**Microglial contribution to synaptic uptake in the prefrontal cortex in schizophrenia**

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**Keywords: synapse; gliosis; phagocytosis; psychiatric disorder; post-mortem; immunohistochemistry**

**The data that support the findings of this study are available from the corresponding author upon reasonable request.**

**Abstract word count:** 178

**Manuscript word count:** 2,793

**Number of Figures:** 2

**Number of Tables:** 1

Efficient synaptic communication is crucial to maintain healthy behavioural and cognitive processes. In neurodevelopmental diseases, like schizophrenia, affected individuals can exhibit behavioural symptoms like psychosis, hallucinations and alterations in decision-making. A reduction in cortical grey matter volume and enlarged ventricles in the brains of schizophrenia cases has been consistently reported [1,2]. This reduction in cortical volume is likely to be an outcome of neuronal and synaptic loss, which has also been reported in schizophrenia but the results have varied between brain area and synaptic markers examined [3–7]. A meta-analysis of the expression of synaptic markers in the disease has shown reduced levels of pre-synaptic markers in the frontal cortex which are heavily implicated in schizophrenia, but not in unaffected areas like the temporal and occipital lobes [8]. Synapses are crucial mediators of brain communication [9], and so, such synaptic alterations can have an impact on brain network connectivity, a process known to be affected in schizophrenia [10]. There are several factors during brain development that influence brain connectivity, with non-neuronal contributors playing an important role in synaptic formation and network maturation [11,12]. One of these non-neuronal contributors are microglia, the resident brain immune cells and primary phagocytes of the brain [12,13]. Gliosis is commonly observed during loss of brain homeostasis. Microglia have also been shown to facilitate neural network shaping in development by phagocytosing synapses using the complement system [14]. However, microglia can be aberrantly involved in synaptic elimination in non-physiological contexts, as observed in animal models of Alzheimer’s disease [15]. Here, we performed a human post-mortem study to investigate the role of microglia in synaptic engulfment in schizophrenia. We examined microglial burden using Iba1 which labels the microglial cytoplasm and reflects microglial motility and homeostasis. Iba1 is considered as a pan-microglial marker and has been observed to be increased in a subset of neurodegenerative diseases [16]. Our other microglial marker, CD68, labels the lysosomal compartment of microglia [17].

We studied post-mortem brains from 10 control and 10 schizophrenia cases from the dorsolateral prefrontal cortex (DLPFC) which is affected in schizophrenia [1]. Cortical sections were stained with Iba1 and CD68 to label microglia (Figure 1A-D). We observe that there was no difference in either Iba1 (p=0.315) or CD68 (p=0.794) area coverage of the cortex (burden) between the schizophrenia and control cohorts (Figure 1E,F) (full statistical results found in Supplementary Table 1). Furthermore, there was no difference in the co-localisation between CD68 and Iba1 in controls and schizophrenia brains (p=0.639), suggesting the co-expression of the two markers per single cell is unchanged (Figure 1G).

Though no difference in microglial burdens between the two cohorts was observed, we aimed to assess whether microglia were involved in synaptic engulfment in schizophrenia. To do this, we quantified the amount of co-localisation between synapsin I and CD68 (% area), as a measure of engulfed synaptic material in the microglial phago-lysosomal compartment (Figure 1H-M). Firstly, in our cohort we did not find a significant difference in the cortical area occupied by synapsin I staining between the schizophrenia and control groups (p=0.956) (Figure 1N). Furthermore, we found no difference in synaptic engulfment by microglia between the schizophrenia and control cases (p=0.413) (Figure 1O). Additionally, when we normalised this co-localisation to their respective CD68 or Iba1 burdens, there was still no statistical difference between schizophrenia and control tissue (p=0.167 and p=0.964 respectively) (Figure 1P&Q). Of note, we have also shown microglia are capable of ingesting other pre-synaptic proteins like synaptophysin, as well as the post-synaptic protein PSD-95 (Supplementary Figures 1 and 2). Our data therefore suggest that at the time of death, microglia do not appear to be involved in aberrant synaptic internalisation in patients with schizophrenia.

In human post-mortem tissue from both patients with schizophrenia and age-matched controls, we found pre-synaptic proteins inside microglial cells in the frontal cortex of the brain, but no difference in the levels of synaptic internalisation between the two groups.

A limitation of our post-mortem tissue is that the use of human post-mortem tissue it provides a snapshot of the disease many years after onset, which does not address the mechanism involved in synaptic internalisation by microglia. A greater sample size in an independent cohort will be useful to assess the reproducibility of these results and to stratify by confounding variables like sex and age. This would also allow us to assess whether confounding factors like depression, psychosis, systemic inflammation, and use of antipsychotic drugs affect these microglial and synaptic interactions. Furthermore, we have looked through all six cortical layers in a non-biased manner but we cannot exclude layer specific differences in gliosis, synapse loss, or synaptic engulfment by microglia. However, this study is unique by the type of assessment performed on schizophrenia tissue is scarce.

