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Oligodendrocyte precursor cells in ageing and Alzheimer’s disease

Irene Chacón de la Rocha

This thesis is submitted in partial fulfilment of the requirements of the award of the degree of Doctor of Philosophy of the University of Portsmouth

University of Portsmouth
School of Pharmacy and Biomedical Sciences

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Abstract

Oligodendrocyte precursor cells (OPCs) are a population of dividing cells and their main role is to generate oligodendrocytes, the myelinating cells of the CNS, throughout life. They express a wide range of neurotransmitter receptors and ion channels. A key feature of oligodendrocyte precursor cells is that they contact neuronal synapses and nodes of Ranvier. Synaptic communication is thought to control the dynamics (proliferation, differentiation and survival) of oligodendrocyte precursor cells. Oligodendrocyte precursor cells are essential for myelin replacement through natural ageing. In many neurodegenerative diseases such as multiple sclerosis or Alzheimer’s disease myelin is disrupted, and oligodendrocyte precursor cells fail to regenerate the myelin lost. The causes of oligodendrocyte precursor cells impairment in these cases are poorly understood. In this thesis, I analysed oligodendrocyte precursor cells in tissue from mice injected with botulinum neurotoxin in the hippocampus to investigate whether the synaptic silencing that this toxin causes influenced the morphology and dynamics of oligodendrocyte precursor cells, resulting in myelin loss. In addition, I examined oligodendrocyte precursor cells in the APP/PS1 mouse model of Alzheimer’s disease. Finally, I analysed the dynamics of oligodendrocyte precursor cells in the adult and ageing brain of the Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mice. The results showed that synaptic silencing by botulinum neurotoxin causes reduction in oligodendrocyte precursor cells number, cell shrinkage and loss of complexity (branching, length and volume of their processes), but not loss of myelin. In the APP/PS1 mouse model, oligodendrocyte precursor cells were analysed in 9 months and 14 months old mice. The results showed decrease in oligodendrocyte precursor cells number, increase in immature oligodendrocytes, cell shrinkage and a trend towards an increase in myelination at 9
months, whereas at 14 months there was loss of oligodendrocyte precursor cells, as well as of immature oligodendrocytes and myelin, and an increase in cell complexity consistent with reaction to damage. In addition, the results showed a decrease in cells of the oligodendrocyte lineage and a slow down in the process of differentiation in the ageing brain. Overall, this thesis provides new knowledge on regulation of oligodendrocyte precursor cells by synaptic signalling and how this is related to changes in oligodendrocyte precursor cells in ageing an Alzheimer’s disease.
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Chapter 7

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C – Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Word count: 38948
D – Acknowledgements

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E – Dissemination

Publications:


Meetings attended:

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**FENS Regional meeting.** Thessaloniki, Greece. (October 2015). Poster: ‘Potassium channel profile of white matter glia’.


**XIII European Meeting on Glial Cells in Health and Disease.** Edinburg, UK. (July 2017). ‘Synaptic silencing by BonT/A results in OPCs dysregulation in vivo’.
### F - Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>3rd</td>
<td>Third</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>B-amylod</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BoNT/A</td>
<td>Botulinum neurotoxin type A</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis 1</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis 3</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus callosum</td>
</tr>
<tr>
<td>CNPase</td>
<td>2’,3’-Cyclic nucleotide 3’-phosphodiesterase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>Cspg</td>
<td>Chondroitin sulphate proteoglycan</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillar acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPR17</td>
<td>G protein-coupled receptor 17</td>
</tr>
<tr>
<td>GRIP</td>
<td>Glutamate receptor interacting protein</td>
</tr>
<tr>
<td>HB</td>
<td>Hoechst blue</td>
</tr>
<tr>
<td>HCH</td>
<td>Hyperpolarization-activated cyclic nucleotide–gated channels</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------------</td>
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</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>Kir4.1</td>
<td>Inwardly rectifying potassium channel Kir 4.1</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mo</td>
<td>Months old</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>NaN3</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NG2</td>
<td>Neuron-glia 2</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>ns</td>
<td>No-significant</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>OL</td>
<td>Oligodendrocyte</td>
</tr>
<tr>
<td>Olig1/2</td>
<td>Oligodendrocyte transcription factor 1/2</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGFαR</td>
<td>Platelet-derived growth factor receptor alpha subtype</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PLP</td>
<td>ProteoLipid protein</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SL</td>
<td>Stratum lacunosum</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor activating protein receptor</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TgN</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TS</td>
<td>Trizma saline</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>vGAT</td>
<td>Vesicular γ-aminobutyric acid transporter</td>
</tr>
<tr>
<td>vGluT1</td>
<td>Vesicular glutamate transporter 1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Chapter 1

Introduction
1.1. Overview of glial cells

Two main types of cells, neurons and glia, constitute the central nervous system (CNS). Neurons are electrically excitable cells that receive, process and conduct electrical and chemical information throughout the CNS. The term glia from the Greek ‘γλια’ was firstly coined by Rudolf Ludwig Karl Virchow (1821-1902). Glia are classified into astrocytes, oligodendrocytes (OLs), oligodendrocyte precursor cells (OPCs), microglia and ependymal cells. Initially, glial cells were considered to be connective tissue in the brain, but they perform a wide range of functions that are essential for the correct functioning of the CNS (Verkhratsky and Butt, 2013). Astrocytes and oligodendrocytes (and therefore, oligodendrocyte progenitor cells) constitute the macroglia and are derived from a common embryonic multipotent neural progenitor originated from the neuroepithelium, while microglia (firstly named by Pio Del Rio Hortega) are derived from embryonic mesodermal macrophages that penetrated the brain before the formation of the blood brain barrier (BBB) (Kettenmann et al., 2011; Verkhratsky and Butt, 2013) (Fig. 1.4).

1.1.1. Astrocytes

Astrocytes are stellate cells that can be identified by their expression of glial fibrillary acidic protein (GFAP) (Fig. 1.1) and they perform several roles essential for the correct functioning of the CNS (Kimelberg, 2004). Astrocytes provide physical support as well as maintenance of the homeostasis by K⁺ and glutamate uptake avoiding the toxicity generated by accumulation of these factors. They express neurotransmitter receptors and participate in the modulation of the synaptic transmission. In addition, astrocytes contribute to brain development, regulating neurogenesis and gliogenesis, allowing neuronal migration, formation of new synapses, maintaining the integrity of the BBB and regulating the blood flow. Moreover, they play a role as neuroprotective cells by
formation of the ‘glial scar’ in response to damage to the CNS (reviewed in Verkhratsky and Butt, 2013). As described previously, one of the main functions of astrocytes is the modulation of synaptic transmission. Upon electrical or chemical (direct or spill over) stimuli from neurons, the concentration of Ca$^{+2}$ increases in astrocytes causing the release of the neurotransmitter glutamate. This glutamate acts on neurons as a negative feedback causing the inhibition of the neurotransmission. Between adjacent astrocytes, Ca$^{+2}$ waves can propagate and the glutamate released can modulate distant synapses (reviewed in Araque et al., 1999). Astrocytes also maintain OPCs and oligodendrocytes functional integrity by release of protective factors that act through ERK and Akt pathways (Arai and Lo, 2010) as well as in an integrin dependent manner, as reported in experiments with astrocytes and oligodendrocytes co-cultures (Corley et al., 2001). Astrocytes provide support to OPCs that use their extracellular matrix to migrate during development (Schnadelbach and Fawcett, 2001) and release several factors such as, Pdgf-AA that promotes survival and proliferation of OPCs, BMPs (Bone morphogenic proteins) that inhibit the differentiation of OPCs into myelinating OLs and chemokines that promotes OPCs proliferation and inhibit their migration (reviewed in Clemente et al., 2013).

Figure 1.1. Confocal image of an astrocyte from mouse optic nerve expressing GFAP-EGFP (green) (Vanzulli and Butt, 2015).
1.1.2. Oligodendrocytes and oligodendrocyte progenitor cells

Oligodendrocytes (OLs), (Fig. 1.2) are the myelinating cells of the CNS and their main role is to wrap axons with a myelin sheet, isolating them and allowing the fast and saltatory conduction of action potentials. OLs are generated by oligodendrocyte progenitor cells (OPCs), also called NG-2 glia, throughout life (Miller, 2002). OPCs are the main proliferating cells in the adult brain. Apart from the production of OLs, they perform other functions such as, contacting nodes of Ranvier where they sense K+ ions (Butt et al., 1999) and neuronal synapses from which they receive excitatory and inhibitory inputs (S. C. Lin and Bergles, 2004). OLs have been reported to also communicate with neurons by release of exosomes with trophic factors (mainly RNAs and proteins) that support myelinated axons. The secretion of these exosomes is glutamate dependent and is mediated by Ca\(^{2+}\) that enters the oligodendrocytes through NMDA and AMPA receptors (Frühbeis et al., 2013). This PhD focuses on OPCs and cells of the oligodendrocyte lineage.

![Figure 1.2. Oligodendrocyte precursor cell immunostained for NG2 (Chacón de la Rocha, I., Unpublished).](image)

**Figure 1.2. Oligodendrocyte precursor cell immunostained for NG2** (Chacón de la Rocha, I., Unpublished).
1.1.3. Microglia

Microglia (Fig. 1.3) are 10-15% of the glial cells and they are the primary immune cells of the CNS. In physiological conditions they are resting cells, but when there is an insult to the CNS, they activate and migrate to the site of injury to attack and phagocytize pathogens (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011). Besides their defensive function, microglia have been reported to have other important roles such as being the primary iron source for OLs and OPCs during development, which is an essential function since iron is crucial for myelin formation, and the secretion of factors that control axon outgrowth and branching, as well as secretion of IGF-1 that is essential for myelin repair and remyelination and FGF-2 that it has been reported to promote OPCs migration (reviewed in Clemente et al., 2013).

Figure 1.3. Microglial cells DAB (Diaminobenzidine)-stained with IBA-1 antibody. (Fernández-Arjona et al., 2017).
1.1.4. Ependymal cells

Ependymal cells are from neuroectodermal origin (Jacquet et al., 2009) and are present in the ventricles of the brain and the central canal of the spinal cord. Their role is to control the production and monitoring of the cerebrospinal fluid (CSF) (reviewed in Bruni, 1998).

Figure 1.4. Glial cells in the healthy CNS (modified from Xiao et al, 2014).
1.2. Oligodendrocytes and myelination

Oligodendrocytes (OLs) (Fig. 1.5) are specialised cells that form the insulating myelin sheaths around the axons in the CNS. The myelin sheaths are formed by lipids (70%) and proteins (30%) such as Myelin Basic Protein (MBP), Proteolipid Protein (PLP), 2’,3’-Cyclic nucleotide 3’-phosphodiesterase (CNPase), Myelin-Associated Glycoprotein (MAG), Myelin Oligodendrocyte Glycoprotein (MOG), among others. Myelin sheaths are separated along the axons and form the nodes of Ranvier, which allows the saltatory propagation of action potentials (reviewed in Verkhratsky and Butt, 2013). Each OL can myelinate 20-30 axons within a short distance of their body (Butt and Ransom, 1989). Myelin disruption is a common factor observed in neurodegenerative diseases such as Multiple sclerosis (MS) and Alzheimer’s disease (AD) (Bartzokis et al., 2003).

![Myelinating oligodendrocyte illustrating their morphology](image)

**Figure 1.5.** Myelinating oligodendrocyte illustrating their morphology. Oligodendrocyte processes contact several axons (from Butt and Berry, 2000).
1.2.1. Oligodendrocyte lineage

Oligodendrocytes are generated throughout life by oligodendrocyte progenitor or precursor cells (OPCs) that originate from multipotent neural stem cells (NSC) in the subventricular zone (SVZ) of the lateral ventricles (Richardson et al., 2006). OPCs migrate to their final destination where they proliferate and differentiate into myelinating OLs (Fig. 1.6). OPCs are defined by their expression of the chondroitin sulphate proteoglycan (Cspg) neuron-glia 2 (NG2) and the platelet-derived growth factor receptor alpha subtype (PDGFαR) (Nishiyama et al., 1991, 1996). OPCs undergo different stages until they differentiate into myelinating OLs. Each one of these stages can be identified by specific markers. OPCs differentiate from NSC and express the transcription factors Olig1/2, Sox10 and Nkx2.2 (Arnett et al., 2004; Li et al., 2007; Dimou et al., 2008; Talbott et al., 2013). OPCs lose the expression of NG2 and PDGFαR and they become immature OLs (Verkhratsky and Butt, 2013), which are identified mainly by expression of the P2Y-like receptor G-protein coupled receptor (GPR17) (Lecca et al., 2008; Chen et al., 2009; Boda et al., 2011; Viganò et al., 2016). Similarly, they lose expression of GPR17 and start expressing adenomatous polyposis coli (APC), MBP, PLP, MAG, MOG, (CNPase), which are myelin related proteins, and they become myelinating OLs (Verkhratsky and Butt, 2013). A population of OPCs persist in the adult brain and they are known as adult OPCs. They are the major proliferating cell population in the adult CNS (Dawson et al., 2003), although their cell cycle is slower than during development (Psachoulia et al., 2009), and are found in grey and white matter where they produce OLs at a slow rate throughout life. The rate of OL generation is increased upon injury or demyelination or when learning a new motor skill (Dimou et al., 2008; Rivers et al., 2008; Zhu et al., 2008; Kang et al., 2010; Xiao et al., 2017). OPCs divide asymmetrically to form ‘sister cells’, one of them remains as OPC to keep the OPC population and the other
differentiate into myelinating OL (Boda et al., 2015). OPCs are essential for remyelination and they proliferate in the injury sites and generate new OL, although they form a higher number of shorter internodes (Guo et al., 2008; Tripathi et al., 2010; Young et al., 2013)

Figure 1.6. Stages of oligodendrocyte differentiation. Neural stem cells differentiate into mature OLs through a series of developmental stages characterised by specific markers. (Modified from Verkhratsky and Butt, 2013).
1.2.2. Oligodendrocyte precursor cells (OPCs): origin, function and regulation of their differentiation.

OPCs constitute 5-10% of glial cells in the developing and adult CNS (Trotter et al., 2010). They distribute evenly and form a mosaic covering the entire brain (Butt et al., 2002) and they proliferate even in the adult brain (Psachoulia, Jamen, Kaylene M. Young, et al., 2009). OPCs are generated in different areas of the brain such as the ventral germinal zones and the lateral ganglionic eminences and they expand generating the majority of OPCs in the forebrain, corpus callosum (CC) and neocortex. From late embryonic stages to postnatal period they generate from the dorsal ventricular zone and later, from the subventricular zone (SVZ) (Hill and Nishiyama, 2014). OPCs are characterised by the expression of the proteoglycan NG2 (Nishiyama et al., 1991). The role of NG2 is not well known, but it presents binding sites for proteins implicated in the regulation of cell migration and proliferation, and it binds in the extracellular domain to integrins and growth factors that regulate proliferation and migration, such as PDGFA and FGF-2 (Stallcup and Huang, 2008; Trotter et al., 2010; Azim, Raineteau and Butt, 2012) (Fig. 1.7). The cytoplasmic domain of NG2 has a PDZ-binding domain and interacts with proteins such as glutamate receptor interacting protein (GRIP), postsynaptic density protein (PSD95), which anchors to the membrane AMPA receptors and inwardly rectifying potassium channel Kir4.1 (Fig. 1.4) (Stegmüller et al., 2003; Trotter et al., 2010; Kucharova and Stallcup, 2015; Brasko et al., 2017). OPCs express a wide variety of molecules, as reported in RNAseq studies. They express ion channels such as Kir4.1, which is also expressed in astrocytes and OLs and is implicated in maintaining the resting membrane potential of the cell, although it could have a role in buffering K⁺, similarly to the role of these channels in astrocytes (Larson et al., 2016; Brasko et al., 2017). It has been shown recently that Kir4.1 is important for development and ischemia-related myelin loss (Duan et al., 2018). Voltage-gated Na⁺ channels are also
expressed by OPCs and their expression decreases as OPCs differentiate into OLs (De Biase et al., 2010; Larson et al., 2016), as well as voltage-gated Ca\textsuperscript{2+} channels, which are important for proliferation, migration, process extension, and myelination, among other channels such as voltage-gated Cl\textsuperscript{-} channels, HCN channels and TPR channels (Larson et al., 2016). OPCs express several receptors such as ionotropic and metabotropic glutamate and GABA receptors, as well as adrenergic receptors and receptors for acetylcholine, dopamine, glycine, serotonin and purinergic receptors. The expression of these genes were different among the different stages of differentiation within the oligodendrocyte lineage and they may have a role in the regulation of oligodendrocyte generation and differentiation (Larson et al., 2016). One of the major factors regulating OPCs proliferation is PDGF, produced by astrocytes and neurons and acting in OPCs via PDGFR\textalpha (Raff et al., 1988). FGF2 also have a role in OPCs proliferation maintaining their undifferentiated state (Baron et al., 2000).
Figure 1.7. Functional interactions of NG2. NG2 proteoglycan interacts with intracellular and extracellular proteins that determine its function in OPCs (from Trotter et al., 2010).
1.2.3. Myelination

Myelin sheaths electrically insulate axons. They are formed by lipids and proteins such as PLP, MBP, MOG, MAG and (CNPase). This proteins can be used as markers for myelin (Dubois-Dalcq et al., 1986). The myelination process in the mouse brain starts at birth in the hindbrain and continues caudally and rostrally (Foran and Peterson, 1992; Baumann and Pham-Dinh, 2001), and requires a series of processes such as contact between axon and immature OL, loss of non-myelinating processes by OLs, generation and wrapping of the axon with myelin and formation of internodes and nodes of Ranvier, and growth of myelin sheaths to the adult axonal diameter and myelin compaction (Butt and Berry, 2000). This process is controlled and initiated by integrins and adhesion molecules (Laursen and Ffrench-Constant, 2007). Oligodendroglial process growth and initiation of axon wrapping is known to be mediated by Fyn kinase, and neuroligin is believed to be responsible for the myelination of a particular axon by an OL (Sherman and Brophy, 2005). The myelination process is not restricted to postnatal life, since in humans, myelin volume increases until the fourth decade (Bartzokis, 2001). The existence of adult OPCs indicates that myelination is an active process that occurs throughout life (Rivers et al., 2008; Young et al., 2013). Myelin is essential to maintain the cognitive functions and myelin deficits as well as age-related or injury-related myelin breakdown are related to psychiatric and neurodegenerative disorders, such as bipolar disorder, schizophrenia, Multiple sclerosis and Alzheimer’s disease (Bartzokis et al., 2003; Bartzokis and Lu, Po H., Mintz, 2007; Bartzokis, 2011; Hill et al., 2018). Myelination capacity decreases with age and this could be due to the age-related decrease in the capacity of OPCs to replace loss OLs, since their cell cycle increases and their differentiation capacity slows down with age (Rivers et al., 2008; Psachoulia et al., 2009). The failure in myelination in the ageing brain and demyelinating pathologies has
also been reported to be related to a failure in OPCs recruitment (Shields et al., 1999; Franklin et al., 2002).

