University of Dundee

DOCTOR OF PHILOSOPHY

A high-resolution assessment of human Motor Neuron Disease and its association with clinical presentation

Sanchez Avila, Anna

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A high-resolution assessment of human Motor Neuron Disease and its association with clinical presentation

Anna Sanchez Avila
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ALSci</td>
<td>Amyotrophic lateral sclerosis with cognitive impairment</td>
</tr>
<tr>
<td>ALSnoci</td>
<td>Amyotrophic lateral sclerosis with no cognitive impairment</td>
</tr>
<tr>
<td>ASO</td>
<td>Anti-sense oligonucleotide</td>
</tr>
<tr>
<td>AT</td>
<td>Array tomography</td>
</tr>
<tr>
<td>BA17/19</td>
<td>Brodmann area 17/19, visual cortex</td>
</tr>
<tr>
<td>BA44/45</td>
<td>Brodmann area 44/45, Broca’s area</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic protein assay</td>
</tr>
<tr>
<td>CD68</td>
<td>Cluster of differentiation 68</td>
</tr>
<tr>
<td>CF</td>
<td>Correction factor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPR</td>
<td>Dipeptide repeat</td>
</tr>
<tr>
<td>ECAS</td>
<td>Edinburgh cognitive and behavioural ALS screen</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ESRIC</td>
<td>Edinburgh super-resolution imaging consortium</td>
</tr>
<tr>
<td>fALS</td>
<td>Familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
</tr>
<tr>
<td>FUS</td>
<td>Fused in sarcoma</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRE</td>
<td>Hexanucleotide repeat expansion</td>
</tr>
<tr>
<td>ISM</td>
<td>Image scanning microscopy</td>
</tr>
<tr>
<td>MND</td>
<td>Motor neuron disease</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>PALM</td>
<td>Photoactivated localisation microscopy</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PET</td>
<td>Position emission tomography</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>POST</td>
<td>Post-synaptic density sample</td>
</tr>
<tr>
<td>PRE</td>
<td>Pre-synaptic density sample</td>
</tr>
<tr>
<td>PSD</td>
<td>Post-synaptic density</td>
</tr>
<tr>
<td>pTDP-43</td>
<td>Hyperphosphorylated TAR DNA-binding protein 43</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA-recognition motif</td>
</tr>
<tr>
<td>sALS</td>
<td>Sporadic amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SEQUIN</td>
<td>Synaptic evaluation and quantification by imaging nanostructure</td>
</tr>
<tr>
<td>SIM</td>
<td>Structured illumination microscopy</td>
</tr>
<tr>
<td>SNS</td>
<td>Synaptically-enriched sample</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated emission depletion</td>
</tr>
<tr>
<td>STORM</td>
<td>Stochastic optical reconstruction microscopy</td>
</tr>
<tr>
<td>SV2</td>
<td>Synaptic vesicle glycoprotein 2</td>
</tr>
<tr>
<td>SYN3</td>
<td>Syndecan3</td>
</tr>
<tr>
<td>SYP</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>SYPO</td>
<td>Synaptopodin</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA-binding protein 43</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TH</td>
<td>Total homogenate sample</td>
</tr>
<tr>
<td>UNC13A</td>
<td>Uncoordinated 13 A</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WS</td>
<td>Window size</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
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First and foremost, I would like to thank my supervisor, Dr Chris Henstridge, for his support, understanding and help throughout this project. He has been an excellent teacher and mentor and has helped me grow more confident and has helped me never lose my curiosity and love for science. It is because of his encouragement that I have had so many opportunities to develop as a researcher and communicator, and for that I will always be so grateful to him.

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Candidate’s declaration

I declare that I am the author of this thesis, and that it is a true record of the work performed by myself. This thesis has not previously been submitted in application for a higher degree. All of the references used in the preparation of this thesis have been consulted and are cited appropriately. This work was carried out in the cellular and systems medicine division at the University of Dundee, supervised by Dr Chris Henstridge and funded by the Euan MacDonald Centre.

Anna Sanchez Avila
Supervisor's declaration

I certify that Anna Sanchez Avila has completed nine terms of experimental research and that she has fulfilled the conditions of Ordinance 39, University of Dundee, such that she is eligible to submit the following thesis in application for the degree of Doctor of Philosophy.

Chris Henstridge
Abstract

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease and affects up to 5000 people a year in the UK. Unfortunately, it is a fatal neurodegenerative disease, with an average lifespan of 3 years after diagnosis. Even though ALS is characterised by its progressive paralysis, half of people with ALS also develop cognitive impairment. Those who are cognitively impaired tend to have a worse prognosis and a faster developing disease. The mechanisms underlying cognitive impairment are still not known, but unravelling the drivers of this dysfunction could prove crucial to the people living with ALS. Previously, factors such as synapse loss, presence of protein aggregates and inflammation have been associated with cognitive decline, but their regional specificity and correlation with the presence of symptoms remains to be assessed.

The main aim of this project was to generate a database of comprehensive patient information including detailed clinical cognitive profiling, post-mortem high-resolution synapse density measurements as well as pathology presence and astrocytic and microglial burden. I believe this study is the first of its kind, given all these factors have been assessed in the same group.

Moreover, molecular biology techniques as well as high-resolution and super-resolution microscopy techniques have been used to assess the synaptic localisation of ALS-associated proteins TDP-43 and FUS. I believe this study provides the first evidence of the synaptic presence of TDP-43 and FUS in the human synapse in post-mortem tissue.

In summary, this project provides a unique human dataset combining detailed cognitive assessment, regional neuropathology, and single synapse analysis to try and uncover the underlying pathology
associated with cognitive decline in ALS. It also provides evidence of the physiological synaptic presence of ALS-associated proteins, which could be crucial for the field, highlighting them as potential therapeutic targets.
Chapter 1: Introduction

1.1 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a lethal and progressive neurodegenerative disease that primarily affects motor neurons and is the most common form of motor neuron disease (MND).

Described in 1869 by Jean-Martin Charcot, it is characterised by the loss of both upper and lower motor neurons (Charcot and Joffroy, 1869; Oskarsson et al., 2018). Upper motor neurons originate in the cerebral motor cortex (in the brain) and project their axons through the brain stem and into the spinal cord, where they connect with lower motor neurons. Lower motor neurons reside in the spinal cord and project their axons into the periphery and innervate the muscles (Zayia and Tadi, 2023). Gradual neuronal loss leads to muscle fasciculations and weakness, which escalates to paralysis as well as difficulty swallowing (aphagia) and breathing (asphyxia), with respiratory failure being the most common cause of death, usually within 3 years after ALS diagnosis (Al-Chalabi and Hardiman, 2013; Oskarsson et al., 2018).

ALS is usually an adult-onset disease, with the average age of onset around 60 years (Longinetti and Fang, 2019). In European populations, it affects 2-3 people per year per 100,000, with the incidence being slightly higher in men than women (Al-Chalabi and Hardiman, 2013; Logroscino et al., 2010). At any one time, there are 5000 people living with ALS in the UK (Opie-Martin et al., 2021), and the lifetime risk of developing ALS is surprisingly high at 1 in 350 for men and 1 in 400 for women (Al-Chalabi and Hardiman, 2013; Johnston et al., 2006).
Despite these astonishing numbers, there is currently only one approved drug for ALS in Europe, Riluzole, which has been approved since 1995 and only increases life expectancy by about 2-3 months (Bellingham, 2011; Miller et al., 2002). Riluzole, originally developed as a muscle relaxant, acts by reducing neuronal firing (Bellingham, 2011; Beltran-Parrazal and Charles, 2003; Doble, 1996; Faustino and Donnelly, 2006; Pieri et al., 2003). This might sound counterintuitive, but the mechanisms behind why reducing firing could be beneficial will be discussed later. Edaravone is another drug that has been approved for use in Japan, South Korea and the US and has been shown to also slow disease progression, albeit only in a select subgroup of patients (Abe et al., 2014; Oskarsson et al., 2018; Rokade et al., 2022; Writing Group and Edaravone (MCI-186) ALS 19 Study Group, 2017). Edaravone is a free radical scavenger, meaning it acts as an antioxidant, by lowering free radicals and reactive oxygen species, which are highly-reactive, unstable molecules that are toxic to the cell (Lobo et al., 2010).

There is, however, cause for optimism when it comes to treatments and clinical trials (Johnson et al., 2022; Shefner et al., 2022). There are several approaches being taken to develop effective treatments, but one of particular interest is anti-sense oligonucleotides (ASOs) (Johnson et al., 2022). ASOs are synthetic molecules consisting of 8-50 single-stranded nucleotides, which bind to RNA and thus modify protein expression (Rinaldi and Wood, 2018). This approach has tremendous potential, as it could help modify the expression of disease-associated proteins, such as fused in sarcoma (FUS), chromosome 9 open reading frame 72 (C9ORF72) or superoxide dismutase 1 (SOD1) (Johnson et al., 2022), which will be discussed in detail later.
It is also worth mentioning MND-SMART, a one-of-a-kind multi-arm, multi-stage, parallel-group, multi-centre, randomised controlled trial based in the UK, which shows huge potential due to the flexibility of being able to add arms and targets as soon as they are flagged as potentially beneficial (Parker et al., 2023). It will test repurposed drugs highlighted as potentially beneficial by cell-based and animal-based screenings. The first two drugs to be tested against placebo are memantine and trazodone, but more will be added as the screening progresses. Memantine is an N-Methyl-D-Aspartate (NMDA) receptor antagonist that has been previously used as a treatment for dementia (Matsunaga et al., 2018, 2015); whereas trazodone is a serotonin antagonist and reuptake inhibitor that has been used for depression and anxiety (Al-Yassiri et al., 1981; Cuomo et al., 2019).

ALS can be categorised as familial (fALS) or sporadic (sALS), with only 5-10% of ALS cases having a hereditary background. ALS-associated mutations have been discovered in over 30 genes (Al-Chalabi and Hardiman, 2013; Mejzini et al., 2019; Renton et al., 2014; Zou et al., 2017). The most common genetic alterations are a hexanucleotide repeat expansion in the non-coding region of the chromosome 9 open reading frame 72 (C9orf72), and mutations in the superoxide dismutase gene (SOD1), as well as in the transactive response DNA-binding protein (TARDBP) and fused in sarcoma (FUS) genes. However, the exact aetiology for the majority of ALS cases still remains unknown (Figure 1.1).
Figure 1.1 Proportion of ALS cases explained by different genetic mutations. Percentage of fALS or sALS cases explained by different genetic mutations in European populations. Data adapted from Renton et al., 2014 and Zou et al., 2017.

1.1.1 ALS genetic mutations

1.1.1.1 SOD1

Dominant mutations in the superoxide dismutase-1 (SOD1) gene were identified as being causative of ALS 30 years ago (Rosen et al., 1993), providing the first evidence of a genetic cause of ALS. SOD1, the protein encoded by the SOD1 gene, is an antioxidant enzyme crucial for oxidative stress regulation and is present in the cytosol of neurons (Field et al., 2003; McCord and Fridovich, 1969; Trist et al., 2021). Mutations in SOD1 are causative of almost 15% of fALS cases and over 1% of sALS cases (Figure 1.1). Misfolding of SOD1 protein has been hypothesised to diminish its crucial antioxidant activity and thus renders neurons extremely vulnerable against stressors (Benkler et al., 2018; Duranti and Villa, 2023; Trist et al., 2021). Interestingly, characteristic TDP-43 aggregates – the most common pathological feature of ALS, which will be discussed later - are absent in SOD1-ALS, however SOD1-positive aggregates have been described in SOD1-ALS (Benkler et al., 2018; Blokhuis et al., 2013; Duranti and Villa, 2023). Recently there has been a shift in the field to
understanding SOD1-ALS pathology as distinct from all other types of ALS (Renton et al., 2014). There have been several attempts at modifying the expression of pathological SOD1 using ASOs. In fact, results of a phase 3 clinical trial using Tofersen, an ASO against SOD1 have been somehow promising, albeit with significant side effects (Meyer et al., 2023; Miller et al., 2022). This was approved by the FDA for use in SOD1-ALS patients in the USA April 25th, 2023 (Research, 2023). The ATLAS project, is now attempting treatment with Tofersen in pre-symptomatic SOD1 carriers, which could improve outcome (Benatar et al., 2022).

1.1.1.2 TARDBP

In 2006, the presence of neuronal cytoplasmic aggregates containing a protein named TAR DNA-binding protein 43 (TDP-43) was discovered (Arai et al., 2006; Neumann et al., 2006). TDP-43 is a DNA and RNA-binding protein encoded by the TARDBP gene. Not too long after this discovery, mutations in TARDBP were found to be causative of around 4% of fALS cases and 1% of sALS cases (Figure 1.1) (Kabashi et al., 2008; Sreedharan et al., 2008). Even though mutations in TARDBP account only for a small percentage of overall cases, TDP-43 aggregates are found in 97% of people with ALS, independently of their aetiology, making their presence a major hallmark of this disease (Loganathan et al., 2020).

Structurally, TDP-43 has 2 RNA-recognition motifs (RRM), a nuclear localisation signal (NLS), a nuclear export signal (NES), a prion-like structure in the C-terminal domain, as well as Q/N and G-rich domains (Prasad et al., 2019). TDP-43 can be cleaved in 2 sites, to form what is called C-term fragments, of 35 and 25KDa each (Arai et al., 2010; Berning and Walker, 2019; Neumann et al., 2009, 2006; Tsuji et al., 2012; Xiao et al., 2015). These fragments are the most important forms
of pathological TDP-43 since they contain TDP-43’s intrinsically disordered domain (prion-like), as well as serines 404/405 and 409/410, the main site for hyperphosphorylation (Arai et al., 2006; Fang et al., 2014; François-Moutal et al., 2019; Hasegawa et al., 2008; Kametani et al., 2016; Neumann et al., 2009; Zhang et al., 2019). Hyperphosphorylated C-term fragments are the most common pathological marker for aggregated forms of TDP-43 (Berning and Walker, 2019; Prasad et al., 2019) (Figure 1.2).

TDP-43 is a widely expressed protein and, due to its involvement in RNA and DNA processing as well as transcription and splicing regulation, it is predominantly nuclear (Ayala et al., 2008; Buratti et al., 2001; Mackenzie et al., 2010; Winton et al., 2008). Its levels are very tightly regulated by TDP-43 itself, and over or under-expression of TDP-43 is neurotoxic (Ayala et al., 2011; Polymenidou et al., 2011). TDP-43 is involved in many aspects of RNA metabolism, both in and out of the nucleus, including but not limited to transcription regulation, micro-RNA processing, splicing, and of particular interest recently, cryptic exon splicing (Ayala et al., 2008, 2006, 2005; Bose et al., 2008; Buratti et al., 2001; Buratti and Baralle, 2008, 2001; François-Moutal et al., 2019; Lagier-Tourenne et al., 2010; Ling et al., 2015; Mercado et al., 2005; Ou et al., 1995). Cryptic exons are intronic sequences that – in physiological circumstances – are effectively cleaved during splicing, but in disease remain included in the mature RNA (Ling et al., 2015). Thus, TDP-43’s impaired splicing can result in novel RNA isoforms, which are often unstable and end up degraded, leading to an overall loss of functional protein (Mehta et al., 2023). A particular cryptic exon event that has been uncovered recently is that of UNC13A. Mutations in UNC13A were identified as a risk factor for ALS (Calvo et al., 2022; van Es et al., 2009), and the absence of UNC13A was found to be fatal.
UNC13A, is the protein resulting from *UNC13A* (Engel et al., 2016), it is widely expressed in the central nervous system (CNS) and crucially for this project, it plays a role in synaptic vesicle docking as well as correct neuromuscular junction function (Mullins et al., 2022). Last year, two publications uncovered TDP-43’s physiological role in repressing the inclusion of a cryptic exon in UNC13A (Ma et al., 2022), which is present in UNC13A when TDP-43 is depleted or loses its function (Brown et al., 2022). Moreover, the single nucleotide polymorphisms found in UNC13A that have been linked to an increased risk of ALS, are found to be in TDP-43’s binding site to UNC13A, which could explain why they confer an increased risk of disease (Brown et al., 2022; Mehta et al., 2023).

Another evidence of TDP-43’s many roles in RNA processing is the fact that TDP-43 has also been shown to have a physiological role in axonal transport and correct dendritic transport and translation (Chu et al., 2019; Wang et al., 2008a). This topic will be explored further in chapter 5.

Mislocalisation of TDP-43 to the cytoplasm has been hypothesised as one of the main pathways of neurodegeneration in ALS, with evidence pointing towards both a loss of function of the physiological protein (Broeck et al., 2014), which gets sequestered in aggregates, as well as a potential toxic role of the aggregates themselves (Broeck et al., 2014; Lee et al., 2012; Xu, 2012). This is shown by the fact that TDP-43 aggregates are neurotoxic *per se*. Oligomerised full-length TDP-43 can induce cell death in primary neuronal cultures and *in vivo*, an injection of oligomerised full-length TDP-43 showed neuron loss in the mouse hippocampus (Fang et al., 2014). Moreover, mutated forms of the protein can also gain toxic functions, with neurotoxicity linked to TDP-43 mutation using *in vitro* in primary cultured rodent neurons and human-induced stem cell-derived neurons (Alami et al., 2014; Liu-
Yesucevitz et al., 2014). The results are replicated in *in vivo* studies using zebrafish (Kabashi et al., 2010) and rodent (Arnold et al., 2013; White et al., 2018) models harbouring mutations in TDP-43.

Another avenue of research is trying to understand the widespread presence of TDP-43 pathology irrespective of genotype as well as its presence in several areas of the brain and spinal cord (Brettschneider et al., 2013; Jucker and Walker, 2013). One of the theories that could explain this presence across brain areas is that pathological and aggregation-prone forms of TDP-43 spread through the brain in a prion-like manner (Nonaka and Hasegawa, 2020; Polymenidou and Cleveland, 2011).

This idea is supported by the fact that TDP-43, besides its 2 RNA recognition motifs (RRM, Figure 1.2), also has a prion-like domain in its structure (Figure 1.2), which makes TDP-43 prone to aggregate *in vitro* (Nonaka and Hasegawa, 2020; Smethurst et al., 2016). Moreover, TDP-43 has been shown to have the ability to spread from neuron to neuron both using *in vitro* (Feiler et al., 2015; Ishii et al., 2017; Porta et al., 2021; Smethurst et al., 2016) and *in vivo* (Ding et al., 2021; Porta et al., 2021) models. This “prion-like” behaviour of aggregation-prone proteins has been linked to several other neurodegenerative diseases, and proteins such as α-synuclein and Tau, involved in Parkinson’s Disease and Alzheimer’s disease respectively, also have the same spreading abilities (Jucker and Walker, 2013).
Figure 1.2 TDP-43 and FUS structure. Diagram showing the structure of both TDP-43 and FUS protein, the domains where the highest concentration of mutations can be found, as well as TDP-43's cleavage sites and hyperphosphorylation sites. Data adapted from Berning and Walker, 2019; François-Moutal et al., 2019; Prasad et al., 2019; Vance et al., 2013. Several similarities can already be seen in structure, as both proteins have Q and G-rich domains, RRs as well as a NLS; FUS, however, does not have a NES, nor does it have a hyperphosphorylation site.

1.1.1.3 FUS

Very soon after the discovery of mutations in TARDBP in ALS, mutations in the FUS gene were also found to be causative of the disease (Kwiatkowski et al., 2009; Vance et al., 2009). FUS-ALS is entirely mutation-driven and characterised by an earlier onset and more aggressive phenotype (Bäumer et al., 2010; King et al., 2015). Similar to TDP-43, FUS is a DNA and RNA-binding protein. Given they both have similar structures (Lagier-Tourenne et al., 2010; Vance et al., 2013) and functions, particularly regarding RNA processing, they could be considered homologues (Lagier-Tourenne et al., 2010). They are also responsible for roughly the same...
percentage of ALS cases (Mackenzie et al., 2010; Nolan et al., 2016; Rademakers et al., 2010; Renton et al., 2014).

FUS also seems to play a crucial role in several stages of RNA processing, like regulating transcription initiation and repression, splicing regulation and microRNA processing (Bertolotti et al., 1998, 1996; Chansky et al., 2001; Devoy et al., 2017; Hallier et al., 1998; Hartmuth et al., 2002; Lagier-Tourenne et al., 2010; Law et al., 2006; Powers et al., 1998; Rapp et al., 2002; Rappsilber et al., 2002; Uranishi et al., 2001; Wu and Green, 1997; Yang et al., 2000; Zhou et al., 2002). FUS also has several cytoplasmic functions, for instance, it is an integral component of stress granules (Lagier-Tourenne et al., 2010; Vance et al., 2013), which is a topic that will be discussed later.

FUS is also found in RNA-transporting granules, which translocate to dendritic spines to promote local translation (Belly et al., 2005; Fujii et al., 2005) as well as correct synaptic function (Deshpande et al., 2019; Fujii et al., 2005; Fujii and Takumi, 2005; Salam et al., 2021; Schoen et al., 2016; Sephton et al., 2014).

FUS inclusions can be seen in the CNS of FUS-ALS cases (Lagier-Tourenne et al., 2010; Mackenzie et al., 2010; Vance et al., 2013). Strikingly, TDP-43 aggregates are not present in FUS-ALS, leading to the hypothesis that FUS aggregation might be downstream of the TDP-43 pathological cascade (King et al., 2015; Nolan et al., 2016).

When it comes to pathology, FUS seems to drive motor neuron degeneration mostly through a toxic gain of function, rather than loss of function mechanisms, given the lack of nuclear FUS clearance in heterozygous NLS-mutated models and the toxicity of FUS overexpression models (Devoy et al., 2017; Mitchell et al., 2013; Nolan et al., 2016; Qiu et al., 2014).
1.1.1.4 C9orf72

It wasn’t until 2011 that the most common genetic cause of both fALS and sALS to date was discovered (DeJesus-Hernandez et al., 2011; Renton et al., 2011). It is a hexanucleotide (GGGGCC) repeat expansion (HRE) in a non-coding region of chromosome 9 open reading frame 72 (C9orf72).

Physiologically, C9orf72 is hypothesised to play a role in endosomal trafficking (Farg et al., 2014) and nucleocytoplasmic transport. C9orf72 knock-out models suggest C9orf72 also plays a role in endocytosis and autophagosome formation (Almeida et al., 2013; Fang et al., 2014; Gendron and Petrucelli, 2018). Interestingly, similar to TDP-43 and FUS, there is now evidence suggesting C9orf72 might also be present in the synapse and play a role in the regulation of post-synaptic receptor levels (Xiao et al., 2019). Moreover, a very recent publication showed that C9orf72 interacts with synapsin and can regulate correct function of excitatory synapses (Bauer et al., 2022). This interaction is impaired in ALS, where a marked decrease in synapsin levels has been shown in the hippocampus of both C9orf72 knockout mice and post-mortem brain of ALS/FTD patients with C9orf72 haploinsufficiency (Bauer et al., 2022).

Pathologically, the mechanisms of action are quite different from those previously discussed for other ALS-associated proteins. The hexanucleotide repeats can be transcribed to mRNA which form RNA foci. The mRNA can be translated to dipeptide repeats (DPRs) through a non-canonical mechanism named repeat-associated non-ATG translation (RAN translation) (Balendra and Isaacs, 2018; Zu et al., 2013) (Figure 1.3). There are five possible DPRs that can result from RAN translation of the HRE (Figure 1.4), and they all have varying levels of toxicity, as shown by several in vivo (Wen et al., 2014) and in...
vitro (Swaminathan et al., 2018; Wen et al., 2014) models, but what is crucial is that the presence of two of those DPRs (GR and PR) is enough to cause toxicity, irrespective of HRE presence, in a length-dependent manner (Bennion Callister et al., 2016; Kwon et al., 2014; Mizielinska et al., 2014; Swaminathan et al., 2018).

**Figure 1.3 C9orf72 pathological mechanisms.** Figure adapted from Gendron and Petrucelli, 2018. Blue bars represent the exons of the gene, in light blue are the non-coding exons and in dark blue, the coding ones. The HRE can be transcribed bidirectionally, with both sense and antisense RNA generating foci. Those RNAs can then be translated into dipeptide repeats, were 5 different ones can be generated, PolyGA (Glycine and Alanine), PolyGP(or PG, Glycine and Proline), PolyGR (Glycine and Arginine), PolyPR (Proline and Arginine) and PolyPA (Proline and Alanine). These DPRs can be toxic and aggregate into pathological inclusions. Image generated using Biorender.com.
Neuronal death seems to be driven by a loss of function due to the repeat expansion leading to lower levels of protein (Shi et al., 2018) but also to a toxic gain of function of both the RNA foci, which can sequester important proteins (Sareen et al., 2013; Shaw et al., 2018) and the DPRs themselves (Balendra and Isaacs, 2018).

Loss of function does not seem to be enough to induce degeneration, however, given the induced loss of function via ASOs (Donnelly et al., 2013; Lagier-Tourenne et al., 2013), as well as haploinsufficiency (O’Rourke et al., 2016), does not seem to generate any symptoms in vivo. Interestingly though, full C9orf72 null mice do show widespread immune system dysfunction, including microglial impairment, and consequent neuroinflammation (Balendra and Isaacs, 2018; O’Rourke et al., 2016).
Usually, some form of foci or DPR-associated toxicity is necessary to cause neurodegeneration even in the presence of loss of function. Although there is a lack of knowledge as to the mechanisms by which RNA foci and DPRs are able to generate pathology (Balendra and Isaacs, 2018; Gendron and Petrucelli, 2018), there is evidence that RNA foci presence alone (Balendra and Isaacs, 2018; Zhang et al., 2015), as well as DPRs alone (Bennion Callister et al., 2016; Kwon et al., 2014; Mizielinska et al., 2014; Swaminathan et al., 2018), are able to induce neurodegeneration and that they might act synergistically as well (Balendra and Isaacs, 2018). Therefore, much more research is required to fully understand the physiological and pathological role of C9orf72.

1.1.2 ALS Pathology

The exact pathogenesis of ALS is still unknown, but here are several hypotheses as to what mechanisms are at play in disease (Oskarsson et al., 2018).

The main focus of research regarding pathology is TDP-43 and its aggregates, mainly due to their widespread presence across cases. However, how TDP-43 aggregates link to neuronal loss is not certain. As discussed before, several mechanisms involving the loss or toxic gain of function may play a role, one of them being disruption of normal RNA metabolism (Oskarsson, 2018). This theory is further supported by the fact that TDP-43 homologue proteins involved in RNA-processing are also linked to disease, such as FUS - discussed above - and others like TIA1 (Mackenzie et al., 2007) and TAF15 (Kapeli et al., 2016; Ticozzi et al., 2011). Moreover, all these proteins are involved in a step central to cell survival, which is stress granule formation. In response to cellular stress, these granules containing RNA-binding proteins are formed and mRNA recruited, so mRNAs not
needed for survival are effectively silenced and only the essential ones are translated. Crucially, after the stressful event subsides, stress granules must disassemble appropriately, but they fail to do so in disease (Mackenzie et al., 2007; Monahan et al., 2016; Oskarsson et al., 2018).

