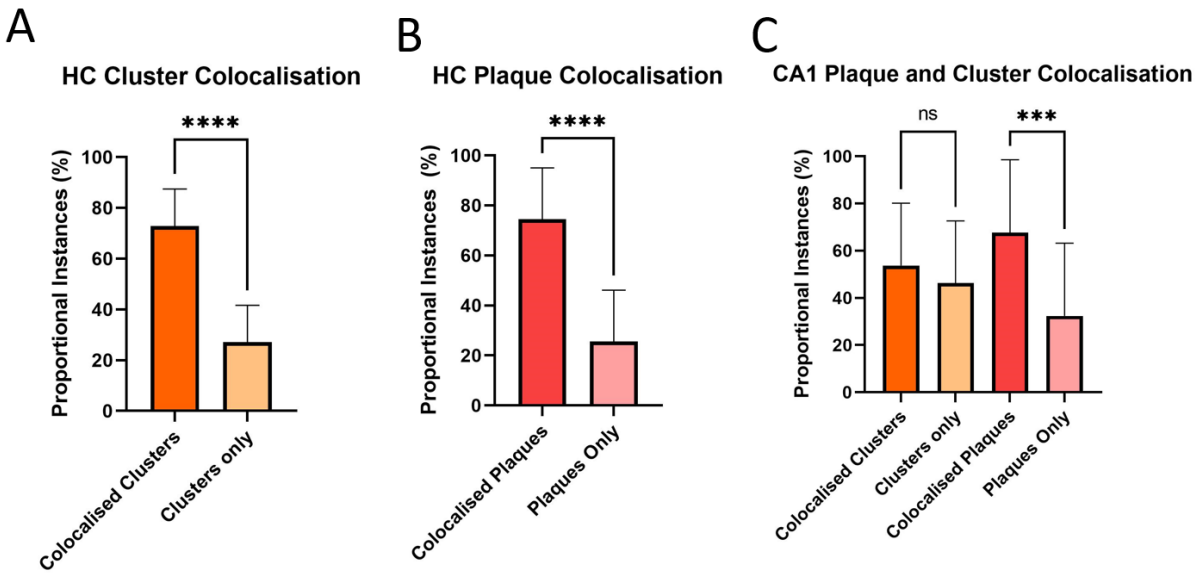

Figure 5: Aβ plaque and GFAP cluster colocalization analysis. The proportion of colocalised to non-colocalised clusters and plaques throughout A) the hippocampus and B) the CA1. C) A regional colocalization analysis showing only instances of overlap within all hippocampal regions. A) colocalised plaques and clusters are indicated in bright orange and non-colocalised plaques and clusters are indicated in paler orange. B) CA1 is indicated in grey, CA3 in pale orange and DG in bright orange. C) colocalised plaques and clusters are indicated in bright orange and non-colocalised plaques and clusters are indicated in paler orange. * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$; ns indicates $p > 0.05$. N = 23

Discussion

Astrocytes become reactive in concentrated clusters and colocalise with Aβ plaques

We found that astrocytes become reactive in concentrated clusters, as there was a significant difference between selected clusters and near, far and WT selections. This is supported by the fact that there was no significant difference between far and WT selections indicating that there is likely no difference between WT and APP/PS1 slices except for in specific cluster selections. Moreover, astrocyte reactivity colocalises significantly more frequently with Aβ plaques than without, supporting the probable relationship between the two. This is not an unexpected finding and has been ascertained previously (14). Interestingly, the analysis did not show a 100% overlap between plaques and clusters, indicating that, though significantly less, there are instances where plaques or reactive astrocytes have formed without a colocalised counterpart. This is especially noticeable within the CA1, where the proportion of non-colocalised to colocalised clusters was not significant. To our knowledge, this observation has not been made in other studies. It should be noted that the quality of the 6E10 images was suboptimal as some images were underexposed, many were not focused on the GFAP image, and Aβ plaques are found at a different focal length. It is therefore possible that plaques may have been missed, even if present. A solution would be to, not only adjust the focus within the same slice, but to also image adjacent slices depending on the depth of the plaque. Considering another study found fewer Aβ plaques in the CA1, it is possible that missing even a small number of plaques was sufficient for the colocalization analysis to no longer be significant, though a reduced number of plaques in CA1 cannot be claimed with absolute certainty (23,24). We investigated plaque quantities, but the suboptimal imaging meant that it could not be used in this analysis. It is important to consider that this may have also introduced a certain bias in the plaques that were selected for this analysis. Thus, it would be interesting to quantify the number of plaques and clusters rather than comparing proportional differences in the future. Another reason for the lack of colocalised clusters might be that plaques have already been sequestered by the reactive astrocytes at the time of analysis, meaning they were no longer visible even if present, hence