1.3. Neuron-OPC interactions

OPCs (also called NG2-glia) have a complex stellate morphology with multiple processes that they extent to contact neurons at the nodes of Ranvier (Butt et al., 1999) and at synapses sites (Bergles et al., 2000; S. C. Lin and Bergles, 2004; S. Lin and Bergles, 2004). At nodes, they sense $K^+$ released during action potential via Kir4.1 channel, as well as activating extrasynaptic receptors expressed at that area. At synapses they form synaptic junctions similar to the ones formed by neuron-neuron, with a presynaptic component which in this case is the neuron and a postsynaptic component which is the OPC that receives the synaptic inputs (reviewed in Butt, 2017) (Figure 1.8). In more detail, Bergles and collaborators described different types of synapses. In the first type, the OPC processes were apposed to the presynaptic sites of neurotransmitters release as well as to dendritic spines, presenting the postsynaptic specialized region of the OPC a thinner membrane than in the case of neuron-neuron interactions. In the second type of association, the OPC processes were aligned with an axo-dendritic junction (Bergles et al., 2000).

OPCs are associated with pre- and post-synaptic proteins such as the vesicular GABA ($\gamma$-aminobutyric acid) transporter (vGAT), the vesicular glutamate transporter (vGluT), the postsynaptic density protein 95 (PSD-95) and Gephyrin (Bergles et al., 2000; Lin and Bergles, 2004; Wigley and Butt, 2009; Orduz et al., 2015; Sahel et al., 2015). There are evidences that OPCs proliferation and differentiation are regulated by neuronal activity. Inhibition of electrical communication with Tetrodotoxin (TTX) decreases OPCs number in the optic nerve (Barres and Raff, 1993), and the number of OPCs increases when
electrical activity is activated using optogenetics, as well as the oligodendrogenesis and myelination (Gibson et al., 2014). Glutamatergic and GABAergic neurons signal onto OPCs in the hippocampus and other areas of the brain (Bergles et al., 2000; Paukert and Bergles, 2006; Bergles, Jabs and Steinhäuser, 2010). Direct glutamatergic synapses onto OPCs persist throughout life, while GABAergic neurons form direct synapses with OPCs in the postnatal brain and they change to spill over in the adult, in which OPCs do not contact synapses directly but they still receive neurotransmitters from GABAergic synapses in their vicinity (Vélez-Fort et al., 2010). Effects of glutamatergic signalling onto OPCs reported in the literature are contradictory, since pharmacological blockade of AMPA receptors promotes proliferation (Fannon et al., 2015) but knocking out AMPA receptors has no effect on OPCs proliferation (Kougioumtzidou et al., 2017). In contrast, GABAergic signalling reduces proliferation, reducing OLs numbers and decreasing myelination via GABA_A receptors (Zonouzi et al., 2015; Hamilton et al., 2016). Moreover, the NG2 proteoglycan does not seem to be necessary for neuron-OPC communication (Passlick et al., 2016), although disruption of OPCs causes deficits in excitatory neurotransmission in NG2-KO mice (Sakry et al., 2014).
**Figure 1.8. Neuron-OPC interactions** (modified from Otis and Sofroniew, 2008). OPCs (also called NG-2 cells) contact myelinated and unmyelinated axons receiving inputs from inhibitory and excitatory synapses. OPCs also contact nodes of Ranvier in which they sense K+ ions that come from action potentials that pass throughout the axon.
1.4. OPCs long-life generation of oligodendrocytes

Generation of new OLs by OPCs is essential for replacement of myelin lost during normal ageing (Rivers et al., 2008; Young et al., 2013). Several fate-mapping studies have been done recently to elucidate the fate of OPCs in development and ageing. It has been shown that OPCs are cycling cells and they are mitotically active throughout life, although the duration of their cell cycle changes with age increasing from 2 days at P6 to 9 days at P60 (2 months), 70 days at P240 (8 months) and P540 (18 months) in the corpus callosum, while in the cortex the cell cycle increased from 2 days at P6 to more than 150 days in P240 and P540 (Psachouli et al., 2009). It has also been shown that OPCs generate mature OLs in the adult and ageing brain (Young et al., 2013), but the capacity of OLs generation decreases with age and only 18% of OPCs differentiate into mature OL at P240 in CC and cortex (Psachouli et al., 2009), while in the spinal cord white matter 41% of OPCs differentiated into OLs at P60 and 31% in the spinal cord white matter. In the corpus callosum and optic nerve 64% and 69% of OPCs formed OLs at P60, respectively. At P120 (4 months) 1/3 of OPCs differentiated into OLs in the mouse optic nerve. This indicates a decrease in the differentiation rate with age and variability within the different areas of the CNS (Young et al., 2013). Fate mapping studies have also shown that OLs produce myelin throughout life and that late-born OLs generate shorter internodes and these are more numerous that the generated by early-born OLs, being the mean length of internodes 76 µm at P60 and 22 µm at P185 (Young et al., 2013). It has been shown that OPCs start generating OLs the first postnatal week (Huang et al., 2014). Another study demonstrated lifelong generation of OLs and plasticity of myelin internodes formation in mice from P30 (1 month) to P950 (31 months), with myelin internodes increasing at a steady pace until P650 (21 months) when it started decreasing. This suggests that the remodelling of myelin occurs until at least 2 years of age in the mouse brain (Hill et al., 2018). Studies using NG2 inducible reporter mice have shown
that while at P2 dividing and differentiating OPCs produce clusters formed mainly by mature OLs and some OPCs, at P60 these clusters are composed almost exclusively by OPCs and few OLs, indicating a decline with age in oligodendrogenesis (Zhu et al., 2011). Immature OLs dynamics have also been studied using fate-mapping with GPR17 inducible reporter mice, showing that immature OLs identified by NG2\(^+\)GPR17\(^+\) markers do not differentiate into myelinating mature OLs within 3 months unless a damage or insult occurs to the CNS. As a reaction to damage, immature OLs rapidly start the differentiation process into mature OLs, indicating that these cells serve as a pool of adult progenitors with the role of maintaining and repairing the CNS (Viganò et al., 2016). All these studies confirm the capacity of OPCs to proliferate and differentiate throughout life in normal conditions and in the event of an injury or insult to the CNS, although in some particular conditions such as MS OPCs fail to remyelinate and differentiate in the demyelinated lesions (reviewed in Zuo et al., 2013).

1.5. Oligodendrocyte lineage and synaptic impairment in Alzheimer’s disease

The most common type of dementia is Alzheimer’s disease (AD) and it is characterised by formation of neurofibrillary tangles (NFTs), extracellular β-amyloid plaques and neuronal loss (Braak and Braak, 1991; Gomez-Isla et al., 1997; Braak et al., 2006). Synaptic failure is also present in AD and it starts in the early stages of the disease, with loss of dendritic spines in areas related to Aβ plaques (Selkoe, 1991; Merino-Serrais et al., 2011). Most studies in synaptic pathology in AD in humans focus on the hippocampus and specifically in the CA1 and various layers of the dentate gyrus (reviewed in Scheff and Price, 2003); therefore, in this thesis, I focussed my analysis of synaptic proteins and OPCs morphology in the CA1 of the hippocampus so my data can be comparable to previous studies. Several studies have shown significant loss of synaptic density in
patients with advanced AD (specifically, in post-mortem tissue from AD patients with an average duration of the disease of 9 years) (reviewed in Scheff and Price, 2003). An increase in the length of synaptic contacts was also found in ultrastructural studies of tissue from AD subjects. This has been shown also in transgenic mice and it is believed to be a compensatory mechanism for the loss of synaptic contacts (Reviewed in Scheff and Price, 2003). The causes of the synaptic pathology in AD are not known. It has been shown that there is not a clear correlation between synaptic loss and β-amyloid plaques deposition since subjects with heart disease and presence of amyloid plaques did not present synaptic loss nor dementia (reviewed in Scheff and Price, 2003). Other causes of synaptic loss in AD contemplated in several studies are oxidative stress (reviewed in Scheff and Price, 2003 and Overk and Masliah, 2014) and the accumulation of hyperphosphorylated Tau in the neuronal terminals for long periods of time interfering with the vesicular traffic (Morsch et al., 1999). Other studies report that Tau and Aβ are believed to be related to this failure by dysregulation of glutamatergic neurotransmission through destabilization of NMDA and AMPA receptors and impairment of molecules related to synaptic vesicles transport (reviewed in Overk and Masliah, 2014) (Fig. 1.9).

Several animal models have been developed to study AD, from invertebrates (Drosophila melanogaster and Caenorhabditis elegans) to rodents. Mice models are the main animals used in AD studies and they contain several mutations in genes related to AD such as APP (amyloid precursor protein), PSEN1 and PSEN2 (presenilin 1 and 2) and Tau (MAPT, microtubule associated protein that forms NFTs when is hyperphosphorylated) (reviewed in Götz and Ittner, 2008). APP is a transmembrane protein that when is enzymatically cleaved by β-secretase and γ-secretase complex generates β-amyloid (Aβ), the mayor component of amyloid plaques and one of the main lesions found in AD (reviewed in Götz and Ittner, 2008 and Chow et al., 2010). Presenilin 1 and 2 are part of the γ-secretase complex together with other proteins, while the β-secretase is a single
enzyme, being BACE1 (Beta-site APP cleaving enzyme 1) the major β-secretase in the brain (reviewed in Chow et al., 2010). Contrary, α-secretase cleaves APP within the Aβ domain generating non-amyloidogenic fragments (Götz and Ittner, 2008). Mutations in APP, PS1 and PS2 have been identified in familiar AD (FAD) which is only 1% of the total number of AD cases (Götz and Ittner, 2008) and this may be the reason why successful clinical trial in AD animal models do not have the same results in humans (reviewed in Drummond and Wisniewski, 2017). A widely used mouse model of AD is the APP/PS1 mouse which combines the mutations in APP and Presenilin 1, and presents β-amyloid plaques but not neurofibrillary tangles (NFTs) (reviewed in Bilkei-Gorzo, 2014; Li et al., 2015; Drummond and Wisniewski, 2017). Due to the wide use of the APP/PS1 mouse model and its availability for our lab, this is the model that I have chosen to study in my thesis. NFTs are not present in mice, therefore, to generate mouse models that contains this feature of AD, forms of human tau containing mutations associated to frontotemporal dementia (FTD) are expressed in mice (Drummond and Wisniewski, 2017). The problem with this human mutated tau is that there are not tau mutations known related to AD and therefore, the cognitive and motor deficits that this mutation produces in mice does not correlate with real human pathology of AD (reviewed in Götz and Ittner, 2008; Drummond and Wisniewski, 2017). A mouse model with APP, PS1 and Tau mutations is the 3xTg, which presents Aβ and Tau pathology as well as synaptic failure (Oddo et al., 2003). Other genes present in cases of sporadic AD (SAD), such as APOE (Apolipoprotein E) have been identified (reviewed in Bertram and Tanzi, 2005). It has been reported in previous studies that cholesterol in the brain is produced by astrocytes and oligodendrocytes, and they also secrete apolipoprotein E that is essential for lipid metabolism, synaptogenesis and, as described before, is related to sporadic AD. In fact, there is a strong myelin breakdown in patients with mutation APOE4 (reviewed in De Strooper and Karran, 2016). White matter breakdown in frontal lobes is an early feature
of AD (Bartzokis et al., 2003; Sjöbeck and Englund, 2003; Ihara et al., 2010) and it has been reported to be accompanied by loss of OLs in AD patients (Sjöbeck and Englund, 2003) and in mice, especially in the core and surroundings of Aβ plaques (Mitew et al., 2010). It is believed that the OLs damage and myelin breakdown could be due to neuroinflammation, oxidative stress and apoptosis, although the failure in OPCs replacement and differentiation due to NFTs and Aβ toxicity it is thought to accelerate the progress of AD (Cai and Xiao, 2015).

Figure 1.9. Schematic representation of progression of the mechanisms of synaptic damage in AD. At early stages of the disease, oligomers interfere with synaptic communication. In intermediate stages, there is failure of axonal transport and at late stages appears neuronal loss (Overk and Masliah, 2014).
1.6. Hypothesis and Aims

There is abundant evidence of regulation of OPCs proliferation and differentiation by synaptic activity in physiological conditions (Barres and Raff, 1993; Vélez-Fort et al., 2010; Gibson et al., 2014; Fannon et al., 2015; Zonouzi et al., 2015; Hamilton et al., 2016; Kougioumtzidou et al., 2017). However, it is not known whether failure in synaptic communication causes impairment in OPCs proliferation and differentiation processes as well as repercussion in myelin impairment in ageing and pathologies such as Alzheimer’s disease. The hypothesis underpinning this thesis is that synaptic failure leads to impairment in OPCs capacity to differentiate into myelinating OLs and this causes loss of myelin in ageing and Alzheimer’s disease affected brains. To address my hypothesis, the aims are:

1. Identify changes in OPCs morphology, proliferation and differentiation that could be caused by synaptic failure.
2. Examine changes in OPCs morphology, proliferation, and differentiation, as well as myelination in the APP/PS1 mouse model of AD.
3. Examine OPCs proliferation and differentiation processes in normal ageing using the Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse.
Chapter 2

Materials and methods
2.1. Animals

2.1.1. Declaration

All animals were killed humanely by schedule one procedure in accordance with the regulations issued by the Home Office of the United Kingdom under the Animals (Scientific Procedures) Act 1986.

2.1.2. Animal strains.

Mice of various ages and strains were used throughout the project. Several transgenic mice lines were used to identify OPCs, and to study Alzheimer’s related changes in glial cells:

- SOX10-lox-GFP-STOP-lox-DTA mice in which expression of the Enhanced Green Fluorescent Protein (EGFP) is under the control of the SOX10 gene (Matsuoka et al., 2005; Kessaris et al., 2006) were used to identify cells in the oligodendrocyte lineage. The lox recombination was not induced in this case, using the mice as a reporter (Sox10-GFP) in order to identify the cells from the oligodendrocyte lineage and stain them using different antibodies against each subtype of cell within the lineage. In this thesis I used NG2 immunolabeling to identify OPCs (Fig. 3.9 A). This strain was a gift from William D Richardson and Nicoletta Kessaris’ lab.

- Pdgfra-CreERT2-Rosa26R-YFP in which a tamoxifen-inducible form of the Cre recombinase (CreERT2) is expressed under the transcriptional control of PDGFRA in a PAC (phage artificial chromosome) and combined with Rosa26-YFP reporter line. As a result, expression of yellow fluorescent protein (YFP) can be induced in oligodendrocyte precursor cells (OPCs), allowing the identification of these cells and
their progeny (Rivers et al., 2008). Pdgfra-creER\textsuperscript{T2} and Rosa26-YFP strains were kindly provided by Professor William D. Richardson.