In healthy cells, proteins also misfold, but cells have mechanisms that either degrade the misfolded protein before it is able to recruit others and form aggregates, or clear already established aggregates. In ALS – and in other neurodegenerative diseases characterised by protein aggregation - this mechanism stops working. The fact that protein aggregates persist in disease as well as the existence of ALS causing mutations in genes that encode proteins which are crucial in protein degradation pathways, points to impaired protein metabolism as a key pathological phenomenon in ALS (Oskarsson et al., 2018; Ramesh and Pandey, 2017).

Mitochondrial dysfunction and oxidative damage have also been linked to motor neuron death in ALS (Oskarsson et al., 2018; Rothstein, 2009), and are highlighted in SOD1-ALS. SOD1 mutations are hypothesised to be neurotoxic by generating reactive hydroxyl radicals, which can generate oxidative damage and be detrimental to neurons (Rothstein, 2009; Wiedau-Pazos et al., 1996). This could explain the effectiveness of free radical scavenger Edaravone as a treatment for ALS. Mitochondrial dysfunction is an early event in motor neuron degeneration (Bernal et al., 2023; Nemtsova et al., 2023; Rothstein, 2009). Proper mitochondrial function is crucial, even more so in energy-demanding cells such as motoneurons, and the fact that ALS-linked mutations associate with mitochondrial defects also points to this pathomechanism as a potential key player in neurodegeneration (De Vos et al., 2012; Smith et al., 2019).
However, ALS in not exclusively a disease of motor neurons. In fact, other cell types, like microglia (Lall and Baloh, 2017), astrocytes (Kia et al., 2018; Meyer et al., 2014; Tripathi et al., 2017), and even oligodendrocytes (Ferraiuolo et al., 2016), have also been shown to play an active part in the degeneration process (McCauley and Baloh, 2019; Vahsen et al., 2021). Microgliosis – an increase in reactive, activated microglia – and an increase in reactive astrocytes has been observed in the upper motor neurons of both TDP-43 mouse models and human ALS brains (Jara et al., 2019). Interestingly, the addition of mutated TDP-43 to cultured microglia, induced microglial activation and an increase in pro-inflammatory markers (Zhao et al., 2015). Microglia are hypothesised to be both neuroprotective and neurotoxic. At the beginning of disease, microglia might be protective by phagocytosing debris from degenerating neurons. However, later in disease, it might become too reactive and thus toxic by engulfing healthy synapses and neurons or by releasing neurotoxic factors (Vahsen et al., 2021). Astrocytes are thought to play a crucial role in disease as well, mutations in astrocytic TDP-43 in a rodent model, induced astrogliosis, which resulted in neuronal loss (Tong et al., 2013). Moreover, diseased astrocyte-conditioned media was enough to cause neurodegeneration, pointing to secreted factors being involved in astrocyte-induced pathogenesis (Rojas et al., 2014). Overall, there is now growing evidence that non-neuronal cells also play a crucial and active role in disease (Vahsen et al., 2021), and highlights the importance of taking glial influence into account.

While some of the pathomechanisms described above are still debated as key players or not, one convergent feature observed across ALS models as well as human post-mortem tissue, is the loss of synapses (Cantor et al., 2018; Fischer et al., 2004; Fogarty et al., 2015; Henstridge et al., 2018; Malpetti et al., 2021; Nagao et al., 1998; Noto et al., 2011; Shoichi Sasaki and Iwata, 1996; S. Sasaki and Iwata,
1996; Sasaki and Maruyama, 1994a, 1994b; Shahidullah et al., 2013; Starr and Sattler, 2018; Tang et al., 2022) preceding motor neuron loss. This topic will be explored later in more detail.

1.1.3 Cognitive impairment in ALS

ALS was considered a disease with an exclusively motor phenotype until recently (Goldstein and Abrahams, 2013). Now, there is increasing evidence that up to 50% of ALS patients show cognitive or behavioural impairment, with language, verbal fluency and executive dysfunction being the areas most affected in those impaired (Goldstein and Abrahams, 2013).

Cognitive impairment in ALS can be measured with the Edinburgh Cognitive and Behavioural ALS Screen (ECAS). The ECAS is a 15-20 min cognitive and behavioural impairment test that has been designed specifically for people with ALS (Goldstein and Abrahams, 2013). It consists of a multidomain assessment that assesses both ALS-specific functions (ALS-specific ECAS, language, verbal fluency, and executive dysfunction) as well as non-specific functions (memory and visuospatial functions). The overall score has a max of 136 and the impaired cut-off is below 105. Each independent task within the ECAS also has its own cut-off value for impairment (Table 1.1).
<table>
<thead>
<tr>
<th>Task</th>
<th>Max. score</th>
<th>Cut-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>Executive function</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>Language function</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>Verbal fluency</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>ALS-specific ECAS</td>
<td>100</td>
<td>77</td>
</tr>
<tr>
<td>Memory function</td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>Visuospatial function</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>ALS Non-specific ECAS</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>ECAS total</td>
<td>136</td>
<td>105</td>
</tr>
</tbody>
</table>

Table 1.1. ECAS scores and cut-off values. Data extracted from Goldstein and Abrahams, 2013, showing all the different ECAS tasks, their maximum score as well as the cut-off value. A score equal or below the cut-off indicates impairment.

Importantly, cognitive impairment correlates with a worse disease prognosis (Elamin et al., 2011; Goldstein and Abrahams, 2013), therefore understanding the pathomechanisms behind cognitive decline is crucial.

Interestingly, both synapse loss and the presence of TDP-43 aggregates have been linked to cognitive impairment. Synaptic loss occurs in the dorsolateral prefrontal cortex, a brain area thought to be involved in executive function, in ALS with cognitive impairment (Henstridge et al., 2018), and TDP-43 presence in brain areas specifically involved in executive function, language and verbal fluency correlated with cognitive impairment (Gregory et al., 2020).

1.1.4 ALS as part of a disease spectrum

Half of ALS patients show some level of cognitive impairment, but more importantly, up to 15% also receive a frontotemporal dementia (FTD) diagnosis. Conversely, up to 50% of FTD patients display motor symptoms and 15% are also diagnosed with ALS (van Es et al., 2017).
This puts both diseases at either end of a disease spectrum involving cognitive, behavioural, and motor symptoms.

ALS and FTD not only share clinical overlap. ALS-causing mutations are also known to cause FTD, such as the C9ORF72 HRE and mutations in TARDBP. Moreover, TDP-43 aggregates, present in 97% of ALS cases, are present in 45% of FTD cases and FUS aggregates can be found in 9% of people with FTD (Ling et al., 2013), this was the first publication to link the two diseases.

Pathomechanisms involved in ALS such as DPR toxicity in C9-ALS are also conserved in C9-FTD (Bennion Callister et al., 2016; Gendron and Petrucelli, 2018; Shi et al., 2018; Zu et al., 2013), as well as noxious effects linked to TDP-43 aggregates (Berning and Walker, 2019; Chiò et al., 2010; Fang et al., 2014; Mackenzie et al., 2010; Neumann et al., 2006) and synaptic dysfunction preceding neuronal loss (Schoen et al., 2016; Starr and Sattler, 2018). This highlights ALS should be understood as more than just a disease of motor neurons and focus on the overlapping pathology between FTD and ALS could prove very influential in the quest for effective treatment. One important shared feature between ALS and FTD is synapse loss, which has also been observed to occur early in disease and precede cell death in FTD (Malpetti et al., 2022, 2021).

### 1.2 Synapses

Synapses are minute, delicate structures through which neurons communicate with one another. They often do so by releasing chemicals, known as neurotransmitters, that can activate or stall the firing of the target cell (Dharani, 2015). Broadly, chemical synapses are classified as excitatory versus inhibitory based on the neurotransmitter they release as a result of the arrival of an action
potential to the presynaptic terminal (Rollenhagen and Lübke, 2015). The neurotransmitter released will determine whether the postsynaptic cell increases – excitatory- or decreases – inhibitory - its chance to fire. Excitatory neurotransmitters depolarise the postsynaptic cell, letting positive charges in, and bringing it closer to the threshold, whereas inhibitory neurotransmitters hyperpolarise the cell, letting negative charges in and reducing its chances to fire.

Briefly, an example of an excitatory chemical synapse consists of (Figure 1.5):

- A pre-synaptic terminal, in which vesicles filled with neurotransmitter (in this case, glutamate) are formed and docked to the membrane ready to be released via a calcium-dependent fusion once the action potential arrives.
- A post-synaptic terminal, containing post-synaptic receptors, which are channels that open in response to neurotransmitter presence to allow charged ions to pass through. These receptors are secured by the postsynaptic density (PSD), a scaffold of several proteins.
- The synaptic cleft is the space between the two neurons, where the neurotransmitter is released. Interestingly, though the two neurons do not physically touch, adhesion proteins connect the two terminals.
- Glial end feet, which are there to provide support, help with synapse integrity as well as the reuptake of the neurotransmitter (Rollenhagen and Lübke, 2015).
Figure 1.5 Structure of the excitatory synapse. A) Excitatory synapses occur between two neurons, a presynaptic one and a post-synaptic one. Astrocytes and other glial cells are also involved in correct synaptic function. B) Diagram showing the synaptic structure, which involves pre-synaptic vesicles filled with neurotransmitter, which is released to the synaptic cleft to then bind to the post-synaptic receptors held in place by the post-synaptic density. Glial end feet are also present to provide support and help with neurotransmitter reuptake. Figure created using BioRender.com.

Inhibitory synapses have a similar structure, though they do not contain such a prominent PSD (Stewart et al., 2014), and the main inhibitory neurotransmitter released is γ-aminobutyric acid (GABA), which induces hyperpolarisation of the postsynaptic cell by opening Cl⁻ channels.

While neuron number tends to remain constant in healthy adulthood (Kumar et al., 2019), synapse density and morphology are highly dynamic, since synapses change over time in response to continual changes in brain function. Synaptic plasticity is therefore crucial for memory and learning, as well as the ability to adapt to new environments.
Besides these general descriptions, there are also specialised synapses, which do not necessarily adhere to those definitions and perform a very specific function. Some examples are ribbon synapses, found in sensory neurons (Odermatt and Lagnado, 2009) and of interest to this thesis, the neuromuscular junction (NMJ). The NMJ (Figure 1.6) is a specialised chemical synapse that occurs between a motor neuron and a muscle fibre. Following neurotransmitter release (in this case, acetylcholine, ACh), the motor end plate’s (which acts as a post-synapse) ACh receptors open Na\(^+\) channels, which elicits muscle contraction (Sousa-Soares et al., 2023).

**Figure 1.6 Structure of the neuromuscular junction.** Diagram showing the structure of a neuromuscular junction, which involves pre-synaptic vesicles filled with ACh, which is released to the synaptic cleft to then bind to the post-synaptic receptors in the motor end plate. These receptors then let in an influx of Na\(^+\), which leads to depolarisation and muscle contraction. Figure created using a template from BioRender.com.
1.2.1 Synapses in disease

Synapse alteration is at the core of many neurological diseases, which can be grouped into what is now known as synaptopathies, with over 100 diseases of the brain having alterations in their synaptic proteins, or proteome (Grant, 2012). Disrupted synaptic machinery and function are present in the most common neurodegenerative diseases, with altered synaptic receptor trafficking being the most affected pathway (Henstridge et al., 2016).

Moreover, synapse loss has been associated as a key early feature of many neurodegenerative diseases (Henstridge et al., 2016). Alzheimer’s Disease (AD) is the most common form of dementia, and it is characterised by memory loss as well as the presence of amyloid-\(\beta\) positive dense core plaques and neurofibrillary tangles of Tau protein. Synapse loss has been a topic of interest in AD since the 1990s, when DeKosky and Scheff’s seminal work using electron microscopy (EM) to assess synaptic density highlighted synapse loss occurs in Brodman area 9 and correlates with cognitive severity (DeKosky and Scheff, 1990; Terry et al., 1991). This was the first evidence of synapse loss being a better correlate for cognitive decline than the presence of hallmark pathology. With recent advances in technology, this important finding has been validated and expanded. For example, using array tomography (AT) in human post-mortem tissue, it has been recently shown that synapse loss happens predominantly near amyloid-\(\beta\) plaques (Koffie et al., 2009, 2012a; Kurucu et al., 2022; Mecca et al., 2020; Polydoro et al., 2014; Spires-Jones and Hyman, 2014).

Parkinson’s Disease (PD) is the second most common neurodegenerative disease and is characterised by the presence of bradykinesia, tremor and rigidity, as well as the presence of Lewy
bodies – α-synuclein positive aggregates – in the substantia nigra (Tolosa et al., 2021). Synapse loss is also a very early phenomenon in this disease that has been observed in several rodent models, parkinsonian monkeys and post-mortem human tissue (Day et al., 2006; Mathai et al., 2015; Matuskey et al., 2020; Stephens et al., 2005; Villalba et al., 2015; Zaja-Milatovic et al., 2005). Dementia with Lewy bodies is also characterised by the presence of these α-synuclein positive aggregates. Symptoms of dementia with Lewy bodies include hallucinations, cognitive impairment, confusion, and similar tremor to PD. This time, the presence of α-synuclein aggregates is more widespread, and synapse loss is observed in rodent models too (Kramer and Schulz-Schaeffer, 2007; Lim et al., 2011).

Huntington’s disease (HD) is a disease characterised by the presence of a trinucleotide (CAG) repeat in the gene encoding Huntingtin. This disease is known for its chorea – movement disorder – as well as cognitive and psychiatric deficits. Interestingly, synapse degeneration and loss preceding neuronal death have also been very well established in this disease using Golgi stains in mouse models as well as human post-mortem tissue (Ferrante et al., 1991; Nithianantharajah and Hannan, 2013; Sotrel et al., 1993; Spires et al., 2004).

Lastly, another aspect that highlights the importance of synapses in neurodegenerative diseases is that synaptic accumulation of aggregation-prone proteins can be seen. All the diseases mentioned above are characterised by the presence of protein aggregates, which are most striking in the neuronal cytoplasm, but also found in more discrete compartments, such as the synapse.

In AD, aggregates of amyloid-β and Tau are characteristic of the disease, and have been found to be present in the AD synapses using AT and super-resolution imaging approaches in human post-mortem
tissue and rodent models (Koffie et al., 2012b, 2009; Kurucu et al., 2022; Pickett et al., 2019, 2016).

α-synuclein is present in Lewy bodies found in both PD and dementia with Lewy bodies and has been shown in synapses using immunofluorescence and AT in human post-mortem tissue (Colom-Cadena et al., 2017, 2013).

In HD, there is evidence of synaptic accumulation of huntingtin in the post-synapse of mouse models, observed using synaptic fractionation and molecular biology (Suopanki et al., 2006).

Taken together, it is clear that early synapse loss is a consistent feature across the majority of neurodegenerative diseases, therefore discovering the processes leading to synapse loss could have a significant impact on these devastating conditions.

1.2.2 Synapses in ALS

Mounting evidence suggests that synapse dysfunction and subsequent synaptic loss precedes motor neuron death (Fischer et al., 2004; Fogarty et al., 2015; Henstridge et al., 2018; Nagao et al., 1998; Sasaki and Maruyama, 1994a, 1994b; Shahidullah et al., 2013; Tang et al., 2022). The importance of synapses in the context of ALS is highlighted by the fact that the two main hypotheses as to why motor neurons die – die back vs. die forward - have synapses at their core.

The die forward hypothesis, also known as excitotoxicity, postulates than an increase in upper motor neuron excitability (Devlin et al., 2015; Fogarty, 2018; Fogarty et al., 2015; Frey et al., 2000; Geevasinga et al., 2016; Kanai et al., 2006; Kuo et al., 2004; Pieri et al., 2003; Starr and Sattler, 2018; Vucic and Kiernan, 2006; Wainger et al., 2014)
might be the driver of cell death, by causing excess generation of intracellular calcium, which is toxic to neurons (Grosskreutz et al., 2010). This increase in excitability might be due to an increase in glutamate release by the presynaptic neuron (Rothstein et al., 1992, 1990), a decrease in glutamate reuptake by the surrounding glia (Rothstein et al., 1995; Sasabe et al., 2012, 2007; Van Damme et al., 2007), or by changes in the postsynaptic glutamate receptors (Bursch et al., 2019; Couratier et al., 1993; Hideyama et al., 2012, 2010; Kawahara et al., 2006, 2004, 2003; Kwak and Kawahara, 2005; Rembach et al., 2004; Selvaraj et al., 2018; Spalloni et al., 2011; Takuma et al., 1999; Tortarolo et al., 2006; Vandenberghhe et al., 2000), meaning several key components of the synapse might play a pathological role. This hypothesis is supported by the fact that the only approved drug in Europe to treat ALS, Riluzole, is thought to work by lowering glutamate levels (Bellingham, 2011).

Also supporting this hypothesis, synapse degeneration has been shown in the ALS central nervous system – both in the cortex and in the spinal cord - prior to cell loss, pointing to it as a key early step in the degeneration cascade (Fogarty et al., 2015; Henstridge et al., 2018; Nagao et al., 1998; Qiu et al., 2014; Sasaki and Maruyama, 1994a, 1994b). Moreover, a recent publication selectively mislocalised TDP-43 in the motor cortex of a rodent model (Reale et al., 2023) and observed 20 days after mislocalisation induction, increased cortical neuron firing. After 30 days, changes in postsynaptic proteins occurred in the spinal motor neuron as well as loss of lumbar motor neurons. This provides evidence that presynaptic changes in the upper motor neurons elicit postsynaptic changes in the spinal cord motor neurons that drive their death (Reale et al., 2023).

The die back hypothesis claims that degeneration first occurs at the peripheral NMJ synapse, following a toxic insult (yet to be identified),
which leads to axonal retraction and subsequent cell death (Fischer et al., 2004; Frey et al., 2000). There is also ample evidence of presymptomatic NMJ loss in both animal models and humans that backs this hypothesis (Felice, 1997; Fischer et al., 2004; Fogarty, 2018; Frey et al., 2000; Killian et al., 1994; Lépine et al., 2022).

Lastly, ALS-causing mutations can occur in FUS and TDP-43, which, as discussed before, are RNA-binding proteins thought to play a crucial role in synaptic function (Alhindi et al., 2022; Chu et al., 2019; François-Moutal et al., 2019; Lépine et al., 2022; Ling et al., 2013; Piol et al., 2023; Portz et al., 2021). Therefore, the synaptic dysfunction seen in those familial cases could potentially be due to the malfunction of these proteins.

1.3 Studying synapses

It is estimated that there are more synapses in one adult brain than stars in the milky way galaxy (Tang et al., 2001). Their sheer number implies their size must be in the nanoscale. This means that all the complex machinery needed to synthesise, release and respond to neurotransmitters needs to be encapsulated in microscopic compartments (Sanchez Avila and Henstridge, 2022).

In-depth synaptic study in the context of neurodegeneration, is essential. However, understanding synapses at a physiological and pathological level requires an understanding of both their number and their composition.

1.3.1 Imaging

There are two main challenges one faces when wanting to study synaptic number. The first one is that synapses are incredibly delicate
and fragile, therefore conserving an intact ultrastructure during sample processing – particularly when dealing with post-mortem tissue – is a difficult task. There are ways to help this, such as shortening the interval between death and tissue fixation, working at very low temperatures as well as embedding tissue in hard resin. These methods will be explored here.

The most limiting step with synaptic study, however, is their size. They are so small that they are in fact smaller than the resolution of light microscopes. The resolution is the minimum distance between two points needed to distinguish them as two separate entities instead of one. In most conventional microscopes, filtered light is used as the source to excite fluorophores. Light exists as a wave and diffracts when it passes through a sample. This means that it spreads wider than the real size of the object of interest (Figure 1.7).

![Light diffraction in light microscopy](#)

**Figure 1.7. Light diffraction in light microscopy.** Diagram from Nikon MicroscopyU showing the behaviour and path light takes when imaging a sample. Left to right, the light source (in fluorescence work, a coloured laser) shines light which is condensed by the lens to focus on the sample of interest and then diffracts until it reaches the objective. The numeric aperture of the objective depends on the angle the light forms with the vertical line to the objective. Figure created using BioRender.com.

There is a formula that can be used to establish the resolution of any light microscope, and that is: resolution = \( \frac{\lambda}{2*\text{NA}} \) where \( \lambda \) is the size
of wavelength used to excite the sample and the numeric aperture (NA) of the objective, which indicates its light gathering power. Given the two variables are defined by the components of a microscope, each light microscope, with each objective set up, will have a different resolution.

Figure 1.8. Resolution limit in light microscopy. Diagram showing the size of light scatter and therefore what a light microscope can resolve relative to synapse size. If two synapses are very close together in the vertical (z) plane, the microscope will not resolve them as two separate objects but instead a single, much bigger, one. Figure created using BioRender.com.

Confocal microscopes achieve better resolution than widefield fluorescence microscopy through the use of pinholes which reduce the detection of scattered light and allow only the collection of in-focus light on a particular focal plane. However, even with the most powerful confocal microscope, the lowest resolution that can be achieved is around 200nm in the x-y plane, but of 0.5-1 μm on the z-plane (Fouquet et al., 2015). Taking into consideration that the average diameter of a post-synaptic density is around 250 nm, the resolution would be just enough to resolve 2 synapses together in the xy plane but not enough to resolve synapses in the z plane. This means that conventional light microscopy approaches cannot be used to accurately study the structural integrity of individual synapses (Sanchez Avila and Henstridge, 2022) (Figure 1.8).
1.3.1.1 Electron microscopy

One solution to this problem is using EM. In EM, a beam of electrons is used as a source of illumination instead of light, which allows for resolutions of less than 1nm. There are two types of electron microscopes:

- Transmission electron microscope (TEM): In TEM the beam of electrons is shone through an ultrathin tissue section and provides a two-dimensional image. It is analogous to a conventional light microscope but using electrons instead of light.

- Scanning electron microscope (SEM): In SEM the electrons are detected after bouncing off the sample, rather than going through it, resulting in an incredibly detailed image of the surface of the sample, in 3D.

In both cases the resulting image is monochrome and heavy metals can be used to increase the contrast for a more precise image. TEM has been extensively used for synaptic study, and it is still considered the gold standard approach for synaptic density studies. It was used in the ground-breaking work of DeKosky and Scheff to first highlight synapse loss in human AD brains (DeKosky and Scheff, 1990; Scheff et al., 1993). Soon after, it was also used to assess the loss of synapses in the spinal cord of people with ALS (Shoichi Sasaki and Iwata, 1996; S. Sasaki and Iwata, 1996; Sasaki and Maruyama, 1994a, 1994b) and more recently in the human cortex (Henstridge et al., 2018) as well as mitochondrial accumulation in human AD synapses (Picket et al., 2018).

Another way to add targeted specificity to EM is by performing immuno-EM. In immuno-EM, antibodies with electron-dense gold
particles attached are used to stain the sample and provide a high-contrast and specific labelling of a particular protein of interest. This allows detailed study of synaptic localisation of a protein, down to subsynaptic compartments. This technique has been used in AD to assess the synaptic presence of Tau and amyloid-β in a transgenic mouse model (Katsuse et al., 2006), and of amyloid-β synaptic presence in cultured neurons (Takahashi, 2004).

A caveat of this technique is that antibodies often have very low penetration and epitope access due to the sample processing, which requires embedding in solid resins. This can be helped with several etching protocols, but it is still a major drawback of this approach (Petralia and Wang, 2021; Phend et al., 1992; Tao-Cheng et al., 2021).

Another hurdle of this imaging technique, however, is retaining ultrastructural integrity. Inadequate fixation, use of detergents or the wrong buffers can all lead to the deterioration of tissue quality, which is amplified at the EM level. Cryo-EM preserves ultrastructure by performing EM in flash-frozen samples at very low temperatures, which allows for resolutions of 3.5Å. Cryo-EM is even able to resolve protein structures, which is why it has been used to study the different isoforms of Tau filaments from an AD brain (Fitzpatrick et al., 2017) as well as the ultrastructure of TDP-43 aggregates (Cao et al., 2019) and C9orf72 DPRs, where it was seen DPRs have the ability to sequester proteasomal subunits (Guo et al., 2018).

Overall, different forms of EM have been used to study synapse structure and density for decades, including in neurodegeneration contexts and more importantly in ALS. It is thus an incredibly powerful technique and still considered the gold standard approach for synapse density studies. However, it is a very technically demanding and time-consuming technique and requires the use of very specialised and
expensive equipment, as well as the health and safety risk of using heavy metals to enhance contrast. Moreover, the number of synapses that can be studied with this technique is very limited, given it requires manual synapse count and the images captured are small.

1.3.1.2 Array tomography

Developed in 2007 (Micheva and Smith, 2007) and later adapted for use in human post-mortem tissue (Kay et al., 2013), the high-resolution imaging technique of AT can overcome some of the challenges EM has. AT is a light microscopy-based approach that solves the axial resolution problem by cutting ultrathin (70nm) serial sections of resin embedded tissue (Sanchez Avila and Henstridge, 2022). This technique will be the focus of chapter 3 and will be discussed in more detail there.

1.3.1.3 Super-resolution imaging

Instead of relying on physical dissection to solve the resolution limit of light microscopy, super-resolution techniques selectively excite a limited number of fluorophores at one given time. There are various super-resolution techniques one can perform, and each has its own intricacies. Briefly, techniques such as stochastic optical reconstruction microscopy (STORM, Rust et al., 2006) and photoactivated localisation microscopy (PALM, Betzig et al., 2006), achieve nanometre-scale resolutions by switching on and off the fluorescent state of single fluorophores at a time. Meanwhile, approaches like stimulated emission depletion (STED, Hell and Wichmann, 1994), use two lasers to excite a set of fluorophores and deplete the ones around them. Other techniques such as structured illumination microscopy (SIM, Gustafsson, 2000) image the sample several times using different non-uniform illumination patterns each
time and then reconstruct a high-resolution image through an algorithm based on the combination of all the images obtained. All of these techniques can allow for a detailed study of synaptic protein composition as well and have been successfully used to study the synaptic localisation of ALS-associated proteins in cell cultures (Deshpande et al., 2019) as well as *drosophila* larvae (Bademosi et al., 2018). Though the potential of super-resolution in the field of synaptic degeneration is undeniable, their need for specialised equipment and very experienced users still restricts their widespread use (Hindley et al., 2023).

Image scanning microscopy (ISM) can improve the resolution obtained by conventional confocal microscopes by replacing the detector of the microscope, which contains a photosensitive surface that can capture the photons emitted by the sample, with an array detector which contains many pixels. In ISM, each pixel of the detector acts effectively as a camera that records an image of the scanned sample, so the final result is a composite of all the pixels in the array detector. These can then be merged into one super-resolution image using an algorithm that accounts for the imaging angle differences of each pixel (Gregor and Enderlein, 2019).