colocalised, before. Finally, it is possible that astrocyte clusters were a result of another inflammatory cause than Aβ plaques, which would not have been stained for and therefore not observed. Regardless, it might be interesting to delve into the relationship between plaques and clusters, especially in the CA1 to gain further understanding of the correlation between the two. This could be done by taking images of the CA1 at a higher magnification for more detail, quantifying the plaque and cluster analysis and considering plaques at different focal lengths as well. If this finding is a true reflection of AD, and there is no significant colocalization in CA1, we might gain a deeper understanding of their relationship.

Astrocyte reactivity appears concentrated to the DG

Astrocytes in the APP/PS1 model had a significantly higher fluorescence within the DG, which was also significantly higher than the other two regions. This is not a necessarily surprising finding, as a higher plaque load, as well as astrocyte reactivity in the DG has been observed previously (25). Functionally, the DG is known for its diverse cell population, its role in working memory, pattern separation and completion, novelty detection and binding information to spatial context (26). The localised reactivity in the DG suggests that it is subject to severe excitotoxic and inflammatory stimulation and its diverse yet prominent role in memory tasks might explain poor memory performance in AD (25). Receiving input from the entorhinal cortex, the DG connects to the other subregions through strong forward networks, recognised as the 'tri-synaptic circuit'. Mostly involved in memory acquisition, this circuit has been suggested to be a particularly vulnerable pathway in AD, which could justify other regions being affected as a result (27). Research targeting the DG might therefore be a good step towards further understanding AD pathology and finding a possible therapeutic target. This could be accomplished by performing another exploratory analysis and conducting a more detailed analysis of astrocyte morphology within the DG. Additionally, it might be interesting to repeat an analysis focusing on the relationship between the DG and the CA3. It is known that the DG projects to the CA3 through the granule cell layer, which is clearly identifiable in IHC stainings. Perhaps a more detailed analysis of astrocyte reactivity (comparing count, fluorescence, and morphology) focusing on the granule cell layer might provide more information regarding the effect of DG impairments on the other two regions. Especially since impairments in this layer have been found, and related to astrocytes, in the past (28,29).

Why the difference in fluorescence is not reflected in the hippocampus-wide analysis, is not entirely clear. One explanation is that the reactive response is concentrated to the DG, and that this area alone is not sufficient to yield a significant response throughout the entire hippocampus. Another explanation may be related to changes in astrocytic morphology further away from Aβ plaques. Indeed, a study using similar methods found hypertrophy in astrocytes exclusively associated to plaques but hypotrophy of overall GFAP-positive astrocytes in the DG and CA1 (30). If this were the case, a significant fluorescent difference between far and WT selections would be expected. As we did not find that difference, it seems unlikely that hypotrophy counterbalances the increased fluorescence observed near plaques. Our lack in difference between the two selections could be explained by the background subtraction in the image analysis. Perhaps this process removed a possible difference in the morphology of astrocytes in non-reactive clusters, and hence a more optimised fluorescence analysis would be necessary for such a specific comparison. Another possibility might be that the far selections were in a closer proximity to a different nearby cluster, which would also affect this finding. Regardless, it may be beneficial to conduct a more detailed analysis of astrocytes located further away from reactive clusters to justify a possible hypotrophic response. This could include a similar analysis with a higher magnification, a higher minimum distance from clusters or it could exclude astrocyte clusters completely to solely focus on the difference between far and WT slices. Additionally, it would be beneficial to analyse hippocampal

slices of mice of varying ages, as the supporting study found that hyper- and hypotrophic differences were age-dependent (30).