- TgN APP/PS1 (APP KM670/671NL (Swedish), PSEN1 L166P) containing human transgenes for both APP with the Swedish mutation (double mutation adjacent to the β-secretase site in APP resulting in a substitution of two amino acids, lysine (K) and methionine (M) to asparagine (N) and leucine (L) and PSEN1 containing an L166P mutation, both under the control of the Thy1 promoter. This causes the expression of the human APP transgene to be approximately 3-fold higher than endogenous murine APP (Radde et al., 2006; Maia et al., 2013). Tissue from these mice was kindly provided by our collaborators Professor Hugh Perry and Dr. Diego Gómez-Nicola (University of Southampton, UK).

- C57BL/6N adult mice (WT). Tissue from these animals previously injected ipsilaterally in the hippocampus with Botulinum neurotoxin A (BoNT/A) and their respective controls, injected with saline solution (Antonucci et al., 2008), was kindly provided by our collaborators Professor Hugh Perry and Dr. Diego Gómez-Nicola (University of Southampton, UK) and Dr. Matteo Caleo (University of Pisa, Italy). Intrahippocampal injections were performed as described in (Antonucci et al., 2008). Briefly, 0.2µl of a 10nM solution of BoNT/A (or saline solution in the case of controls) were injected with a glass micropipette connected to an injector in the coordinates (in mm with respect to Bregma) -2.0 (anteroposterior), 1.5 (mediolateral) and 1.7 (below dura). Animals were anesthetised with chloral hydrate and intracardially perfused with 4% PFA in 0.1M phosphate buffer. Dissected brains were postfixed for 2h at 4°C. After this, 40µm brain sections were cut with a freezing microtome (Antonucci et al., 2008). All this procedure was performed in Dr. Matteo Caleo’s lab. Finally, the brain slices were sent to our collaborators in Southampton.
and to us. Lastly, I performed the immunostaining of the slices, the imaging and the analysis of the data obtained.

All transgenic animals used in this study were fertile and showed no unusual phenotype or abnormal behaviour.

2.1.3. Animal ages

Animals of both sexes were used at different ages:

- 3 and 18 months old Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP were used for immunohistochemistry.
- Tissue from 9 months old and 14 months old wild type and APP/PS1 mice was used for immunohistochemistry.
- Tissue from adult wild type mice (injected with Botulinum neurotoxin A or saline solution) was used for immunohistochemistry.

2.1.4. Breeding and genotyping of CreERT\textsubscript{2}/lox mice

Pdgfra-CreER\textsuperscript{T2} heterozygous mice were crossed with Rosa26R-YFP, homozygous or heterozygous, to generate the strain Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP used in this study. A piece of tissue from the ear of each mice (breeders and experimental mice) was collected from the Bioresources unit and DNA was extracted and prepared for PCR using REDExtract-N-Amp\textsuperscript{TM} tissue PCR kit (Sigma). Animals were genotyped routinely using the following primers to detect the presence of CreER\textsuperscript{T2} and Rosa26R-YFP:
- CreER\textsuperscript{T2}:
Forward primer sequence: 5’ TGTCATTTGCAGGTCTCAGGAGCTATG 3’
Reverse primer sequence: 5’ GGTGTTATAAGCAATCCCCAGAA 3’

- Rosa26R-YFP:
  Forward primer sequence: 5’ AAAGTCGCTCTGGAGTTAT 3’
  Reverse primer sequence 1: 5’ GCGAAGAGTTTGTCTCAACC 3’
  Reverse primer sequence 2: 5’ GGAGCGGGAGAAATGGATATG 3’

2.2. Chemicals and solutions

All chemicals were from Sigma-Aldrich or Fisher, unless otherwise stated, and were of analytical grade.

2.3. In vivo tamoxifen injections

3 months and 18 months old Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP mice were injected intraperitoneally twice a day for 5 consecutive days with 0.1ml of a 10mg/Kg solution of tamoxifen (Sigma) prepared in ethanol and corn oil. Ten days after the last injections the mice were anesthetised using Isoflurane and Pentobarbital and perfused intracardially with 0.1 M saline solution to eliminate the blood from the blood vessels, followed by perfusion with 1% Paraformaldehyde (PFA) during 10 min (Fig. 2.1).

Figure 2.1. Diagram of experiments performed with Pdgfra-CreERT2:Rosa26R-YFP mice.
2.4. Immunolabelling technique

2.4.1. Preparation of the tissue and immunohistochemistry

Mice of different ages and strains (described in section 2.1.1 and 2.1.2) were anesthetised and perfused intracardially with 1% PFA. Brains and optic nerves were dissected and post-fixed by submersion in 1% PFA overnight. After fixation, tissues were washed 3 times in PBS and stored at 4°C, in PBS containing 0.05% NaN3 (Sigma) until used. Coronal brain sections were cut using a vibratome (Leica) at a thickness of 60 μm and used immediately or stored at 4°C in PBS/NaN3 in 24 well plates. Optic nerves were cryoembedded in a recipient made with foil and stored at -80°C until needed, when they were sliced at 25 μm using a cryostat (Leica) and collected onto poly-L-lysine coated slides. Samples were stored at -80°C until they were used for immunohistochemistry (IHC). Different immunohistochemical protocols were performed depending on the antibodies used. Tissue was blocked with 10-20% normal goat serum or normal donkey serum (NGS or NDS) or 0.5% bovine serum albumin (BSA) for 1-2 hrs, washed 3 times in PBS, and incubated overnight in the antibody solution which comprised of the primary antibody diluted in blocking solution containing Triton-X. Tissues were then washed 3 times in PBS and incubated with secondary antibody diluted in blocking solution for 1-2 hrs. at room temperature on an orbital shaker and protected from the light by wrapping the 24-well plate in foil. Following secondary antibody incubation, tissues were washed 3 times with PBS before being mounted on glass slides and covered with mounting medium (Fluoromount-G) (Southern Biotech) and glass coverslips ready for imaging.

In the case of IHC for NG2 sections were rinsed with 0.1 M PB for 5 min and incubated in 1% Sodium borohydride (Sigma) for 30 min, and then rinse abundantly with PB and incubated for 10 min with Trizma base saline (TS). After, brain sections were incubated in 0.5% bovine serum albumin (BSA) (Sigma) in 0.1 M TS+0.25% Triton (Sigma) for 30
min. Then, brain sections were incubated overnight with primary antibody at room temperature in an orbital shaker and protected from the light. Sections were then washed and incubated for 1-2 hours with secondary antibody at room temperature in an orbital shaker and covered from light. Finally, tissues were washed and mounted as previously described for confocal microscopy.
2.4.2. Antibodies

All antibodies used for immunohistochemistry staining are listed below with relative concentrations at which they were applied.

2.4.2.1. Primary antibodies

2.4.2.1.1. Glial markers

<table>
<thead>
<tr>
<th>Antibody epitope</th>
<th>Host</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olig2</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Millipore</td>
</tr>
<tr>
<td>NG2</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Millipore</td>
</tr>
<tr>
<td>GPR17</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Cayman Laboratories</td>
</tr>
<tr>
<td>APC</td>
<td>Mouse</td>
<td>1:700</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>MBP</td>
<td>Rat</td>
<td>1:300</td>
<td>Millipore</td>
</tr>
</tbody>
</table>

2.4.2.1.2. Synaptic markers

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<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
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<td>PSD95</td>
<td>Goat</td>
<td>1:300</td>
<td>Abcam</td>
</tr>
<tr>
<td>vGAT</td>
<td>Guinea pig</td>
<td>1:300</td>
<td>Frontier Institute co. Ltd</td>
</tr>
<tr>
<td>vGluT1</td>
<td>Guinea pig</td>
<td>1:300</td>
<td>Frontier Institute co. Ltd</td>
</tr>
<tr>
<td>Gephyrin</td>
<td>Goat</td>
<td>1:300</td>
<td>Synaptic systems</td>
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</table>

2.4.2.1.3. Potassium channels

<table>
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<th>Host</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir4.1</td>
<td>Rabbit</td>
<td>1:400</td>
<td>Alomone</td>
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</table>

2.4.2.1.4. Other antibodies

<table>
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<th>Antibody epitope</th>
<th>Host</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Chicken</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
</tbody>
</table>
### 2.4.2.2. Secondary antibodies

<table>
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<tr>
<th>Conjugate</th>
<th>Reactivity</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa fluor®-488</td>
<td>Gt-a-Rb; Gt-a-Ck; Dk-a-Rb</td>
<td>Life technologies/ Jackson ImmunoResearch</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa fluor®-568</td>
<td>Gt-a-Rb; Gt-a-Ck; Gt-a-Ms; Gt-a-Rat; Gt-a-GP</td>
<td>Life technologies</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa fluor®-594</td>
<td>Dk-a-GP</td>
<td>Jackson ImmunoResearch</td>
<td>1:1000</td>
</tr>
<tr>
<td>Alexa fluor®-647</td>
<td>Gt-a-Rb; Gt-a-Ck; Gt-a-Ms; Gt-a-GP Dk-a-GP</td>
<td>Life technologies/ Jackson ImmunoResearch</td>
<td>1:500</td>
</tr>
</tbody>
</table>

### 2.5. Confocal microscopy

Images were captured using a Zeiss Axiovert LSM 710 VIS40S confocal microscope and maintaining the acquisition parameters constant to allow comparison between samples within the same experiment. The brightness and contrast of flattened confocal sections were adjusted slightly to obtain clear visible images, and flattened confocal micrographs are of equal thickness and magnification in images used to do comparisons unless otherwise stated.

Acquisition of images for cell counts was done with x20 objective. Images of synaptic markers were captured using x100 objective plus 0.2 zoom to increase detail of the image. Images for OPC reconstruction were taken using x100 objective and capturing z-stacks formed by 80-100 single plains with an interval of 0.3 µm. Images for colocalization and area measurements were capture with x40 objective.

Specifically, the images used in each section of this thesis were taken as follows:

- Imaging of OPCs and synaptic markers: Confocal images of the 3D morphology of OPCs were generated from 15 z-sections with 1µm interval using x100 oil objective and are illustrated as 2D flattened images and single z-sections, together with 90° rotations illustrating transects in the x-x plane, y-y plane and a
representation of voxels through 360º in the x-y-z planes. In addition, isosurface rendering of images was performed to illustrate equalized intensity of all voxels to provide 3D representations of short lengths of single OPC processes and their contacts with puncta of PSD95, vGlut1, vGAT and Gephyrin.

- Imaging for cell counts: Confocal images were captured using 710 LSM confocal microscope at 20x objective from 15-20 z-sections to make flattened 2D representations and cell counts were performed in a determined area (for more detail, see section 2.6.1).

- Imaging of single OPCs for morphometric analysis using Neurolucida: Confocal images were captured using 80-100 z-sections, each of 0.3μm thickness and using a x100 oil objective and reconstructed and analyzed using Neurolucida 360 and Neurolucida 360 Explorer.

- Imaging of Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP for cell counts: Confocal images of 10 z-sections with 1 μm interval were captured and cell counts performed in a constant FOV of 200μm x 200μm or 100μm x 100μm, depending on the area analysed.

**2.6. Quantification of glia and synaptic markers**

**2.6.1. Cell counts**

For chapters 4 and 5, OPCs were counted in projected images of z-stacks within each region analysed and the number of cells was divided by the area of the region to obtain the numerical density.

The rest of cells analysed in this thesis were counted in a constant field of view (FOV) of 100 μm x100 μm or 200 μm x 200 μm, depending on the area analysed, in projected
flattened images from z-stacks formed by 10 or 15 z-single plain images with 1µm interval between them.

2.6.2. Quantification of synaptic markers

Quantification of OPC associations with pre- and post-synaptic membranes was performed by counting the total number of PSD95+, vGLUT1+, vGAT+ or Gephyrin pixels relative to NG2+ pixels within the process fields of individual OPCs, to provide a relative index of immunostaining density. This immunostaining density was measured using ImageJ. For PSD95 the number of puncta in direct contact with OPCs was also counted, but for vGluT1, vGAT, and Gephyrin the overall density of immunostaining was too great for accurate quantification by this method.

2.6.3. Relative density

NG2 and MBP relative density (number of pixels stained by NG2 or MBP within a certain FOV) was measure in a constant field of view using ImageJ. A threshold was set for all the images to allow comparison between them.

For synaptic markers density index, the density of each marker was measure as above and divided by the density of the cell to which they were related.

2.7. Morphometric analysis

2.7.1. Quantitative morphological analysis with Neurolucida 360

Confocal images of OPCs were captured as explained in section 2.5.4. Single cells were manually drawn in detail using Neurolucida 360 and their morphology was analysed
using Neurolucida 360 explorer. For Sholl analysis, the interval between Sholl shells was 5µm. All the analysis was performed in Dr. Olivier Rainateau lab in Lyon, France.

2.7.2. Measurement of area occupied per cells (Cell coverage)

The coverage index (area occupied by cell in relation to the area of the region analysed) of OPCs was measured using ImageJ by drawing a line around the cell processes and measuring the area enclosed within the line. The area measured was related to the area of the region analysed in each case.

2.8. Statistical analysis

All data were analysed for significance using GraphPad Prism 6.0 and expressed as Mean±SEM. For comparison between 2 groups (2 different ages, ipsilateral and contralateral hippocampus, etc.), Unpaired t-test was used. For Sholl data analysis, Mann-Whitney test or Two-way ANOVA were performed.
Chapter 3

Organisation of oligodendroglial components in the adult brain
3.1. Introduction and Aims

OPCs present a characteristic stellate morphology with several branches. They are distributed throughout the brain and form a network with other cellular components, contacting synapses and nodes of Ranvier (Butt et al., 1999). The main role of OPCs is to form OLs throughout life by a process of differentiation that requires several stages (Dimou et al., 2008; Young et al., 2013). OPCs express a wide variety of channels and receptors that are thought to be important for their physiology, playing a role in their proliferation and differentiation processes (Chittajallu et al., 2004; Larson et al., 2015). The ultimate purpose of their differentiation is to produce myelinating OLs that will make myelin around neuronal axons to enable rapid electric communication between different areas of the CNS (Bunge, 1968; Griffiths et al., 1998). Despite the numerous studies on OPCs, no papers describe their exact distribution throughout the hippocampus, which forms the basis of my thesis. Hence, the aim of this chapter is to provide knowledge of how OPCs and other components of the oligodendrocyte lineage organize within the hippocampus of the adult mouse, since most of the experiments conducted in this thesis are focused on this specific area, in addition to other regions of the brain.
3.2. Results

3.2.1. Morphology and distribution of OPCs in the adult brain

OPCs were studied using immunohistochemistry for NG2-chondroitin sulfate proteoglycan (Cspg), an intermembrane protein expressed in their surface by which OPCs can be identified (Nishiyama et al., 1991). NG2 immunolabeling shows the distribution of OPCs all over the brain, in which they are arranged occupying a certain area without invading the field of the neighbor cell and with a light overlap of their processes (Fig. 3.1A; Fig. 3.2 A-F). In the CA1 (Fig. 3.1A; Fig. 3.2 A; Fig. 3.3 A) and the SL (Fig. 3.2E; Fig. 3.3B) OPCs present stellate morphology with long radial processes separated between them and a central irregular cell body, with the difference that in the CA1 the processes seem to be longer and less branched than in the SL. In the latter, OPCs are heavily packed in comparison with the CA1 where they are more spaced. The disposition of OPCs in the CA3 (Fig. 3.2B), DG (Fig. 3.2C) and Hilus (Fig. 3.2D) is similar to the one in the SL, with highly packed cells that present heavily tangled processes making it difficult to distinguish one cell from another. Interestingly, OPCs are rare in the granular cellular layers, to which they send few processes and OPC cell bodies are hardly ever found. This characteristic disposition makes possible the differentiation of the hippocampal regions by NG2 staining. Moreover, in the CA3 area, OPCs seem to follow the neuronal disposition in the form of a C. The distribution of OPCs in the CC (Fig. 3.2F) is notably different from the rest of the areas being less numerous and with their processes in a parallel disposition instead of radial, following the direction of the axons. This morphology is similar in the optic nerve (Fig. 3.6F), where the processes of the OPCs follow the axons. This suggests two different structures for OPCs depending on their location in grey or white matter. Consistent with this, OPCs in the Cortex (Fig. 3.6C) are
similar to the ones in the CA1. Figure 3.3 illustrates the apparent different morphologies that OPCs present at high magnification in the different areas of the hippocampus, although no specific measurements have been undertaken. On average, the dimensions of OPCs are 80µm x 60µm x 25µm aprox. (x, y and z dimensions). As described above, OPCs in the CA1 present a less complex morphology than in the rest of areas, with a central irregular cell body and between 4-8 radial primary processes with several branches each and multiple spines (Fig. 3.3A). In the SL (Fig. 3.3B), Hilus (Fig. 3.3C), DG (Fig. 3.3D) and CA3 (Fig. 3.3E) the cell bodies of OPCs seem to be bigger and the branches shorter and more ramified, with a high number of spines per branch. In addition, OPCs are the main proliferating glial cells in the adult brain (Dawson et al., 2003) and characteristically form duplets and triplets after division (Fig. 3.1B). These cells are called sister or daughter cells and they arrange their cell bodies facing each other and the processes together and towards one side of the cell.
Figure 3.1. Adult oligodendrocyte precursor cells (OPCs). (A) OPCs immunolabeled for NG2 in the CA1 of the hippocampus of the adult brain. White arrows show duplets of sister cells. (B) High magnification confocal image of a sister cells duplet in the CA1 of the adult hippocampus. Scale bars: 50 µm (A), 10 µm (B).
Figure 3.2. Distribution of adult oligodendrocyte precursor cells (OPCs) in different regions of the brain. (A-F) Low magnification confocal images of OPCs immunolabeled for NG2 in the CA1 (A), CA3 (B), DG (C), Hilus (D), SL (E) and CC (F) of the hippocampus of the adult brain. Scale bars: 50 µm.
Figure 3.3. Morphology of oligodendrocyte precursor cells (OPCs) in different regions of the brain. (A-E) High magnification confocal images of OPCs immunolabeled for NG2 in the CA1 (A), SL (B), Hilus (C), DG (D) and CA3 (E) of the hippocampus of the adult brain. Scale bars: 10 µm.
3.2.2. Physiological and synaptic characteristics of OPCs in the adult brain

The Kir4.1 potassium channel is known to be expressed by glial cells and specifically astrocytes, oligodendrocytes and OPCs (Kalsi et al., 2004). Immunolabeling for Kir4.1 and GFP was performed in brain slices of adult Pdgfra-CreERT2:Rosa26R-YFP mice. As explained above (see Materials and Methods chapter), Pdgfra-CreERT2:Rosa26R-YFP mouse express permanently YFP reporter protein in OPCs by induction of Cre recombinase expression under the control of Pdgfra promotor when they are injected with tamoxifen. OPCs were identified in different areas of the hippocampus, such as CA1 (Fig. 3.4A), DG (Fig. 3.4B), Hilus (Fig. 3.4C) and SL (Fig. 3.4D) by their stellate morphology and the immunolabeling for GFP. To determine the expression of the Kir4.1 channel by OPCs, a colocalization channel was generated using Volocity software. Kir 4.1 is colocalised with GFP in the cell body and processes of OPCs (Figure 3.4; yellow).