Synaptic evaluation and quantification by imaging nanostructure (SEQUIN), is a recently developed approach that combines image scanning microscopy with localisation-based analysis. It can identify synapses by both the presence of synaptic markers as well as the space between said markers based on the ultrastructural parameters of synapses in the chosen model. In the original publication, the authors were able to use this technique to study synapse density changes near amyloid-β plaques in three different Alzheimer’s disease rodent models (Sauerbeck et al., 2020). Their results recapitulate previously published literature, proving the robustness of their new
approach (Koffie et al., 2009) and point its potential use as a tool for high-throughput multiplexed synaptic density study once it is more easily accessible.

Taken together, the advances in imaging technologies are beginning to shine a new light on synaptic structure and composition, however, their complexities still prevent mainstream use. AT is a good compromise on technical demands and data output and will be discussed as our chosen method in Chapter 3.

1.3.1.4 In vivo imaging of human synapses

All the previously discussed approaches are invaluable for synaptic density analysis and have been well established. However, they restrict their findings to post-mortem use. A recently explored area is the study of synapses in the brain in vivo.

This can be achieved through positron-emission tomography (PET). A PET scan is a nuclear medicine imaging test in which a radiotracer is administered to the person, the radiotracer then emits photons which are then detected in a scanner.

Synaptic imaging can be performed after exposure to a radioligand bound to a synaptic marker, such as synaptic vesicle glycoprotein 2 (SV2) (Cai et al., 2019; Finnema et al., 2016). PET scans are fairly non-invasive, and they can give great insight into disease progression and how synapse density changes in the disease course. Moreover, it could be a fantastic tool in clinical trials to monitor disease progression and treatment effectiveness.

In the context of ALS and FTD research, $^{18}$F-SynVesT-1 – an SV2 radiomarker – was used as contrast in PET scans of people with ALS
to assess synaptic density changes in different areas of the brain (Tang et al., 2022). Crucially, their results found differences between cognitively impaired and unimpaired brains in the frontal lobe, which matches previously published literature in post-mortem tissue (Henstridge et al., 2018). SV2A-PET has also been used to study synaptic changes in people with behavioural variant FTD, where they see a marked synaptic loss in the prefrontal cortex (Malpetti et al., 2022) as well as in pre-symptomatic carriers of the C9orf72 repeat expansion mutation, where there is evidence of synapse loss at presymptomatic stages, with one carrier predominantly losing synapses in the motor cortex and occipital lobe and another with a markedly prefrontal loss (Malpetti et al., 2021).

Synaptic PET imaging in the spinal cord has been very recently attempted too (Rossano et al., 2022), where they show the feasibility of this approach, which could prove crucial for motor neuron disease research as well as spinal cord injury and regeneration research.

This approach will undoubtedly be incredibly useful in our understanding of how synapses change throughout disease, but low tracer specificity and sensitivity as well as not enough resolution for accurate whole brain study at a synaptic level mean there is still improvements to be made in the current protocols.

1.3.2 Molecular biology approaches to study synaptic composition

Changes in synaptic density are an important characteristic of neurodegenerative diseases, but there is more synaptic information needed to fully elucidate the pathomechanisms underlying these conditions. Using imaging approaches restricts the focus to a small number of target proteins of interest, but we need a much broader...
understanding of protein changes at the synapse. One approach one could take to assess molecular synaptic composition is by taking advantage of synaptic fractionation protocols followed by various proteomic analyses.

Synaptically-enriched samples can be obtained from post-mortem tissue through various protocols, such as a series of filtration and centrifugation steps, through gradient centrifugation or fluorescent activated synaptosome sorting. It is important to note that the sample obtained, though synaptically-enriched nevertheless, can vary depending on the method used.

Synaptosomes contain a resealed presynaptic sac as well as the postsynaptic density attached. Synaptoneurosomes, however, contain presynaptic and postsynaptic resealed sacs. In both cases the synaptic junction is present, as well as any presynaptic markers in the sac. Both preparations also contain a small percentage of glial fragments, which are often present in the synapse (Gulyássy et al., 2020). The difference lies on the reseal of the whole postsynaptic compartment, encapsulating far more than just the postsynaptic density, which can also provide very useful information and puts synapse study in the wider, more complex context of the whole brain. It is an important difference to take into consideration (Figure 1.9) and the use of one model or the other should depend in the question that is being assessed (Ahmad and Liu, 2020; Hindley et al., 2023)
Figure 1.9 Synaptosomes vs. synaptoneurosomes. Diagram adapted from Hindley et al., 2023 showing the differences between synaptosomes and synaptoneurosomes. As highlighted in the figure, synaptosomes lack a resealed postsynaptic sac as well as the presence of glial endfeet. Figure created using BioRender.com.

This approach can then be taken one step further and, through further lysis and centrifugation steps, the pre- and post-synaptic compartments can be separated in what is called synaptic fractionation. To assess synaptic localisation of particular proteins of interest, molecular biology approaches such as western blotting can be performed using these samples. However, using this approach you are limited to a small number of proteins you can study, and you have to know which ones you want to analyse prior to experimental set up.

Proteomic analysis provides an unbiased hypothesis-generating and quantifiable summary of many thousands of proteins in your sample (Hindley et al., 2023). In fact, it has now been used in the context of ALS to study the synaptic proteomic content of BA4 and BA9 in healthy, ALS and C9-ALS brains (Laszlo et al., 2022), where they saw up to 500 proteins with different expression patterns between all the
conditions studied and a more post-synaptic driven proteomic change in both ALS with cognitive impairment as well as C9-ALS. The same approach has now also been used for studying synaptic change in the ALS spinal cord, revealing remarkable convergence on presynaptic dysfunction (Aly et al., 2023).

Moreover, a recently published technique called “mass synaptometry” is able to provide single-synapse proteomic information. It performs cytometry by time-of-flight mass spectrometry to synaptosomes. This approach uses antibodies tagged with metal ions, rather than fluorophores, which means several antibodies can be used at once without being limited by laser availability. This protocol has now been validated in mouse and human brain tissue and could show great potential in the field of synaptic proteomics (Gajera et al., 2022, 2019).

1.4 Objectives

Synapse loss is an established early feature of ALS. However, its regional specificity and, more importantly, how that correlates with changes in cognition as well as other demographic and pathological data is still not fully elucidated. Thus, the main objective of this thesis will be to study synaptic density changes in different areas of the brain of people with ALS using human post-mortem tissue.

One of the techniques used to perform synapse density analysis in this project will be AT. This technique is only performed in a few select laboratories across the world, therefore the first goal will be to establish this technique at the University of Dundee as well as optimising and streamlining the protocol using the resources available in this institution.
Once this technique is established, both AT and EM images will be used to study synapse density changes in ALS. Cortical thinning in the same areas will also be measured. Then, the correlation between these parameters and changes in cognition, presence of pathology, genetic status and other demographic information will be assessed.

Lastly, the synaptic localisation of the ALS-associated proteins discussed above is still not fully understood, particularly in human post-mortem tissue. Hence, the final goal of this project will be to study the localisation of TDP-43 and FUS in ALS synapses by using AT as well as western blotting of synaptic fractionation samples obtained from frozen human brain.
Chapter 2: Methods

2.1 Human samples

Post-mortem tissue was obtained from the Edinburgh brain bank. All donors used were assessed using the revised El Escorial criteria for diagnosing ALS (Brooks et al., 2000). Patients were recruited through the Scottish Motor Neurone Disease Register and data collected in the CARE-MND database (Clinical Audit Research and Evaluation). Ethical approval for this project was obtained from Scotland A Research Ethics Committee 10/MRE00/78 and 15/SS/0216.

The genetic and cognitive status of ALS patients was obtained via the CARE-MND database. Cognition was assessed using the Edinburgh Cognitive and Behavioural ALS Screen (Goldstein and Abrahams, 2013).

Resin-embedded tissue was obtained from Broca’s area (BA44/45) and the visual cortex (BA17/19) from 23 ALS cases and 11 non-neurological controls. Resin-embedded tissue was also obtained from the motor cortex (BA4) from 10 ALS cases (5 labelled TDP-43 negative and 5 labelled TDP-43 positive based on paraffin section staining for pTDP-43) and 5 non-neurological controls. All cases were anonymised using SD numbers and I was blinded as to their demographic information, cognitive status and group (ALS vs. control) until data analysis.

Non-neurological controls were part of the Lothian birth cohort and were selected given there was no reporting of any neurological condition, and their cognition was intact. Their Braak stages were between 0-2. Their age, sex, PMI and cause of death information can be found in Appendix 1 Figure 2.
The samples were processed by the Edinburgh brain bank and embedded as outlined in Henstridge et al., 2018. Briefly, fresh post-mortem tissue was dissected into cortical columns that were fixed in 4% paraformaldehyde (PFA) and 2.5% sucrose in phosphate-buffered saline (PBS) for 2-3h. The tissue was then dehydrated via ascending ethanol washes (50%, 70%, 90% and 100%, for 10min each), then into a 50/50 solution of ethanol and LR White resin (agar scientific) for 10 more mins and into pure resin and left overnight at 4°C. Tissue blocks were then placed into resin-filled capsules and left to polymerise at 60°C overnight.

For neuropathology analysis, paraffin-embedded tissue was obtained from Broca’s area (BA44/45) and the visual cortex (BA17/19) from the same cohort as the resin blocks.

A summary of the demographic data of the donors can be found in Table 2.1. A more detailed and comprehensive table with genetic status included can be found in Appendix 1 Figure 1. Briefly, the ALS noci/ci groups were taken entirely from the ALS cohort; ECAS information from 2 ALS cases was not available so they were taken out of the cognitive split cohort.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PMI (hrs)</th>
<th>Age at death (yrs)</th>
<th>ECAS Total</th>
<th>Gender split</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>61.27±13.73</td>
<td>79.45±1.57</td>
<td></td>
<td>4 F, 7 M</td>
</tr>
<tr>
<td>ALS</td>
<td>23</td>
<td>75.96±26.71</td>
<td>62.65±9.62</td>
<td>110.11±11.06</td>
<td>8 F, 15 M</td>
</tr>
<tr>
<td>ALSnoci</td>
<td>14</td>
<td>81.79±29.35</td>
<td>65.14±9.45</td>
<td>116.71±4.98</td>
<td>5 F, 9 M</td>
</tr>
<tr>
<td>ALSsci</td>
<td>7</td>
<td>65.71±22.17</td>
<td>58.71±10.42</td>
<td>97.57±8.28</td>
<td>2 F, 5 M</td>
</tr>
<tr>
<td>ALS TDP+ve</td>
<td>5</td>
<td>73.8±14.34</td>
<td>63.8±7.05</td>
<td>101.2±14.87</td>
<td>3 F, 2 M</td>
</tr>
<tr>
<td>ALS TDP-ve</td>
<td>5</td>
<td>68±17.2</td>
<td>56.2±13.14</td>
<td>115.11±5.92</td>
<td>1 F, 4 M</td>
</tr>
<tr>
<td>C9+ve</td>
<td>5</td>
<td>66.40±24.37</td>
<td>56±9.03</td>
<td>109.6±7.27</td>
<td>3 F, 2 M</td>
</tr>
<tr>
<td>C9-ve</td>
<td>5</td>
<td>90±27.34</td>
<td>66.2±13.26</td>
<td>97.8±11.84</td>
<td>2 F, 3 M</td>
</tr>
</tbody>
</table>
Table 2.1 Summary demographics. Table summarising the demographic information of the different groups studied throughout the thesis. PMI, Age and ECAS total score represented by mean±SD.

2.2 Mouse samples

2.2.1 5xFAD mice

In this experiment, the hemibrains from 3 5xFAD (2 female, 1 male) and 3 WT (2 female, 1 male) 6-month-old mice were obtained and fixed by our collaborators Dr Trevor Bushell and Dr Shannon Gilchrist from the University of Strathclyde. The 5xFAD mice are a very well-characterised model of Alzheimer’s disease containing 5 AD-causing mutations in 2 genes: amyloid and presenilin known for the development of amyloid plaques very early on (Oakley et al., 2006; Oblak et al., 2021).

Once in Dundee, they were dissected into cortical columns by Dr Chris Henstridge and then processed and embedded in resin by me. Columns from the motor cortex were dehydrated via ascending ethanol washes (50%, 70%, 90% and 100%, for 10min each), then into a 50/50 solution of ethanol and LR White resin (agar scientific) for 10 more mins and into pure resin and left overnight at 4°C. Tissue blocks were then placed into resin-filled capsules and left to polymerise at 60°C overnight.

2.2.2 C57BL/6

Hippocampi from 3 non-transgenic, wild type C57BL/6 mice (2 years old, 2 male 1 female) were dissected into cortical columns by Dr Chris Henstridge and then processed and embedded in resin by me as outlined previously.
2.2.3 FUSDelta14 mice

Spinal cords from 5 WT (4 female, 1 male) and 4 FUSDelta14 (2 female, 2 male) 2 year old mice (Devoy et al., 2017) were dissected, processed and embedded in resin by our collaborator Dr Anny Devoy from University College London as outlined previously.

2.3 Array Tomography

AT ribbons were obtained from the resin-embedded tissue blocks. At least 20 serial sections of 70nm thickness were cut using an ultramicrotome (Leica) with a Histo Jumbo Diamond knife (Diatome, Hatfield, PA) and collected on gelatine-coated glass coverslips (0.1% cold fish skin gelatine (Sigma, UK), 0.01% chromium potassium sulphate (Merk, USA)) (Figure 2.1).

To remove residual aldehydes from the resin, ribbons were incubated using 50mM glycine (Melford Laboratories, UK) in Tris-buffered saline (TBS) solution (pH 7.4) for 5 mins at room temperature and then blocked for 30min at room temperature with AT blocking buffer (0.1% fish skin gelatine, 0.05% Tween-20 in TBS). The ribbons were then incubated at 4°C overnight with the appropriate primary antibodies (Table 2.2).

Next day, primary antibody solution was washed off for 20s with a continuous flow wash of TBS and three washes of 5min each. Secondary antibodies (Table 2.2) were then added (1:50 dilution in block buffer) for 30min at room temperature and the ribbons were washed 5 times for 5min each in TBS after. 4’,6-diamidino-2-phenylindole (DAPI) (Abcam, UK, 1:1000 in AT block buffer) solution was added for 5 min to stain for nuclei and washed once more with
TBS for another 5 min. The coverslips were then mounted onto slides using ImmuMount (Thermo Fisher, UK) mounting media.

All the antibodies were diluted in AT block buffer and centrifuged for 2 min at 22238G prior to use (Eppendorf centrifuge 5424).

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Species</th>
<th>Company (code)</th>
<th>Dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
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<tr>
<td>Synaptophysin</td>
<td>Mouse</td>
<td>Abcam (Ab8049)</td>
<td>1:50</td>
<td>Donkey α-mouse AF594 (ab150108) Donkey α-mouse AF488 (ab150105)</td>
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<tr>
<td>PSD95</td>
<td>Guinea pig</td>
<td>Synaptic Systems (124014)</td>
<td>1:50</td>
<td>Goat α-guinea pig AF647 (ab150187)</td>
</tr>
<tr>
<td>PSD95</td>
<td>Rabbit</td>
<td>Cell Signalling (3450S)</td>
<td>1:50</td>
<td>Donkey α-rabbit AF488 (ab150073) Donkey α-rabbit AF594 (ab150076)</td>
</tr>
<tr>
<td>FUS</td>
<td>Rabbit</td>
<td>Merk (HPA008784)</td>
<td>1:50</td>
<td>Donkey α-rabbit AF594 (ab150076)</td>
</tr>
<tr>
<td>Delta-FUS</td>
<td>Goat</td>
<td>Courtesy A Devoy</td>
<td>1:50</td>
<td>Donkey α-goat AF488 (ab150129)</td>
</tr>
<tr>
<td>TDP - 43</td>
<td>Rabbit</td>
<td>Abcam (ab133547)</td>
<td>1:50</td>
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<tr>
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<td>Species</td>
<td>Vendor</td>
<td>Dilution</td>
<td>Secondary Antibody</td>
</tr>
<tr>
<td>----------------------------</td>
<td>---------</td>
<td>-------------------</td>
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<td>--------------------</td>
</tr>
<tr>
<td>pTDP - 43</td>
<td>Rabbit</td>
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<tr>
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<td>Rabbit</td>
<td>Proteintech</td>
<td>1:50</td>
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<td>Rabbit</td>
<td>Milipore</td>
<td>1:50</td>
<td>Donkey α-rabbit AF594 (ab150076)</td>
</tr>
</tbody>
</table>

**Table 2.2 Antibodies used for array tomography.** Table showing the different antibodies and the concentrations used for AT.

**Figure 2.1. Array tomography pre-imaging workflow.** Diagram showing the steps involved pre-imaging in AT. Tissue is extracted from the brain, fixed, and embedded in resin. Then ribbons of serial sections can be cut using a Leica ultramicrotome and collected onto a coverslip where it can be immunostained.

**2.3.1 Array tomography imaging**

Two locations per ribbon were found by manual searching using DAPI patterns as landmarks and a stack of at least 15-20 consecutive images per location was obtained using a DeltaVision Elite widefield
fluorescence microscope (Image solutions) equipped with a CoolSnap digital camera and softWoRx software. High resolution images were obtained with a 63x 1.4NA Plan Apochromat objective. At least two image stacks were captured for each ribbon and ribbons were collected from at least two blocks per case. This means 4 images were collected per region, per case (Figure 2.2).

Further detail into image processing is given in the optimisation steps discussed in Chapter 3. Briefly, stacks were aligned using the ImageJ MultistackReg plugin (courtesy of Brad Busse and P. Thevenaz, Stanford University) and an in-house MATLAB script was used to segment the channels and to calculate the densities (https://github.com/arraytomographyusers/Array_tomography_analysis_tool). An in-house python script was also used to perform colocalization analysis (https://github.com/lewiswilkins/Array-Tomography-Tool). Data was then analysed using GraphPad Prism9 and R studio software (Figure 2.2).

Figure 2.2. Array tomography imaging workflow. Diagram showing the steps involved in imaging in AT. The same location is imaged along the ribbon (red square) using a widefield epifluorescence microscope. The images are then stacked to generate a 3D model.
2.4 Electron microscopy

TEM images from Brodmann areas 44/45 and 17/19 of 18 ALS patients and 5 controls were provided by Prof. Tom Gillingwater at the University of Edinburgh. Samples were obtained and images processed as discussed in Henstridge et al., 2018. Briefly, post-mortem samples were fixed in 4% PFA and 2.5% glutaraldehyde in 0.1M PB for 3h. Blocks were then washed in PB and 70μm sections cut with a vibratome. The sections were then treated with osmium tetroxide (1% in 0.1M PB) for 30 mins and then dehydrated through an ascending series of ethanol and propylene oxide washes before being embedded in Durcupan. Once embedded, 70nm sections were obtained using an ultramicrotome (Leica) with a Histo Jumbo Diamond knife (Diatome, Hatfield, PA) and collected onto grids where they were stained with lead citrate before being imaged on a Philips CM12 TEM equipped with a Gatan digital camera.

Synapses were manually counted and identified by the presence of pre-synaptic vesicles and a post-synaptic density. 20 images were analysed for each case and all 20 datapoints averaged to give a synapsee number per each region and case.

2.5 Cortical thickness

Cortical thickness was measured in the same cases as AT from H&E-stained paraffin embedded-tissue obtained from the Edinburgh brain bank and imaged using a digital tile scanner (Hamatsu) by the Glasgow Tissue Research Facility. Gray matter thickness was calculated using NanoZoomer Digital Pathology software to measure the distance between the grey/white matter border to the pial surface. 10 measurements were taken per case and averaged to give one single cortical thickness for each region and case.
2.6 Neuropathology

Sections from paraffin-embedded tissue in the same cases as AT were obtained from the Edinburgh brain bank and DAB-stained as described in Henstridge et al., 2018 for pTDP-43 (dual phosphorylated at Ser409/410), amyloid-β plaques, Tau, CD68 and GFAP (Table 2.3).

Briefly, tissue blocks were fixed in 10% formalin for at least 24 h. Tissue was dehydrated in an ascending alcohol series (70–100%), followed by three xylene washes, 4h each. Next, three paraffin waxing stages, 5h each, were performed for full penetration of the embedding wax. Tissue sections were cut on a Leica microtome at 4 μm, collected on glass slides and dried at 40 °C for at least 24 h before staining. Immunohistochemistry was performed by the brain bank using standard protocols, enhanced using the Novolink Polymer detection system and visualized using DAB as chromogen. Slides were finally counterstained with hematoxylin for 30 s to stain cell nuclei. For each staining run, positive and negative control tissue for each pathological marker were used to ensure specificity and success of each experiment. All staining was performed by experimenters blind to clinical diagnosis.

For pathology assessment, images were observed using NDP Server and manually graded low, medium, or high by the researcher based on pathology presence. Looking at a magnification of 10x, if there were 0-2 aggregates per ROI, it was considered low; if there were 3-10, medium and if there were over 10, it was considered high.

Astrocyte (GFAP) and microglia (CD68) burden was measured using QuPath software. ROIs were obtained along the cortical grey matter and positive cell detection performed to obtain the percentage of stain-positive pixels. Once the parameters providing optimal coverage and
detection of the different stains were set using two example sections, these were used for all the analysis. The parameters used were:

- GFAP: Downsampling factor 2, Gaussian Sigma (signal to noise ratio) 1, negative threshold 0.7, positive threshold 0.2.
- CD68: Downsampling factor 1, Gaussian Sigma (signal to noise ratio) 2, negative threshold 0.7, positive threshold 0.1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company (code)</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-amyloid (BA4)</td>
<td>Dako (M087201-2)</td>
<td>1:100</td>
</tr>
<tr>
<td>p-TDP-43</td>
<td>2B scientific (CAC-TIP-PTD-MO1)</td>
<td>1:4000</td>
</tr>
<tr>
<td>pTau (AT8)</td>
<td>Thermofischer (MN1020)</td>
<td>1:2500</td>
</tr>
<tr>
<td>GFAP</td>
<td>Dako (Z0334)</td>
<td>1:800</td>
</tr>
<tr>
<td>CD68</td>
<td>Dako (M08769)</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Table 2.3 Antibodies used for histopathology. Table showing the different antibodies and the concentrations used for pathology analysis.

2.7 STED

STED was performed on an AT ribbon collected from BA9 from a TDP-43+ve case. Ribbon was cut as per the AT protocol and collected onto the same gelatin-coated coverslip. The ribbon was then outlined using a barrier tip pen, and, to remove residual aldehydes from the resin, incubated using 50mM glycine (Melford Laboratories, UK) in TBS solution (pH 7.4) for 5 mins at room temperature and then blocked for 30min at room temperature with AT blocking buffer (0.1% fish skin gelatine, 0.05% Tween-20 in TBS). The ribbon was then incubated at 4°C overnight with primary antibodies against synaptophysin and pTDP-43 (Table 2.1).

Next day, primary antibody solution was washed for 20s with a continuous flow wash of TBS and three washes of 5min each. 50μL of
secondary antibodies (Abberior STAR RED, goat anti-mouse IgG, STRED-1001-500UG; Abberior STAR 580, goat anti-rabbit IgG, ST580-1002-500UG) were then added 1:25 in AT block buffer for 1h at room temperature and the ribbon washed 3 times for 5min in TBS after. DAPI (Abcam, UK, 1:500 in AT block buffer) solution was added for 5 min and washed once more with TBS for another 5 min. The coverslips were then mounted onto slides using ProLong Diamond Antifade mountant (Thermo Fisher, UK, P36961).

The slide was then sent to the Edinburgh Super-Resolution Imaging Consortium (ESRIC) to be imaged. STED was performed on a Leica SP8 TCS SP8 3X STED microscope with a Leica HC PL APO CS2 93x/1.30 Glycerol STED WHITE objective (Leica 11506417). Samples were excited using a Supercontinuum White Light Laser at 635 nm for STAR RED and 590 nm for STAR 580 or a Leica SP8 405 nm laser for DAPI. Images were detected using a Leica HyD hybrid detector with a detection window of 650-730 nm for STAR RED, 600-630 nm for STAR 580 and 415-550 nm for DAPI. STED depletion was performed on STAR RED and STAR 580 with a Leica 775 nm depletion laser with time gating of 0.2-11ns. All images were captured with a pixel size of 10nm.

Two locations were found by manual searching using DAPI patterns as landmarks and a stack of 10 consecutive images per location obtained. The images were then stacked and aligned using the ImageJ MultistackReg plugin (courtesy of Brad Busse and P. Thevenaz, Stanford University).
2.8 Synaptic fractionation

2.8.1 Synaptic enrichment

The sample enrichment for synapses was performed as previously described (Laszlo et al., 2022) to obtain synaptoneuroosomes. In short, fresh frozen tissue was homogenized using a Dounce homogenizer in 1mL of Buffer A solution (25mM HEPES, Sigma; 120mM NaCl, Sigma; 5mM KCl, Sigma; 1mM MgCl$_2$, Sigma; 2mM CaCl$_2$, Sigma; phosphatase inhibitors, Thermo fisher; and protease inhibitors, Roche). The resulting sample was filtered through an 80µm nylon filter (Merk), which yielded the total homogenate, 100µL of which was aliquoted and snap frozen.

The sample was then filtered through a 5µm filter (Merk millex$^R$ SV) and centrifuged twice for 5 mins at 1000G (Eppendorf centrifuge 5424). After each centrifugation, the supernatant was removed, and the pellet resuspended into 1mL of Buffer A. The resulting pellet was the synaptically-enriched fraction (SNS).

For protein extraction, the SNS sample was resuspended 1:5 based on pellet weight into protein extraction buffer (100mM Tris -pH 7.6-, 4% SDS (Severn Biotech), protease inhibitor cocktail (Thermo fisher)). After homogenization, the sample was centrifuged at 17,000G at 4°C for 20 mins, and the supernatant contained the extracted proteins from the synaptically-enriched fraction.

2.8.2 Synaptic fractionation

To separate the pre-synaptic fraction from the post-synaptic fraction, the synaptoneurosome pellet was resuspended 1:5 based on pellet weight in a 1:1 mix of lysis buffer (1% triton X (VWR), 12mM Tris, pH
8.1) and resuspension buffer (0.32mM sucrose (Sigma), 1mM NaHCO₃ (Sigma)) and end-over-end rotated for 15min at 4°C. Then it was centrifuged at 23000G for 40mins at 4°C. The supernatant was the presynaptic fraction and the pellet the post synaptic fraction, which was resuspended 1:5 in protein extraction buffer as explained before.

2.9 Western blotting

Protein concentration of the obtained samples was assessed using a BCA protein assay kit (Thermofisher scientific). Briefly, a standard curve using 2mg/mL BSA was generated via serial dilutions (concentrations used 2, 1, 0.5, 0.25, 0.125 and 0 mg/mL). The sample was diluted 1:10 into water. The pierce™ BCA Protein Assay Kit was then used for the reagent. 10μL of sample or standard were added onto a 96-well plate, as well as 240μL of reagent. The well plate was incubated at 37°C for 1h and then absorbance read using a Modulus II Microplate Multimode Reader spectrophotometer.