GFAP-Positive astrocyte count increases gradually around clusters

Whether the number of astrocytes increases in APP/PS1 mice is still debated. Within the DG, we found significantly more GFAP-positive astrocytes, as well as a similar hippocampus-wide trend. The cluster analysis also suggested a more gradual, and perhaps systemic, rather than localised increase, as there was no significant difference between cluster and near selections, but there was a significant difference between far and WT selections.

Literature on increased GFAP-positive cell count in AD is conflicting. A study on human brain tissue found that the significant increase in proliferation marker Ki-67 was mostly due to astrocytes (31). Additionally, a study on a different transgenic AD mouse model found that the significant cell increase in the neocortex was also due to astrocytes suggesting they proliferate at an increased rate in AD (32). But opposing studies using human tissue found that, though astrocytes do go into a reactive state, no significant hippocampus-wide differences could be found in the number of GFAP-positive astrocytes (33). They also did not observe a significant increase in the previously mentioned proliferation marker Ki-67 meaning their insignificant results were not because of simultaneous cell death (33). This was supported by another study on the human AD neocortex, in which there was no significant difference in the number of astrocytes between AD and control patients (34). It should be noted however, that this study differentiated between cells based on morphological features rather than staining.

It is also possible that astrocytes migrate, which would explain the lack of significant differences in the hippocampus-wide analysis and account for the increased number of cells within the DG as well as the significant differences between clusters. This does seem unlikely as a study using a computer model and quantitative spatial analysis found that astrocytes only migrate minimally and only with very large plaque loads. The migration also appeared to move astrocytes away from plaques rather than towards them (35). Astrocyte migration away from plaques would explain why no significant difference was found between clusters and near selections though a higher amount of migration than suggested.

The above discussion indicates that it is still not entirely clear whether the number of astrocytes increase in AD or not. Our findings suggest that there is indeed an increase which is gradually higher around astrocytic clusters. As we did not stain for any proliferation markers, this could either be a result of increased proliferation or migration but could also be due to changes in cell death rates. It would be interesting to further delve into these observed differences as our results suggest that astrocytic reactivity is incongruent to astrocytic count. Solely based on our findings, it seems as though the number of GFAP-positive astrocytes increase gradually, though still localised, throughout the hippocampus.

There were no sex-based differences

We did not find any significant sex differences which is somewhat surprising given earlier described sexual dimorphisms in AD. Our results also oppose another study which used the same mouse model and found sex discrepancies in parenchymal Aβ, vascular Aβ burden, tau pathology, neuroinflammation and loss of neurons and synapses (36). Sex differences in our plaque analysis might have emerged if plaque and cluster quantities were compared rather than proportions, as there may have been quantitative differences. Therefore, sexual differences might still be found in the plaque and cluster colocalization analysis if quantities are compared, rather than proportions. Because oestrogen may play an important part in astrocyte reactivity and AD, it is important to

consider fluctuating oestrogen levels throughout a female's lifetime. We used 9-month-old mice, meaning females reached the endocrine equivalent of human perimenopause (37). Given the fact that their reproductive senescence transition occurs between 9 and 12 months of age, and the study with significant findings used 12-month-old mice, it might be beneficial to repeat our study on slightly older mice. It has also been suggested that the mouse model significantly impacts sex differences, but our selected model was expected to show those differences in the DG (38). As we also observed significant overall changes within the DG, a study more specifically looking into the DG might be interesting with regards to sex-based comparisons as well. Regional differences are especially relevant in this analysis because, within the WT cluster analysis, a significantly lower fluorescence was found within the CA3. As all regions were pooled within the sex analysis, it is possible that a difference within a particular region (such as the DG) may still arise.