Synaptic proteins were identified by immunohistochemistry using antibodies against vGAT, Gephyrin, vGluT1 and PSD-95 (Figs. 3.5-3.8). vGAT and PSD-95 present a punctate distribution, while vGluT1 and Gephyrin form a mat all over the tissue (Figs. 3.5A, 3.6A, 3.7A, 3.8A). At high magnification, cross-section images of OPCs processes showed the direct apposition of these synaptic proteins to the OPCs (Figs. 3.5B, 3.6B, 3.7B, 3.8B), with hundreds of synapses located within ≤1 μm of OPC processes (Fig. 3.5B, 3.6B, 3.7B, 3.8B some indicated by arrows), which could mediate direct or spill-over synaptic signalling (Vélez-Fort et al. 2010). 3D isoform reconstruction shows OPCs processes embedded in a mass of synaptic proteins in the case of vGluT1 (Fig. 3.7C) and Gephyrin (Fig. 3.6C) and direct contact with synaptic puncta in the case of vGAT (Fig. 3.5C) and PSD-95 (Fig. 3.8C), which are considered to mediate rapid and direct neuron-OPC synaptic signalling (Orduz et al. 2015). Colocalisation channel (Figs. 3.5D, 3.6D, 3.7D, 3.8D; white dots) was generated for the synaptic proteins and the OPCs that they
were surrounding, and it showed clear points of contact between the OPCs and vGAT (Fig. 3.5D), Gephyrin (Fig. 3.6D), vGluT1 (Fig. 3.7D) and PSD-95 (Fig. 3.8D) not only along the processes, but also in the cell body of OPCs. Therefore, the results demonstrate that there are innumerable synapses within the process domains of individual OPCs (Fig. 3.5A, 3.6A, 3.7A). These results indicate that OPCs contact both glutamatergic and GABAergic synapses in the hippocampus, which supports electrophysiological studies (Bergles et al. 2010; Bergles et al. 2000; Lin & Bergles 2004).
Figure 3.4. Oligodendrocyte precursor cells (OPCs) express Kir4.1 potassium channel in different areas of the brain. (A-D) High magnification confocal images of OPCs immunolabeled for GFP (green) and Kir4.1 (red) in the CA1 (A), DG (B), Hilus (C), SL (D) of the hippocampus of the adult brain from a Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP mouse. Top inset: Detail of the OPCs (green). Bottom inset: Colocalisation channel (yellow) generated using Volocity (only the cell bodies of the cells were selected as regions of interest to generate the colocalization channel in this figure). Scale bars: 10 µm.
Figure 3.5. Oligodendrocyte precursor cells (OPCs) are interconnected to the presynaptic protein vGAT. (A) High magnification confocal image of an OPC immunolabeled for NG2 (green) and immunostained for vGAT (red) in the CA1 of the adult hippocampus. (B) Cross-section showing the relation of vGAT with OPCs. (C) 3D isoform of OPCs process (green) in contact with vGAT. (D) Synaptic proteins distribution around OPCs and points of colocalisation with the cell (white dots). White arrows show points of contact of synaptic proteins with OPCs. Scale bars: 10 μm.
Figure 3.6. Oligodendrocyte precursor cells (OPCs) are interconnected to the postsynaptic protein Gephyrin. (A) High magnification confocal image of an OPC immunolabeled for NG2 (green) and immunostained for Gephyrin (blue) in the CA1 of the adult hippocampus. (B) Cross-section showing the relation of Gephyrin with OPCs. (C) 3D isoform of OPCs process (green) in contact with Gephyrin. (D) Synaptic proteins distribution around OPCs and points of colocalisation with the cell (white dots). White arrows show points of contact of synaptic proteins with OPCs. Scale bars: 10 µm.
Figure 3.7. Oligodendrocyte precursor cells (OPCs) are interconnected to the presynaptic protein vGluT1. (A) High magnification confocal image of an OPC immunolabeled for NG2 (green) and immunostained for vGluT1 (red) in the CA1 of the adult hippocampus. (B) Cross-section showing the relation of vGluT1 with OPCs. (C) 3D isoform of OPCs process (green) in contact with vGluT1. (D) Synaptic proteins distribution around OPCs and points of colocalisation with the cell (white dots). White arrows show points of contact of synaptic proteins with OPCs. Scale bars: 10 µm.
Figure 3.8. Oligodendrocyte precursor cells (OPCs) are interconnected to the postsynaptic protein PSD95. (A) High magnification confocal image of an OPC immunolabeled for NG2 (green) and immunostained for PSD95 (blue) in the CA1 of the adult hippocampus. (B) Cross-section showing the relation of PSD95 with OPCs. (C) 3D isoform of OPCs process (green) in contact with PSD95. (D) Synaptic proteins distribution around OPCs and points of colocalisation with the cell (white dots). White arrows show points of contact of synaptic proteins with OPCs. Scale bars: 10 µm.
3.2.3. OPCs differentiation

OPCs undergo a process of differentiation in order to produce the myelin forming cells in the CNS. To study this process, I used IHC for several markers of different stages of OPCs differentiation in various mouse models such as Sox10-EGFP and Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP. Confocal images were taken and analysed using Volocity and ImageJ softwares to study co-expression of the reporter proteins in each of the animals used and the marker corresponding to each differentiation stage. Sox10\textsuperscript{+} cells in the mouse Hilus were always co-stained for NG2 (Fig. 3.9A). In the case of Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mice, there was also co-staining with NG2, identifying the OPCs (Fig. 3.9B, C). To study OPCs differentiation into immature OLs, Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP brain slices were immunolabelled for GPR17 (Fig. 3.9D). There was co-staining of GFP\textsuperscript{+} cells with GPR17 indicating the differentiation of OPCs, initially expressing Pdgfra. Some of this GFP\textsuperscript{+}/GPR17\textsuperscript{+} cells were found in duplets with GFP\textsuperscript{+} cells indicating that they were coming from sister OPCs that divided recently (Fig.3.9D). Antibodies against APC were used to identify cells that differentiated into mature oligodendrocytes and they were present and co-immunolabelled with GFP mainly in white matter tracts such as the CC (Fig. 3.9E) and optic nerve (Fig. 3.9F), since these mature APC\textsuperscript{+} oligodendrocytes are the cells in charge of the myelin formation.
Figure 3.9. Oligodendrocyte precursor cells (OPCs) express different lineage markers and differentiate into immature and mature OLs in different areas of the adult CNS. (A, F) Confocal images of OPCs immunolabeled for (A) NG2 (red) in a Sox10-EGFP mouse (green) CA1, (B) NG2 (red) and GFP (green) in a Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse optic nerve, (C) NG2 (red) and GFP (green) in a Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse Cortex, (D) GPR17 (red) and GFP (green) in a Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse CA1, (E) APC (red) and GFP (green) in a Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse CC and (F) APC (red) and GFP (green) in a Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse optic nerve. Scale bar: 50 µm in Ai, Aiii, Aiv and Av; 20 µm in Aii and Avi.
3.2.4. Morphology and distribution of other cells of the oligodendrocyte lineage in the adult brain

Immature OLs represent an intermediate stage between OPCs and mature OLs and are identified by immunostaining for GPR17. Immature GPR17+ OLs are distributed throughout the brain and, as OPCs, they present a stellate morphology, with a rounded cell body in the center and radial processes to the periphery (Fig. 3.10 A-H; Fig. 3.11 A-F). On average, their dimensions are 80µm x 60µm x 25µm (x, y and z dimensions). In the CA1 (Fig. 3.10A; Fig. 3.11A), CA3 (Fig. 3.10B), DG (Fig. 3.10C; Fig. 3.11D), Hilus (Fig. 3.10D; Fig. 3.11C), and Cortex (Fig. 3.10G; Fig. 3.11F), GPR17+ cells present long, fine and ramified radial processes (usually 4, but more in the case of the Dentate Gyrus) of around 50 µm long with several bifurcations ending in rounded terminals. In the case of the SL (Fig. 3.10E; Fig. 3.11B), the processes seem to be shorter and more ramified than in the rest of areas. Immature OLs in the CC (Fig. 3.10F; Fig. 3.11E) present primary processes that ramify and are disposed following the direction of the axons. They seem to adopt the same disposition in the optic nerve (Fig. 3.10H). In relation to the number of GPR17+ cells in each area analysed, this seems to be variable. GPR17+ cells seem to be less numerous and spaced far apart in the CA1 (Fig. 3.10A), CA3 (Fig. 3.10B), DG (Fig. 3.10C), Hilus (Fig. 3.10D) and optic nerve (Fig. 3.10C), while they appear to be more abundant and closer together in the SL (Fig. 3.10E), CC (Fig. 3.10F) and Cortex (Fig. 3.10G). Additionally, adult brain slices were immunostained with APC/CC1 to identify mature OLs, which are distributed throughout the brain (Fig. 3.12 A-H), although some areas seem to be more populated than others. In the CA1 (Fig. 3.12A), DG (Fig. 3.12C) and Hilus (Fig. 3.12D) there are few mature OLs, infrequently spaced without any evident pattern. In contrast, in the CA3 (Fig. 3.12B), SL (Fig. 3.12E), Cortex (Fig. 3.12B), CC (Fig. 3.12F) and optic nerve (Fig. 3.12H), APC+ cells are more abundant, especially in
CC and optic nerve, which are highly myelinated areas, where they appear close together and form rows of 3-5 OLs following the axons (Fig. 3.12F; H).
Figure 3.10. Distribution of immature oligodendrocytes in different regions of the adult brain. (A-H) Low magnification confocal images of immature OLs immunolabeled for GPR17 in the CA1 (A), CA3 (B), DG (C), Hilus (D), SL (E) and CC (F), Cortex (G) and Optic nerve (H) of the hippocampus of the adult brain. Scale bars: 50 µm.
Figure 3.11. Morphology of immature oligodendrocytes in different regions of the adult brain. (A-F) High magnification confocal images of immature OLs immunolabeled for GPR17 in the CA1 (A), SL (B), Hilus (C), DG (D), CC (E) and Cortex (F) of the hippocampus of the adult brain. Scale bars: 10 µm.
Figure 3.2. Distribution of mature oligodendrocytes in different regions of the adult brain. (A-H) Low magnification confocal images of mature OLs immunolabeled for APC (red) in the CA1 (A), CA3 (B), DG (C), Hilus (D), SL (E) and CC (F), Cortex (G) and optic nerve (H) of the hippocampus of the adult brain. Cells were counterstained with Hoechst blue (blue). Insets show only APC staining. Scale bars: 50 µm.
3.2.5. Myelin distribution in the adult brain

Myelin is essential for the correct function of the CNS and it is generated in the murine CNS from birth and throughout adulthood by mature OLs (Foran and Peterson, 1992). To study myelin disposition in the adult brain, slices from adult mice were immunolabeled with antibody against MBP, which is one of the main components of myelin, and imaged using confocal microscopy (Fig. 3.13). Of all the areas analysed, the CA1 (Fig. 3.1A) had the less MBP⁺ staining, presenting more MBP levels in the stratum oriens than in the stratum radiatum. The stratum lacunosum-moleculare of the CA1 was highly myelinated with bundles of myelinated axons, heavily positive for MBP, in the superior part (Fig. 3.13E). In the CA3, MBP was disposed in the granular cell layer and in the molecular layer (Fig. 3.13B), whereas in the DG (Fig. 3.13C) MBP⁺ areas were mainly in the molecular layer, and the granular cell layer was free from MBP immunostaining. The Hilus was also packed with MBP⁺ myelinated axons in the molecular region (Fig. 3.13D). The CC (Fig. 3.13F) was packed with longitudinal MBP⁺ myelinated axons, being the region with stronger MBP staining. The Cortex (Fig. 3.13G) was also heavily myelinated with MBP⁺ axons running from the inner cortical layers to the outer ones. In the case of the Striatum (Fig. 3.13H), the MBP⁺ myelinated axons were disposed in thick bundles.
Figure 3.13. Distribution of myelin in different regions of the adult brain. (A-H) Low magnification confocal images of MBP immunolabeling (red) in the CA1 (A), CA3 (B), DG (C), Hilus (D), SL (E), CC (F), Cortex (G) and Striatum (H) in the adult brain. Scale bars: 50 µm.
3.3. Discussion

My results show that OPCs are stellate cells with a complex branched morphology and that they are disposed ubiquitously throughout the adult brain. Moreover, I show in this chapter that OPCs express the Kir4.1 potassium channel, as well as that OPCs have intimate relations with synapses as identified by well-known glutamatergic and GABAergic synaptic markers. I have also shown that they proliferate and form duplets or sister cells and differentiate into immature and mature OLs, expressing different lineage markers in each differentiation stage in the adult brain. My results also illustrate the different distribution of immature and mature OLs in the different regions of the hippocampus and other parts of the brain.

3.3.1. Oligodendroglial cells have different morphology and distribution in different regions of the adult brain

OPCs have a characteristic morphology with several radial branches and irregular cell bodies, as my NG2 immunolabeling shows. OPCs have been shown to express simultaneously the lineage markers NG2 and Pdgfra (Nishiyama et al., 1991, 1996). They also express, as the rest of cells of the oligodendrocyte lineage do, the transcription factors Sox10 (Li et al., 2007; Takada et al., 2010) and Olig2 (Dimou et al., 2008; Zhu et al., 2012). OPCs are distributed throughout the brain, in grey and white matter (Dawson et al., 2003; Hill et al., 2011), although their morphology in both of these areas is different, being radial in grey matter areas and longitudinal but following the axons direction in white matter areas. My NG2 immunolabeling shows the existence of duplets, also called sister or daughter cells in the adult brain. It has been shown in previous publications that OPCs represent the major population of cycling cells in the adult brain (Dawson et al., 2003) and although their cell cycle slows down with age (Psachoulia, Jamen, Kaylene M.
Young et al., 2009) they undergo asymmetric division to assure the replacement of OLs throughout life (Boda et al., 2015). Immature oligodendrocytes present a morphology similar to OPCs, with several branches but a more rounded cell body. They are identified by GPR17 expression and they have been reported to be generated from OPCs (Lecca et al., 2008; Chen et al., 2009; Fumagalli et al., 2011; Viganò et al., 2016). GPR17+ cells appear to be less abundant in the brain than OPCs, although they appear to be more numerous in heavy myelinated areas than in grey matter regions. Mature OLs are identified by expression of APC (CC1) (Lang et al., 2013) and their distribution pattern is quite similar to the one for GPR17+ cells, since they appear to be more numerous in areas where myelination was abundant but not specific measurements have been done.