Once the protein concentration of each sample was known, 10μg of protein were solubilized in 4X Laemmli sample buffer (Bio-Rad, 1.610.747), β-mercaptoethanol and diluted in water to reach a final volume of 15μL. Samples were then denatured at 95°C for 5 min (Grant QBA2).

Samples were then loaded onto 4 to 20% Tris-Glycine 1.0 mm polyacrylamide precast gels (Invitrogen, WXP42020BOX). The gels were set up in the running buffer (25mM Tris, 200mM Glycine, 0.1% SDS) and ran using a Fischerbrand blotting system, for 10 mins at 80mV then for 60-70 mins at 180mV. Once electrophoresis was completed, the proteins were then transferred onto a nitrocellulose membrane using precast transfer stacks (Invitrogen, IB23001) and an i-Blot™2 gel transfer device (Invitrogen, IB21001) for 5 mins at 25 mV.
The membranes were then stained for total protein with REVERT™ 700 Total Protein stain (LI-COR, 926-11.020) and washed using a LI-COR kit (LI-COR, 926-11.020). The membrane was then imaged with the LI-COR Odyssey CLx system and de-stained based on manufacturer instructions (LI-COR, 926-11.020) and then blocked with either 5% BSA or 5% milk at room temperature for 1h.

The membrane was then incubated with primary antibodies in block solution overnight at 4°C. A full list of antibodies and concentrations used can be found in table 2.4. Membranes were washed with TBST 3 times for 5 mins each and secondary antibodies in block solution (1:5000) incubated for 1h at room temperature. Lastly, the membrane was washed with TBST 5 times for 5 min each and imaged using Odyssey CLx (LI-COR).

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Species</th>
<th>Block</th>
<th>Company</th>
<th>Dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptophysin</td>
<td>Mouse</td>
<td>5% Milk or 5% BSA</td>
<td>Abcam (Ab8049)</td>
<td>1:1000</td>
<td>Donkey α-mouse IRDye800CW (Li-cor 926-32212)</td>
</tr>
<tr>
<td>PSD95</td>
<td>Rabbit</td>
<td>5% Milk or 5% BSA</td>
<td>Cell Signalling (3450S)</td>
<td>1:1000</td>
<td>Donkey α-rabbit IRDye700CW (Li-cor 926-32212)</td>
</tr>
<tr>
<td>TDP - 43</td>
<td>Rabbit</td>
<td>5% Milk</td>
<td>Abcam (ab133547)</td>
<td>1:500</td>
<td>Donkey α-rabbit IRDye700CW (Li-cor 926-32212)</td>
</tr>
<tr>
<td>Antibody</td>
<td>Species</td>
<td>Concentration</td>
<td>Blocking Buffer</td>
<td>Dilution</td>
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<tr>
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<td>---------------</td>
<td>-----------------</td>
<td>----------</td>
<td>--------------------</td>
</tr>
<tr>
<td>pTDP - 43</td>
<td>Rabbit</td>
<td>5% BSA</td>
<td>Proteintech</td>
<td>1:500</td>
<td>Donkey α-rabbit IRDye700CW (Li-cor 926-32212)</td>
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<tr>
<td>FUS</td>
<td>Rabbit</td>
<td>5% BSA</td>
<td>NovusBio</td>
<td>1:500</td>
<td>Donkey α-rabbit IRDye700CW (Li-cor 926-32212)</td>
</tr>
</tbody>
</table>

**Table 2.4 Antibodies used for western blotting.** Table showing the different antibodies and the blocks and concentrations used for western blotting.
Chapter 3: Establishing and optimising array tomography

Some of the data presented in this chapter has been published in two collaborative articles. Only experiments conducted solely by myself are included in this chapter.


3.1 Introduction

AT relies on ultra-thin sectioning of resin-embedded tissue to overcome the axial resolution limit of light microscopy (Figure 3.1) (Kay et al., 2013; Micheva and Smith, 2007; Sanchez Avila and Henstridge, 2022).

**Figure 3.1 Array tomography.** Diagram showing how 4 synapses in a block of brain tissue would be resolved using confocal microscopy and AT. AT can resolve synapses in very close proximity due to the ultrathin sectioning. A synapse must be present in 2 consecutive sections to be classed as real. xyz axis show the resolution in each direction of both imaging approaches. Figure created using BioRender.com.

The serial sections – also called ribbons - can then be immuno-stained using standard immunofluorescence protocols and imaged with widefield fluorescence microscopes. This already proves advantageous over EM since there is less need for specialised equipment. Images are taken on the same spot along the ribbon and stacked into a 3D model *post hoc*. The image stack is then aligned and segmented, meaning only synaptic puncta present in at least two consecutive sections are retained, to achieve a binarized image in which synapse density count is performed.

AT has become an indispensable tool given it allows for the high-throughput study of thousands of synapses, in a more accessible and less time-consuming manner than other imaging approaches. Its multiplexing potential means that the presence of multiple proteins can...
be assessed in the same individually resolved synapses. Even though theoretically this process of stripping antibodies and re-staining could be repeated as many times as needed, the tissue quality does seem to degrade over repeated cycles. However, it has now been used with 6 separate rounds of staining, which allowed the visualisation of up to 18 markers (Kleinfeld et al., 2011). This enables the study of several proteins of interest with subsynaptic resolution, which is a significant advantage over EM. Some antibodies might also have low penetrance, but several antigen retrieval and etching protocols have been published to help solve that issue (Sanchez Avila and Henstridge, 2022).

AT has been the technique of choice in many studies assessing synaptic neurodegeneration. Dr Kristina Micheva, who along with Prof. Stephen Smith pioneered this technique (Micheva and Smith, 2007), has mostly used AT in the context of mouse models of Fragile-X Syndrome. For instance, AT has allowed her team to reveal layer-specific synaptic changes in disease as well as differences in how astrocytes associate with excitatory synapses (Simhal et al., 2019). This article showcases one of the main advantages of this approach: multiplex imaging of intact tissue allows synapse study with incredibly detailed spatial and molecular resolution, which would have been extremely challenging at this scale using any other technique.

Prof. Stephen Smith is now working at the Allen Institute, where he has also established this technique and has been using it to study activity-based plasticity in mouse models (Weiler et al., 2014) as well as connectomic studies and developing new adaptations for AT (Smith, 2018).

Moreover, Prof. Tara Spires-Jones was one of the leads in taking advantage of this technique to study human post-mortem tissue (Kay
et al., 2013). Her previously discussed work has been crucial in the field of synaptic pathology in AD, describing the synaptic presence of both amyloid-β and Tau – the two main pathological aggregates of AD – as well as synaptic loss near amyloid-β plaques (Koffie et al., 2012b, 2009; Kurucu et al., 2022; Pickett et al., 2019, 2017, 2016; Polydoro et al., 2014; Zhou et al., 2017).

Lastly, another advantage of AT is its versatility, which allows for its combination with other approaches. AT can be combined with EM, called conjugate AT (Bloss et al., 2018; Collman et al., 2015; Lane et al., 2021). The ultrathin ribbons are stained and imaged as per conventional AT protocols then stripped and prepped for EM imaging. This can combine the anatomical structural insight of EM with the multiplexing of AT, generating anatomical and molecular data at a single synapse resolution. AT and EM are technically demanding, and ribbons are delicate, so both protocols in one sample can affect ultrastructure, meaning only a few select groups have optimized this approach. With processing techniques improving by the day, the power of combining these anatomical and molecular imaging modalities will soon be fully realised (Sanchez Avila and Henstridge, 2022).

Another possible combination is the use of super-resolution imaging on AT ribbons. It was using this approach – imaging ribbons using STED - that a complete amyloid-β plaque in human post-mortem tissue was reconstructed (Querol-Vilaseca et al., 2019). This combined the xy super-resolution that STED provides, with the sub-μm resolution in the axial plane of AT. AT has also been combined with SIM, to reveal distinct cortical synaptic input from different areas of the thalamus in mouse brain (Kim et al., 2021). The authors named this approach structured illumination microscopy on the putative region of interest on ultrathin sections (SIM-PRIUS). Thus, combining super-
resolution imaging and AT appears to be an attractive method for gaining 3-dimensional data at the nanoscale, but given the limitations of super-resolution microscopy discussed before, it does mean it is not a particularly accessible technique.

Lastly, it is worth mentioning the very recent studies performing AT on physiologically characterised synapses (Micheva et al., 2021a, 2021b; Valenzuela et al., 2016). They can do this by filling neurons with a dye using the recording electrode. Once the recording is done, the tissue can be embedded in resin as per AT protocols. These studies provide exquisite detail on the synaptic connectivity between paired cells. Moreover, Holderith et al., have devised an improved protocol for the imaging of physiologically characterised synapses in epoxy resin-embedded tissue (Holderith et al., 2020). This paper also describes several advances in the utility of AT and an improved methodology. They also performed STED on characterised synapses. Their publication is a fantastic example of correlated function and molecular composition at a single synapse scale.

These studies highlight AT as a powerful yet accessible imaging tool. Despite its clear potential, it is undeniable that AT is still a technically demanding technique only set up in a select few laboratories across the UK. However, the fact it can be performed with widefield epifluorescence microscopes, which are often easily available in most imaging facilities, makes it more convenient and accessible than EM and super-resolution techniques.

This chapter will explore the extensive steps needed to establish this technique in a new setting as well as ensuring the quality control checks and optimisation processes necessary to ensure accurate analysis takes place.
3.2 Results

3.2.1 Optimisation of array tomography

To perform the optimisation experiments, BA44/45 ribbons from a control sample were cut and AT staining for synaptophysin was performed (Outlined in Chapter 2, page 41).

As seen in Figure 3.2, the first step is to trim the excess resin from the tissue block (Figure 3.2A) until only a rectangle of tissue is facing the outside (Figure 3.2B). Then, the tissue can be cut into ultrathin serial sections (Figure 3.2C) and the ribbon collected onto a coverslip. This step, though technically challenging, requires no optimisation except visually checking that all the sections have the same colour – which is indicative of consistent thickness – and there are no visible tears in the ribbon. It takes a lot of patience and practise to master this essential step in the process.
Figure 3.2 Example images of tissue capsule blocks and ribbons. Panel A shows tissue (pointed by arrow) inside a resin capsule. The excess resin needs to be trimmed until only tissue is revealed. Panel B shows an example of a capsule without excess resin. Tissue is signalled by arrow. After that is done, ribbons – serial sections – can then be cut. Panel C shows a ribbon being cut into the Histo Jumbo knife. Note the straight sections and even colour through them, Brackets show beginning and end of example sections.

The next step requires standard immunofluorescence protocols (Outlined in Chapter 2, page 41). Antibodies like synaptophysin, our gold standard, require no further optimisation and produce beautifully clear staining consistently. That is not the case for some antibodies, which might require some antigen retrieval protocols. One approach that can be used is to treat with citrate buffer. This extra step consists of incubating the slides in boiling citric acid (from 100x citrate - Vector Labs H-3300 – pH6) solution for around 20 mins in between the glycin and block steps. This was attempted a few times throughout my PhD to try and improve staining, but as seen in Figure 3.3, it never provided any significant improvement and in fact, made synaptophysin staining weaker (Figure 3.3C). Figure 3.3 shows an example of a GNG stain that was attempted to be optimised for use in the proteomics validation study discussed in 3.2.3.
Figure 3.3 Example images of citrate-treated vs. untreated staining. Panel A shows example image of synaptophysin stain using standard staining with no antigen retrieval. Panel B shows example image of GNG stain using standard staining with no antigen retrieval, visual inspection revealed no 3-dimensional objects. Panel C shows example image of synaptophysin stain after citrate treatment, no improvement was perceived. Panel D shows GNG stain snapshot after citrate treatment.

3.2.1.1 Optimising hardware

In their published protocols, the groups of both Dr Kristina Micheva and Prof. Tara Spires-Jones use a 63x objective lens to take the AT images. However, the widefield epifluorescence microscopes we had access to had either a 100x lens or a 63x lens. The first step, then,
was to use synaptophysin stain – consistent throughout – to assess which objective produced the best quality images. Given the ultra-thinness of the ribbon, correct focus throughout the image was more difficult to obtain using the 100x objective (Figure 3.4A). Nonetheless, two stacks from two different ribbons from the same case, were obtained using both objectives (Figure 3.4A-B). The only variable in this experiment was the objective used to capture the images. A stack is the collection of images taken in the same location along the ribbon. The same location along the ribbon was found by manually searching using DAPI patterns as landmarks.

As seen in Figure 3.4C, synaptic density measured using the 100x objective was significantly higher than the one obtained using the 63x. In fact, it was also higher than previous publications had established as the normal synaptic density in the brain. Moreover, the issues surrounding focus could prove a great challenge down the line. Thus, it was decided to continue using the 63x objective.
Figure 3.4 Objective optimisation. Panel A shows an example image of a Synaptophysin stain obtained using 100x objective. Panel B shows an example image of a Synaptophysin stain obtained using 63x objective. Panel C shows significant differences in synaptic density when analysing images taken with a 100x or 63x objective. Graphs show median ± interquartile range. Data was not normally distributed, so a Mann-Whitney test was performed. p-value 0.0286.
3.2.1.2 Optimising analysis

Figure 3.5 Array tomography workflow. Diagram showing the workflow of AT, from obtaining and staining the images, through staining, to all the different image processing steps needed after image collection. Three different analyses can be performed on the datasets obtained: overall density analysis, as well as density near and far from plaques, and colocalization analysis, including distance and overlap.

I was now confident that the steps required to collect AT images (Figure 3.5) were optimised and so the next experiments were the optimisation of image processing and analysis.

The first one was alignment. All the images were taken roughly in the same place (by eye) along the ribbon, but the location had been selected manually, and for accurate segmentation, the images must fit seamlessly, with no visible jumps. To achieve that, the Multistackreg ImageJ plugin was used. This plugin has 4 different aligning algorithms (Figure 3.6):
- Translation: Only includes moving the image left-right top-bottom.
- Rigid body: Translation + rotation of the image
- Scaled rotation: Rigid body + scaling, meaning changing size of objects.
- Affine: Scaled rotation + skewing, meaning allowing slight deformation of objects.

**Figure 3.6 Aligning algorithms.** Diagram showing the different aligning algorithms our code can perform and the different transformations they can do to the images to ensure alignment is as seamless as possible. Translation only moves the object; rigid body can also rotate it; scaled rotation can also change object size; and lastly affine is also able to slightly skew objects to ensure perfect fit.
Alignment can be performed using any of the algorithms just mentioned as well as any of the channels in a stack as reference. The channels are the colours used in imaging, for instance, the secondary antibody for synaptophysin is bound to an Alexafluor488 fluorophore, which means it will be imaged using the green channel. In this case, the affine algorithm used on the first image on the synaptophysin channel was found to give the best results (Figure 3.7A). However, all aligned stacks were quality checked by the researcher throughout all the experiments enclosed in this thesis, if the alignment was not satisfactory, several troubleshooting approaches were taken:

- Change starting section (Figure 3.7B): sometimes not starting the alignment on the first section helped resolve the problem.
- Change reference channel (Figure 3.7C): DAPI could also be used as a reference channel for alignment if changing the starting synaptophysin section would not work.
- Shortening ribbon (Figure 3.7D): sometimes the issue would be that an image of the stack was blurry or not aligned well at the microscope level, when that was the case, shortening the stack was used as a last resort when other troubleshooting did not work.
Figure 3.7 Aligning workflow. Diagram showing the different aligning options. At first, images with both DAPI and Synaptophysin (SYP) are obtained. The stacks are then split by channel, meaning a SYP stack and a DAPI stack. First, you try aligning using the first image on the SYP channel (A). If it works, you use it as a template to align DAPI. If it does not work, you try starting alignment from another section (B). If it works, you use it as a template to align DAPI. If it does not work, you try aligning using the first image on the DAPI channel (C). If nothing works, you crop out the unaligned sections (D).

To make sure that even with ribbon shortening there were still enough sections to yield an accurate result, a long ribbon of 45 sections was used. As normal, two image stacks were generated by taking images along the 45 sections. To assess how synaptic density measurements changed with the different number of serial sections (2-45), the stacks were then cut one section at a time and synapse density analysis was performed at each step. As seen in Figure 3.8, any ribbon below 10 sections would be inaccurate, given the high synaptic density obtained, which would be double than that previously published (Henstridge et al., 2018). Thus, a ribbon between 10-15 was considered adequate and any ribbon above 15 sections, optimal. Given that above 20 sections the synaptic density reaches a plateau, there is no need to add any more information, and imaging more sections than necessary would not be efficient. Therefore, all stacks used in this thesis were aimed to be between 15-20 sections long wherever possible. Though some stacks with only 11-14 sections were included, no stack under 10 was deemed appropriate for analysis, and a new ribbon had to be cut, stained, and imaged again.
Figure 3.8 How number of sections impacts synaptic density results. Graph showing how the same ribbon but with different number of sections in it, yields different synapse density results. Both stacks plateau after 15-20 sections, meaning a ribbon over 15 sections will give an accurate representation of synaptic density. Ribbons with 10-15 sections can also be used as they are adequate though not optimal.

Once the images are properly aligned, the next step in the analysis workflow is segmentation. To perform this next step, an in-house MATLAB script was used. Segmentation is an image analysis approach that forms a 3-dimensional boundary around objects in the image – in this case, synapses – and transforms the image into a binarized output for simplified quantification. The algorithm is trained to only segment objects that are present in at least 2 consecutive images, thus removing non-specific background (Figure 3.9). There are 2 variables in our segmentation script that can be controlled, window size and correction factor.
Figure 3.9 Segmentation. Diagram showing how segmentation works. Once your stack is aligned, the code runs through all the images and whenever a puncta is present in 2 consecutive sections it appears in the binarized, segmented stack. If, however, it is not in consecutive sections, it will be removed from the segmented image.

Window size refers to the pixels around the centre of an object being analysed, that is, how big in pixels you expect your synaptic markers to be. It is important to set a correct window size, since a window size too big will be less stringent and not correctly segment objects leading to false overlap in the segmentation algorithm. Too little of a window size, will do the opposite, and not detect real overlap, meaning positive objects would be lost. Figure 3.10A highlights how changing window size changes the synaptic density output. If you are too restrictive, you might miss some synapses, but after you reach a certain size, density plateaus. In Figure 3.10B you can also see window size affects the segmented output, a very low window size will not detect all the
synapses, but too big of a window size might skew the overlap data later, since the objects will be artificially bigger (highlighted by the arrow on Figure 3.10B). Even though at first glance it might seem that WS30 detects more puncta, those were not 3-dimensional based on visual inspection. A window size at the start of the plateau would therefore be the most suitable, which is why the window size used for all analysis following this was 15.
**Figure 3.10 How window size impacts synaptic density results.** Panel A shows a graph highlighting how different window sizes (WS) used on the same ribbon can yield different synapse density results. Synapse density plateaus after WS 10-15 meaning it will give an accurate representation of synaptic density. Panel B has examples of the raw original image as well as images of segmentation done in the same section but using different WS. Arrows show non-3-dimensional puncta detected using WS30. Graphs show mean±SEM.

Correction factor indicates how strict you are when considering a stain positive for synaptic marker, a real object. This is based on the amount of pixel overlap between the object in one section and the next one. Again, it is important to set this factor right, as otherwise you might either lose objects or the algorithm will generate objects that are false positives. Figure 3.11A shows how changes in correction factor change the synaptic density output. Same as with window size, if you are too restrictive, you might miss some synapses, but crucially in this case, being too lenient could massively increase background noise. In Figure 3.11B it is very clearly represented how that affects the segmented output, a very low correction factor will not detect all the synapses, but a very high one might severely increase background (background stains detected pointed by arrow on Figure 3.11B) In this case, a correction factor nearing the plateau would be too high as that would mean so much background noise is filtering through, that no matter what parameters you use, density remains the same. In this case, the appropriate correction factor was decided by manually checking what most closely resembled what could be seen in the raw files, which was determined to be 0.5.
Figure 3.11 How correction factor impacts synaptic density results. Panel A shows a graph highlighting how different correction factors (CF) used on the same ribbon can yield different synapse density results. Panel B has the raw original image as well as example images of segmentation done in the same section but using different CF. There is a marked increase in number of objects recognised as CF increases. Even though CF2 has more objects, some were not 3-dimensional and thus it was increasing background. Arrows show non-3-dimensional puncta detected using CF2. To accurately decide which CF more closely represents reality, manual checks with the raw image should be done. Graphs show mean±SEM.
After the aforementioned steps, the output should be a properly aligned and segmented binarized image in which synaptic density can be performed. Before density analysis, the MATLAB script generates an inclusion/exclusion mask that excludes the nuclei area from the analysis (Figure 3.12). That is because synapses are not found in the nucleus and including that much “negative” space would skew the results. Thus, synapse density is only measured in the neuropil.

**Figure 3.12 Example of neuropil mask.** Panel A shows an example image of a synaptophysin stain. Panel B shows the resulting neuropil mask generated by MATLAB. Synaptic density will only be performed in the “grey” area.

Our MATLAB script measures synaptic density using the following formula: 

\[
\frac{\text{Objects detected/pixels analysed}}{(0.1075^2 \times 0.07)} \times 10^9.
\]

Where the objects are the synapses detected, and pixels indicate the neuropil size. 0.1075 indicates pixel size, in microns (squared because pixels are square, size will be determined by the microscope) and 0.07 is the section thickness this part of the formula allows to go from pixel – area – to voxel – volume.
3.2.2 Positive control for synaptic density: synapse loss near amyloid-β plaques

After all the steps involved in staining, imaging and image processing were optimised, and the correct parameters set, the next step in the optimisation process was to use a known case of synapse loss as a positive control to ensure our density measurement paradigm accurately detects synaptic density changes.

Since synapse loss around amyloid-β plaques in Alzheimer’s disease has been well documented (Koffie et al., 2009), a mouse model of AD, 5xFAD was used as our positive control, in which synaptic density near and far from plaques was measured. The 5xFAD mouse model carries five different mutations in two genes (APP and PSEN1) that have been linked to AD and has been very well-characterised. One of its main characteristics is that it starts developing amyloid-β plaques very early, at around 1.5 months old (Oblak et al., 2021).

In this experiment, the motor cortex from 3 5xFAD and 3 WT mice was stained for synaptophysin and amyloid-β fibrils (See Table 2.2 for details). Two image stacks containing a mature amyloid-β plaque (in the case of the 5xFAD mice) were taken from each ribbon, meaning 4 image stacks per case were obtained.

Images of dense core amyloid-β positive plaques and the surrounding synaptophysin stain were taken in the 5xFAD mice (Figure 3.13A) and synaptic density analysis near and far from the plaque core was performed. This was performed by our MATLAB script, which used the amyloid-β plaque as the centre point. From that, concentric bins were generated every 10 μm (Figure 3.13B) from the edge of the plaque, and their densities analysed separately. As expected, WT mice did not display amyloid-β plaques, so images were taken of the motor cortex...
in a similar layer. To control for regional differences in the synaptic density of the bins, a “fake” plaque was added artificially, and synapse density analysed as described previously.

As shown in Figure 3.13 there is substantial synapse loss within 10 μm of the plaque. These results match those of existing literature and therefore highlight our method accurately detects synapse density changes that recapitulates previously published data.

**Figure 3.13 Synapse loss occurs near amyloid-β plaques in the 5xFAD mouse model.** Panel A shows a maximum intensity projection of a segmented 20 section stack with the amyloid-β plaque in red and the synaptophysin stain in green. Panel B shows the segmented image and each 10μm synaptophysin bin in which synapse density was performed from 10 to 50μm from the plaque (red) edge. Panel C shows a graph displaying how synapse density changes near and far from plaques. All data showed normal distribution and a two-way ANOVA with multiple comparisons showed a significant decrease in synaptic density only in the bin closest to the plaque (p-value 0.0430). Graphs show mean±SEM.

### 3.2.3 Positive control for synaptic localisation analysis: validation of synaptic proteomics results

Another very valuable use of AT is studying synaptic localisation of proteins of interest. To assess whether our methods and colocalization code were able to detect overlap between synaptic markers and
potentially synaptic proteins, we used AT to validate synaptic proteomics results from a previous study in the lab.

As published in Laszlo et al., 2022, proteomics analysis of synaptically-enriched samples from the dorsolateral prefrontal cortex of human post-mortem brain tissue from ALS cases and healthy controls was performed. The data was then stratified based on C9orf72 status of the ALS cases. Results showed an increase of expression of a protein named synaptopodin exclusively in c9orf72-positive cases relative to C9-ve cases. Synaptopodin is a known postsynaptic scaffolding protein (Yap et al., 2020). AT was used to validate these results and to assess if it was able to detect the markedly post-synaptic expression pattern of the protein.

To do so, ribbons from 5 c9orf72-positive, 5 c9orf72-negative and 5 control cases were obtained and stained for synaptophysin and synaptopodin (Figure 3.14A). 3-dimensional rendering of the stain already revealed a marked post-synaptic expression pattern, as clear synaptopodin-positive puncta could be observed directly opposed to synaptophysin puncta (Figure 3.14B). This 3D rendered image clearly shows the resolution power that AT has.

Density analysis was then performed for both markers. as shown in Figure 3.14C, though not statistically significant, the percentage increase in synaptopodin density very closely resembled that of the proteomics results. This shows AT is a very useful validation tool for proteomics analysis as it can also quantitatively measure positive puncta and detect the same percentage difference as proteomic studies.

Moreover, a colocalization analysis was then performed. The colocalization code used allows for two types of study, overlap or
distance. Overlap looks at the percentage of positive pixel overlap between puncta from 2 different markers, whereas distance looks at the percentage of puncta from one marker that are within a specified distance to puncta from another marker. The distance considered “close” can be manually set by the experimenter, and based on previously published synaptic structure data (Colom-Cadena et al., 2023), the distance between the presynapse and postsynapse is approximately 0.5μm. Since synaptopodin is thought to be postsynaptic (Figure 3.14B) and synaptophysin is a presynaptic marker, a distance study was performed, and as seen in Figure 3.14D, there is also an increase in synaptophysin puncta that are in close proximity (0.5μm) of synaptopodin puncta in c9orf72-positive cases compared to c9orf72-negative ones.
Figure 3.14 Synaptopodin shows a synaptic staining pattern and density analysis recapitulates proteomics results. Panel A shows example images of synaptophysin (SYP) and synaptopodin (SYNPO) staining of AT ribbons from BA9. Single channel images suggest increased density of SYNPO puncta in c9orf72-positive tissue. Panel B shows an example of a segmented 3D rendering of the AT images in panel A generated using the free imaging tool Paraview (paraview.org). Panel C contains two graphs showing the density of SYP or SYNPO in each group using 5 cases per group. SYNPO shows a similar change in c9orf72-positive samples that proteomics data showed. Groups were compared with one-way ANOVA using Tukey’s multiple comparison test, no significant difference was found. Graphs show mean±SEM. Panel D shows the percentage of synaptophysin puncta that were directly opposed to synaptopodin puncta, groups were compared with one-way ANOVA using Tukey’s multiple comparison test, but no statistically significant changes were observed.