Limitations

Though most findings support existing hypotheses, several limitations should be addressed. The first has to do with the imaging process. To keep the difference between staining as constant as possible, all factors were kept the same, as well as the focal length. Given that Aβ plaques are generally found at a different length, the 6E10 staining was not always in focus for analysis. Plaques remained clearly identifiable, but the overall image quality was suboptimal. As a result, plaque and cluster quantities could not be compared and, instead, only proportions of colocalization could be analysed, which may have introduced certain biases. Additionally, the algorithm for the count analysis was likely not an exact reflection of the number of GFAP-positive astrocytes. A substantial amount testing went into finding and optimising the counting process, but it should be mentioned that the algorithm provides an approximation rather than an identical reflection of number of GFAP-positive astrocytes.

Another limitation was an inherent regional fluorescence difference within the cluster analysis. When comparing clusters, near and far selections within WT samples, the CA3 consistently showed a significantly lower fluorescence than the CA1 and, occasionally, the DG (supplementary Fig3B). Why the CA3 had a lower fluorescence is not entirely clear but should still be noted. As a WT regional difference was only observed in the cluster analysis, and not in the general fluorescence analysis, this limitation is not deemed too influential of our existing conclusions. It might however be interesting to repeat the cluster analysis per sexes, while differentiating per region, as they were pooled and no significant results were found in this analysis. With regards to fluorescence, it is also important to note some unanticipated data points. These were fluorescence levels found to be negative. This is particularly noticeable in the DG in Fig 3B/C, where an APP/PS1 sample, had a negative fluorescence of approximately -4. Though this data point is not entirely logical, it can be explained by the fact that the selected background for this region, in this slice, had a higher fluorescence than the selection itself. All negative fluorescence points had a thorough second inspection and were noted but, because no issues were found, they were kept within this analysis. This is an indication that the background subtraction process might need refining, so that no negative fluorescence levels are found in the future.

Finally, it is important to consider the relevance of this study regarding its translatability to humans. Though APP/PS1 has been widely validated as an AD model, there are some important differences between the mouse model and AD itself. The most noted being the lack of TAU pathology, which is a microtubule associated protein, in the APP/PS1 model (39). This is often overlooked due to the popularity of the amyloid cascade hypothesis but, as it is considered a main feature of AD, should be taken into consideration. Additionally, this model has been shown to more strongly mimic familial AD rather than sporadic AD, a form that occurs far more frequently. Though generally, both familial and sporadic AD have the same major pathological features, such as plaque

accumulation and neurofibrillary tangles (39). Another important limitation is the inherent in astrocytic differences between humans and mice. A study comparing morphological and transcriptomic differences found several significant differences, which have already been outlined as potential obstacles in translating astrocytic changes in models to human AD (39,40). Indeed, its predictive validity remains questionable as positive pre-clinical trials have not always resulted in successful clinical trials (41). A possible improvement to this limitation might be to use wild-derived mice, rather than a C57BL/6 strain (42). That being said, this model aligns with numerous important features of AD, such as the accumulation of Aβ plaques and reactive astrocytes, as well as behavioural similarities (43). For an exploratory analysis investigating the disease progress, this model is thus still suitable. To account for possible translational differences, it might be beneficial to repeat this study on post-mortem, human tissue.

Conclusion

This analysis has validated the concept of localised astrocyte reactivity and their spatial relationship to plaques. We have found a moderate difference in intensity which was not widespread throughout the hippocampus but rather localised to the DG, further emphasising its potential role in AD. Our findings in the GFAP-positive astrocyte count analysis suggests that astrocytes might proliferate locally, though further investigation is recommended. Unexpectedly, we did not find any sex difference which may be age-related. Still, a more thorough analysis is recommended here as well, especially in the plaque and cluster colocalization analysis.