3.3.2. OPCs are intimately related to synapses

My results show how several inhibitory and excitatory synaptic markers, such as vGAT, Gephyrin, vGluT1 and PSD-95 are associated to OPCs processes and cell body and they share several points of colocalization with NG2 in the CA1 of the hippocampus of the adult brain. vGAT is a vesicular GABA transporter localized to synaptic vesicles in the presynaptic terminal of GABAergic and Glycinergic neurons (Chaudhry et al., 1998), while vGluT1 it is a vesicular glutamate transporter localized to synaptic vesicles in the presynaptic terminal of glutamatergic neurons and it has been shown to be present in CA1-3, DG and Hilus of the hippocampus among other regions of the brain (Vigneault et al., 2015). Furthermore, both vGAT and vGluT1 have been shown to colocalize in the hippocampus (Herzog et al., 2006). vGluT1 has been reported to be related to OPCs in several areas of the brain such as hippocampus and CC (Ziskin et al., 2007), similarly to the postsynaptic protein gephyrin which it has been shown to be associated to OPCs in the locus coeruleus (Seifi et al., 2014). Additionally, it has been shown the close relation
of neurons and OPCs (Wigley and Butt, 2009) and that the latter contact nodes of Ranvier in the adult CNS (Butt et al., 1999). I have shown in this chapter, using immunohistochemistry, the expression of Kir4.1 inwardly rectifying potassium channel in OPCs with clear colocalization between the OPCs and the channels. Kir4.1 is also expressed in other glial cells such as astrocytes and OLs and their role is to maintain the membrane resting potential in the cell, which is important for myelin stability as well as to eliminate the excess of K⁺ after action potentials (Kalsi et al., 2004; Brasko et al., 2017). The role of Kir4.1 in OPCs is not totally understood, but it might be to sense K⁺ levels (Maldonado et al., 2013) and it has been reported during the writing of this PhD that Kir4.1 in OPCs is essential to maintain the integrity of myelin in development and ischemia in studies with a conditional knockout mice (Duan et al., 2018). Moreover, RNA-seq studies in OPCs have shown that Kir4.1 potassium channel expression in OPCs is higher than the expression of any other channel, demonstrating also the expression by OPCs of many other molecules such as other ion channels - Na⁺, Ca²⁺, Cl⁻, HCN and TRP - and receptors for glutamate and GABA among those for other neurotransmitters and neuromodulators and this has been also analysed using microarray studies (Cahoy et al., 2008; Zhang et al., 2014; Larson et al., 2015). The expression of different ion channels and receptors and the close relation of OPCs with neurons and synapses suggest that they may respond to synaptic inputs.

3.3.3. OPCs differentiate into mature myelinating OLs in the adult brain

My results show that the hippocampus is densely populated by oligodendrocyte lineage cells and is highly myelinated, which is not generally appreciated for this grey matter region in the literature, although OLs and myelin are much denser and more packed in white matter tracks, such as CC and optic nerve. I also showed the differentiation of OPCs
into immature OLs and mature OLs in the adult brain and optic nerve. This is consistent with previous studies that showed OPCs proliferation and differentiation in the adult and ageing brain (Psachoulia et al., 2009). The signals that make OPCs proliferate or differentiate are not entirely known, but previous studies show that OPCs respond to glutamate and GABA and these influence OPCs proliferation and differentiation (Lin and Bergles, 2004). In the case of glutamate, it has been shown to be released in vesicles in the white matter unmyelinated axons and act in OPCs, generating electrical currents through AMPA receptors (Kukley et al., 2007; Ziskin et al., 2007), and Kainate receptors are also implicated in OPCs response to glutamate (Kukley and Dietrich, 2009). Moreover, it has been reported in previous publications that proliferation of OPCs is dependent on the electrical activity of axons and suppression of this electrical activity inhibits OPCs proliferation (Barres and Raff, 1993). Contrary, other studies report an increase on OPCs proliferation after inhibition of neuronal activity, AMPA receptors (Fannon et al., 2015) or vesicular release (Gautier et al., 2015). GABA has also been reported to influence OPCs, producing depolarization of their membrane by acting on GABA$_A$ receptors through direct synapsis between OPCs and interneurons in the hippocampus (Lin and Bergles, 2004). Additionally to direct synapses between OPCs and neurons, these cells can also communicate by spillover of neurotransmitters released without necessity of direct contact (Maldonado et al., 2011). OPC differentiation into mature myelinating OLs has also been reported to be controlled by neuronal activity and previous studies show that blockade of neuronal electrical activity using TTX in developmental mice (Fannon et al., 2015) or in models of MS (Gautier et al., 2015) impairs myelination and remyelination. Interestingly, it has been reported that blockade of neuronal activity and AMPA receptors has an effect on OPCs morphology reducing the lengths of their processes and their branching (Fannon et al., 2015).
3.4. Summary and Conclusions

Overall, my results in this chapter show the distribution of OPCs and their progeny in the hippocampus, which is the focus of this thesis, together with other areas of the brain. This chapter also confirms that recently divided OPCs can be identified by the presence of cycling sister cells in the adult hippocampus and other brain regions. Additionally, I show differences in the morphology and distribution of OPCs, immature OLs and mature myelinating OLs. The differentiation of OPCs into immature OLs and mature OLs in the adult brain is also confirmed in this chapter. Further, I analysed the distribution of myelin in several areas of the adult brain. The colocalization of pre- and post-synaptic proteins with OPCs demonstrate that OPCs are closely related to synapses, but I did not detect clear expression of these proteins in OPCs, although it has been shown in the literature using RNAseq techniques that OPCs express all of them (Zhang et al., 2014). Finally, I demonstrate the expression of Kir4.1 potassium channel by OPCs.

This chapter sets the basis for the subsequent chapters in this thesis with the description of several characteristics related to OPCs in normal adult brain and some of these features will be analysed further.
Chapter 4

Effect of synaptic silencing on oligodendrocyte precursor cells in the adult hippocampus
4.1. Introduction and Aims

Oligodendrocyte precursor cells (OPCs) are a significant population of cells in the adult brain and can be identified by their expression of PDGFαR and NG2 (Cspg4), which has led to them often being referred to as NG2-glia (reviewed in Polito and Reynolds, 2005; Trotter et al. 2010). The function of OPCs is to generate new oligodendrocytes throughout life, which are required to myelinate new connections formed in response to new life experiences and to replace myelin lost through natural ‘wear and tear’ and disease (Young et al. 2013; Hill et al. 2014). The life-long generation of OLs from OPCs is regulated by multiple factors, both intrinsic and extrinsic, including growth factors such as PDGF and FGF2, which promote OPC proliferation, and transcription factors such as Sox10 and Olig2, which are essential for OL differentiation (Miller 2002; McTigue and Tripathi, 2008).

Notably, OPCs have a complex branched morphology and extend process to contact synapses and nodes of Ranvier, by which they sense neuronal electrical and chemical activity (Butt et al. 1999; Bergles et al. 2000; Hamilton et al., 2009). The effect of neuronal activity on OPCs is unclear, but there is evidence that OPC proliferation and differentiation are regulated by neuronal electrical activity, since inhibition of neuronal activity by TTX in the optic nerve has shown to decrease OPCs number, the activation of neuronal activity using optogenetics in cortex increased OPCs proliferation and differentiation into mature OLs, as well as myelination (Barres & Raff 1993; Gibson et al. 2014), and the neurotransmitters glutamate and GABA play an important role in OPCs proliferation and differentiation since blocking AMPA receptors promotes proliferation and differentiation and GABA acting on GABA_A receptors reduces the number of oligodendrocyte lineage cells (Fannon et al. 2015; Hamilton et al. 2016). Significantly, the NG2 CSPG complexes with AMPA-type glutamate receptors and is important for glutamatergic synaptic signalling (Stegmüller et al. 2003; Sakry et al. 2014), and
disruption of NG2 reduces OPC proliferation, migration and differentiation (Biname et al., 2013; Huang et al. 2014; Huang et al., 2016; Kucharova and Stallcup, 2010; Kucharova & Stallcup 2015). Thus, the balance of evidence leads to the hypothesis underpinning this section, that synaptic signalling has an important modulatory effect on OPCs, potentially regulating their morphology, proliferation and their differentiation into myelinating OLs. The aim of this section is to test this directly, by examining the response of OPCs to synaptic silencing in the adult mouse hippocampus following in vivo injection of BoNT/A, which causes prolonged blockade of neuroexocytosis by cleaving the SNARE protein SNAP-25 (Fig. 4.1) and disrupting the presynaptic machinery (Antonucci et al. 2008; Dolly, 2003; Dolly and Aoki, 2006).

**Figure 4.1. Diagram of neuroexocytosis.** The SNARE protein SNAP-25 interacts with other SNARE proteins (Syntaxin and VAMP, respectively) facilitating the fusion of the synaptic vesicle full of neurotransmitters with the presynaptic membrane, and their release into the synaptic cleft where the neurotransmitters bind to their respective receptors in the postsynaptic membrane. BoNT/A cleaves SNAP-25 (cleavage site illustrated by broken lines) preventing the interaction of the v-SNARE VAMP and SNAP-25 and producing blockade of neuroexocytosis by disruption of the presynaptic machinery (Modified from Dolly, O., 2003; Dolly, J.O. and Aoki, K. R., 2006).
4.2. Results

4.2.1. Effect of synaptic silencing by BoNT/A on OPC synaptic contacts in the CA1 region of the hippocampus

Unilateral injection of BoNT/A has been shown to result in persistent blockade of hippocampal synaptic activity for up to 120 days (Antonucci et al. 2008). To examine whether synaptic silencing affects OPC synaptic connectivity, hippocampi were analysed 14 days after injection of BoNT/A or vehicle in controls (tissue provided by Prof Hugh Perry and Dr Diego Gómez-Nicola, University of Southampton, originally from Prof Matteo Caleo, Institute of Neuroscience, Pisa, Italy). Both ipsilateral (injected) and contralateral (non-injected) hippocampi were analysed (Fig. 4.2; Fig. 4.3; Fig. 4.4; Fig. 4.5), since BoNT/A is retrogradely transported and cleaves SNAP-25 in the contralateral hippocampus after unilateral delivery (Antonucci et al. 2008). Hippocampal sections were immunofluorescence labeled and examined by confocal microscopy, as described above (see Materials and methods chapter). Compared to controls, PSD95 immunostaining appeared heavier following synaptic silencing by BoNT/A, both ipsilaterally (Fig. 4.2Ai, Aii) and contralaterally (Fig. 4.2Aiii, Aiv); similar observations were made for vGluT1 and vGAT immunostaining (Fig. 4.3Ai-Aiv and Fig. 4.4Ai-Aiv, respectively). Consistent with this, the relative PSD95+/NG2+ index was significantly higher in both ipsilateral and contralateral hippocampi (Fig. 4.2Av); this was supported by the analysis of the number of PSD-95 puncta per OPC, which were also increased proportionally in both hippocampi, but was statistically significant at the 95% confidence limit contraterally and not ipsilaterally (Fig. 4.2 Avi). For vGluT1 and vGAT, there were no clear differences between the groups and the data were too variable to draw conclusions, although the immunostaining indexes for vGluT1/NG2 (Fig. 4.3B) and vGAT/NG2 (Fig. 4.4B) were significantly increased contralaterally following BoNT/A. Overall, the results indicate
that immunostaining for synaptic markers is increased following synaptic silencing with BoNT/A and the PSD95 data indicates there is a corresponding increase in associations of OPCs with these synapses.
Figure 4.2. Effect of synaptic silencing by BoNT/A on OPC associations with the synaptic protein PSD95 in the CA1 of the hippocampus. Hippocampi were examined following injection of BoNT/A to silence synaptic activity, compared to vehicle treated controls, using double immunofluorescence labelling for NG2 to identify OPCs and the postsynaptic protein PSD95. (Ai-iv) Representative confocal projections of individual OPCs in the ipsilateral injected hippocampus (Ai, Aii) and contralateral non-injected hippocampus (Aiii, Aiv), in vehicle injected controls (Ai, Aiii) and BoNT/A injected (Aii, Aiv), illustrating NG2 (green) and PSD95 (blue). Scale bars = 10µm. (B) Box-whisker plots of PSD95/NG2 index (n=10 cells from 4 animals; 1 slice/animal and 2-3 cells/slice). (C) Box-whisker plots of PSD95 puncta/NG2 cell (n=10 cells from 4 animals; 1 slice/animal and 2-3 cells/slice). Data are mean ± SEM, ns= no significant, **p<0.01, Unpaired t-test.
Figure 4.3. Effect of synaptic silencing by BoNT/A on OPC associations with the synaptic protein vGlut1 in the CA1 of the hippocampus. Hippocampi were examined following injection of BoNT/A to silence synaptic activity, compared to vehicle treated controls, using double immunofluorescence labelling for NG2 to identify OPCs and the presynaptic protein vGlut1. (Ai-iv) Representative confocal projections of individual OPCs in the ipsilateral injected hippocampus (Ai, Aii) and contralateral non-injected hippocampus (Aiii, Aiv), illustrating NG2 (green) and vGlut1 (red). Scale bars = 10µm. (B) Box-whisker plots of vGLUT1/NG2 index (B, n=10 cells from 4 animals; 1 slice/animal and 2-3 cells/slice). Data are mean ± SEM, ns= no-significant, *p<0.05, Unpaired t-test.
Figure 4.4. Effect of synaptic silencing by BoNT/A on OPC associations with the synaptic protein vGAT in the CA1 of the hippocampus. Hippocampi were examined following injection of BoNT/A to silence synaptic activity, compared to vehicle treated controls, using double immunofluorescence labelling for NG2 to identify OPCs and the presynaptic protein vGAT. (Ai-iiv) Representative confocal projections of individual OPCs in the ipsilateral injected hippocampus (Ai, Aii) and contralateral non-injected hippocampus (Aiii, Aiv), in vehicle injected controls (Ai, Aiii) and BoNT/A injected (Aii, Aiv), illustrating NG2 (green) and vGAT (red). Scale bars = 10µm. (B) Box-whisker plots of vGAT/NG2 index (B, n=10 cells from 4 animals; 1 slice/animal and 2-3 cells/slice). Data are mean ± SEM, ns= no-significant, **p<0.01, Unpaired t-test.
4.2.2. Effect of synaptic silencing by BoNT/A on numbers of OPCs and sister cells in the CA1 region of the hippocampus

I focused my analysis of OPCs on the CA1 region of the hippocampus (Fig. 4.5), because complete synaptic silencing has been demonstrated both ipsilaterally and contralaterally in CA1 following BoNT/A mediated cleavage of SNAP-25 (Antonucci et al. 2008), and OPCs have been shown to contact and respond electrically to synaptic signaling in this region (Bergles et al. 2000; Lin & Bergles 2004), consistent with my data above (see Chapter 3 Fig. 3.5, Fig. 3.6, Fig. 3.7, Fig. 3.8). Additional analyses were performed in the CA3 region and DG (Table 4.1), which also display significant SNAP-25 cleavage following unilateral BoNT/A injection (Antonucci et al. 2008); the DG is also of interest as a potential site of adult oligodendrogenesis following altered neuronal activity in stress and epilepsy models (Chetty et al., 2015; Luo et al., 2015), although parenchymal OPCs appear to be the primary source of new oligodendrocytes not the DG (Bonaguidi et al., 2011).