3.2.4 Negative control for synaptic localisation analysis: assessing potential synaptic localisation of a non-synaptic protein, Syndecan3

Syndecan3 is a transmembrane protein thought to be expressed in the axons of CA1 excitatory neurons (Hsueh and Sheng, 1999). These hippocampal excitatory neurons play a crucial role in memory, in particular regarding formation, consolidation and retrieval of memories (Bartsch et al., 2011). As part of a collaborative project looking at Syndecan3’s potential involvement in epilepsy through the GDNF pathway, we used AT to confirm the non-synaptic localisation of Syndecan3 in CA1 excitatory neurons. To do so, hippocampi from 3 C57BL/6 mice were stained for both synaptophysin and Syndecan3.

As seen in Figure 3.15, Syndecan3 staining did not co-localise with synaptophysin in fact, analysis showed that on average only 0.07 % of synaptophysin puncta overlapped with Syndecan3 and less than 10% was directly opposed. This, as well as the staining pattern that can be observed, corroborates previous literature that points to the expression of Syndecan3 in axons but not synaptic terminals (Hsueh
and Sheng, 1999). Our approach is therefore able to accurately distinguish between synaptic and non-synaptic proteins.

Figure 3.15 Syndecan3 does not show a synaptic staining pattern and does not colocalise with synaptophysin. Panels A-D shows example 3D rendered
images from CA1 stratum pyramidale. Panel A shows DAPI and Syndecan3 (SYN), Panel B synaptophysin (SYP) and DAPI, Panel C a merge of the three channels and panel D a magnified inlay from C. Colocalisation analysis based on distance shown in panel E and based on overlap in panel F. Graphs show mean±SEM.
3.3 Discussion

AT is a high-resolution imaging technique that can prove very advantageous when studying synapses. Its multiplexing ability and its accessibility make it a very attractive tool for neurodegeneration researchers looking at synaptopathies.

Here, the necessary steps taken to establish this technique in a new setting have been highlighted. From getting good quality ribbons, and clear staining to optimising all the analysis and image processing steps making sure at each step that the outputs our code generated closely matched reality. Different positive and negative controls were also used to prove the set-up was able to accurately and reliably measure changes in synaptic density as well as having a high sensitivity to detect the synaptic localisation of a protein of interest based on overlap and/or distance with synaptic markers.

Throughout the project, no major issues were had when it comes to cutting the ribbons, as long as they were quality checked consistently. Cutting ribbons is quite a delicate step, it is very vibration-sensitive, which is why it should be done in an isolated space with little to no foot traffic. If those conditions are met, and the tissue and blade are aligned correctly, there should be no major issues cutting ribbons besides the steep learning curve on how to set everything up and delicately collect them without ripping them.

One of the major advantages of AT is the fact that standard immunofluorescence methods can be used. However, similar to any other immunostaining, several optimisations can be required. I tested antigen retrieval via citrate treatment, which is a well-established and widely used approach. However, it didn’t seem to improve the quality of the staining, and it did weaken the DAPI signal, which is crucial for
finding the right location on the ribbon, so it was deemed unnecessary and not continued.

When it comes to image processing, there are several steps involved, and it is important to note the researcher must quality control the images after each one. Automatization via code and algorithms is a fantastic tool but they should not be blindly trusted.

The first crucial step of image processing is correct alignment. That is because the next step – segmentation – relies on the presence of a marker in at least two consecutive sections. Therefore, if the alignment is not precise, it might introduce errors by not detecting synapses, or mistakenly labelling puncta as such, due to accidental overlap. There are several steps one can – and should - take when aligning. These are choosing the right alignment mode (in this case, affine, as it gives the most flexibility to ensure accurate overlap of objects); the right reference channel (for this project, synaptophysin, due to its clear labelling and presence all throughout the image); as well as going through the troubleshooting steps previously suggested when the alignment does not progress as expected.

The segmentation algorithm forms a boundary around objects through the entire stack and, whenever a stain is present in at least two consecutive sections along the ribbon, it segments it, generating a positive (white) puncta. This relies on several parameters that guide where you set the threshold. Whereas the different settings and correct values could be argued for and against, what is most important is that all images pertaining to one set of experiments are processed using the same parameters. Since all the studies in this thesis are based on comparisons between groups processed the same way, rather than overall absolute synapse quantification analysis, as long as all the parameters are set the same and quality control is in place,
accurate conclusions can be reached from the comparisons. Having said that, it is still important to try and set the parameters that best depict reality, bearing in mind some concessions will need to be made. Results shown in this chapter suggest that a window size of 15 and a correction factor of 0.5 for synaptophysin are optimal. However, the same optimisation and manual checks for correction factor accuracy should be made whenever another marker is introduced. For conciseness, only the optimisation of synaptophysin has been depicted, however, window size remained constant at 15 but correction factor was optimised for each antibody and kept constant for all images with that particular stain.

To further validate this analysis setting, known models have been used to prove the robustness of this approach. To show AT in this setting can accurately measure synapse density loss, a model of AD was used. The 5xFAD mouse model has been very well established and characterised in AD research, and it very closely recapitulates major hallmarks of AD, such as its characteristic β-amyloid-β plaques (Oblak et al., 2021). It does so by adding five different mutations in two genes (APP and PSEN1) that have been linked to AD. These mice start showing plaques very early, at around 1.5 months old (Oakley et al., 2006), and the images obtained show clear presence of plaques throughout the motor cortex. Though synapse loss near plaques had not been studied in this particular model, it has been well established in other mouse models and in humans (Dong et al., 2007; Koffie et al., 2009; Spires et al., 2005; Spires-Jones et al., 2007; Tsai et al., 2004). The results from this chapter show a clear and significant decrease in synapse density near plaques. Therefore, these results not only validate our method as an accurate way to measure synapse loss near plaques, but also this model as a good representation of human pathology as well.
To further corroborate AT as a tool to study synaptic localisation of proteins of interest, two different models were used: a positive control, in which AT was used to validate results seen in proteomics, and a negative control, where samples were stained for a marker not thought to be synaptic.

In the first case, not only was AT able to accurately measure density changes in synaptopodin puncta that were remarkably similar to that of the proteomics result (Laszlo et al., 2022), but to accurately detect the post-synaptic localisation of synaptopodin with the 3D rendering of both stains generating beautiful directly opposed synaptophysin and synaptopodin puncta. This highlights the exquisite detail that AT images are able to provide. Furthermore, colocalization analysis also was able to accurately detect a marked decrease in the percentage of postsynaptic synaptopodin in C9-negative cases compared to C9-positive. Meaning it is a reliable method for synaptic protein detection that provides subsynaptic compartment (pre- vs. post-synapse) resolution.

Regarding the negative control, AT was also able to detect the non-synaptic nature of syndecan3. Again this shows that AT is a great resource to evaluate the synaptic localisation of proteins of interest with high accuracy and that is also sensitive enough to detect non-synaptic markers as such.

Overall, this chapter has explored the intricate details of setting up such a technique in a new laboratory and the results show that it can consistently and robustly generate valuable and credible data.
Chapter 4: Neuropathological assessment of post-mortem ALS brain

4.1 Introduction

Cognitive and behavioural deficits are present in up to 50% of people with ALS, with 15% of them reaching an FTD diagnosis (Ling et al., 2013; Strong et al., 2017). The most common cognitive changes in people with ALS are executive dysfunction, language impediments and decreased verbal fluency, which are all thought to be mediated by different areas of the prefrontal cortex (Goldstein and Abrahams, 2013). The underlying pathomechanisms driving this cognitive decline, however, are still not yet understood. This is crucial, given people with ALS and cognitive impairment not only suffer with the added pressure of extra-motor symptoms, but they also have a worse prognosis and reduced survival (Elamin et al., 2011; Goldstein and Abrahams, 2013; Henstridge et al., 2018).

Several factors have now been linked to cognitive impairment in ALS. For instance, functional changes have been found in the brains of people with ALS and cognitive impairment. There are activation differences in the middle and inferior frontal gyri and the anterior cingulate gyrus when taking a functional magnetic resonance (fMRI) while completing a verbal fluency task in the brains of people with ALS compared to healthy individuals (Abrahams et al., 2004). Resting-state fMRI studies also revealed a more marked progressive deactivation of the frontoparietal and frontostriatal networks in people with ALS and executive dysfunction (Castelnovo et al., 2020). Moreover, another resting-state fMRI study also showed frontal hypoconnectivity in ALS with cognitive impairment compared to those unimpaired (Temp et al., 2021).
These brain changes go beyond function, though. There are also anatomical changes that can be observed in those areas too. Frontotemporal white matter changes have been observed between unimpaired ALS and ALS with cognitive impairment (Abrahams et al., 2005). Cortical thinning in those cognitively impaired was observed in similar areas to functional impairment using MRI studies (Schuster et al., 2014). This suggests that in a late stage of disease progression, in which so much cell loss has taken place, cortical shrinking occurs. This would be similar to what takes place in the motor cortex of all ALS brains, where there is a marked motor cortex shrinking (Henstridge et al., 2018), regardless of cognition, given it is the most affected area.

Human post-mortem tissue studies have also shown that presence of TDP-43 pathology in different areas of the prefrontal cortex correlates with cognitive impairment, with more pathology being found in brain areas that are involved in language, verbal fluency and executive function in impaired cases (Gregory et al., 2020). Beyond pathology, inflammation also seems to play a role (McCauley and Baloh, 2019). Microglial activation has been shown to correlate with disease progression in human post-mortem tissue (Brettschneider et al., 2012). Moreover, microglial burden also associates with language impairment, since a more reactive microglial phenotype is observed in human post-mortem tissue from Brodmann Area 39, which is involved in language and speech production, in impaired cases (Rifai et al., 2022).

In fact, transcriptomical profiling of human post-mortem tissue from several areas of the prefrontal cortex revealed an overall pro-inflammatory phenotype in cognitively impaired cases (Banerjee et al., 2022). All in all, these post-mortem studies suggest pathology presence and a pro-inflammatory phenotype in different areas of the prefrontal cortex seem to be predictive of cognitive decline.
Synapse loss has been highlighted as the best correlate for cognitive impairment in AD (DeKosky and Scheff, 1990; Koffie et al., 2009; Terry et al., 1991). In ALS, synapse loss has been very well documented in the motor system (Fischer et al., 2004; Fogarty, 2018; Fogarty et al., 2015; Frey et al., 2000; Nagao et al., 1998; Qiu et al., 2014; Shoichi Sasaki and Iwata, 1996; S. Sasaki and Iwata, 1996; Sasaki and Maruyama, 1994a, 1994b).

However, there have not been many studies focusing on the non-motor synaptic changes taking place in ALS, and moreover their correlation with cognitive changes. A recent study in human post-mortem tissue revealed synapse density loss in the dorsolateral prefrontal cortex associated with cognitive impairment in people with ALS (Henstridge et al., 2018). Similar changes have also been seen using in vivo synaptic PET studies, which revealed synapse loss occurs in presymptomatic C9orf72 HRE carriers (Malpetti et al., 2021).

These findings in the prefrontal cortex of ALS with cognitive impairment very closely mimic what happens in FTD. MRI studies show cortical shrinking does also take place in the prefrontal cortex of people with FTD (Malpetti et al., 2023; Whitwell, 2019) and in vivo PET analysis also reveal protein aggregation, synapse loss and microglial activation are key factors of disease (Bevan-Jones et al., 2020; Cagnin et al., 2004; Malpetti et al., 2023, 2022), and more importantly they can be predictive of cognitive changes too (Malpetti et al., 2023).

This not only further consolidates the idea of FTD and ALS existing in a spectrum, where similar pathology in the frontal cortex correlates with similar cognitive changes, albeit with different severity, but it also further highlights the multifactorial nature of said impairment.
However, all of the evidence from these previous studies has looked at one or two of these factors individually. This means there is still much research needed into the mechanisms underlying neuropathology in ALS, but more importantly how they correlate with each other as well as their regional specificity and how that associates with specific cognitive impairments.

To date, there is no study that has looked at how synaptic disfunction, anatomical changes, pathology presence and inflammation exist in the same cohort. This study set up would reveal how all these variables might interact with each other to drive cognitive impairment. This leads to the goal of this chapter, which is to perform this in-depth neuropathological analysis in post-mortem human tissue from two different areas of the brain. The areas studied will be:

- Brodmann areas BA44/45, also known as Broca’s area, involved in language and speech production (Stinnett et al., 2023), which is one of the most affected phenotypes in cognitively impaired ALS.
- Brodmann areas BA17/19, corresponding to the visual cortex, which is thought to be spared in ALS and would act as an internal control (Cechetto and Topolovec, 2002).

Synaptic density analysis will be conducted using two complimentary high-resolution imaging techniques, AT and EM. Cortical thickness measures will also be performed as well as detailed pathology analysis. Presence of aggregation-prone proteins, such as TDP-43, Tau and amyloid-β will be assessed. Moreover, microglia and astrocytic burden will also be analysed. All of this neuropathology data will then be complemented with detailed cognitive and demographic information of the donors, to investigate possible associations that could shine a light on synaptic neuropathology in ALS.
This chapter, therefore, aims to build a database of comprehensive information of two biologically relevant areas of the ALS brain, expanding from detailed clinical cognitive profiling to high-resolution synaptic density measurements, with the goal of furthering our understanding of the underlying neuropathology in the ALS brain.
4.2 Results

4.2.1 Synaptic density assessment in BA44/45 and BA17/19 using two complimentary high-resolution imaging techniques

The first goal of this chapter was to assess whether synapse loss occurs in different regions of the brain and whether that correlates with cognition. To do so, two complimentary high-resolution imaging techniques were used. AT has now been established as a reliable tool to measure synaptic density in a high-throughput manner. In fact, it was used to study synapse density changes in the motor cortex and dorsolateral prefrontal cortex of this same cohort (Henstridge et al., 2018).

For this project, synaptic density was measured with AT in 23 ALS cases and 11 non-neurological controls using post-mortem tissue from areas BA44/45 and BA17/19 using AT. As seen in Figure 4.1, no differences in synaptic density were observed in either of the brain regions.
Figure 4.1 Array tomography reveals no changes in synaptic density. Panels A-D show example images of projections of the 20 aligned images stained for synaptophysin. A is an example image of BA44/45 from a control, B is an example image of BA44/45 from an ALS case, C is an example image of BA17/19 from a control, D is an example image of BA17/19 from an ALS case. Panel E shows there are no differences in synaptic density in BA44/45 between ALS and controls. Panel F shows there are no differences in synaptic density in BA17/19 between ALS and
controls. Data points represent the averaged density values for each case. Graphs show mean±SEM. Groups were normally distributed, and t-tests performed. n = 11 Ctrl, 23 ALS.

To see whether there were any cognitive-related changes, the ALS cohort was then stratified based on cognition given their scores on the ECAS test, a cognitive and behavioural impairment test adapted for the requirements of people with ALS (for information on ECAS threshold refer to Table 1.1). There are several values obtained from the ECAS score (Table 1.1), in Figure 4.2, the cohort is stratified based on their overall ECAS score as well as the ALS-specific ECAS, which takes away any memory and visuospatial ability tasks. No differences in synaptic density are seen in either of the brain areas even after stratification.

BA44/45 is thought to be involved in language and speech production, to assess if there are task-specific cognitive impairments driven by synapse loss, the cohort was further stratified based on the different ALS-ECAS tasks, which include language, verbal fluency and executive function. There were no significant differences in synaptic density in either of the brain areas when stratified by specific cognitive task scores (Appendix 2, ECAS task split).
Figure 4.2 Array tomography reveals no changes in synaptic density based on cognition. Panel A shows there are no differences in synaptic density in BA44/45 between controls, ALS with no cognitive impairment and ALS cognitively impaired when stratified based on overall ECAS scores. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows there are no differences in synaptic density in BA17/19 between controls, ALS with no cognitive impairment and ALS cognitively impaired when stratified based on overall ECAS scores. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel C shows there are no differences in synaptic density in BA44/45 between controls, ALS with no cognitive impairment and ALS cognitively impaired when stratified based on ALS-specific ECAS scores. n= 11 Ctrl, 15 ALSnoci, 6 ALSci. Panel D shows there are no differences in synaptic density in BA17/19 between controls, ALS with no cognitive impairment and ALS cognitively impaired when stratified based on ALS-specific ECAS scores. n= 11 Ctrl, 15 ALSnoci, 6 ALSci. Data points represent the averaged density values per case. Graphs show mean±SEM. Groups were normally distributed and one-way ANOVA performed.
To further solidify the robustness of AT as a valid tool for synaptic density measurement, as well as the replicability of the results, manual synapse count on EM images from a different cohort of 20 ALS cases and 5 non-neurological controls was performed. EM images were obtained by Prof. Tom Gillingwater at the University of Edinburgh, but the manual synapse counts and analysis were performed by me. As seen in Figure 4.3, no differences in synaptic density were observed between ALS cases and controls in either of the brain areas, which closely recapitulates the AT findings. A slight trend towards a synaptic density decrease in BA17/19 could be observed, but due to the low n-number significance won’t be reached. This result shows that using just a presynaptic marker as a proxy for synapse number in the AT experiments cab provide accurate synaptic data. Information on the cognitive status of this ALS cohort was not available and thus stratification based on cognition could not be performed.
Figure 4.3 Electron microscopy reveals no changes in synaptic density. Panels A-D show example EM with the synapses circled in green. Scale bars 2μm. A is an example image of BA44/45 from a control, B is an example image of BA44/45 from an ALS case, C is an example image of BA17/19 from a control, D is an example image of BA17/19 from an ALS case. Panel E shows there are no differences in synaptic density in BA44/45 between ALS and controls. Panel F shows there are no differences in synaptic density in BA17/19 between ALS and controls. Data points represent the averaged synapse number values for each case. Graphs show mean±SEM. Groups were normally distributed, and t-tests performed. n = 5 Ctrl, 16 ALS.
4.2.2 Cortical thickness measurements in BA44/45 and BA17/19

Previously published data, saw a decrease in synaptic density in the dorsolateral prefrontal cortex of people with ALS (Henstridge et al., 2018). Stratification by cognition revealed that synapse loss only occurred in the cognitively impaired group (Henstridge et al., 2018). The same study also looked at the motor cortex, where they did not see synapse loss, but did see cortical thinning instead (Henstridge et al., 2018). This suggests the motor cortex is at a more advanced stage in disease progression, in which neurons are also being lost, not just synapses. This conclusion is reached by the fact that in the motor cortex of controls compared to ALS, there is equal synaptic density but lower volume. The same density in a lower volume means an overall decreased number of synapses, but potentially also neurons, as that would explain the volume loss.

To assess if that was also the case for BA44/45 and BA17/19, cortical thickness measurements were performed using H&E-stained paraffin-embedded sections from the same cases as the AT. Interestingly, a significant decrease in cortical thickness was observed in both BA44/45 and BA17/19 in ALS compared to controls (Figure 4.4).
Figure 4.4 There is cortical thinning of BA44/45 and BA17/19 in ALS brains. Panels A-D show example images of H&E stains with a red line indicating the distance between grey/white matter edge and exterior, taken as a measurement of cortical thickness. All images are in the same magnification (scale bar 1mm). A is an example image of BA44/45 from a control, B is an example image of BA44/45 from an ALS case, C is an example image of BA17/19 from a control, D is an example image of BA17/19 from an ALS case. Panel E shows there is a decrease in cortical thickness in BA44/45 between ALS and controls. p-value 0.041. Panel F shows there
is a decrease in cortical thickness in BA17/19 between ALS and controls. p-value 0.023. Data points represent the averaged density values for each case. Graphs show mean±SEM. Groups were normally distributed, and t-tests performed. n = 11 Ctrl, 23 ALS.

To see if that change in thickness was explained by cognitive impairment, the ALS group was once again stratified based on overall ECAS and ALS-specific ECAS. Figure 4.5 shows that in BA44/45, it appears all changes in cortical thickness are driven by the cognitively impaired group, however, that does not seem to be the case for BA17/19, where all significance is lost after stratification.

The next step was to assess whether regional cortical thickness changes relate to specific cognitive deficits. Broca’s area is heavily involved in language and speech production, so a further stratification into the different ECAS tasks was performed, to see if it was specifically language cognitively impaired cases driving that change. Furthermore, to see if there were also any particular impairments driving that change in BA17/19, the same was done with that dataset. Surprisingly, as seen in (Appendix 2, ECAS task split), the changes in cortical thickness in BA44/45 seem to be driven exclusively by impaired executive function, rather than the other two, more language-based tasks. When it comes to BA17/19 (Appendix 2, ECAS task split), there was one significant difference, between language unimpaired and controls, this was not expected and most likely a result of a very low n number in the impaired cohort.
Figure 4.5 Cortical thinning in BA44/45 takes place in the cognitively impaired group. Panel A shows there is a marked decrease in cortical thickness in BA44/45 between controls and ALS cognitively impaired when stratified based on overall ECAS scores. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. p-value 0.034. Panel B shows there are no differences in cortical thickness in BA17/19 between controls, ALS with no cognitive impairment and ALS cognitively impaired when stratified based on overall ECAS scores. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel C shows there is a marked decrease in cortical thickness in BA44/45 between controls and ALS cognitively impaired when stratified based on ALS-specific ECAS scores. n= 11 Ctrl, 15 ALSnoci, 6 ALSci. p-value 0.014. Panel D shows there are no differences in synaptic density in BA17/19 between controls, ALS with no cognitive impairment and ALS cognitively impaired when stratified based on ALS-specific ECAS scores. n= 11 Ctrl, 15 ALSnoci, 6 ALSci. Data points represent the averaged density values per case. Graphs show mean±SEM. Groups were normally distributed and one-way ANOVA performed.
4.2.3 Assessing pathology presence in BA44/45 and BA17/19

The presence of pTDP-43 aggregates is a hallmark of ALS, and their presence in non-motor areas of the brain is hypothesised to correlate with cognition (Gregory et al., 2020). To assess this, presence of pTDP-43-positive pathology was analysed in both BA44/45 and BA17/19. Two assessments were made, one is whether there is presence of aggregates or not, and the other was a manual grading of pathology based on burden. Unsurprisingly, as seen in Figure 4.6, no pTDP-43 pathology was observed in controls, in either area. Interestingly, Table 4.1 shows there is a higher burden of pathology in BA44/45 than in 17/19.

![Example images of pTDP-43 presence and burden](image)

*Figure 4.6 Example images of pTDP-43 presence and burden.* Example images of pTDP-43 stains for both control (left column), ALS cases (right column) and BA44/45 (top row) and BA17/19 (bottom row). All images are in the same magnification (scale bar 50μm).
Table 4.1 pTDP-43 burden. Table showcasing the number of cases that were positive for pTDP-43 inclusions (presence) as well as the burden of those inclusions. Looking at a magnification of 10x, if there was an average of 0-2 aggregates per ROI, it was considered low burden; if there were 3-10, medium and if there were over 10, it was considered high.

Stratification by cognition based on overall ECAS showed a higher percentage of positive cases in cognitively impaired ALS (Table 4.1), but interestingly, a similar burden in BA44/45 between ALS and ALSci and a lower burden of pathology in BA17/19. As shown in (Table 4.1), the impaired n numbers are already low, and even more so after grading, which could skew the data. Stratification into different ECAS tasks was also performed and can be found in Appendix 2, ECAS task split.

pTau aggregates are a key hallmark of AD, as well as present in up to 45% of FTD cases (Ling et al., 2013), and in the context of AD, pTau pathology follows a very similar pattern to synapse loss and cognitive deficits (Tanner et al., 2022). pTau aggregates can also be found in ALS cases (Strong et al., 2020; Yang et al., 2003; Yang and Strong, 2012), so their presence was also studied in this cohort. As seen in Figure 4.7, pTau aggregates were present in both areas of the brain, but ALS cases had a higher presence in BA44/45, whereas controls had a higher presence in BA17/19. When it comes to the graded analysis, overall, both ALS and controls tended to have a lower burden of pathology.
Figure 4.7 Example images of pTAU presence and burden. Example images of pTAU stains for both control (left column), ALS cases (right column) and BA44/45 (top row) and BA17/19 (bottom row). All images are in the same magnification (scale bar 50 μm).

Table 4.2 pTAU burden. Table showcasing the number of cases that were positive for pTAU inclusions (presence) as well as the burden of those inclusions. Looking at a magnification of 10x, if there was an average of 0-2 aggregates per ROI, it was considered low burden; if there were 3-10, medium and if there were over 10, it was considered high.
Stratification by cognition based on overall ECAS (Table 4.2) showed that in BA44/45, the cognitive impaired ALS group had a higher percentage of positive cases as well as a higher burden of pathology. In BA17/19, however, the control group had a higher percentage of positive cases but interestingly, the cognitive impaired group showed a higher pathology burden as well. Stratification into different ECAS tasks was also performed and can be found in Appendix 2, ECAS task split, where it is shown impaired cases tend to have a higher presence and burden of Tau in both areas.

Amyloid-β-positive dense-core plaques are a hallmark of AD, but also take place in other diseases as well as ageing (Arenaza-Urquijo and Vemuri, 2018). Even though they do not correlate with cognitive decline as well as synapse loss or Tau presence, they are thought to be toxic, and cause synapse loss around them (Koffie et al., 2019). Therefore, they are a factor worth studying in this cohort, so pathology analysis of amyloid-β plaques was also performed.

As shown in Figure 4.10, the percentage of amyloid-β positive cases was higher than the other two pathologies, and amyloid-β plaques were present in up to 80% of non-neurological controls (Table 4.3). In fact, plaque presence is similar between cases and controls as well as areas of the brain (Table 4.3). When looking at amyloid-β burden, both a high and low burden were more common in both areas, with controls arguably having a higher burden in BA17/19. Still, it is remarkable that non-neurological controls had such a high amyloid-β burden (Table 4.3).
**Figure 4.8 Example images of amyloid plaque presence and burden.** Example images of amyloid-β stains for both control (left column), ALS cases (right column) and BA44/45 (top row) and BA17/19 (bottom row). All images are in the same magnification (scale bar 50μm).

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**Table 4.3 Amyloid-β burden.** Table showcasing the number of cases that were positive for amyloid-β plaques (presence) as well as the burden of those inclusions. Looking at a magnification of 10x, if there was an average of 0-2 aggregates per ROI, it was considered low burden; if there were 3-10, medium and if there were over 10, it was considered high.
Stratification by cognition (Table 4.3) showed no substantial differences in the percentage of positive cases in either of the brain areas. Grading, however, unveiled a higher burden of pathology in BA17/19 in ALS with cognitive impairment compared to their unimpaired counterparts. No major differences between groups was found in BA44/45. Stratification into different ECAS tasks was also performed and can be found in Appendix 2, ECAS task split, where no major differences were seen either.