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Supplementary Materials

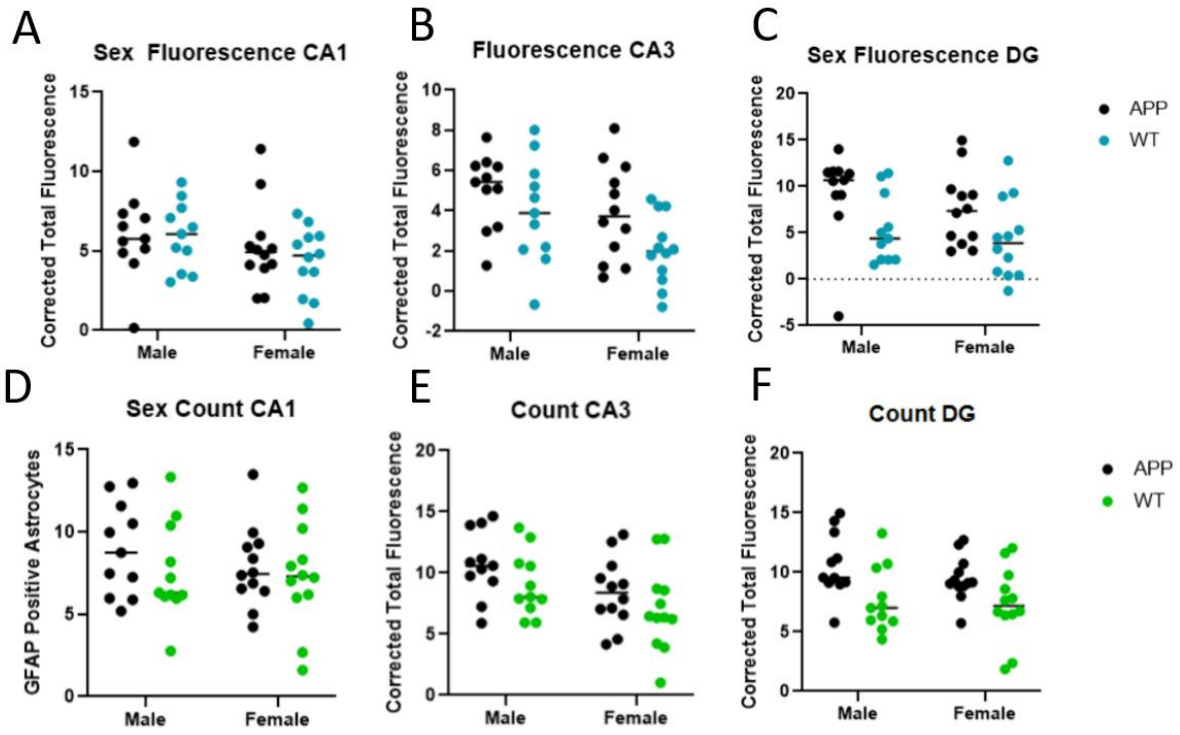

Figure 1: hippocampal fluorescence and count analysis per sex. Corrected total fluorescence comparing WT and APP/PS1 differences between males and females in A) the CA1, B) the CA3 and C) the DG. The same analysis was repeated using corrected GFAP-positive astrocyte count, comparing males and females in A) the CA1, B) the CA3 and C) the DG. A/B/C) APP/PS1 is indicated in grey; WT is indicated in blue. D/E/F) APP/PS1 is indicated in grey; WT is indicated in green. * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$. $p > 0.05$. Mean and SEM are indicated. WT: N = 23; APP/PS1: N = 23.