There was an evident decrease in the number of OPCs in both the ipsilateral and contralateral CA1 region 14 days after BoNT/A injection compared to controls (Fig. 4.5Ai-ii, Bi-ii), and this was equivalent and statistically significant ipsilaterally (Fig. 4.5 Aiii; $p<0.01$, Unpaired t-test) and contralaterally (Fig. 4.5 Biii; $p<0.001$, Unpaired t-test). Likewise, the numbers of OPCs were decreased in the ipsilateral and contralateral CA3 and DG (Table 4.1); this was statistically significant in both ipsi- and contralateral CA3, whereas although a similar decrease in cell number was observed in both ipsi- and contralateral DG, only the former was statistically significant (Table 4.1). Similar to stem cells, OPCs self-maintain by undergoing asymmetric divisions generating sister cells that initially maintain NG2 expression prior to one of them proceeding to differentiation (Fig. 4.5Aii, inset), and the number of NG2$^+$ sister cells is therefore used as a measure of the number of OPCs that have recently divided (Boda et al. 2015). Counts of sister cells
revealed no significant effect of synaptic silencing in the ipsilateral or contralateral CA1 (Fig. 4.5Bi, Biv) or the other regions analysed (Table 4.1). The results demonstrate a significant decrease in the number of OPCs in the hippocampus by approximately 40% after 14 days of synaptic silencing, but no evidence of altered cell division was detected at this time point.
Figure 4.5. Effect of synaptic silencing by BoNT/A on OPC numbers in the CA1 of the hippocampus. Hippocampi were examined following injection with BoNT/A to silence synaptic activity, compared to vehicle treated controls, using immunofluorescence labelling for NG2 (green) to identify OPCs and counterstained with Hoechst blue (blue) for nuclei (red line shows delimitation of the región analysed). Representative confocal projections of (Ai, Aii) the ipsilateral injected hippocampus, and (Bi, Bii) the contralateral non-injected hippocampus, (Ai, Bi) in vehicle injected controls, (Aii, Bii) and BoNT/A injected. Insets in Ai, Bi, Bii indicate individual OPCs and their process domain (white line) and inset in Aii illustrates NG2+ sister cells. Scale bars: 50 µm in main panels, 20 µm in insets. (Aiii-Aiv,Biii,Biv) Box-whisker plots of the total number of NG2+ OPCs (Aiii, Biii) and number of sister cells (Aiv-Biv) per mm2 in the ipsilateral and contralateral hippocampus. Data are expressed as Mean±SEM. **p<0.01; ***p<0.001; Unpaired t-test, n=4 animals per each group; 2 slices/animal.
Table 4.1. Effect of synaptic silencing by BoNT/A on numbers of OPCs in the hippocampus

<table>
<thead>
<tr>
<th></th>
<th>OPCs number</th>
<th>Sister cells</th>
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<tbody>
<tr>
<td></td>
<td>Ipsilateral</td>
<td>Contralateral</td>
</tr>
<tr>
<td>Vehicle</td>
<td>289 ± 17.36</td>
<td>206.5 ± 13.3</td>
</tr>
<tr>
<td>CA1</td>
<td><strong>p&lt;0.01</strong></td>
<td>*<strong>p&lt;0.001</strong></td>
</tr>
<tr>
<td>DG</td>
<td>284.5 ± 16.53</td>
<td>221.4 ± 13.66</td>
</tr>
<tr>
<td></td>
<td>*p&lt;0.05</td>
<td>*p&lt;0.05</td>
</tr>
<tr>
<td>CA3</td>
<td>291.3 ± 16.41</td>
<td>226.8 ± 12.22</td>
</tr>
</tbody>
</table>
4.2.3. Effect of synaptic silencing by BoNT/A on OPC process domains and coverage in the CA1 region of the hippocampus

As above, my analysis of OPCs was focused on the CA1 region of the hippocampus (Fig. 4.6), together with the CA3 and the DG (Table 4.2). Confocal examination indicated that OPC process domains appeared shrunken and their coverage of the hippocampus appeared reduced following BoNT/A compared to controls (Fig. 4.6). To examine this, the area of the process domain of each OPC was measured by drawing a line by hand from the end of one process to the end of the next one until the cell was surrounded (Fig. 4.6 Ai-Aii, Bi-ii), the area surrounded by the line was measured as OPC coverage and in addition this was normalized to the total area analysed, to provide a measure of the overall coverage by OPCs. Additionally, the total area covered by OPCs was measured using ImageJ (Relative NG2 density). In the CA1 region, OPC process domains and overall coverage were reduced following synaptic silencing, but statistical significance at the 95% confidence level was demonstrated only for process domains (coverage) in the contralateral hippocampus (Fig. 4.6 Aiii-iv, Biii-iv; p<0.001, Unpaired t-test). In the CA3, OPC coverage was decreased in both ipsi- and contralateral hippocampi, but the decrease was greater and statistically significant only in the former (Table 4.2); OPC coverage was not altered in the DG, although there was an apparent significant decrease in their process fields in the contralateral DG (Table 4.2; p<0.001, Unpaired t-test). Overall, the quantitative analysis supported qualitative observations that OPC process domains and coverage were reduced following synaptic silencing.
Figure 4.6. Effect of synaptic silencing by BoNT/A on OPC process domain and coverage in the CA1 of the hippocampus. Hippocampi were examined following injection with BoNT/A to silence synaptic activity, compared to vehicle treated controls, using immunofluorescence labelling for NG2 (green) to identified OPCs. High magnification confocal projections of OPCs and their processes domain (indicated by broken White lines) in the ipsilateral injected hippocampus (Ai, Aii), and the contralateral non-injected hippocampus (Bi, Bii), in vehicle injected controls (Ai, Bi), and BoNT/A injected (Aii, Bii). Scale bars: 20 µm. (Aiii-Aiv-Biii,Biv) Box-whisker plots of the area covered by OPC process domain/area analysed (Aiii, Biii) and density of the CA1 area covered by OPCs (Aiv-Biv) per mm2 in the ipsilateral and contralateral hippocampus. Data are expressed as Mean±SEM. ***p<0.001; Unpaired t-test, n=4 animals per each group; 2 slices/animal; 2-3 cells/slice for NG2 cell coverage index.
Table 4.2. Effect of synaptic silencing by BoNT/A on OPC process domains and coverage in the hippocampus

<table>
<thead>
<tr>
<th></th>
<th>Process domain</th>
<th></th>
<th>OPC density</th>
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<tr>
<td></td>
<td></td>
<td>Ipsilateral</td>
<td>Contralateral</td>
<td>Ipsilateral</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>BoNT/A</td>
<td>Vehicle</td>
<td>BoNT/A</td>
</tr>
<tr>
<td>CA1</td>
<td>0.01874 ± 0.0007163</td>
<td>0.01697 ± 0.0007908</td>
<td>0.01968 ± 0.001071</td>
<td>0.01429 ± 0.0007217</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>***p&lt;0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DG</td>
<td>0.01715 ± 0.0008413</td>
<td>0.0169 ± 0.00119</td>
<td>0.02199 ± 0.0009142</td>
<td>0.01489 ± 0.0007552</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>****p&lt;0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CA3</td>
<td>0.01591 ± 0.000919</td>
<td>0.01506 ± 0.0008513</td>
<td>0.01516 ± 0.001013</td>
<td>0.01373 ± 0.0004486</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>**p&lt;0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>
4.2.4. Effect of synaptic silencing by BoNT/A on OPC morphology in the CA1 region of the hippocampus

OPCs contact synapses via their processes and display dynamic changes in their morphology in response to injury, demyelination and other changes in their environment (Levine 1994; Rhodes et al., 2006). Hence, the apparent shrinkage observed above may reflect a retraction of processes in response to synaptic silencing. To examine this possibility, the morphology of OPCs was examined in the ipsilateral CA1 area following BoNT/A treatment compared to controls. I performed the Neurolucida cell tracing in Prof. Olivier Raineteau’s lab (University of Lyons, France) and due to insufficient time only the ipsilateral CA1 was analysed. It is evident that OPCs displayed a range of morphologies in controls and following BoNT/A treatment, but the simplest cells were observed in the BoNT/A group and the most complex cells in controls (Fig. 4.7). Quantification (n=12 cells from 4 animals/sections, 3 cells per section/animal from BoNT/A treated and control; tested for statistical significance by Mann Whitney tests) confirmed that the number of processes per cell (Fig. 4.8A), process lengths (meaning sum of the length of the processes in a cell) (Fig. 4.8B), number of end points (Fig. 4.8C), and number of nodes or branch points (Fig. 4.8D) were all decreased following synaptic silencing with BoNT/A compared to controls (p values provided on graphs in Fig. 4.8) (for clarification about these terms, see Fig. 4.9). These data clearly indicate that OPC morphology was altered by synaptic silencing and this was examined further using a Sholl analysis (Sholl, 1953); a grid formed by concentric circles located equidistantly 5μm apart was placed on the cell, with the cell body in the centre (Fig. 4.9). Multiple morphological parameters were measured, as indicated in Figure 4.10, and tested for statistical significance using two-way ANOVA followed by Sidak’s multiple comparisons test (p values provided on graphs in Fig. 4.10). The data indicate that the most marked changes in OPC processes were within 20-30 μm of the cell body, with significantly reduced
number of nodes or branch points (Fig. 4.10A), number of process terminals (Fig. 4.10B) and process lengths (Fig. 4.10C). In addition, the processes length in the different branch orders (1\textsuperscript{st} order branches closest to the cell body, 2\textsuperscript{nd} order branches the ones after the first ramification point, etc) revealed a marked shrinkage in the distal branches following synaptic silencing, and the maximum branch order was decreased to 13 compared to 15 in controls (Fig. 4.10D). Overall, the multiple morphological analyses confirm the qualitative observation that OPCs are shrunken following synaptic silencing and indicate this is due to reduced branching and decreased process length.
Figure 4.7. Heterogeneous morphology of OPCs in the CA1 hippocampus. Confocal images of OPCs in the ipsilateral CA1 of the hippocampus injected with vehicle (A-C) or BoNT/A (D-F) ranked from most complex (left-hand in each panel) to simplest (right-hand in each panel). (Ai-Aiii and Di-Diii) Images created with Neurolucida 360 (MBF Bioscience). (Bi-Biii and Ei-Eiii) Projections of OPCs created with Volocity software (PerkinElmer). (Ci-Ciii and Fi-Fiii) 3D isosurface rendering of OPCs created with Volocity software (PerkinElmer). Scale bars: 10µm.
Figure 4.8. Effects of synaptic inhibition on OPC morphology in the ipsilateral CA1 after BoNT/A injection. Data were generated by Neurolucida analysis of cells, as illustrated in Fig 4.5A, D. Box-whisker plots of (A) Number of processes per cell. (B) Length of processes. (C) Number of end points. (D) Number of nodes (points of ramification of the processes). Data are Mean ± SEM. *p<0.05; Mann Whitney test, n=11 or 12 cells as indicated from 4 animals (sections) per group.
Figure 4.9. Schematic representation of a Sholl analysis. The red dot in the centre represents the cell body and the red lines represent the cell processes. The concentric circles are termed Sholl shells and are placed at an equidistant position apart. The points of branching of the processes are called nodes (blue dots) and the points where the processes intersect the Sholl shells are named intersections (green dots). This method allows to detect morphological changes in the cell. Adapted from Sholl 1953 and Rietveld et al. 2015.
Figure 4.10. Morphological changes in OPCs in the ipsilateral CA1 of the hippocampus following synaptic inhibition with BoNT/A. Data were generated by Sholl analysis of confocal microscopic images using Neurolucida 360. (A) Number of nodes or branch points. (B) Number of process terminals or end points. (C) Processes length. (D) Length of processes of different branch orders. Data are Mean ± SEM. **p<0.01, ***p<0.001, two-way ANOVA followed by Sidak’s multiple comparisons test; n = 12 cells from 4 animals (sections) per group.
4.2.5. Effect of synaptic silencing by BoNT/A on OPC differentiation and myelination

As OPCs differentiate into mature OLs, they pass through a number of distinct stages that can be identified: first, OPCs lose expression of NG2 and Pdgfra and gain GPR17, which identifies post-mitotic immature OLs, and these cells lose GPR17 as they begin to express myelin proteins, such as MBP, and differentiate into mature myelinating OLs (Boda et al. 2011; Fumagalli et al. 2011). GPR17+ OLs provide a pool of undifferentiated cells that can differentiate rapidly into OLs following injury or demyelinating insults (Viganò et al. 2016). In this section, I analysed the number of Gpr17+ cells and the density of MBP immunostaining using ImageJ to examine whether OPC differentiation and myelination are altered following synaptic signalling. As above, I focused on the CA1 region (Figs. 4.11 and 4.12), together with the CA3 and DG (Table 4.3); for ease of comparison, cell counts for NG2+ OPCs from Table 4.1 are also included in Table 4.3. In contrast to OPCs, which were decreased in all the regions, no significant differences were found for the number of GPR17+ cells following BoNT/A injection compared to controls (Fig. 4.11, Table 4.3, Unpaired t-test). In contrast, MBP density appeared increased throughout the hippocampus following synaptic silencing by BoNT/A, both ipsilaterally and contralaterally, as illustrated for the CA1 region (Fig. 4.12), but none of these changes were statistically significant at the 95% confidence level (Table 4.3); these findings are promising, but due to limited availability of tissue the ‘n’ values are low (n=3 or 4 animals; 1 slice per animal) and further replicates are required.
Table 4.3. Effect of synaptic silencing by BoNT/A on OPC differentiation in the hippocampus

<table>
<thead>
<tr>
<th></th>
<th><em>NG2+ cells</em></th>
<th>GPR17+ cells</th>
<th>MBP immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ipsilateral</td>
<td>Contralateral</td>
<td>Ipsilateral</td>
</tr>
<tr>
<td>Vehicle</td>
<td>289 ± 17.36</td>
<td>206.5 ± 13.3</td>
<td>288.9 ± 9.821</td>
</tr>
<tr>
<td>BoNT/A</td>
<td><strong>p&lt;0.01</strong></td>
<td><strong>p&lt;0.001</strong></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4.078 ± 4.078</td>
<td>20.16 ± 9.119</td>
<td>11.47 ± 2.676</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.363 ± 0.01377</td>
<td>0.4865 ± 0.04759</td>
<td>0.3857 ± 0.03315</td>
</tr>
<tr>
<td>BoNT/A</td>
<td><em>p&lt;0.05</em></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DG</td>
<td>284.5 ± 16.53</td>
<td>221.4 ± 13.66</td>
<td>257 ± 16.36</td>
</tr>
<tr>
<td></td>
<td><em>p&lt;0.05</em></td>
<td><em>p&lt;0.05</em></td>
<td>16.21 ± 2.571</td>
</tr>
<tr>
<td></td>
<td>66.54 ± 14.7</td>
<td>63.63 ± 17.75</td>
<td>0.5229 ± 0.05298</td>
</tr>
<tr>
<td></td>
<td>0.4354 ± 0.08543</td>
<td>0.6854 ± 0.06647</td>
<td>NS</td>
</tr>
<tr>
<td>CA3</td>
<td>291.3 ± 16.41</td>
<td>226.8 ± 12.22</td>
<td>312.7 ± 13.02</td>
</tr>
<tr>
<td></td>
<td>0.6509 ± 0.06603</td>
<td>0.8431 ± 0.03507</td>
<td>0.6794 ± 0.04616</td>
</tr>
<tr>
<td></td>
<td><strong>p&lt;0.01</strong></td>
<td>NS</td>
<td>NS</td>
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</table>

*same data presented in Table 1; **n=2*
Figure 4.11. Effect of synaptic silencing by BoNT/A on number of GPR17+ immature oligodendrocytes in the CA1 of the hippocampus. (Ai-Aii, Bi-Bii) Representative confocal images of Gpr17 immunopositive cells (green) in the ipsilateral (Ai, Aii) and contralateral (Bi, Bii) CA1 region of the hippocampus, in controls injected with saline (Ai, Bi) or BoNT/A (Aii, Bii). (Aiii, Biii) Number of GPR17+ cells (per mm$^2$) in the ipsilateral (Aiii) and contralateral (Biii) CA1 region of the hippocampus, comparing controls (blue bars) and BoNT/A (red bars). Data expressed as Mean ± SEM. ns = not significant, Unpaired t-test. Scale bars=50µm.
Figure 4.12. Effect of synaptic silencing by BoNT/A on myelination in the CA1 of the hippocampus. (Ai-Aii, Bi-Bii) Representative confocal images of MBP immunostaining (red) in the ipsilateral (Ai, Aii) and contralateral (Bi, Bii) CA1 region of the hippocampus, in controls injected with saline (Ai, Bi) or BoNT/A (Aii, Bii). (Aiii, Biii) Density of MBP immunostaining measured as the number of MBP⁺ pixels per constant FOV, using ImageJ, in the ipsilateral (Aiii) and contralateral (Biii) CA1 region of the hippocampus, comparing controls (blue bars) and BoNT/A (red bars). Data expressed as Mean ± SEM. ns = not significant, *p<0.05; Unpaired t-test. Scale bars=50µm.
4.3. Discussion

A key characteristic that distinguishes OPCs from other glia is that OPCs form direct synapses with neurons and sense glutamatergic and GABAergic synaptic activity via their neurotransmitter receptors (Bergles et al. 2000; Bergles et al. 2010; Lin & Bergles 2004). Several lines of indirect evidence support the possibility that neurotransmission may regulate OPC proliferation and differentiation. To test the importance of synaptic signalling on OPCs directly, I examined the hippocampus of adult mice that received unilateral injection of BoNT/A, which causes cleavage of SNAP-25 and has been proven to silence synaptic signalling for up to 120 days (Antonucci et al. 2008). The key findings of this section are that synaptic silencing results in a decrease in the number of OPCs and cell shrinkage after 14 days of synaptic inhibition, indicating they retract their processes in the absence of synaptic inputs. Interestingly, I also found evidence that synaptic silencing may increase the GPR17+ OLs and promote myelination (although this is not entirely clear in the CA3 and DG and more measurements need to be done), raising the possibility that the decline in OPCs is related to increased differentiation (Table 4.4). Overall, the results provide new evidence that synaptic signalling plays an important role in regulating OPC cell dynamics, morphology and possibly differentiation.

<table>
<thead>
<tr>
<th>Table 4.4. Summary of effects of synaptic silencing on OPCs</th>
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<td></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td><strong>Synaptic contacts</strong></td>
</tr>
<tr>
<td><strong>Cell number</strong></td>
</tr>
<tr>
<td><strong>Cells coverage</strong></td>
</tr>
<tr>
<td><strong>Cell complexity</strong></td>
</tr>
<tr>
<td><strong>Differentiation / Myelination</strong></td>
</tr>
</tbody>
</table>
4.3.1. Synaptic inhibition alters the association of OPCs with synaptic proteins

Prior studies have noted that glutamatergic and GABAergic neurones signal onto OPCs in the hippocampus and other areas of the brain, by direct or spill-over synaptic neurotransmission (Bergles et al. 2000; Lin & Bergles 2004; Paukert & Bergles 2006; Kukley & Dietrich 2009; Bergles et al. 2010; Sun and Dietrich 2013; Butt et al., 2014). In this section, I show that markers for pre- and post-synaptic neuronal membranes are directly apposed to OPC processes at multiple sites. These findings clearly illustrate the potential importance of synaptic signalling on OPCs.