4.2.4 Assessing glial burden in BA44/45 and BA17/19

There are several non-cell autonomous mechanisms linked to ALS and motor neuron death. Moreover, a higher activated microglia burden in the prefrontal cortex seems to associate with cognitive decline in ALS (Rifai et al., 2022). To assess whether that was the case in this cohort, CD68-positive pixel percentage was measured. CD68 is a lysosomal marker often used as a proxy for microglial activation (Chistiakov et al., 2017). Figure 4.9 shows that no significant differences in activated microglia were found in either of the two brain areas studied. Furthermore, stratification by cognitive status also showed no differences between groups. It is worth noting the high variability in the groups as well as the low positive pixel percentage. Stratification into different ECAS tasks was also performed and can be found in Appendix 2, ECAS task split.
Figure 4.9 There are no differences in microglial burden between groups. Panels A-D show example images of CD68 stains. All images are in the same magnification (scale bar 50μm). A is an example image of BA44/45 from a control, B is an example image of BA44/45 from an ALS case, C is an example image of BA17/19 from a control, D is an example image of BA17/19 from an ALS case. Panel E shows no differences in microglial burden in BA44/45 between ALS cases and controls. n=11 ctrl, 20 ALS. Data was not normally distributed and Mann-Whitney test done. Panel F shows there are no differences in microglial burden in BA17/19 between ALS cases and controls. n=11 ctrl, 23 ALS. Data was not normally distributed and Mann-Whitney test done. Panel G shows there are no differences in microglial burden in BA44/45 between controls, ALS unimpaired and ALS impaired. n=11 ctrl, 11 ALSnoci, 7ALSci. Data was not normally distributed and Kruskall-Wallis test done. Panel H shows there are no differences in microglial burden in BA17/19 between ALS cases independent of cognition and controls. n=11 ctrl, 14 ALSnoci, 7ALSci. Data was not normally distributed and Kruskall-Wallis test done.

There are other glial cells, however, that can also become reactive and activated in an inflammatory state. Astrocytes are crucial for tissue homeostasis, and interestingly, astrocytes with ALS-linked mutations have been shown to be enough to cause neuronal degeneration in in vitro models (Birger et al., 2019). To assess astrocytic burden in this cohort, GFAP-positive pixel percentage (Figure 4.10 A-D) was used as a proxy for astrocytic burden. GFAP stands for glial fibrillary acidic protein, which in the CNS, is only present in astrocytes and it is thus a widely used astrocytic marker (Yang and Wang, 2015). No significant differences in astrocytic burden between cases and controls were found in either of the two brain areas studied (Figure 4.10 E,F). However, stratification by cognitive status revealed a significant increase in astrocyte reactivity in BA44/45 of cognitively impaired ALS cases (Figure 4.10G). No such differences, though a marked tendency towards the same results, were found in BA17/19 (Figure 4.10H). Stratification into different ECAS tasks was also performed and can be found in Appendix 2, ECAS task split.
4.2.5 Correlation study in the ALS cohort

Even though all the data collected and presented until this point provides insight and interesting information into the mechanisms underlying cognitive impairment as well as the regional differences between cognitively impaired and unimpaired brains, a crucial factor is the interaction between all the variables previously studied as well as with the demographic information that is available on all of the cases.

To assess that, an unbiased, hypothesis-generating approach was taken, to generate a correlation matrix that compared all the variables between each other. As can be seen in Figure 4.1, the demographic information added was age, sex, post-mortem interval (PMI, refers to the time elapsed between death and tissue retrieval) and presence of the C9orf72 HRE. Besides demographics, all the cognition information was used twice as different variables, the first one including the numerical value of the ECAS score, and the second one (labelled as
impaired), as a binarized version of impaired/unimpaired. A correlogram (Figure 4.11) taking all these factors and the data presented above into account was generated, which revealed many significant correlations between variables that will be explored one by one.

![Correlogram summarising all the correlations between all the variables measured in this dataset. Blank squares indicate non-significant correlations, thus only significant ones have a circle. Colour and size of the circle are determined by the correlation factor, that is, how strong the](image)

**Figure 4.11 Correlation analysis in the ALS cohort.** Correlogram summarising all the correlations between all the variables measured in this dataset. Blank squares indicate non-significant correlations, thus only significant ones have a circle. Colour and size of the circle are determined by the correlation factor, that is, how strong the
correlation is. The scale of colour and correlation factor is found to the right of the correlogram. Figure generated using R studio.

As shown in Figure 4.11, there are significant correlations between the different cognitive tasks and markers. That is, poor performance in one task of the test, correlates with poor performance in another; and good performance in one task associates with good performance overall too. Given it is reasonable that the cognitive tasks would significantly correlate with one another, these were not explored further but each correlation split can be found in Appendix 3, ECAS tasks correlations. It is worth noting that the language task is the only cognition measurement that does not seem to follow that trend.

For ease of data presentation, positive correlations related to cognition will be explored first (Figure 4.12). Unsurprisingly, given the results presented in Figure 4.5, cortical thickness of BA44/45 correlates with cognitive impairment, and in particular executive function. Because this was a result already shown, it was not explored further. Age positively correlates with a better performance in the language task (Figure 4.12A, p 0.0132). Amyloid-β presence in both areas also positively correlates with a better performance in the language task (Figure 4.12B,C p 0.0146). An important caveat of these results is that the correlations with language task are likely skewed by the very low number of language impaired cases (n=7).
Looking now at positive correlations unrelated to cognition, age also correlated with a higher amyloid-β burden in both areas studied (Figure 4.13A,B, p 0.0143, 0.022 respectively). Another positive correlation found is between cortical thickness in BA17/19 and synaptic density in BA44/45, meaning a lower density in BA44/45 also associates with a lower cortical thickness in BA17/19 (Figure 4.13C p 0.0203). Interestingly, a higher microglial burden in BA44/45, correlates with a higher presence of pTDP-43 aggregates in both areas of the brain (Figure 4.13D,E p 0.0351, 0.0155 respectively).
Lastly, pTDP-43 presence in one area, correlates with the pTDP-43 presence in the other (Figure 4.13 F p 0.0283), and the same can be said for amyloid-β plaque presence (Figure 4.13G p <0.0001).
Looking now at negative correlations related to cognition (Figure 4.14), we have sex and language negatively correlate, indicating men tend to have a lower language score (Figure 4.14A p 0.0364); as before, language results might be confounded by the low impaired number, but all impaired cases are male. Interestingly, microglia presence negatively correlates with executive function, meaning those with a lower microglia burden perform better in the executive function tasks (Figure 4.14B p 0.0348). Astrocytes also negatively correlate with cognition, this time with ALS specific ECAS scores; higher astrocyte presence in either of the two brain areas studied significantly associates with an overall poorer performance in ALS-specific tasks (Figure 4.14C p 0.0028), which closely fits the results seen in the stratified cohorts (Figure 4.10). Even though the correlogram suggests that a higher presence of Tau pathology in BA17/19 seems to correlate with a lower score in both verbal fluency and executive function tasks, further detailed exploration revealed no significant correlation, this can be again explained by the low impaired numbers, and the fact one impaired case has an unusually high Tau burden. A pathology that does correlate with executive function, however, is amyloid-β presence in BA17/19; this particular comparison is interesting,
because even though the correlation between score and burden is not significant, when analysed considering ALS and ALSci as two different groups, there is a significant increase in amyloid-β burden in the impaired group, suggesting amyloid-β does not correlate perhaps with the severity of impairment, but on whether the impaired threshold is crossed (Figure 4.14D p 0.0307).

**Figure 4.14 Negative correlations in the ALS cohort based on cognition.** Panel A shows sex differences in the ECAS language task, for data presentation purposes, it has been presented as a group analysis. Data was not normally distributed, so a Spearman correlation was performed. R -0.4590 and p-value 0.0364. Impaired threshold is signalled by the red line. Panel B shows that microglia burden in BA17/19 negatively correlates with scores in ECAS executive tasks. Data was not normally distributed, so a Spearman correlation was performed. R and p-value shown on graph. Impaired threshold is signalled by the red line. Panel C shows that there are significant differences in astrocytic burden in BA17/19 between ALS
without cognitive impairment and ALS impaired. Data was not normally distributed, so Mann-Whitney test was performed. p-value 0.0028. Panel E shows that there are significant differences in amyloid-β burden in BA17/19 between male and female ALS cases. p-value 0.0307.

Regarding negative correlations unrelated to cognition (Figure 4.15), PMI negatively correlates with synapse density in BA17/19, that is, the more time elapsed between death and tissue retrieval, the lower the synaptic density in BA17/19 (Figure 4.15A p 0.0231). Sex also correlates with amyloid-β presence in BA17/19, with females having a higher amyloid-β burden than males (Figure 4.15B p 0.0258). Lastly, there is a negative correlation between astrocytic presence and cortical thickness in BA44/45, meaning a high astrocytic burden associates with a lower cortical thickness (Figure 4.15C p 0.0281).

Figure 4.15 Negative correlations in the ALS cohort unrelated to cognition. Panel A shows that PMI negatively correlates with synaptic density in BA17/19. Data was normally distributed, so a Pearson correlation was performed. Panel B shows...
that there are sex differences in amyloid-β burden in BA17/19. Data was not normally distributed, so Mann-Whitney test was performed. p-value 0.0258. Panel C shows a negative correlation between astrocyte presence and cortical thickness in BA44/45. Data was not normally distributed, so a Spearman correlation was performed. R and p-value shown on graph.

4.2.6 Correlation study in the control group

To assess if some of these correlations were disease independent, the same unbiased approach was performed on the control group (Figure 4.16). No cognition variables were added, as no ECAS data is available for the controls, but they were all non-neurological and part of the Lothian birth cohort and thus subjected to a battery of tests and they had no cognitive impairments.

Figure 4.16 Correlation analysis in the control group. Correlogram summarising all the correlations between all the variables measured in this dataset. Blank squares indicate non-significant correlations, thus only significant ones have a circle. Colour
and size of the circle are determined by the correlation factor, that is, how strong the correlation is. The scale of colour and correlation factor is found to the right of the correlogram. Figure generated using R studio.

Following the same structure as before, there were several positive correlations. The first one was regarding sex and cortical thickness in BA44/45; interestingly, there were significant differences in cortical thickness between the male and female controls (Figure 4.17A p 0.0192). It is worth noting, however, the sample consisted of only 4 females. Moving on to pathology, amyloid-β presence in BA44/45 positively correlated with three other variables: plaque presence in BA44/45 associates with plaque presence in BA17/19 (Figure 4.17C p 0.0058) – recapitulating what was seen in the ALS group (Figure 4.13G). Moreover, amyloid-β burden in BA44/45 correlated with astrocytic burden in both BA44/45 (Figure 4.17B p 0.0049) as well as BA17/19 (Figure 4.17D p 0.0006). The last positive correlation revealed an association between astrocytic burden in both areas, meaning a high burden in BA44/45 associates with a high burden in BA17/19 and vice versa (Figure 4.17E p 0.0015).
**Figure 4.17 Positive correlations in the control group.** Panel A shows sex differences in cortical thickness in BA44/45. Data was normally distributed, so t-test was performed. p-value 0.0192. Panel B shows a positive correlation between astrocytic and amyloid-β burden in BA44/45. Panel C shows a positive correlation between amyloid-β presence in BA44/45 and BA17/19. Panel D shows astrocytic burden in BA17/19 positively correlates with amyloid-β presence in BA44/45. Panel E shows a positive correlation between astrocytic burden in BA44/45 and BA17/19. Unless stated, data was not normally distributed, so a Spearman correlation was performed. R and p-value shown on graph.
There were only two negative correlations in this matrix. There was a very significant negative correlation between synaptic density in BA44/45 and age, meaning older controls tended to have a lower synaptic density (Figure 4.18A p 0.0045). Lastly, there were significant differences in PMI between the male and female controls (Figure 4.18 B p 0.0487). Again it is worth noting that there were only 4 female controls, with very little variability between them in regards to PMI.

Figure 4.18 Negative correlations in the control group. Panel A shows age negatively correlates with synaptic density in BA44/45. Data was normally distributed, so a Pearson correlation was performed. R and p-value shown on graph. Panel B shows sex differences in PMI. Data was normally distributed, so t-test was performed. p-value 0.0487.

4.2.7 Correlation study in the full cohort

To tie it all together, and to see if by increasing the sample size some non-disease associated correlations became significant, a correlation matrix was also generated using both ALS and controls, and taking ALS (i.e. Ctrl vs. ALS) as its own variable (Figure 4.19). Given no ECAS information is available on the controls, this was also taken out of this matrix.
This study revealed several positive correlations. For ease of data presentation, pTDP-43-related positive correlations will be studied first (Figure 4.20). The first one, to be expected, was regarding ALS status and pTDP-43 presence in BA44/45, meaning there was a statistically significant difference in pTDP-43 burden between ALS cases and controls (Figure 4.20A p 0.031). pTDP-43 presence in BA44/45 not only associated with disease status, but also with a higher microglial burden in BA44/45 (Figure 4.20B p 0.0228) and also with presence of
pTDP-43 pathology in BA17/19 as well (Figure 4.20C p 0.0045). A High microglial burden in BA44/45 also associated with a higher pTDP-43 burden in BA17/19 (Figure 4.20D p 0.0097).

Looking now at other pathologies, the correlogram suggested there might be a positive correlation between age and amyloid-β burden, but further analysis revealed it was not enough to reach statistical
significance. Amyloid-β presence in BA17/19 seem to associated with several variables, such as pTau (Figure 4.21A p 0.0147) and amyloid-β presence in BA44/45 (Figure 4.21B p <0.0001), as well as a higher astrocytic burden in BA17/19 (Figure 4.21C p 0.0381). A high astrocytic burden in BA17/19 also seemed to correlate with both a higher amyloid-β (Figure 4.21D p 0.0174) and astrocytic (Figure 4.21E p <0.0001) burden in BA44/45.
Figure 4.21 Positive correlations in the full cohort unrelated to TDP-43. Panel A shows amyloid-β presence in BA17/19 positively correlates with Tau presence in BA44/45. Panel B shows a positive correlation between amyloid-β burden in BA44/45 and BA17/19. Panel C shows a positive correlation between astrocyte and amyloid-β burden in BA17/19. Panel D shows amyloid-β presence in BA44/45 positively correlates with astrocytic burden in BA17/19. Panel E shows a positive correlation between astrocytic burden in BA44/45 and BA17/19. Unless stated, data was not normally distributed, so a Spearman correlation was performed. R and p-value shown on graph.

Regarding negative correlations, the first one was between age and ALS status, revealing the control group was significantly older than the ALS group (Figure 4.22A p <0.0001), however, the controls were cognitively intact. The next two associations have already been assessed in (Figure 4.5), but they are regarding cortical thickness in both areas with ALS status, this does show that this correlation matrix is sensitive enough to pick up associations previously highlighted. Recapitulating what was seen in the ALS group, in the entire population there also seemed to be a negative correlation between PMI and synaptic density in BA17/19 (Figure 4.22B p 0.0273), where the more time elapsed between death and tissue retrieval significantly decreased synaptic count. Lastly, there was a suggested correlation between sex and Tau presence, but when taken the cohort as two groups based on sex, there were no significant differences.
Figure 4.22 Negative correlations in the full cohort. Panel A shows significant age differences between cases and controls. Data was not normally distributed, so a Mann-Whitney test was performed. p-value <0.0001. Panel B shows that PMI negatively correlates with synaptic density in BA17/19. Data was normally distributed, so a Pearson correlation was performed. R and p-value shown on graph.
4.3 Discussion

Cognitive impairment occurs in 50% of people with ALS, and besides the added toll that extra-motor symptoms imply, they also have a faster progressing disease (Goldstein and Abrahams, 2013). Thus, elucidating the underlying mechanisms driving cognitive impairment is of the upmost importance, not only for the research field, but for the people affected by this disease.

This chapter aimed to explore several factors that could play a role in cognitive decline in ALS, including synapse density, cortical thickness, pathology presence and glial burden. Taking advantage of this complex dataset from the same cohort, this chapter also explores the intricate correlations that could occur between these variables.

The two main areas of focus for this study were BA44/45 (Broca’s area) and BA17/19 (visual cortex), due to the involvement in language and speech production of the former, and the hypothesised disease resistance of the latter. The results herein, as well as a few other very recent publications, will show however, the potential need for a change of paradigm when it comes to considering the visual cortex in the context of ALS.

The first variable to be studied was synaptic density. As discussed in the previous chapter, there are now several approaches one can take to study synaptic density, each with advantages and drawbacks. Here, I used AT, due to its high-throughput capacity when it comes to density analysis. The marker used as a proxy for synapses was synaptophysin, a membrane glycoprotein present in synaptic vesicles, and that has been used in multiple occasions by several groups for these purpose, given its high reliability (Colom-Cadena et al., 2023; Henstridge et al., 2018, 2015; Koffie et al., 2012b, 2009; Pickett et al.,
Synaptophysin density analysis was thus performed in the two areas of study and no significant differences were found. Further stratification showed that there were also no differences in synaptic density between cognitively impaired and intact ALS cases. This might have proven surprising, especially in the context of BA44/45, since changes in synaptic density were seen in the dorsolateral prefrontal cortex (BA9), an area nearby and thought to be involved in executive function, another affected task of cognitive impairment (Henstridge et al., 2018).

AT relies on the presence of synaptic markers for the antibodies to bind to, in order to perform density analysis. Theoretically, there could be post-synaptic loss taking place that would not be detected by this method. EM synaptic density analysis, however, relies on the manual assessment of synapse counts in their totality and thus would provide important validation for the data generated with AT. EM images are still the gold standard for synapse density analysis of post-mortem tissue, and this technique has been previously used to look at synapse loss in the context of ALS, albeit exclusively in motor areas and without taking into consideration cognition as a factor (Shoichi Sasaki and Iwata, 1996; S. Sasaki and Iwata, 1996; Sasaki and Maruyama, 1994a, 1994b). Manual count in the areas of study revealed no differences in density either. On the one hand, this proves that AT results agree with the gold standard technique, pointing to AT as a more accessible and high throughput approach, and yet equally reliable. On the other hand, this further corroborates the fact there are indeed no differences in synaptic density between ALS cases and controls in these two areas.

The same publication that saw a decrease in synaptic density in BA9, did not see a change in density in the motor cortex, the area known to
be most affected in disease. What they did see, however, was a marked decrease in cortical thickness (Henstridge et al., 2018). Equal density in less volume, does overall translate to fewer synapses, and the authors also hypothesised that this might point to a further stage in disease progression, in which not only synapses are lost, but there is effective neuronal degeneration taking place. To see if that was also the case, cortical thickness measurements were performed here.

Perhaps unsurprisingly, a significant decrease in cortical thickness in BA44/45 was found, but more importantly, that decrease seemed to be exclusively driven by the cognitively impaired group. This might point to BA44/45 being at a more advanced stage of disease progression in the cognitively impaired group, which could indicate pathology spreading anteriorly from the motor cortex, with BA44/45 being affected before BA9. This would fit the current understanding if we assume degeneration follows the same spread pattern as TDP-43 does (Brettschneider et al., 2013; Jucker and Walker, 2013). Interestingly, further breakdown into tasks revealed that executive function impairment, rather than language or verbal fluency, was the task that was associated with said changes. This might seem incongruent at first glance, but one must take into consideration that Broca’s area is not the only area involved in language and speech production, in fact several others have been known to play a role, but moreover recent evidence suggests there might be more to language production that was previously thought (Tremblay and Dick, 2016). This could also point to executive function taking place in more areas than simply the prefrontal cortex (Takeuchi et al., 2013). An important caveat is that once the cohort is split into cognitive tasks, the n numbers decrease significantly. So much so, that the n number for impaired cases in executive function and verbal fluency is 4. This is a limitation of this study, especially given the known variability and heterogeneity in ALS cases (Cooper-Knock et al., 2012; Takeda et al.,
However, it is a very common issue when working with human post-mortem tissue, since these precious samples are not easily accessible and overall numbers tend to be limited. Still, preliminary conclusions can be drawn, but for more broad conclusions regarding the task split, a bigger n number would be necessary.

Cortical thickness analysis in BA17/19 also revealed a significant decrease in ALS cases, however stratification by cognitive impairment suggests that said decrease is independent of cognitive status. The visual cortex has been regarded as largely spared in ALS for decades. However recent evidence, as well as the data presented in this chapter, suggests a more involved and affected role of this region of the brain than was previously thought. MRI studies on C9+ve and C9-ve ALS patients observed a marked impairment in visual networks which was more exacerbated in C9+ve cases (Chipika et al., 2022) and a more severe posterior cortical thinning (Nigri et al., 2023). A cortical thickness MRI study also revealed people with ALS and a higher upper motor neuron burden also had shrinking in the left occipital cortex (Mezzapesa et al., 2013). Moreover, connectivity MRI studies do show a decreased connectivity within the occipital cortex in ALS brains (Loewe et al., 2017). An explanation as to why no other MRI studies besides these very recent ones see the thinning we do, is that, though significant, the overall shrinking of BA17/19 is of below 1mm, which could fall below the resolution of the MRIs used.

The fact that synapse density remains the same and cortical thickness decreases suggests some level of neuronal loss might be taking place. To further corroborate this, cell counts could be performed, as well as potentially an assessment of NeuN staining, a pan-neuronal marker, to assess if neurons are indeed being lost. However due to time constraints this further analysis was not able to be performed.
To further elucidate the mechanisms driving cognitive impairment, pathology analysis was performed. This revealed that pTDP-43 pathology was only present in ALS cases, whereas controls did also have Tau and amyloid-β. This is in accordance with established knowledge, particularly because Tau and amyloid-β have been seen in in “healthy” aged brain (Arenaza-Urquijo and Vemuri, 2018; Braak et al., 2011; I, 2023), whereas TDP-43 pathology is usually only found in diseased brain. Even though it mostly found in the context of ALS/FTD, aggregates have been found in many AD (Josephs et al., 2017, 2014b, 2014a; Josephs and Dickson, 2016), PD (Nakashima-Yasuda et al., 2007) and HD (Schwab et al., 2008) brains as well as what is now known as Limbic-predominant age-related TDP-43 encephalopathy (LATE). LATE is an “Alzheimer’s type” dementia occurring in aged individuals (usually over 80), with cognitive impairment and amnestic dementia, but driven by TDP-43 proteinopathy, starting in the amygdala and hippocampus (Nelson et al., 2019).

Another interesting point, this time regarding pathology burden, is that the overall TDP-43 burden – and even number of positive cases – is quite low, especially considered they are found in the motor cortex and spinal cord of up to 97% of ALS cases (Ling et al., 2013). On the one hand, these results do consolidate several studies now that prove TDP-43 pathology in ALS is widespread throughout the brain; on the other hand, however, it suggests said presence is not as prevalent as in the motor system. Interestingly though, burden seems to be higher in cognitively impaired cases. This agrees with the study that linked TDP-43 pathology with cognitive impairment, in particular verbal fluency (Gregory et al., 2020). In that study, they also saw TDP-43 presence in BA44/45 in other cognitively impaired tasks, also keeping in accordance with these results. However, the fact that cortical thinning and TDP-43 burden in BA44/45 is so high in people with ALS
and executive dysfunction definitely points towards the potential need of steering away from the “1 area = 1 function” theories. That means that even though Broca’s area has been widely thought to be involved in language functions, the evidence here points to it also being involved in executive functions, and that perhaps brain areas are not only involved in one particular function (Genon et al., 2018).

Moreover, the fact that pathology was present in BA17/19, albeit at a low burden, matches with other pathological studies of BA17/19 (Gregory et al., 2020) and further highlights that it might not be as spared as it was previously thought and potentially renders the mostly forward spread model (Jucker and Walker, 2013), invalid.

Another interesting finding in the pathology study was that amyloid-β burden was very high in both ALS and non-neurological control cases, with no differences regardless of cognitive status. The amyloid-β hypothesis in the AD field has been no short of controversies in recent years, in particular with the potential introduction of anti-amyloid-β therapies. The fact plaques are present in almost 80% of the cases, even though they showed no cognitive impairment or overall synapse loss, further validates the hypothesis that amyloid-β is not the primary driving factor of cognitive impairment, in ageing or in AD (Arenaza-Urquijo and Vemuri, 2018; Cerasuolo et al., 2023). An important control that could have been added here would have been post-mortem tissue from people with AD, as this would highlight differences in amyloid burden between “healthy aged” brains, ALS brain and how that compares to the plaque burden of AD brains, however that tissue was not available for our use during this project.

Once the potential role of pathology was studied, the next step was to assess the role of glia in cognition. Microglia burden is increased in motor and extra-motor areas of the ALS brain (Brettschneider et al.,
Moreover, higher microglial activation has been seen as a good predictor of cognitive impairment in C9-ALS cases (Rifai et al., 2022) and seems to associate with a faster, more severe progression of disease in ALS cases, regardless of C9 status (Brettschneider et al., 2012). In vivo PET imaging of FTD cases also revealed activated microglia correlates with cognitive decline (Malpetti et al., 2023). Here, microglia burden was assessed with CD68 staining, an important caveat of this is that CD68 is a lysosomal marker, which is a great proxy for activated microglia (both IHC studies above also used it), but it does not exclusively stain microglia, it can also stain other phagocytic cell types (Chistiakov et al., 2017). Regardless, it can still be a fantastic marker for immunoreactivity, and it is indeed mostly expressed in microglia in the context of the brain. The results of this study, however, show no differences in microglial activation between control and ALS brains in any of the areas studied, independent of cognition. It is important to note, however, that there is a huge variability in CD68 expression in ALS cases, which could be a confounding factor.

Astrocytes have also been known to play a crucial role in ALS. The presence of astrocytes from ALS patients is enough to cause neurodegeneration in healthy motor neurons in vitro (Birger et al., 2019; Haidet-Phillips et al., 2011; Meyer et al., 2014). Moreover, they are key players of the excitotoxicity hypothesis, since one of the factors is decreased astrocytic glutamate transporter, which is in charge of the reuptake of excessive glutamate in the synapse (Rothstein et al., 1995). Thus, they might also play a role in cognitive impairment, which is why astrocyte activation was studied. Interestingly, there was a significant increase in astrocytic activation in BA44/45 of the cognitively impaired group. This could potentially suggest that it might be astrocytes, rather than microglia, that associate with cognitive impairment, but it most likely indicates that the cognitively impaired group have a more pro-inflammatory phenotype throughout the brain.
Once all of that data was collected, a correlation study was performed taking all of the aforementioned variables into consideration, as well as demographic information. This showcases the biggest strength of this approach. While the influence of all these factors have been studied previously in isolation, they have not all been studied together in the same cohort, making my approach novel and important to the field. This could shine a light into previously unnoticed associations that could prove crucial towards our understanding on ALS pathology, as well as the underlying mechanisms driving neurodegeneration. This approach was performed in an unbiased, hypothesis-generating way, with the hopes it might reveal new associations. There are a few considerations needed, the first one is that, as much as the overall ALS group has a good n-number, once it is split into cognitive tasks the group size does become small, especially taking into account the huge variability present in ALS. Another is that this correlation study should be seen as a pilot, as a guidance to then dictate further, more powered analysis. Lastly, only one on one interactions have been taken into consideration here. A multi-way interaction study would be the next logical step. This would, however, severely increase the complexity of this analysis and due to time constraints, it is outside the scope of this chapter.