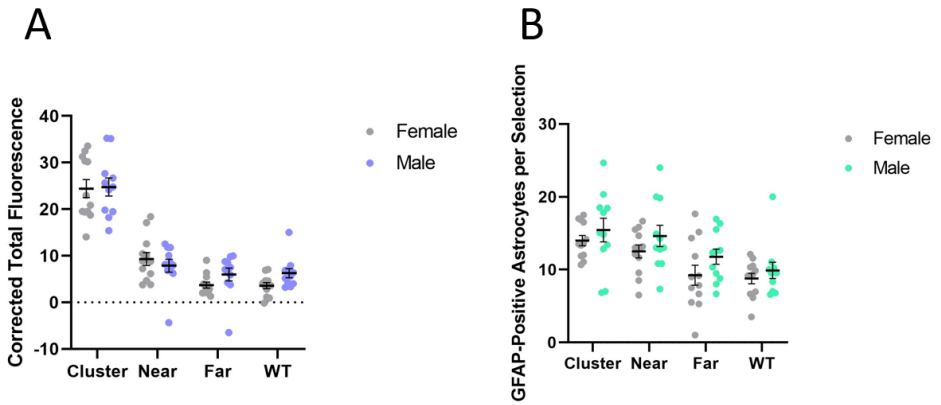

Figure 2. GFAP cluster analysis in per sex. Sex comparisons in the cluster analysis focusing on A) corrected total cell fluorescence and B) GFAP-positive astrocyte count. A) Females are indicated in grey and males are indicated in light purple. B) Females are indicated in grey, males are indicated in light turquoise. * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$; ns indicates $p > 0.05$. Females: N = 12; Males: N = 11.