Notably, my results indicate that the pattern and density of immunostaining for the synaptic protein PSD95 was altered in both ipsilateral and contralateral hippocampi following unilateral injection of BoNT/A, which causes persistent synaptic silencing. BoNT/A is one of 7 proteolytic neurotoxins produced by Clostridium botulinum (Pirazzini et al., 2017) and enters the neurons in the presynaptic terminal by endocytosis binding to the synaptic vesicle protein SV2 (Dong et al., 2006; Verderio et al., 2006). Once inside the cytoplasm, BoNT/A is released from the vesicle following its acidification and cleaves its target, the synaptic protein SNAP-25 (Synaptosomal-associated protein of 25kDa), the predominant SNARE protein that is crucial for the fusion of the synaptic vesicles to the plasma membrane in the presynaptic terminal (Pirazzini et al. 2017; Tighe & Schiavo, 2013; Südhof 2004; Antonucci et al. 2008; Caleo and Schiavo, 2009). Indeed, BoNT/A and /E were used to prove the involvement of SNAP-25 in neuroexocytosis and disrupt activity-dependent release of various transmitters and peptides (for review, see Montecucco et al., 2005). Although glia may also express synaptic machinery, they express SNAP-23 not SNAP-25 (Hepp et al., 1999), and in the hippocampus SNAP25 was found to be absent from glial processes and
typically concentrated in neuronal terminals, whereas SNAP-23 was found in astrocytes (Schubert et al., 2011). The evidence is that botulinum toxins do not cleave SNAP-23 (Sikorra et al., 2016) and have little effects on glia (Caleo et al. 2012; Rojewska et al., 2018). Thus, the results of this chapter are due to cleavage of neuronal SNAP-25 and synaptic silencing.

My data showing that PSD95 is altered in both ipsilateral and contralateral hippocampi is consistent with evidence that BoNT/A is carried anterogradely and retrogradely through axons and transported by transcytosis to cause SNAP-25 cleavage in the contralateral hippocampus (Antonucci et al. 2008; Caleo et al., 2009; Restani et al., 2011; Restani et al., 2011; Restani, Giribaldi, et al., 2012; Restani, Novelli, et al., 2012). Moreover, PSD95 is enriched at glutamatergic synapses and has a key role in modulation of clustering of glutamate receptors, hence my results indicate glutamatergic synapses are not lost following treatment with BoNT/A, which supports previous findings that long-lasting blockade of activity by BoNT/A does not cause synaptic degeneration (Caleo et al. 2012). However, the glutamatergic synapses are not functional and the increase in PSD95 connectivity onto OPCs is not evidence of increased glutamatergic signalling onto OPCs; it has been shown unequivocally that BoNT/A causes persistent cleavage of SNAP-25 and block of hippocampal excitatory activity (Antonucci et al. 2008) and studies on knock-out mice demonstrate SNAP-25 is critical for evoked glutamatergic neurotransmission (Washbourne et al., 2002). In contrast, inhibitory postsynaptic sites lack PSDs and have other scaffolding complexes, a key component of which is gephyrin (Keith 2008). The increase in PSD95 represents an apparent dysregulation or compensatory effect in postsynaptic dendrites in response to presynaptic silencing by BoNT/A, which would be of interest to investigate further.
There were no clear-cut effects of BoNT/A on associations of OPCs with the presynaptic proteins vGLUT1 and vGAT, which were not altered ipsilaterally, but were significantly increased contralaterally. The increase in vGluT is consistent with an accumulation of glutamate neurotransmitter vesicles and enlargement of excitatory presynaptic terminals, which has been demonstrated by EM in the hippocampus following BoNT/A injection (Caleo et al. 2012). The possible increase in GABAergic inputs following SNAP-25 cleavage by BoNT/A injection is of interest, because unlike the essential requirement for SNAP-25 in glutamatergic neurotransmission (Washbourne et al., 2002), the importance of SNAP-25 at GABAergic synapses remains controversial. Studies by Mateolli and colleagues found no immunohistochemical evidence of SNAP-25 in adult hippocampal GABAergic neurons and using BoNT/A indicated that neurotransmission occurs following SNAP-25 cleavage (Frassoni et al. 2005; Verderio et al. 2004), whereas another group found SNAP-25 immunoreactivity to colocalize with vGAT in CA1 of the adult hippocampus and genetic ablation of SNAP-25 resulted in a complete loss of evoked GABAergic transmission (Tafoya et al., 2006, 2008). Nonetheless, based on the studies by Mateolli and colleagues, who provided us with the BoNT/A tissue, the evidence would suggest that there is continued GABAergic signalling onto OPCs and this may even be increased in the contralateral hippocampus, although no evidence for this was found in the ipsilateral hippocampus, hence this was not a direct effect of the BoNT/A or SNAP-25 cleavage. Electrophysiological experiments are required to test whether the GABAergic synapses identified by vGAT immunostaining in my study are functional.

4.3.3 Synaptic inhibition decreases OPC numbers but not immature OLs or myelination

My results show that synaptic silencing by BoNT/A results in a decrease in the overall number of OPCs. It has been reported previously that OPC proliferation and survival of
newly formed oligodendrocytes depends on neuronal activity (Barres & Raff 1993; Barres et al., 1993). The decrease in OPCs following synaptic silencing indicates they depend on neuronal activity to maintain their numbers and this decrease was not compensated for by increased proliferation, which is the usual response of OPCs to insults (McTigue and Sahinkaya, 2011). As noted above, BoNT/A blocks glutamatergic synaptic signalling, but probably not GABAergic signalling. Studies on the effects of glutamate signalling onto OPCs is contradictory, with pharmacological blockade of AMPA receptors being shown to promote OPC proliferation in one study (Fannon et al. 2015), whereas targeted KO of AMPA receptors in OPCs had no effect on their proliferation or number, but oligodendrocyte survival was reduced, resulting in decreased myelination (Kougioumtzidou et al. 2017). In contrast, GABAergic signalling acting on GABA_A receptors on OPCs greatly reduces their proliferation and cell survival, resulting in reduced numbers of OLs and decreased myelination (Hamilton et al. 2016; Zonouzi et al. 2015). Hence, the decrease in OPCs observed following SNAP-25 cleavage by BoNT/A may not represent a lack of glutamatergic signalling onto OPCs per se, but an imbalance in glutamatergic/GABAergic signalling, resulting in reduced numbers of OPCs, either through decreased proliferation and/or decreased cell survival. Notably, however, my data did not indicate that synaptic silencing blocked OPC differentiation or decreased myelination. On the contrary, the number of GPR17^+ cells and myelination may have been increased, although this requires further studies because of low number of replicates. This does not agree with previous studies showing pharmacological or genetic blockade of AMPAR or NMDAR reduces myelination (Fannon et al. 2015; Kougioumtzidou et al. 2017; Li et al., 2013). Thus, the balance of evidence is that glutamate signalling is required for OPC differentiation and myelination. Conversely, GABA signalling is reported to decrease oligodendrocyte number and myelination (Hamilton et al. 2016; Zonouzi et al. 2015). Overall, previous studies are consistent with my finding that SNAP-
cleavage by BoNT/A disrupts the balance of glutamatergic/GABAergic input onto OPCs, resulting in a decrease in their number. However, I found no evidence this resulted in a decrease in the reservoir of GPR17\(^+\) immature OLs or the extent of myelination in the adult hippocampus. Indeed, if the number of GPR17\(^+\) cells and myelination are increased after BoNT/A, it is possible synaptic silencing promotes OPC differentiation and myelination, which requires further examination.

### 4.3.3. Synaptic silencing results in OPC shrinkage

My results show that synaptic silencing by BoNT/A alters several aspects of OPC morphology, most notably resulting in cell atrophy, marked by decreased branching and retraction or shrinkage of their distal processes, resulting in an overall decrease in their coverage. This may suggest a retraction of processes from silent synapses, although the contacts of OPCs with PSD95, vGluT and vGAT did not indicate synaptic contacts were decreased overall. The shrinkage of OPCs is entirely consistent morphological data showing that blocking neuronal activity or AMPAR impairs the morphological development of OPC (Fannon et al. 2015) and time-lapse imaging has shown that NG2\(^+\) OPCs are highly dynamic, surveying their local environment with motile filopodia (Hughes et al., 2013). In contrast, the characteristic response of OPCs to CNS disruption is proliferation and hypertrophy, with increased process branching and NG2 expression (Franklin and Goldman, 2015). This is opposite to what I observed following synaptic silencing, which again is consistent with there not being any neurodegenerative or inflammatory effects of BoNT/A (Caleo et al. 2012). Notably, beside its role in neurotransmitter release, SNAP-25 is involved in neurite outgrowth in neurons (Hepp and Langley, 2001). However, the evidence to date is that glial cells do not express SNAP-25 and it seems unlikely that BoNT/A acts directly on OPCs to reduce process growth.
Overall, my data indicate that process outgrowth in OPCs is regulated by synaptic signalling.

4.4 Summary and Conclusions

In summary, the results of this chapter show that synaptic inhibition by dismantling the presynaptic machinery influences OPCs. Thus, loss of synaptic input causes a reduction on OPCs number, which is not compensated for by increased proliferation, suggesting the latter may be regulated by synaptic transmission. Furthermore, OPCs morphology is altered by synaptic inhibition, as the results show a shrinkage and loss of complexity of these cells. In terms of myelination, my results and previous studies support the possibility that synaptic inhibition promotes myelination, or at the least, does not cause demyelination. Regarding the differentiation process, further studies are necessary, but again it is evident that persistent loss of synaptic signalling did not result in a loss of immature OLs or myelin. As a final conclusion for this chapter, the results support the hypothesis that synaptic signalling is required to maintain OPC numbers and morphology but does not support my initial hypothesis that synaptic silencing may decrease myelination.
Oligodendrocyte precursor cells in the APP/PS1 mouse model of Alzheimer’s disease
5.1. Introduction and Aims

Alzheimer’s disease (AD) is the most common type of dementia and it is characterized by the formation of intracellular neurofibrillary tangles (NFTs), extracellular amyloid-β (Aβ) plaques (Braak & Braak 1991) and neuronal loss (Gomez-Isla et al. 1997). Aβ plaques are usually surrounded by neuronal, astrocytic and microglial processes (Kidd, 1964; Itagaki et al., 1989; Bouvier et al., 2016). Importantly, white matter disruption is indicated at an early stage of AD pathology and this appears to occur mainly in areas of the brain that myelinate late in life, such as the frontal lobes (Sjöbeck et al. 2005; Bartzokis et al. 2003; Ihara et al. 2010). Post-mortem analyses indicate a loss of oligodendrocytes in AD patients (Sjöbeck & Englund 2003) and this appears to be more severe at the core of Aβ plaques (Mitew et al. 2010). Myelin loss has also been indicated in studies on transgenic mouse models of AD and this is more pronounced surrounding Aβ plaques (Mitew et al. 2010). Furthermore, in vitro studies have reported impairment of the myelin sheet formation due to Aβ damage to oligodendrocytes (Horiuchi et al., 2012). In the adult brain, myelin loss is offset by the generation of newly formed myelinating oligodendrocytes from OPCs (Franklin et al. 2002; Young et al. 2013), which raises the possibility that OPCs are dysfunctional in AD. Notably, synaptic transmission has been proposed to regulate the life-long generation of oligodendrocytes from OPCs and synaptic loss is a hallmark of AD and cognitive decline (Selkoe, 2002; Terry et al., 1991). The results of Chapter 4 demonstrate that blockade of synaptic signalling results in a decrease in the number of OPCs and cell shrinkage, which is consistent with evidence of changes in OPC morphology in post-mortem AD tissue and in vitro when they are exposed to different forms of Aβ (Nielsen et al. 2013). This leads to the hypothesis underpinning this section that OPCs are disrupted in AD and this is related to synaptic signalling.

Numerous animal models have been developed to study AD pathology. These animals are mainly mice, although other animal groups like Zebrafish and invertebrates such as
Caenorhabditis elegans and Drosophila melanogaster have been used to study some aspects of the disease (reviewed in Driscoll and Gerstbrein, 2003). Mice models of AD express gene variants that have been shown to be related to AD such as mutations in tau, APP, presenilin (PSEN), BACE and APOE (reviewed in Götz and Ittner, 2008). Some of them, as the widely used APP/PS1 mouse presents combinations of these mutations, that are associated to familial AD (FAD) (reviewed in Drummond and Wisniewski, 2017). The use of this specific mouse strain in my project is due to availability and the presence of synaptic failure in the mouse model that allow us to study its effect on OPCs. The aim of this section is to examine the changes in OPCs in the APP/PS1 transgenic mouse model of AD, which presents Aβ plaque deposition in the hippocampus at 2-3 months of age and extensively throughout the forebrain by 8 months, with associated microgliosis, astrocytosis and dystrophic synaptic boutons, in the absence of Tau pathology (Radde et al. 2006).
5.2. Results

5.2.1. OPC association with synaptic proteins in APP/PS1 mice

Synaptic loss in the APP/PS1 mouse model of AD has been reported to occur around 3 months old, after Aβ plaque formation begins and persists for several months (Bittner et al. 2012). To examine whether there was detectable loss of synapses related to OPCs, hippocampi from 9 and 14 months old APP/PS1 mice were analysed and compared to aged-matched controls, and the immunostaining index for pre- and post-synaptic proteins (vGAT, vGluT1, Gephyrin and PSD-95) relative to NG2 within the process domains of individual OPCs measured, as described in Materials and methods chapter. Compared to controls, no overall differences in immunostaining were observed in APP/PS1 hippocampi for the pre- and post-synaptic GABAergic markers vGAT and Gephyrin, either at 9mo (Fig. 5.1Ai- Aii; Fig. 5.2 Ai-Aii) or 14mo (Fig. 5.1 Aiii-iv; Fig. 5.2 Aiii-Aiv), and there was no significant change in the vGAT:NG2 or Gephrin:NG2 indices (Fig. 5.1 Av; Fig. 5.2 Av). In contrast, there was an apparent decrease in the overall immunostaining for the presynaptic glutamatergic marker vGluT1 at 9 months in APP/PS1 compared to controls (Fig. 5.3 Ai-Aii), and by 14 months both controls and APP/PS1 appeared similarly decreased (Fig. 5.3 Aiii-Aiv); this was not evident for PSD95 (Fig. 5.4 Ai-Aiv). Consistent with this, the vGluT1:NG2 ratio was decreased at 9 months in APP/PS1 hippocampi compared to controls, although this was not statistically significant (Fig. 5.3 Av), and this fell to the same levels in 14 months in controls, although data for APP/PS1 at 14 months was very variable (Fig. 5.3 Av). No changes in the postsynaptic glutamatergic marker PSD-95 were evident (Fig. 5.4 Ai-Aiv). The results suggest there may be a decrease in glutamatergic inputs at 9 months in APP/PS1 that does not occur until 14 months in normal ageing controls, whilst GABAergic synaptic markers were maintained, which requires further investigation. Nonetheless, my analysis did not
reveal a marked change in the relative density of synapses associated with individual OPCs.
Figure 5.1. OPC associations with the synaptic protein vGAT in the CA1 of the hippocampus in a mouse model of Alzheimer’s disease at 9 and 14 months old. Hippocampi were examined in the APP/PS1 mouse model of AD, compared to aged matched controls. (Ai-iv) Confocal images of double immunofluorescence labelling for NG2 to identify OPCs and GABAergic presynaptic marker vGAT. Scale bars = 10μm. (Av) Box-Whisker plots of the immunostaining indices for GABAergic synapses within the process domains of individual OPCs, measured as the ratio of vGAT⁺:NG2⁺ pixels (n=9 cells from 3 slices/3 animals in each case; 3 cells per slice/animal); data are Mean ± SEM, ns= no significant, Unpaired t-test.
Figure 5.2. OPC associations with the synaptic protein Gephyrin in the CA1 of the hippocampus in a mouse model of Alzheimer’s disease at 9 and 14 months old. Hippocampi were examined in the APP/PS1 mouse model of AD, compared to aged matched controls. (Ai-iv) Confocal images of double immunofluorescence labelling for NG2 to identify OPCs and GABAergic postsynaptic marker Gephyrin. Scale bars = 10μm. (Av) Box-Whisker plots of the immunostaining indices for GABAergic synapses within the process domains of individual OPCs, measured as the ratio of Gephyrin$^+$:NG2$^+$ pixels (n=9 cells from 3 slices/3 animals in each case; 3 cells per slice/animal); data are Mean ± SEM, ns= no significant, Unpaired t-test.
Figure 5.3. OPC associations with the synaptic protein vGluT1 in the CA1 of the hippocampus in a mouse model of Alzheimer’s disease at 9 and 14 months old. Hippocampi were examined in the APP/PS1 mouse model of AD, compared to aged matched controls. (Ai-iv) Confocal images of double immunofluorescence labelling for NG2 to identify OPCs and glutamatergic presynaptic marker vGluT1. Scale bars = 10µm. (Av) Box-Whisker plots of the immunostaining indices for glutamatergic synapses within the process domains of individual OPCs, measured as the ratio of vGluT1⁺:NG2⁺ pixels (n=9 cells from 3 slices/3 animals in each case; 3 cells per slice/animal); data are Mean ± SEM, ns= no significant, Unpaired t-test.
Figure 5.4. OPC associations with the synaptic protein PSD95 in the CA1 of the hippocampus in a mouse model of Alzheimer’s disease at 9 and 14 months old. Hippocampi were examined in the APP/PS1 mouse model of AD, compared to aged matched controls. (Ai-iv) Confocal images of double immunofluorescence labelling for NG2 to identify OPCs and glutamatergic postsynaptic marker PSD95. Scale bars = 10µm. (Av) Box-Whisker plots of the immunostaining indices for glutamatergic synapses within the process domains of individual OPCs, measured as the ratio of PSD95 :NG2 pixels (n=9 cells from 3 slices/3 animals in each case; 3 cells per slice/animal); data are Mean ± SEM, ns= no significant, Unpaired t-test.
5.2.2. OPCs and sister cells number in the CA1 of the hippocampus in the APP/PS1 mouse model of Alzheimer’s disease