All the correlations have been highlighted before, but only a select few will be discussed in depth here. One interesting correlation, that is important to take into account, is that PMI seems to negatively correlate with synaptic density in BA17/19, meaning cases with a higher PMI will have a lower synaptic density. At first, there might be then some unease about the validity of the previous results when it comes to the synaptic density study. However, since there are no differences in PMI between ALS cases and controls, and the results
are not taken as an overall quantitative measurement but rather as a comparison, this association should not be a confounding factor. Another potential confounding factor that needs addressed is that ALS cases and controls seem to have significant differences regarding age. This is due to the fact that most of the tissue availability to perform AT studies in controls was from the Lothian Birth Cohort (LBC). The LBC study was a follow-up cohort study designed to investigate cognitive ageing that recruited Edinburgh students in the 1940s. That cohort took cognitive and mental ability tests at age 11 and have been followed up and taken the same test age 70, as well as an ample battery of other tests like brain imaging, blood tests for biomarkers and genetic mutations, etc. (Deary et al., 2007; Taylor et al., 2018). Thus, even though the LBC might be slightly older than the ALS cohort, they have been very well-characterised when it comes to not having any cognitive impairment, and since that was the main focus of this study, it was considered that it would be appropriate to use them. Also, the fact they are older means that they might have a lower synaptic density or lower cortical thickness, which would potentially generate false negatives, rather than false positives, which is what we observe in this study. Moreover, the majority of younger controls whose tissue was available, were sudden deaths, in which suicide was significantly represented. Suicide could be indicative of mental health issues, and also there was no evidence of the cognitive fitness of these cases, so it was deemed that adding those cases would prove as more of a confounding factor than cognitively intact, albeit slightly older controls.

Interestingly, the correlation analysis showed there might be some age and sex-based differences in the language task of the ECAS test. This does actually match what the ECAS creators saw, where age, sex and IQ could be strong predictors of the ECAS score (De Icaza Valenzuela et al., 2018) which gives validity to the approach taken here and also
highlights that these variables are potential confounding factors and should be taken into consideration when doing ECAS-based studies.

Regarding pathology, in particular amyloid-β and TDP-43, their presence in one area seems to very closely associate with their presence in the other. This further validates the theory that pathology spreads throughout the brain and, especially in the case of TDP-43, questions the model that its spread is mostly forward and that the visual cortex is spared in ALS (Jucker and Walker, 2013). In work done regarding the staging of TDP-43 pathology (Brettschneider et al., 2013), TDP-43 inclusions were found in BA17, albeit at a low frequency, which matches the results seen here. Moreover, TDP-43 pathology was also observed in BA19 at a low frequency (Gregory et al., 2020). However, neither publication highlights it as an interesting finding, even though it further suggests that the visual cortex might be impaired at some level in end-stage ALS.

Even though no differences in microglia activation were seen when stratifying the cohort by groups, a strongly significant negative association between microglial burden and executive function was seen in BA17/19. Other studies have shown higher microglial activation in BA39 as a great predictor of language impairment (Rifai et al., 2022). Unfortunately, even though they did measure microglial activation in BA44, their cohort didn’t include executive impaired cases, so no comparisons with our dataset can be performed to see whether they replicate our results. On another study looking at microglial activation they showed a strong correlation between microglial activation and disease duration, and interestingly, that C9+ve cases had a higher microglial burden. However, that study focused on the motor axis and no prefrontal cortex or visual cortex was analysed (Brettschneider et al., 2012). What more closely resembles our results is a very recent publication using PET coupled with a
microglial marker in people with FTD. In this study, they saw that microglial activation correlated with cognitive decline in FTD (Malpetti et al., 2023). Overall, there is evidence showing microglia undoubtedly plays a role in disease, and potentially in cognitive impairment, but more detailed studies focused on looking at extra motor areas and particular task splits would be needed to fully elucidate the extent.

Moreover, a higher astrocytic burden in either of the two brain areas studied correlated with an overall poorer performance in the ALS-specific ECAS test. Even though there is vast evidence about the role astrocytes play in neurotoxicity and neurodegeneration, in particular in ALS (Yamanaka and Komine, 2018), there is fewer studies looking at their role in cognitive impairment overall (excellently reviewed in Santello et al., 2019), even less so in ALS. In that review, the authors discuss that astrocytes – and more accurately pro-inflammatory astrocytes – play a crucial role in memory and sleep impairments in the context of AD. In the study where they identified microglial activation as a predictor of cognitive impairment, they also measured GFAP burden but saw no interesting correlations (Rifai et al., 2022), which contradicts our findings, where we see the opposite taking place, with more correlations highlighting astrocytes, rather than microglia, as key players.

Inflammation not only correlated with cognition, though; microglial burden also correlated with TDP-43 pathology and astrocytic burden with amyloid-β plaques, potentially highlighting the pro-inflammatory role that pathology can play. Lastly, in all the correlations, a high astrocytic burden in one area very significantly correlated with a high burden in the other area. Overall, these results further suggest that cognitive impaired ALS brains have a more inflammatory phenotype throughout the entire brain, whereas inflammation in cognitively intact cases might remain more constrained to the motor axis. This could
agree as well with the evidence that C9+ve ALS has an overall more inflammatory phenotype than C9-ve ALS (Brettschneider et al., 2013) and that RAGE-dependent (receptor for advanced glycation end products) inflammation correlates with a worse disease phenotype in SOD-ALS mice (Lee et al., 2020). Even though no studies specifically looked at extra-motor inflammation and its role in cognitive impairment, studies looking at inflammatory blood biomarkers sometimes see a weak association between increased inflammation markers and worse prognosis (Staats et al., 2022), since cognitive impairment also associates with a worse prognosis, maybe stratifying their data by impairment could then reveal a stronger correlation. This is therefore one of the first few studies specifically looking at the role that extra-motor inflammation might play in cognitive impairment in ALS, and highlights it as a subject worth researching more in.

To summarise, this chapter is the first study in which cognition, pathology presence, gliosis, synaptic density and cortical thickness are assessed in the same cohort, all while taking into consideration other demographic data. This approach has highlighted that BA44/45 is significantly thinner in cognitively impaired cases, in particular in those with executive dysfunction, and might be at an advanced degeneration state. Moreover, it has highlighted that the visual cortex is not as intact as it was previously thought, since there is also shrinking - regardless of cognitive state -, presence of TDP-43 pathology and an increase in inflammatory markers such as astrocytic burden. The unbiased correlation approach also highlighted that TDP-43 spreads throughout the brain both in an anterior and posterior direction and that pathology presence very closely associates with an inflammatory phenotype. All these findings support the fact that cognitive impaired and unaffected brains show distinct neuropathological features, and the importance of taking into consideration several factors as well as their interactions.
Chapter 5: Studying synaptic localisation of ALS-associated proteins

5.1 Introduction

Cytoplasmic TDP-43 aggregates are found in up to 97% of ALS cases. These aggregates, the hallmark of this disease, contain several forms of the protein: full-length physiological TDP-43, truncated c-terminal fragments, as well as ubiquitinated and hyperphosphorylated TDP-43. The different cleavage sites to generate the c-terminal fragments (Berning and Walker, 2019) as well as the serine hyperphosphorylation sites (François-Moutal et al., 2019) can be seen in Figure 1.2. Out of all of those, hyperphosphorylated TDP-43 (p-TDP-43) is canonically used as an aggregate marker (Arai et al., 2006; Lee et al., 2012; Neumann et al., 2006).

TDP-43 has intrinsically disordered regions that make it prone to aggregate in vitro. Beyond that, its prion-like domain (Figure 1.2) might confer the ability to, in a prion-like manner, have seeding activity and promote further aggregation of otherwise healthy protein. Prion stands for "proteinaceous infectious particles", and they are infectious particles made up of misfolded prion protein (PrP) (Jucker and Walker, 2013). What makes them infectious is that the misfolded protein is able to act as a seed and "impose" its misfolded state into physiological protein, meaning it makes benign protein misfold (Jucker and Walker, 2013). There have now been several studies that have even shown that TDP-43 might spread from cell to cell in a prion-like manner. In vitro, synthetic aggregates generated in vitro and insoluble aggregates extracted from diseased brains could act as seeds and propagate aggregation in cultured neurons (Nonaka et al., 2013; Shimonaka et al., 2016). Moreover, cells containing tagged TDP-43 were able to spread it to non-carrying cells when cocultured together. In vivo, TDP-
43 protein extracts from human post-mortem brains injected into a mouse model expressing cytoplasmic TDP-43 were also able to induce a prion-like spread (Porta et al., 2021). And pre-formed TDP-43 fibrils injected in mice expressing humanised TDP-43 induced aggregation and spreading along the axonal tract as well as motor dysfunction (Ding et al., 2021). This theory could explain the progressive spread of TDP-43 aggregation in disease, which starts at the motor axis and then radiates in an anterior as well as posterior direction (Ding et al., 2021; Feiler et al., 2015; Ishii et al., 2017; Jucker and Walker, 2013; Nonaka et al., 2013; Nonaka and Hasegawa, 2020; Polymenidou and Cleveland, 2011; Reale et al., 2023; Sackmann et al., 2020).

Before aggregate formation, however, a mislocalisation of nuclear TDP-43 takes place. This leads to the hypothesis that there might be a two-factor pathological interaction: loss of function of the normal protein once it is cleared from the nucleus, and potential toxic gain of function of the aggregates (Broeck et al., 2014; Lee et al., 2012).

Regardless of the mechanisms behind TDP-43 mediated pathology, what now seems undeniable is that appropriate levels of functional TDP-43 are essential for correct neuronal function. Mutations in the nuclear localisation signal of TDP-43, causing its mislocalisation to the cytoplasm, have been linked to hyperexcitability of cortical neurons and subsequent spinal cord motor neuron death in an in vivo mouse model (Reale et al., 2023). Moreover, TDP-43 deletion in both organotypic hippocampal slice cultures as well as in vivo mouse models caused synaptic loss (Ni et al., 2023). In fact, there are several publications suggesting TDP-43 has an integral role in correct synaptic function and axonal transport (Alami et al., 2014; Chu et al., 2019; Heyburn and Moussa, 2016; Klim et al., 2019; Ling, 2018; Liu-Yesucevitz et al., 2014; Prasad et al., 2019; Wong et al., 2021).
Using rat hippocampal neurons where a TDP-43 mutation had been transduced as well as TDP-43 overexpressing mice, it was shown via immunofluorescence that TDP-43-containing RNA granules were present in the neuronal processes and mutations in TDP-43 impaired that localisation, which could explain why there is synaptic dysfunction, if RNA trafficking to neurites is impaired (Liu-Yesucevitz et al., 2014). That is not the only evidence of mutations in TDP-43 impairing RNA granule transport, however. Using Drosophila, mouse cortical neurons and iPSC-derived neurons from ALS patients, it was shown that disease-causing mutations in TDP-43 lead to impaired axonal trafficking of TDP-43 containing RNA Granules (Alami et al., 2014). Moreover, TDP-43 knockdown in cultured human motor neurons impaired neurite branching and axonal growth (Klim et al., 2019). Not only is TDP-43 involved in correct axonal transport and growth, but there are a very recent publications suggesting TDP-43 might play a physiological role in dendrites (Chu et al., 2019; Wong et al., 2021). Imaging of mouse primary hippocampal neurons showed that TDP-43 might indeed be present in the dendritic compartment, and that it is required, in collaboration with other RNA-binding proteins, for correct dendritic trafficking and thus correct synaptic function (Chu et al., 2019). Using super-resolution imaging in rodent neurons, TDP-43 has been shown to localise in the post-synapse and mediate activity-dependent RNA metabolism at the post-synapse (Wong et al., 2021).

Taking into consideration TDP-43's role in synaptic function, its hypothesised presence in the dendrites, and its potential ability to spread from neuron to neuron, one possible explanation for that phenomenon could therefore be that TDP-43 can spread transynaptically from neuron to neuron in a prion-like manner. There have been two publications that showed TDP-43 presence in the rodent synapse (Chu et al., 2019; Wong et al., 2021) but only one that investigated pTDP-43 presence in the human synapse (Henstridge et
TDP-43 is not the only protein to form aggregates in the context of ALS. Since its link to ALS was discovered in 2009 (Kwiatkowski et al., 2009; Vance et al., 2009), FUS and its aggregates have also been an important research field for ALS. FUS is also a DNA and RNA binding protein associated with ALS. TDP-43 and FUS share several similarities, their structure is fairly similar (Figure 1.2), as they both have an RNA-recognition motif, Q and G-rich domains and of course a nuclear localisation signal, suggesting their primary nuclear role. They are also homologues regarding their function and involvement in several stages of RNA processing, they both autoregulate their own expression and both exist in aggregated forms in the CNS of ALS patients (Ling et al., 2013). Interestingly, they do so in a mutually exclusive manner (Portz et al., 2021; Ratti and Buratti, 2016).

FUS aggregates are also a double-edged sword regarding pathology, with loss of normal function and toxic gain of function mechanisms potentially taking place (Nolan et al., 2016). Similarly to TDP-43 as well, FUS has also been shown to be necessary for correct synaptic function (Ling, 2018; Salam et al., 2021). Cultured neurons from several FUS rodent models have impaired spine maturation and dendritic branching (Fujii et al., 2005; Fujii and Takumi, 2005; Salam et al., 2021; Sephton et al., 2014).

Using immunofluorescence, it was shown that FUS localises in dendrites of mouse hippocampal cultured neurons in an activity-dependent manner (Fuji et al., 2005). Moreover, in hippocampal neurons derived from mutant FUS-containing mice, there was impaired spine morphology and a lower spine number, as evidenced
by staining using a lipophilic dye (Fuji et al., 2005). Interestingly, lower expression of WT FUS in a rodent model, induced a motor phenotype but no visible dendritic effects (Sephton et al, 2014). But the same publication also generated a mouse model carrying a disease-causative mutation in FUS, and saw mice had impaired motor phenotype as well as impaired dendritic spine density, as evidenced by Golgi stain (Sephton et al, 2014); these results indicate that synaptic impairment is mutation-driven rather than downregulation driven, suggesting a toxic gain of function of mutated FUS. This is backed by a recent study using primary rat neurons harbouring disease causing mutations in FUS, where they also saw pre and post-synaptic alterations in both mutated lines (Salam et al., 2021). Moreover, in zebrafish carrying the same mutations, they also saw NMJ dysfunction (Salam et al., 2021). One of those mutations was in the NLS, meaning mutations in the NLS are tied to synaptic alteration. This is supported by another publication, where using a mouse model that was heterozygous for mutations in NLS, they also saw synaptic deficits with age (Scekic-Zahirovic et al., 2021).

Besides the crucial role in synaptic function, there is evidence of the actual presence of FUS in the rodent and human synapse (Deshpande et al., 2019; Fujii et al., 2005; Sahadevan et al., 2021; Salam et al., 2021; Scekic-Zahirovic et al., 2021; Schoen et al., 2016). Immunofluorescent approaches showed FUS localisation within the dendrites of mouse-hippocampal-cultured neurons (Fuji et al., 2005) and the pre and post-synapse of rat primary cortical neurons (Salam et al., 2021). FUS was also found in synaptosomes generated from mice heterozygous for a mutation in FUS's NLS. More recently, super-resolution approaches saw FUS presence in the pre-synapse of rat hippocampal tissue and cultured cortical and hippocampal neurons (Sahadevan et al., 2021; Schoen et al., 2016), as well as post-synaptic FUS presence in human induced pluripotent stem cells (iPSCs,
Deshpande et al., 2019). However, there is no evidence of FUS synaptic localisation in human post-mortem tissue as of the time of writing.

Thus, this chapter will explore the potential synaptic localisation of two disease-relevant ALS-associated proteins, TDP-43 and FUS, with an emphasis on human post-mortem tissue.
5.2 Results

5.2.1 Method validation

To first assess the potential synaptic localisation of TDP-43, synaptic fractionation was performed from frozen tissue samples from BA4 of 5 ALS cases deemed TDP-43 positive based on paraffin sections, 4 ALS cases deemed TDP-43 negative, and 5 non-neurological controls (demographic summary in Table 2.1).

Synaptic fractionation relies on synaptoneurosome generation, which can be performed using an established and reliable protocol. Synaptoneurosomes were generated by me from frozen human post-mortem tissue through a series of homogenisation and filtration steps (Figure 5.1A). Once they were generated, I adapted a protocol from (Xiao et al., 2019) to separate the pre and post-synaptic compartments. Using lysis buffer and long centrifugation (Figure 5.1B), this can be achieved, and as shown in Figure 5.2, western blot shows complete clearance of PSD95 from the presynaptic compartment coupled with significant enrichment in the post synapse, and the opposite taking place with synaptophysin, a presynaptic marker. Each sample was processed separately, but for western blot analysis, the 5 samples from each group were pooled together into one sample (TDP-43 +ve, TDP-43 -ve and Control). The protein content of each sample was then assessed with a BCA protein quantification analysis, and a western blot was performed (Figure 3.1C).
Figure 5.1 Synaptic fractionation workflow. Diagram showing the workflow of synaptic fractionation, from obtaining synaptoneurosomes (A), through synaptic fractionation (B), to western blot validation (C). Briefly, frozen post-mortem tissue from BA4 was obtained, homogenised with a dounce homogeniser and filtered with an 80μm filter to remove debris and yield the total homogenate (TH) fraction. A further filtration step generated the synaptoneurosome (SNS) preparation. The result was then lysed and resuspended, and after a long centrifugation, the supernatant resulted in the presynaptic fraction (PRE) and the pellet, the postsynaptic (POST), which was further resuspended. The protein content of all the samples was then measured using a BCA assay, and the western protocol started.
5.2.2 Assessing synaptic localisation of full-length TDP-43

Once the protocol was established and the successful fractionation optimised and assessed (Figure 5.2), the next step was to use the same approach to study the presence of TDP-43 in these samples. First, the localisation of full-length, physiological TDP-43 was
assessed. As seen in Figure 5.3, here seems to be a marked decrease in expression in SNS compared to TH – indicating the predominant nuclear/cytosolic localisation of TDP-43. However, there is still a significant presence in the SNS, which seems to be mostly driven by the postsynaptic fraction, as signal is very weak in presynaptic fraction. Since the method validation western was done with these same samples, and due to PSD95 and TDP-43 antibody species incompatibility, only SYP was used as reference for this blot.

Figure 5.3 Full-length TDP-43 is present in the human postsynapse. Panel A shows the western blot used to assess the synaptic localisation of full-length TDP-43. Antibodies against a presynaptic marker (Synaptophysin, SYP) and TDP-43 were used. SYP is not present in the POST sample and TDP-43 in only present in the POST. Panel B has a graph showing the intensity of TDP-43 marker normalised to the total protein stain (which can be seen in Appendix 4, total protein stains for WB). Data was normally distributed in all cases and a two-way ANOVA with multiple comparisons performed.

The next step was to use the same approach with an antibody against the hyperphosphorylated form of TDP-43, characteristic of aggregates, to assess aggregate presence in the synapse. Unfortunately, even after several optimisation approaches, changing
the block solution and also the time and temperature of the heat block to denature, this antibody did not seem to work (data not shown). Possible reasons for this will be highlighted in the discussion section.

The western blot approach relies on the homogenisation of the tissue, thus losing all subcellular context of the data. To gain in situ detail on the localisation of TDP-43 in intact human tissue, I used AT to validate and expand the WB findings. AT was performed in ribbons from BA4 from the same cases as the synaptic fractionation. Staining for synaptophysin, PSD95 and full-length physiological TDP-43 revealed an unexpected pattern, as most of the TDP-43 stain seemed punctate and within the neuropil, rather than predominantly nuclear, as would be expected (Figure 5.4). This could be explained by the extremely thin sections cut for AT containing only a small amount of nuclear TDP-43 protein, so a colocalization analysis based on overlap with either or both synaptic markers was performed.

Interestingly, the highest overall percentage of colocalization was with PSD95 (Figure 5.4F,H). 4.3% and 6.4% of control and ALS TDP-43 colocalised with PSD95, respectively; and 2.35% and 2.57% of control and ALS excitatory post-synapses colocalised with TDP-43, respectively, further suggesting its potential postsynaptic role. Regarding pre-synapses, there was a significant increase in TDP-43 puncta colocalising with synaptophysin in ALS (Figure 5.4E, 5.04% controls, 6.02 ALS). Less than 2% of pre-synapses colocalised with TDP-43 (Figure 5.4F, 1.08% controls, 1.60% ALS) and there was also a low percentage of colocalisation of TDP-43 with both markers (Figure 5.4I, 1.22% controls, 1.63% ALS).
Figure 5.4 TDP-43 does not show a nuclear staining pattern but colocalization analysis confirms synaptic presence of TDP-43. Panel A shows synaptophysin (SYP) channel of an example image stain. Panel B shows PSD95 channel of an example image stain. Panel C shows TDP-43 channel of an example image stain. Panel D shows the overlay of SYP, PSD-95, TDP-43 and DAPI staining of AT ribbons from BA4. Scale bars 20µm. Panels E-I contain graphs showing the different colocalization percentages of TDP-43 puncta that were colocalising with both synaptic markers (E), with synaptophysin only, where ALS synapses had a significantly higher colocalization percentage (p-value 0.0415) (F) and with PSD only (G) as well as the percentage of synaptophysin puncta also positive for TDP-43 (H) and the percentage of PSD-95 puncta also positive for TDP-43 (I). Light blue dots represent the TDP-43 negative cases based on paraffin-embedded sections and bars show mean±SEM. Groups were normally distributed, and t-tests performed n=5 Ctrl, 5 ALS+ve, 4 ALS-ve.

5.2.3 Assessing synaptic localisation of hyperphosphorylated TDP-43

Next, AT was performed with the antibody against hyperphosphorylated TDP-43. Opposite to what occurred with the western blots, the pattern observed with this stain seemed closer to that expected (Figure 5.5A-D), with a predominantly nuclear stain, some clear puncta in the neuropil, as well as perinuclear aggregates of considerable size, compared to synapses (Figure 5.5C). Colocalization analysis was also performed as previous. No significant differences were seen between ALS and controls regarding synaptic localisation of pTDP-43 (Figure 5.5 E-I).
Figure 5.5 pTDP-43 does show a nuclear staining pattern and colocalization analysis confirms synaptic presence of pTDP-43. Panel A shows synaptophysin (SYP) channel of an example image stain. Panel B shows PSD95 channel of an example image stain. Panel C shows pTDP-43 channel of an example image stain. Arrow 1 points to distinct nuclear stain. Arrow 2 points to perinuclear aggregates. Panel D shows the overlay of SYP, PSD-95, TDP-43 and DAPI staining of AT ribbons from BA4. Scale bars 20μm. Panels E-I shows the different colocalization percentages of pTDP-43 puncta that were colocalising with both synaptic markers (E), with synaptophysin only (F) and with PSD only (G) as well as the percentage of synaptophysin puncta also positive for pTDP-43 (H) and the percentage of PSD-95 puncta also positive for pTDP-43 (I). Light blue dots represent the TDP-43 negative cases and bars show mean±SEM. Groups were normally distributed, and t-tests were performed but no significant differences between groups were found. n=3 Ctrl, 5 ALS+ve, 4 ALS-ve. Control n number lower as no more than 1 optimal quality ribbon was able to be obtained from two blocks.

Given the fact that pTDP-43 staining had worked in AT ribbons, and as part of a collaboration with the Edinburgh super-resolution imaging consortium (ESRIC), we decided to try STED imaging on AT ribbons. This approach would combine the axial resolution of AT and the super-resolution on the XY plane of STED. Given the optimisation stage was extensive and time-consuming, only one image stack was able to be obtained (Figure 5.6). As shown in Figure 5.6, the staining showed a punctate pattern along the neuropil for Synaptophysin (Figure 5.6A), and a predominantly nuclear pattern for pTDP-43 with also clear puncta in the neuropil (Figure 5.6B). Overlap from both channels (Figure 5.6C) showed direct opposition of synaptophysin and pTDP-43 stain (Figure 5.6D).
Figure 5.6. STED imaging on AT ribbons shows post-synaptic expression of pTDP-43. Panel A shows an example image of synaptophysin (SYP) stain imaged by STED. Panels B shows an example image of pTDP-43 stain imaged by STED. Panel C shows the overlap with both channels, and panel D shows a magnified inlay from Panel C, in which SYP puncta can be seen directly opposed to pTDP-43 puncta. Scalebar 20μm.

Overall, these results show that TDP-43 – both in its physiological and hyperphosphorylated form – is present in the synapses of non-neurological controls and people with ALS, potentially in a predominantly post-synaptic manner.
5.2.4 Assessing synaptic localisation of FUS

Next, the presence of synaptic FUS was assessed. As shown in Figure 5.7, FUS was present in the total homogenate fraction, and in the synaptoneurosome preparation, albeit at a much lower concentration (Figure 5.7). FUS was present in both synaptic fractions and no clear predominantly pre- or post-synaptic expression was observed (Figure 5.7). There appeared to be a second band at 50KDa present in some of the cases, of unknown identity. This could be due to binding non-specifically to proteins with a similar structure or a FUS fragment. Since the method validation western was done with these same samples, and due to PSD95 and FUS antibody species incompatibility, only SYP was used as reference for this blot.

![Western Blot Image]

**Figure 5.7 FUS is present in the human synapse.** Panel A shows the western blot used to assess the synaptic localisation of FUS. Antibodies against a presynaptic marker (Synaptophysin, SYP) and FUS were used. SYP is not present in the POST sample but positive FUS staining observed in all the samples. Panel B has a graph showing the intensity of FUS marker normalised to the total protein stain (which can be seen in Appendix 4, total protein stains for WB). There seems to be a marked decrease in expression in SNS compared to TH – indicating the predominant nuclear/cytosolic localisation of FUS. However, there is still a significant presence in...
the SNS, PRE and POST samples. Data was normally distributed in all cases and a two-way ANOVA with multiple comparisons performed.

The next step was then to assess FUS localisation in the synapse using AT. Given the inconsistency with antibodies on human AT ribbons, we decided to assess FUS in an animal model first, as AT antibodies sometimes work best in mice.

As part of a collaboration project with Dr Anny Devoy from King’s College London, I studied the synaptic localisation of FUS in the spinal cord of wild-type mice as well as a humanised FUS-ALS mouse model. This mouse model, FUSDelta14, is heterozygous for a mutation in the nuclear localisation signal (NLS) and it was generated and characterised by Devoy et al., 2017. Briefly, this mouse line is heterozygous for a human frameshift mutation into mouse FUS’s NLS. In the publication, they show that young heterozygous don’t show any phenotype but as they age, they start displaying motor symptoms and have a lower survival rate than their WT littermates. They also look at the proportion of WT FUS vs. FUSDelta14. Since the NLS is on the C-terminal, antibodies against C-term FUS will bind to WT FUS but not FUSDelta14, while N-term targeting antibodies will bind to both. They see that the heterozygous mice have overall the same N-term FUS but half of C-term, proving that half of the overall FUS expressed in the heterozygous mice is FUSDelta14. They also develop an antibody specific to FUSDelta14. Using those antibodies, they show WT FUS is still predominantly nuclear, but that in FUSDelta14 mice there is an overall decrease in nuclear WT FUS, and that 75% of FUSDelta14, is cytoplasmic in lumbar motor neurons. Given there is a shift into cytoplasmic localisation of FUSDelta14, we wanted to assess whether the mutant protein was expressed in the synapses of the spinal cord.