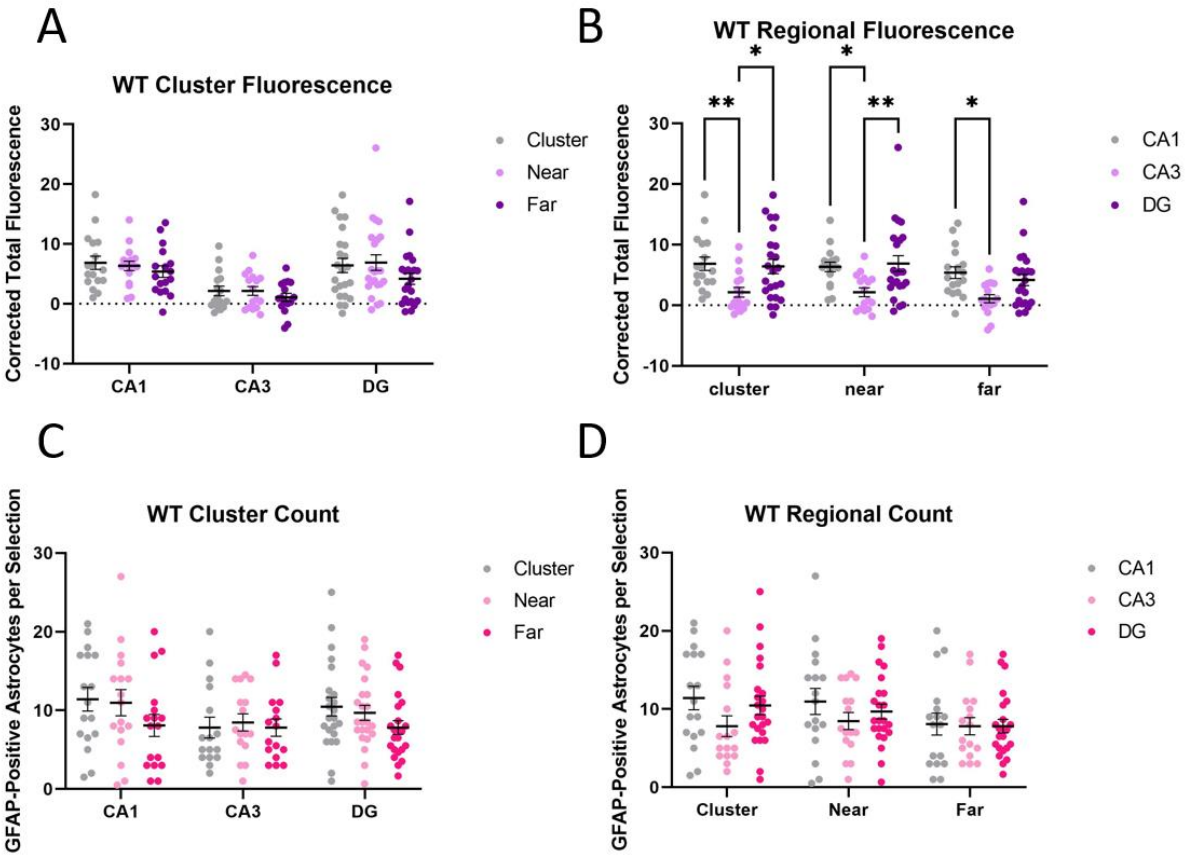

Figure 3. GFAP cluster analysis in GFAP-positive cell count and fluorescence in WT samples. Corrected total cell fluorescence in WT samples comparing differences between A) cluster, near, far and WT selections and B) selections within hippocampal regions. The same analysis was done using GFAP-positive astrocyte count comparing C) cluster, near, far and WT selections and D) selections within hippocampal regions. A) Clusters are indicated in grey, near selections in light purple, far selections in purple and WT selections in dark purple. B) Clusters are indicated in grey; near selections in light pink, far selections in bright pink and WT selections in darker pink. C) CA1 is indicated in grey, CA3 in light purple and DG in purple. D) CA1 is indicated in grey, CA4 in light pink and DG in bright pink. * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$; ns indicates $p > 0.05$. CA1: N = 16; CA3: N = 16; DG: N = 23.

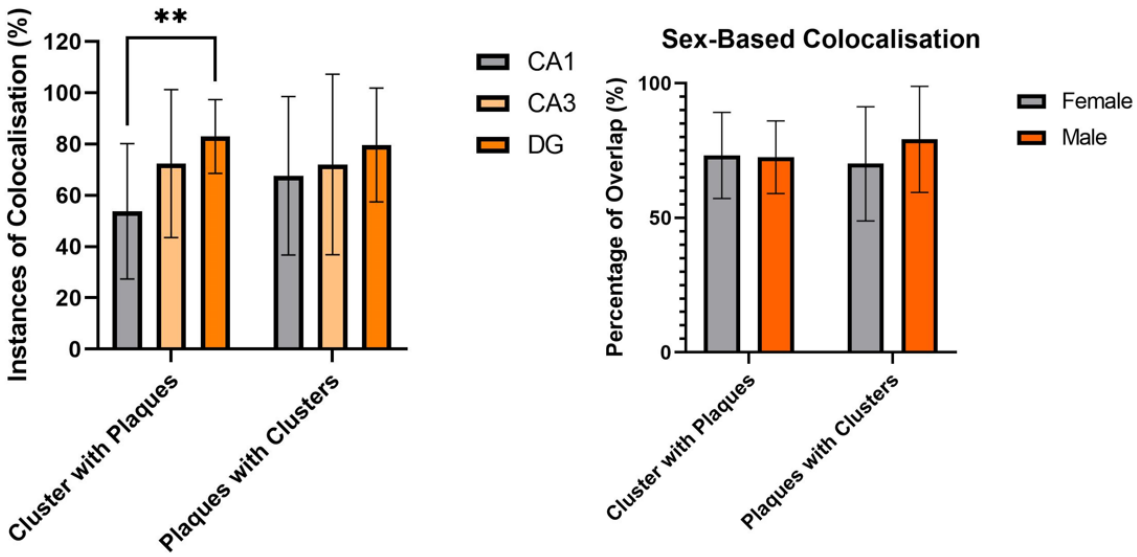

Figure 4: Colocalization analysis per region and sex. The percentage of colocalised plaques and clusters per A) hippocampal region and B) sex. A) colocalised plaques and clusters in the CA1 are indicated in grey, in the CA3 they are indicated in paler orange and in the DG they are indicated in bright orange. B) females are indicated in grey, males are indicated in orange. * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$; ns indicates $p > 0.05$. A) N = 23; B) Females: N = 12; Males: N = 11.