With gliosis being reported in multiple brain disorders, we assessed microgliosis in schizophrenia. As described above, we found no differences in microglial burdens between disease and control groups. This suggests that microglial activation is not a sustained event in chronic schizophrenia, and if any changes do occur in these cells it would instead likely involve functional alterations. Previous literature looking at CD68 expression in control and schizophrenia cases has also reported a similar outcome [18]. It is possible that if any changes in glial dynamics were to occur, they may be seen closer to disease onset, and that by the time the brains were donated ~35 years later, any changes would have subsided. This would be consistent with the observations published to visualise and quantify microglial activation *in vivo* with positron emission computed tomography (PET) using specific ligands of the translocator protein TSPO [19]. The PET studies have revealed that activated microglia are present in patients within the first 5 years of disease onset or during a psychotic state, whereas other PET studies in chronic schizophrenia have shown no difference in microglial activation between healthy controls and these patients. Nevertheless, TSPO signals are not a perfect read-out of microglial-mediated inflammation as they influenced by age and are not microglia-specific [20,21].

Although developmental synaptic alterations, like synapse loss, have been characterised in individuals with schizophrenia [3,8], there are key unanswered questions that remain. For instance, it is not clear how the synapse elimination is mediated, the extent to which it drives behavioural symptoms, or whether it is the outcome of other disease-specific pathologies. Right now, a prominent mechanism for synaptic elimination in development is the use of the classical complement cascade (CCC), where it has been shown to sculpt neural circuits by tagging less electrically active synapses [14]. Recent research has now implicated complement as a signal for aberrant synapse elimination in disease [12,22]. Specifically, variants of C4 of the CCC are associated with a greater risk of developing schizophrenia [23], as well as poorer brain connectivity and schizophrenia-like behavioural deficits in mice [24].

Currently, a suggested mechanism by which complement-tagged synapses are cleared is by microglial recruitment for synaptic removal. In co-cultured neuron and microglia-like cells from human induced pluripotent stem cells from control and schizophrenia lines, increased levels of the excitatory post-synaptic protein PSD-95 was reported phagocytosed in the schizophrenia co-cultures [25]. Interestingly, this increased phagocytic activity was mainly driven by the presence of schizophrenia-derived microglia. Indeed, when schizophrenia neurons were co-cultured with microglia from control patients, the phagocytic index was reduced, indicating that in schizophrenia microglia have intrinsic differences in their phagocytic response. It is worth noting that induced stem cells are a good model for understanding human disease but represent a developmentally earlier phenotype, and not that of the age of the donor. Therefore, this supports a role for phagocytic microglia in early stages of the illness, and may explain why we did not see any changes in phagocytic ability of microglia towards synapses in chronic schizophrenia, since we are not studying the developmental time-frame.

In conclusion, here we report that microglia in human post-mortem tissue internalise pre-synaptic proteins physiologically, and that this does not appear to be altered in the chronic form of schizophrenia, in contrast with our observation in AD. Nevertheless, given the typically early onset of schizophrenia and that synapse loss is likely to have occurred years before brain collection, we cannot make assumptions on the role of microglia in synaptic clearance at the start of the disease. Looking forward, it would be interesting to study difference between young versus older cases in terms of synaptic uptake by microglia, and phenotype these changes in several brain areas to investigate any region-specific differences. Lastly, longitudinal PET imaging of the pre-synaptic marker SV2A [26] and TSPO microglial marker would enable exploration of any microglia-synapse association during the course of the illnesses.

**Acknowledgements**

We would also like to thank our funders, specifically the UK Dementia Research Institute which receives funding from Alzheimer’s Research UK, the Alzheimer’s Society, and the Medical Research Council. We also would like to thank the Wellcome Trust for funding AJS and TLSJ. Tissue samples were obtained from The Corsellis Collection as part of the UK Brain Archive Information Network (BRAIN UK) which is funded by the Medical Research Council and Brain Tumour Research. Ethical approval was provided by BRAIN UK, a virtual brain bank which encompasses the archives of neuropathology departments in the UK and the Corsellis Collection, ethics reference 14/SC/0098. The study was registered under the Ethics and Research Governance (ERGO) of the Southampton University (Reference 19791). Authors contributed in the following ways: MT contributed in study design, performed experiments and imaging, statistical analysis, and manuscript preparation; AJS contributed in statistical analysis and manuscript editing; DB contributed by providing cut paraffin-embedded section, study design, and manuscript editing; TLSJ contributed with study design, statistical analysis, and manuscript editing. TLSJ is on the Scientific Advisory Board of Cognition Therapeutics and receives collaborative grant funding from 2 industry partners. None of these had any influence over the current paper. None of remaining authors declare any conflicting of interest. Figures created with BioRender.

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