To do so, we first attempted to use their antibody specifically targeting FUSDelta14. However even after rounds of optimisation, no stain was
visible (data not shown). Then, we decided to use a FUS antibody that targeted the N-term of FUS, which would then hopefully stain both WT FUS and FUSDelta14. As shown in (Figure 5.8A-D), FUS expression was still predominantly nuclear, with some puncta in the neuropil. The stain performed was N-term FUS and synaptophysin, therefore, to assess pre-synaptic as well as post-synaptic presence, both overlap and distance colocalization were performed. Overlap colocalization measures the percentage of synaptophysin puncta that directly overlap with FUS, indicating presynaptic localisation; whereas distance localisation measures synaptophysin puncta directly opposed (within 0.5μm) to FUS puncta, indicating post-synaptic localisation. Analysis revealed a low percentage of FUS overlapping or opposed to synaptophysin, and an even lower percentage of synapses containing FUS (Figure 5.8D-J). No significant differences were found between WT mice and FUSDelta14 mice (Figure 5.8D-J).
Figure 5.8 FUS shows a marked nuclear staining pattern and there are no significant changes in synaptic localisation in WT versus mutant mice. Panel A shows example images of synaptophysin (SYP), FUS and DAPI staining of AT ribbons from the spinal cord of a FusD14 mouse. Panels B-E shows the different colocalization percentages of FUS puncta that were overlapping with (B) or directly opposed to (C) synaptophysin as well as the percentage of synaptophysin puncta also overlapping with (D) or directly opposed to (E) FUS. Bars show mean±SEM. Groups were normally distributed, and t-tests were performed but no significant differences between groups were found. n= 5 WT, 4 FUSDelta14.
5.3 Discussion

The aim of this chapter was to provide some pilot data on the potential human synaptic localisation of two ALS-associated proteins, TDP-43 and FUS using molecular biology approaches and high-resolution imaging. This method of combining both western blots of synaptic preparations and imaging approaches has also been used by other groups to assess the synaptic localisation of disease-associated proteins. For instance, a different synaptic fractionation protocol was used in conjunction with fluorescence immunostaining and transmission EM of synaptoneurosomes to assess the presence of Tau at the synapse of healthy and AD brains (Tai et al., 2012). Also in AD, a similar approach used synaptoneurosome preparations as well as AT to assess amyloid-β presence in the synapses (Koffie et al., 2012). Congruency between the two techniques further validates the fact that this set-up accurately detects the synaptic presence of candidate proteins.

As for the actual techniques used in this chapter, AT has been used to assess synaptic localisation of proteins in several publications. Its high resolution as well as its multiplexing ability and accessibility make it a great approach for my study. AT has been used to assess the synaptic presence of clusterin (Jackson et al., 2019), amyloid-β (Koffie et al., 2012; Kurucu et al., 2022), and Tau (Pickett et al., 2019) in human post-mortem tissue from AD brains, as well as α-synuclein presence in the synapses of brains with Lewy Body Dementia (Colom-Cadena et al., 2017). Overall, AT is a great resource to perform this assessment, and colocalization analysis with pre and postsynaptic markers can provide information on which subsynaptic compartment that takes place in.
The presence of TDP-43 at the synapse has been a topic of interest in the field, given its hypothesised transsynaptic spread, as well as the fact its presence there might play a role in synaptic function. If that is the case, uncovering a new role of TDP-43 could improve our understanding of the underlying pathomechanisms of disease and highlight new targets.

There is now evidence of TDP-43 presence in different models. The first publication to ever see TDP-43 at the synapse was in 2008, where they used immuno-EM to uncover the post-synaptic localisation of TDP-43 in rat hippocampal neurons (Wang et al., 2008). Moreover, there are two very recent publications that show it might be present in the post-synapse and dendritic spines of cultured hippocampal neurons from a TDP-43 mouse model (Chu et al., 2019; Wong et al., 2021). Regarding TDP-43 expression in human synapses, AT imaging showed synaptic presence of pTDP-43 in human post-mortem tissue (Henstridge et al., 2018) and a very recent synaptic proteomics study also detected TDP-43 presence in human synaptoneurosomes (Laszlo et al., 2022). This chapter, using two established and proven methods, shows the first imaging evidence of TDP-43 at the human synapse. Even more so, synaptic fractionation and colocalization analysis agree with the rodent data that TDP-43 might be predominantly postsynaptic. A caveat of this study is the sample size, which is why it should be considered a pilot. However, there is still very important information that can be taken from it. For instance, if TDP-43 is present in the synapse, even in non-neurological controls, it implies a physical function there. Extensive research is underway to discover what this might be.

Even though there is now evidence that TDP-43 plays a role in correct synaptic function and axonal transport (Alami et al., 2014; Chu et al., 2019; Heyburn and Moussa, 2016; Klim et al., 2019; Ling, 2018; Liu-
Yesucevitz et al., 2014; Ni et al., 2023; Prasad et al., 2019; Reale et al., 2023; Wong et al., 2021), the role that TDP-43 might play in the synapse remains controversial (Ling, 2018). One plausible theory is that, given synapses are dynamic specialised structures with a big RNA processing and translation demand, it could be that TDP-43 plays a key part in dendritic translation and acts as support for RNA processing at the post-synapse. In neurodevelopment, a study in mouse hippocampal culture saw that TDP-43 collaborates with FMRP, a protein linked to Fragile-X syndrome, to regulate expression of mRNAs that are crucial for synaptic plasticity (Majumder et al., 2016). RNA immunoprecipitation showed that TDP-43 could be repressing GluA1 expression, which is crucial for synaptic function and plasticity (Majumder et al., 2016). This is further corroborated by the very recent evidence in mouse hippocampal neurons that TDP-43 binds to FMRP and Straufen to regulate dendritic translation by inhibiting mRNA translation in the dendritic RNA granules (Chu et al., 2019). Even more so, hippocampal neurons generated from mice harbouring mutations in TDP-43 showed impaired dendritic RNA granule disassembly, which directly points to a potential pathological role of TDP-43 mutations (Wong et al., 2021). The evidence discussed here and presented in this chapter could then point to TDP-43 playing a crucial role in translation regulation at the post-synapse, and highlights this as a research field with great potential.

A potential shortcoming of this study is that the AT staining using the full-length TDP-43 antibody, did not provide the expected staining pattern. One possible explanation is that nuclear TDP-43 is dispersed in a diffuse pattern, and given the sections are so thin there is not enough signal for it to be picked up. Alternatively, the AT tissue processing protocol of embedding the material in resin may have blocked the epitope of TDP-43. However, our AT analysis only highlights 3-dimensional objects, so we can be confident that the TDP-
43 positive puncta detected in the neuropil were not random fluorophore background puncta. Super-resolution microscopy approaches have been widely used to assess the synaptic localisation of proteins (Böger et al., 2019; Broadhead et al., 2016; Deshpande et al., 2019; Schedin-Weiss et al., 2016; Schürmann et al., 2020), including TDP-43 (Wong et al., 2021). Thus, this was deemed a potential solution and STED imaging was performed on AT ribbons as part of a collaboration with ESRIC in Edinburgh. Unfortunately, this approach did not work either, as no signal was observed in the full-length TDP-43 channel. This could suggest a technical antibody issue, or tissue-processing issue as discussed above. A future possible step, besides trying other antibodies could be immuno-EM. Immuno-EM provides subsynaptic information on the presence of a protein of interest.

Regarding pTDP-43, no signal was observed in the western blot analysis. This could be explained by its presence in insoluble aggregates. Several protocol changes including changing the temperature at which proteins were denatured were attempted, to no avail. If the problem does reside in pTDP-43 being in the insoluble fraction, this approach would not be feasible, as the synaptic fractionation protocol would need to be heavily modified, and that could potentially affect its effectiveness. Interestingly, however, pTDP-43 staining provided clear images with the expected pattern, using AT. Moreover, we were able to obtain a STED image of pTDP-43 in AT ribbons. For time purposes, after many optimisation steps, only one image was able to be obtained, thus no conclusive information can be extracted from it but it definitely shows potential confirmation of postsynaptic localisation of pTDP-43.

As for the synaptic localisation of FUS, there has been previous evidence, using super-resolution microscopy, of its presence in the
synapses of rodent and human cultured neurons (Deshpande et al., 2019; Schoen et al., 2016). Thus, we wanted to use this established protocol to assess FUS both in the synaptic fractionation samples generated from human tissue, as well as as part of a collaboration with Dr Anny Devoy - the spinal cord of their mouse model harbouring a heterozygous mutation in the NLS of FUS. Western blot results show FUS at the human synapse, with both pre- and post-synaptic localisation, which matches what was seen in rat primary cortical neurons using immunofluorescence (Salam et al., 2021). As far as I know, this is the first evidence of FUS present in the human synapse using post-mortem human tissue and should be explored further and with a bigger cohort. Interestingly, the staining pattern seen in the spinal cord of the rodent model, seems to match their published results in characterisation, where they see a mostly-nuclear stain (Devoy et al., 2017). Since the antibody detects N-term FUS, it would also detect the healthy copy. Several attempts were made to optimise the staining protocol for an antibody generated specifically for the mutated protein – the one used in the publication (Devoy et al., 2017) – but no conclusive stain was obtained. However, more efforts to generate an antibody compatible with AT would provide an interesting insight into the differences in localisation of WT vs. Delta14FUS. Since FUS has a crucial role in synapse function, and disease-causing FUS mutations often target the NLS, it would be very interesting to compare the synaptic presence of both WT and Delta14FUS protein. Differences in synaptic expression of healthy and mutated protein could point to a synaptic pathomechanism happening in disease.

Overall, this chapter proves for the first time that both TDP-43 and FUS are present in the human synapse, even in physiological conditions, and thus could have crucial roles in synaptic function. This makes sense as they are RNA-binding proteins and the transcriptional and energetic demands of the synapse are very high (Cortés-Mendoza et
al., 2013; Roy et al., 2020; Sutton and Schuman, 2006). This evidence should be explored further, with an increased number of cases, and potentially new imaging approaches, to fully elucidate the veracity of these results. It is still, however, very valuable and novel information, that could further the field of ALS research, as it gives a new perspective on the role these proteins might play, both in health and disease.
Chapter 6: Summary discussion

ALS is an irrevocably fatal disease with a predominant motor phenotype. People with ALS suffer from progressive paralysis, which unfortunately often ends in respiratory failure within the first three years after diagnosis. The devastating nature of this disease is even more daunting after realising the overall lifetime risk of 1 in 350-400, and there is no effective treatment, let alone a cure. There is therefore a dire need for more research to finally understand the mechanisms behind this disease.

Moreover, around 50% of people with ALS also display some level of cognitive or behavioural impairment. This is important because it associates with a worse prognosis and an even faster-progressing disease. Unravelling the drivers of cognitive impairment could also then be of great help for people with ALS, since not only does it associate with a worse prognosis, but also cognitive impairment could mean an added burden for people with ALS and their carers.

Several mechanisms are thought to be associated with cognitive impairment, such as synaptic dysfunction, inflammation, and neuropathology. All of these factors have been assessed individually (and now in combination in this thesis) and will be discussed here.

Synapse loss has been extensively proven to precede neuronal loss in the motor system of people with ALS as well as many disease models. However, only recently did studies start looking beyond the motor axis and take into account cognition as a factor. There are several approaches scientists can use to measure synapse number or density, in this thesis, two well-established approaches have been used, EM and AT.
The EM images were kindly provided by a collaborator, but AT was performed exclusively by me throughout this project. The first step, however, was to locally establish this technique, as it had not been performed at the University of Dundee before. In Chapter 3, all the necessary steps involved in setting up this technique have been explored, and I believe the evidence shown proves that AT has been successfully established in this department. Moreover, Chapter 3 also explored the potential of AT to go beyond just synaptic density study to allow assessment of synaptic localisation of proteins of interest. Establishing this technique was essential for the success of my PhD and has already had a huge impact on other work within my host lab, helping to publish papers and secure research funding.

In Chapter 4, AT was used in conjunction with cortical thickness measures, pathology presence, glial burden, cognitive status and demographic information to generate a one-of-a-kind dataset that could interrogate the association of all these variables with cognitive impairment as well as showcase the multifactorial nature of ALS.

In this study, two areas were explored – BA44/45 and BA17/19 – revealing novel information, such as the visual cortex (BA17/19) is more affected than it was previously thought, with cortical shrinking and pathology observed. Interestingly, pathology presence seems to associate with a higher inflammatory burden, which is also more pronounced in the cognitively impaired group. This, coupled with the discovery that Broca’s area (BA44/45) is significantly thinner in ALS with cognitive impairment, highlights there are distinct pathological characteristics in the cognitively impaired group that are regionally different. This extends our knowledge of the underlying brain changes in people with ALS and reinforces multi-system breakdown as a driver of diverse clinical presentation in ALS.
This is the first time that so many factors (pathology burden, synapse density, cortical thickness, microglia and astrocyte burden, age, sex, PMI, C9orf72 status) have been measured in the same cohort, highlighting the importance and novelty of my approach. This allows for the study of how they interact with each other and how that relates to clinical presentation. In Chapter 4, only one-way interactions have been explored, but a very interesting and important next step would be looking at how multi-way interactions could explain changes in cognition. Even though this would increase the complexity of the dataset and the statistical analysis, it could provide very useful information. An approach that could be taken is to use a linear model in which overall changes in cognition are explored as a function of the interaction between all the other variables. The model would then be simplified step by step by removing the non-significant ones until the simplest model with all, but only, significant interactions could be achieved. Given the complexity of the changes I have seen by one-way interaction analysis, it is likely that clinical presentation of ALS will be determined by multiple factors, making this next stage of analysis really relevant.

Another interesting addition to this project would be to expand the dataset. We have access to all of the same information from the dorsolateral prefrontal cortex (BA9) and primary motor cortex (BA4) (Henstridge et al., 2018), so adding them to the matrix would be useful. Thinking much longer term, it could be useful to get a broader understanding of brain changes in ALS by adding more brain areas into the analysis, based on our growing appreciation of multi-system change in ALS. Disease is hypothesised to spread following already established neuroanatomical pathways (Bak and Chandran, 2012). Adding that to the shift to understanding brain function as network-specific rather than brain area-specific, as evidenced by the linked firing and activity of areas of the brain that are physically distant
(Beckmann et al., 2005); a study looked at functional networks in the context of ALS (Mohammadi et al., 2009). In it, they study how different networks differ between ALS and controls and observe differences between those groups mostly in the default-mode network and the sensorimotor network. With regards to this project, however, they do see BA19 as part of 2 networks, one compromising BAs 20-22 and another compromising BA35/36. Interestingly, Brodmann area 22 is part of Wernicke’s area, which is involved in non-verbal semantic processing (Binder, 2015), and BA35/36 corresponds to the perirhinal cortex, involved in memory (Augustinack et al., 2013) and heavily affected in AD (Planche et al., 2022). Since the changes observed here in BA17/19 were somehow unexpected – yet later backed by very recent literature – it would be very interesting to see if similar neuropathology can be observed in these areas too, which would give even more validity to the disease spread hypothesis, especially given their hypothesised function.

Lastly, since there is cortical thinning – less volume – but synaptic density remains unchanged – same objects per volume – there is an overall lower number of synapses and the most logical explanation for this result is that there is neuronal loss, with its subsequent synaptic loss. However, to further prove this is the case, neuronal (and glial) cell counts would be a great experiment to perform. There are several approaches one can take when performing neuronal counts in brain tissue (Coggeshall, 1992; Miller et al., 2014). Some of them rely on tissue homogenisation, and some of them on manual counts in intact tissue. However, publications comparing both, have concluded that they are both comparable and reliable (Collins et al., 2010; Miller et al., 2014). Thus, neuronal cell count can be reliably performed by counting the number of DAPI-stained nuclei in a region of interest (ROI), and the percentage of DAPI and NeuN-positive nuclei in that same ROI. NeuN is a well-established neuronal nuclear marker and
several reliable antibodies against it are commercially available (Mullen et al., 1992). Comparing the neuronal cell count between control and ALS cortexes would shine a light on whether our neuronal loss hypothesis is indeed true. It is worth noting that glia may also be changing in ALS and so counting glial changes would also be useful for understanding ALS pathology and whether glial loss may account for all/some of the cortical thinning we observe. This would be a surprise if true, as it is generally believed gliosis occurs in ALS, in which glia become more numerous and reactive.

An important caveat that is crucial to mention is that all of this work has been done in post-mortem tissue. While working with human post-mortem tissue is extremely valuable, as it provides unique and direct information about human disease, it is necessary to remember that it can only provide a snapshot of end-stage disease, and no causal links can be derived. That is why animal and cell-based models are extremely useful, since once informed about what happens in human disease, they can be used to interrogate the causal link between all the factors. It is also worth mentioning that post-mortem tissue is a very valuable resource, but it is of course very limited in terms of availability, which often leads to low n-numbers, which is the case for this project (in particular the EM controls and ALSci cases). All available cases and controls were used for this study. Even though a bigger sample size would have increased the power of the analysis, as well as the robustness of the results obtained, it was not possible due to the limited nature of post-mortem tissue. Therefore, increasing sample size when it is statistically appropriate to do so, would be a needed next step.

The fact that Chapter 4 showed that pathology presence in BA44/45 is associated with pathology presence in BA17/19 could further give more evidence towards the pathology spread hypothesis. This is why
Chapter 5 further explored this theory by interrogating the synaptic presence of ALS-associated proteins TDP-43 and FUS in the human synapse. While their role in correct synaptic function is now suggested by several publications, their physical presence at the healthy and pathological synapse has been of interest in more recent years, as technologies have progressed.

There have been several studies looking at TDP-43’s synaptic localisation in animal models and cultured cells. Only one study so far (Henstridge et al., 2018) has looked at pTDP-43 in the human synapse. Here, using two different methods I show that full-length physiological TDP-43 is present at the human post-synapse and so is pTDP-43, in both controls and ALS brains. This is interesting and novel information suggesting both forms might play a physiological role in the synapse, since they are present in all conditions. The effects hyperphosphorylation has on pTDP-43 are still unknown. The presence of hyperphosphorylated C-term fragments in aggregates, and the fact ALS-linked mutations either add or remove phosphorylation sites, suggest it is a post-translational modification heavily related to ALS (François-Moutal et al., 2019). However, the fact it’s present in healthy synapses might suggest it also has a role in the correct function of the protein.

A caveat of this project is its very low n-numbers, which is common when working with human post-mortem tissue. A very logical step would be to increase sample size when required, ensuring statistical rigour. In particular, it would be very interesting to further explore the STED work on AT ribbons, since this approach achieves super-resolution in 3D. While full-length TDP-43 antibodies did not work, a beautiful image was obtained using pTDP-43, further validating what was seen with AT and the WB, which is its post-synaptic localisation.
Trying other antibodies for TDP-43, as well as increasing n-numbers for pTDP-43, would strengthen the findings of this work.

Moreover, a potential that could be taken advantage of is the multiplexing abilities AT has, in particular, to explore whether TDP-43 (or pTDP-43) are preferentially present in a particular subset of synapses, and if they are, whether that differs between TDP-43 and p-TDP43 as well as between health and disease. For instance, is it preferentially in excitatory or inhibitory synapses? Synaptophysin is a pan-synaptic marker, so it is expressed in all cortical synapses. However, PSD95 is present only in excitatory ones (Keith and El-Husseini, 2008). So a co-stain with GAD65/67, an inhibitory synaptic marker (Tavazzani et al., 2014), could be performed to assess whether there is more or less GAD65/67-TDP-43 overlap than PSD95-TDP-43.

Another approach would be to perform immuno-EM on brain sections or the synaptoneurosome preparations, and stain for TDP-43. This approach has been done before looking at Tau accumulation in human synapses (Tai et al., 2012), so even though it would require several optimisation steps, that publication serves as a proof of concept that it could be done.

Regarding FUS, which Chapter 5 shows are present in the human synapse, the next logical step would be to perform AT on the same samples to validate these results. If they show promise and validation suggests we can reliably stain the synaptic expression, it would be very interesting to also test that in STED.

Moreover, there now seems to be evidence pointing towards FUS, TDP-43, C9orf72 and SOD1 being present in the synapse (Laszlo et al., 2022; Lum and Yerbury, 2022), and a recent publication saw co-localisation of SOD1, TDP-43 and p62 in human spinal cord motor
neurons using an immunofluorescence approach (Trist et al., 2022). It could be interesting to use our multiplex AT approach to assess colocalization between them. If they colocalise, it would suggest a converging synaptic mechanism between the 4 most common ALS-associated proteins.

To sum up, this thesis uses a modern, high-throughput, high-resolution imaging approach to provide novel insights into the interaction between pathology, inflammation, synapse density and cognition in the context of ALS, as well as highlights the need for a change of thinking regarding the spared state of the visual cortex. It also provides pilot evidence that supports the presence of TDP-43, pTDP-43 and FUS at the human synapse. Overall, these findings point to synapses playing a crucial role in disease, and furthering our knowledge of synaptic pathology could move the field closer to a much-needed treatment or a cure.

To close, it is important to note that, despite the current lack of options for people with ALS, there is hope and cause for optimism, with several clinical trials showing promise, as well as research generating critical new knowledge to help us design new treatments.
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### Appendices

#### 1. Full demographic information

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*Table 1.* Full demographic information of the ALS cohort. Note ECAS information was not available for 2 of the cases.
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**Table 2.** Demographic information of the control group
2. ECAS task split

Figure 1 Synaptic density stratified by ECAS task split. Panel A shows no differences in synaptic density in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows no differences in synaptic density in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows no differences in synaptic density in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows no differences in synaptic density in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows no differences in synaptic density in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows no differences in synaptic density in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Graphs show mean ± SEM. For all of them, data was normally distributed and a one-way ANOVA with Tukey’s multiple comparisons performed.
Figure 2 Cortical thickness stratified by ECAS task split. Panel A shows no differences in cortical thickness in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows no differences in cortical thickness in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C there is a significant decrease in cortical thickness in impaired ALS cases compared to both controls and unimpaired cases. p-values 0.0037 and 0.0177, respectively. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows a significant difference in cortical thickness between controls and ALS unimpaired. p-value 0.0318. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows no differences in cortical thickness in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows no differences in cortical thickness in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Graphs show mean ± SEM. For all of them, data was normally distributed and a one-way ANOVA with Tukey’s multiple comparisons performed.
Figure 3 TDP-43 presence stratified by ECAS task split. Panel A shows TDP-43 presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. \( n= 11 \) Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows TDP-43 presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. \( n= 11 \) Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows TDP-43 presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. \( n= 11 \) Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows TDP-43 presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. \( n= 11 \) Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows TDP-43 presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. \( n= 11 \) Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows TDP-43 presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. \( n= 11 \) Ctrl, 17 ALSnoci, 4 ALSci. Numbers above histogram mean positive cases/total cases.
Figure 4 TDP-43 burden stratified by ECAS task split. Panel A shows TDP-43 burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows TDP-43 burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows TDP-43 burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows TDP-43 burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows TDP-43 burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows TDP-43 burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Numbers above histogram mean positive cases/total cases.
Figure 5 Tau presence stratified by ECAS task split. Panel A shows Tau presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows Tau presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows Tau presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows Tau presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows Tau presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows Tau presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Numbers above histogram mean positive cases/total cases.
Figure 6 Tau burden stratified by ECAS task split. Panel A shows Tau burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows Tau burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows Tau burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows Tau burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows Tau burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows Tau burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Numbers above histogram mean positive cases/total cases.
Figure 7 Amyloid-β presence stratified by ECAS task split. Panel A shows amyloid-β presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows amyloid-β presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows amyloid-β presence in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows amyloid presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows amyloid presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows amyloid presence in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Numbers above histogram mean positive cases/total cases.
Figure 8 Amyloid burden stratified by ECAS task split. Panel A shows amyloid burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows amyloid burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows amyloid burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows amyloid burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel E shows amyloid burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows amyloid burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Numbers above histogram mean positive cases/total cases.
Figure 9 Microglial burden stratified by ECAS task split. Panel A shows no differences in microglial burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows no differences in microglial burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows no differences in microglial burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows no differences in microglial burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows no differences in microglial burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows no differences in microglial burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Graphs show median ± interquartile range. For all of them, data was not normally distributed and a Kruskal-Wallis test with Dunn’s multiple comparisons performed.
Figure 10 Astrocytic burden stratified by ECAS task split. Panel A shows no differences in astrocytic burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel B shows no differences in astrocytic burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel C shows no differences in astrocytic burden in BA44/45 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel D shows no differences in astrocytic burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by language task. n= 11 Ctrl, 14 ALSnoci, 7 ALSci. Panel E shows no differences in astrocytic burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by verbal fluency task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Panel F shows no differences in astrocytic burden in BA17/19 between controls, ALSnoci and ALSci after stratifying by executive function task. n= 11 Ctrl, 17 ALSnoci, 4 ALSci. Graphs show median ± interquartile range. For all of them, data was not normally distributed and a Kruskal-Wallis test with Dunn’s multiple comparisons performed.
3. ECAS tasks correlation

A. ECAS Total vs. ALS-specific ECAS

B. ECAS Total vs. Language

C. ECAS Total vs. Verbal fluency

D. ECAS Total vs. Executive function

E. ALS-specific ECAS vs. Language

F. ALS-specific ECAS vs. Verbal fluency

G. ALS-specific ECAS vs. Executive function

H. Verbal fluency vs. Executive function
Figure 1 Correlations between ECAS tasks. Panel A shows that overall ECAS scores positively correlate with ALS-specific ECAS. Panel B shows that overall ECAS scores positively correlate with language ECAS scores. Panel C shows that overall ECAS scores positively correlate with verbal fluency ECAS scores. Panel D shows that overall ECAS scores positively correlate with executive function ECAS scores. Panel E shows that ALS-specific ECAS scores positively correlate with language ECAS scores. Panel F shows that ALS-specific ECAS scores positively correlate with verbal fluency ECAS scores. Panel G shows that ALS-specific ECAS scores positively correlate with executive function ECAS scores. Panel H shows that verbal fluency ECAS scores positively correlate with executive function ECAS scores. Data was not normally distributed, so a Spearman correlation was performed. R and p-value shown on graph.
4. Total protein stains for WB

Figure 1 Method validation total protein. Total protein stain of the western blot used for the method validation (Figure 5.2)

Figure 2 TDP-43 total protein. Total protein stain of the western blot used for the synaptophysin and full-length TDP-43 stain (Figure 5.3)
Figure 3 FUS validation total protein. Total protein stain of the western blot used for the synaptophysin and FUS (Figure 5.7)