As in Chapter 4, I focused my analysis in the CA1 since synaptic changes in dendritic spines have been reported in this area of the hippocampus in the APP/PS1 mouse model (Merino-Serrais et al. 2011). Additional analyses were performed in other regions such as CA3, DG and Hilus (Table 5.1). Cell counts were performed in the area examined at 9 months (Fig. 5.5 Ai-ii) and 14 months (Fig. 5.5 Bi, Bii). The number of OPCs and sister cells were counted, and their numerical density was calculated by dividing the number of cells in each region by the area of the region analysed (Fig. 5.5 Aiii, Aiv, Biii, Biv). At 9 months, there was significant halving in the number of OPCs in APP/PS1 compared to controls, from 441±70.15 to 215.3±20.36 (Fig. 5.2Aiii; *p<0.05; Unpaired t-test) (Fig. 5.5 Ai-Aiii); a similar decrease was observed in controls at 14 months, with OPC number falling to 312.2±26.68, similar to levels at 9 months in APP/PS1 and with no further decrease at 14 months in APP/PS1 (Fig. 5.5 Ai, Aiii). In comparison, there was no apparent difference in the number of sister cells at 9 months (Fig. 5.5 Aiv), although the number was almost halved at 14 months, from 51.01±5.39 in controls to 28.96±2.068 in APP/PS1 (Fig. 5.2Biv; *p<0.05; Unpaired t-test). The data indicate a decrease in the number of OPCs with age between 9 and 14 months, which occurred prematurely at 9 months in APP/PS1, together with an apparent decrease in OPC proliferation at 14 months in APP/PS1, as indicated by the number of sister cells. Analysis of other hippocampal regions did not detect any clear differences in numerical density or sister cells (Table 5.1). The results indicate a decrease in OPCs with age in the CA1 region of the hippocampus, a process that is accelerated in APP/PS1.
Figure 5.5. OPC quantification in the CA1 of the hippocampus of APP/PS1 mouse model of AD and an aged-matched control. Hippocampi of 9 months old and 14 months old APP/PS1 mice were examined, compared to age-matched controls, using immunofluorescence labelling for NG2 (green) to identify OPCs and counterstaining with Hoechst (blue) for nuclei (red line shows delimitation of the region analysed). Representative confocal projections of 9 months old hippocampus (Ai-Aii), and 14 months old hippocampus (Bi-Bii), in aged-matched controls (Ai, Bi) and in APP/PS1 animals (Aii, Bii). Scale bars = 50µm. (Av) Bar graphs of total number of NG2+ OPCs (Aiii, Biii) and number of sister cells (Aiv, Biv) per mm$^2$ in the APP/PS1 and control hippocampus. Data are expressed as Mean ± SEM, ns= no significant, *p<0,05; Unpaired t-test, n= 3 animals for each group.
5.2.3. OPCs process domain and coverage in the CA1 of the hippocampus of the APP/PS1 mouse model of Alzheimer’s disease

As above, I focused on the CA1 region of the hippocampus. High magnification confocal images of OPCs were captured and the processes domain of individual cells were measured using ImageJ and normalized to the total area of the region analysed in both 9 months old and 14 months old APP/PS1 mouse model and compared to littermate controls (Fig. 5.6). No differences were found in the domains of OPCs at 9mo in the CA1 (Fig. 5.6 Ai, Aii, Aiii), but at 14 months there was evident and statistically significant shrinkage of OPC process in APP/PS1 compared to controls (Fig. 5.6 Bi, Bii, Biii; ***p<0.001, Unpaired t-test). Similarly, measurement of the density of NG2 immunostaining in a constant FOV demonstrates that the OPC coverage of the CA1 was unaltered at 9 months (Fig. 5.6 Aiv) but was significantly decreased at 14 months in APP/PS1 compared to controls (Fig. 5.4 Biv; *p<0.05, Unpaired t-test). The results indicate shrinkage of OPCs at 14 months in APP/PS1 model of AD.
Figure 5.6. OPC process domains and NG2 density in the CA1 of the hippocampus of APP/PS1 mouse model of AD and an aged-matched control. Hippocampi of 9 months old and 14 months old APP/PS1 mice were examined, compared to age-matched controls, using immunofluorescence labelling for NG2 (green) to identify OPCs. High magnification confocal projections of OPCs and their process domains (indicated by broken white lines) in the 9 months old hippocampus (Ai, Aii), and the 14 months old hippocampus (Bi, Bii), in controls (Ai, Bi) and APP/PS1 (Aii, Bii). Scale bars = 20µm. (Aiii-Biii) Box-Whisker plots of the total area of OPC process domains and (Aiv, Biv) bar graphs of the coverage of the CA1 area by OPCs. Data are Mean ± SEM, *p<0.05; ***p<0.001, ns= no significant; Unpaired t-test, n= 3 animals for each group.
5.2.4. Changes in OPC morphology in the CA1 region of the hippocampus of the APP/PS1 mouse model of Alzheimer’s disease

The results above provide evidence of OPC shrinkage and the underlying morphological changes were examined in further detail in the CA1 region, as described in Section 4.2.5. Briefly, between 80-100 z-sections with an interval of 0.3µm were captured using a 100x oil objective and reconstructed using Neurolucida 360 and Neurolucida 360 explorer (Fig. 5.7) and 9 cells from 3 animals/group were examined at each age and tested for statistical significance by Mann Whitney test. Consistent with the results above, no changes in the morphological parameters of OPCs were observed at 9 months in APP/PS1 compared to controls (Fig. 5.8 A-F and Fig. 5.9 A-D). This was confirmed by Sholl analysis, which did not reveal any statistically significant difference between APP/PS1 and controls at 9 months, although there was a trend towards a reduction in the number of nodes (Fig. 5.10 A), terminals (Fig. 5.10 B) and length of processes (Fig 5.10 C) in the APP/PS1 mice; an increased ‘n’ value may result in statistical significance, but the tissue was not available at the time of the project (since we did not have the APP/PS1 mice in our lab and the tissue was donated from our collaborators). In support of this, the process lengths in different branch orders were reduced and this shrinkage was statistically significant in branches of the 3rd order (Fig 5.10 D, *p<0.05, two-way ANOVA followed by Sidak’s multiple comparison test). In contrast, OPCs were morphologically more complex at 14 months in APP/PS1, as indicated by the Neurolucida measurement of cell complexity (Fig. 5.11 E; ***p<0.001, Mann Whitney test) and ramification index (Fig. 5.11 F; **p<0.01, Mann Whitney test), together with increased process lengths (Fig 5.12 B; *p<0.05, Mann Whitney test), number of terminals or end points (Fig. 5.12 C; *p<0.05, Mann Whitney test) and number of ramifications or nodes (Fig. 5.12 D; **p<0.01, Mann Whitney test). Furthermore, the Sholl analysis demonstrates an increase in process branching and overall length, with a statistically significant increase in the number of
nodes or ramification points within 25 µm of the cell body in APP/PS1 compared to controls (Fig. 5.13 A; *p<0.05). These morphological analyses demonstrate an initial shrinkage of OPCs at 9 months in the APP/PS1 mouse model mainly due to a reduction in process length, which is followed by OPC hypertrophy at 14 months, due to increased process branching and processes length.
Figure 5.7. Comparison of complexity of OPC morphologies in the CA1 of the 9 months old and 14 months old APP/PS1 mouse model and an aged-matched control. Images were generated by Neurolucida 360. (Ai-Aiii) Example of complex (Ai), medium (Aii) and simple (Aiii) morphology of OPCs in a 9 months old WT mouse. (Bi-Biii) Example of complex (Bi), medium (Bii) and simple (Biii) morphology of OPCs in 9 months old APP/PS1 mouse. (Ci-Ciii) Example of complex (Ci), medium (Cii) and simple (Ciii) morphology of OPCs in 14 months old WT mouse. (Di-Diii) Example of complex (Di), medium (Dii) and simple (Diii) morphology of OPCs in 14 months old APP/PS1 mouse.
Figure 5.8. OPC morphological changes in the CA1 of the 9 months old APP/PS1 mouse model compared to an aged-matched control. Data were generated by Neurolucida 360 analysis of cells. Box-whisker plots of (A) cell body area, (B) cell body volume, (C) process volume, (D) total cell volume, (E) cell complexity, (F) ramification index. Data expressed as Mean±SEM. ns= not significant; Mann Whitney test. n= 9 cells from 3 animals per group.
Figure 5.9. OPC morphological changes in the CA1 of the 9 months old APP/PS1 mouse model compared to an aged-matched control. Data were generated by Neurolucida 360 analysis of cells. Box-whisker plots of (A) number of processes per cell, (B) length of processes, (C) number of end points, (D) number of nodes (points of ramification of the processes). Data expressed as Mean±SEM. ns= not significant; Mann Whitney test. n= 9 cells from 3 animals per group.
Figure 5.10. Morphological changes of OPCs in the CA1 of the hippocampus in a 9 months old APP/PS1 mouse model of AD compared to aged-matched control. Data were generated by Sholl analysis of confocal images using Neurolucida 360 software. (A) number of nodes or branch points, (B) number of process terminals or end points, (C) processes length, (E) length of processes of different branch orders. Data are Mean±SEM. * p<0.05; two-way ANOVA followed by Sidak’s multiple comparisons test. n= 9 cells from 3 animals per group.
Figure 5.11. OPC morphological changes in the CA1 of the 14 months old APP/PS1 mouse model compared to an aged-matched control. Data were generated by Neurolucida 360 analysis of cells. Box-whisker plots of (A) cell body area, (B) cell body volume, (C) process volume, (D) total cell volume, (E) cell complexity, (F) ramification index. Data expressed as Mean±SEM. ns= not significant; **p<0.01; ***p<0.001; Mann Whitney test. n= 9 cells from 3 animals per group.
Figure 5.12. OPC morphological changes in the CA1 of the 14 months old APP/PS1 mouse model compared to an aged-matched control. Data were generated by Neurolucida 360 analysis of cells. Box-whisker plots of (A) number of processes per cell, (B) length of processes, (C) number of end points, (D) number of nodes (points of ramification of the processes). Data expressed as Mean±SEM. ns= not significant; *p<0.05; **p<0.01; Mann Whitney test. n= 9 cells from 3 animals per group.
Figure 5.13. Morphological changes of OPCs in the CA1 of the hippocampus in a 14 months old APP/PS1 mouse model of AD compared to aged-matched control. Data were generated by Sholl analysis of confocal images using Neurolucida 360 software. (A) Number of nodes or branch points, (B) number of process terminals or end points, (C) processes length. (E) length of processes of different branch orders. Data are Mean±SEM. * p<0.05; two-way ANOVA followed by Sidak’s multiple comparisons test. n= 9 cells from 3 animals per group.
5.2.5. OPC differentiation and myelination in the APP/PS1 mouse model of Alzheimer’s disease

As described in section 4.2.6, GPR17 and MBP immunostaining were used to identify immature OLs and myelin, respectively, to examine whether the differentiation and myelination processes are altered in the APP/PS1 mouse model. As above, I focused in the CA1 region, but other hippocampal regions were also analysed. The number of GPR17\(^+\) cells was greater than controls at 9 months and lower at 14 months, although these differences were not statistically different (Fig. 5.14 Ai, Aii, Aiii; Bi, Bii, Biii). Consistent with this, there were statistically significant increases in the number of GPR17\(^+\) cells in the Hilus and CA3 at 9 months APP/PS1 (Table 5.1; \(*p<0.05\) and \(**p<0.01\), respectively; Unpaired t-test). On the other hand, at 14 months there was an apparent decrease in the number of GPR17\(^+\) in the CA1 region in APP/PS1, although the results are not statistically significant (Figure 5.14 Bi, Bii, Biii); equivalent changes were not observed in the other regions of the hippocampus, in fact, there was a significant increase in GPR17\(^+\) cells in the Hilus (Table 5.1). Measurements of MBP immunostaining density in the CA1 region indicated no significant difference at 9 months in APP/PS1 (Fig. 5.15 Ai-Aiii), whereas MBP was significantly decreased at 14 months in APP/PS1 compared to controls (Fig. 5.15 Bi-Biii; \(*p<0.05\); Unpaired t-test); at 9 months there was an increase in MBP immunostaining in the DG (Table 5.1; \(*p<0.05\); Unpaired t-test), but no more conclusive changes in MBP immunostaining were observed in the other regions analysed (Table 5.1). Overall, data are consistent with a decrease in OL lineage cells and myelination in the CA1 region in the APP/PS1 model.
Figure 5.14. Number of GPR17⁺ immature oligodendrocytes in OPCs differentiation in the CA1 of the hippocampus of the APP/PS1 mouse model of AD and an aged-matched control. (Ai-Aii, Bi-Bii) Representative confocal images of Gpr17 immunopositive cells (green) in the 9 months (Ai, Aii) and 14 months (Bi, Bii) CA1 region of the hippocampus, in controls (Ai, Bi) and APP/PS1 animals (Aii, Bii). Number of GPR17⁺ cells (cells per mm²) in the CA1 region of the hippocampus of 9 months (Aiii) and 14 months (Biii), comparing controls (blue bars) and APP/PS1 animals (red bars). Data are expressed as Mean ± SEM. ns = not significant; Unpaired t-test. n=3-5 slices from 3 animals per group. Scale bars=50µm.
Figure 5.15. Myelination in the CA1 of the hippocampus of the APP/PS1 mouse model of AD and an aged-matched control. (Ai-Aii, Bi-Bii) Representative confocal images of MBP immunostaining (red) in the CA1 region of the hippocampus of at 9 months old (Ai, Aii) and 14 months old (Bi, Bii), in controls (Ai, Bi) and APP/PS1 animals (Aii, Bii). (Aiii, Biii) Density of MBP immunostaining measured as the number of MBP $^+$ pixels per constant FOV, using ImageJ software, in the control (blue bars) and APP/PS1 (red bars) animals. Data are expressed as Mean±SEM. ns= not significant.; *$p<0.05$; Unpaired t-test. n=3-5 slices from 3 animals per group. Scale bars=50µm.
Table 5.1. OPCs process domain and coverage in the hippocampus of the APP/PS1 mouse model of Alzheimer’s disease and its aged matched control

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<td>CA1</td>
<td>441 ± 70.15</td>
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<td>240.9 ± 16.6</td>
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<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Hilar</td>
<td>547.3 ± 39.5</td>
<td>503.8 ± 26.71</td>
<td>760.8 ± 35.19</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>*p&lt;0.05</td>
</tr>
<tr>
<td>CA3</td>
<td>275.4 ± 32.27</td>
<td>272.7 ± 63.22</td>
<td>399.4 ± 19.13</td>
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<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>*p&lt;0.01</td>
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*p same data presented in Table 1
5.3. Discussion

Synaptic loss is a key feature of AD in regions close to amyloid-β plaques (Merino-Serrais et al. 2011; Bittner et al. 2012), as well as demyelination in the periventricular areas (reviewed in Serrano-Pozo et al., 2011) and frontal lobes (Sjöbeck et al. 2005; Bartzokis et al. 2003; Ihara et al. 2010). Since OPCs specifically contact synapses and the results of chapter 4 show that synaptic silencing results in morphological changes in OPCs, I examined whether OPCs are altered in the hippocampus of the APP/PS1 mouse model, which exhibits synaptic loss after 3 months (Bittner et al. 2012). The key findings of this section are that in the APP/PS1 mouse there is a decrease in glutamatergic inputs on OPCs and a decrease in the number and coverage of OPCs at 14 months, combined with cellular hypertrophy and an increase in their morphological complexity (Table 5.2). In addition, there is a significant loss of MBP immunostaining in the CA1 of the APP/PS1 mouse at 14 months. Overall, the results provide new evidence that OPCs and myelination are altered in APP/PS1 mouse and that these are correlated with decreased synaptic input, which is consistent with the results of chapter 4 showing that synaptic signalling plays an important role in regulating OPCs and myelination.

<table>
<thead>
<tr>
<th>Table 5.2. Summary of changes in OPCs in the APP/PS1 mouse</th>
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<tr>
<td></td>
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<tr>
<td>Synaptic contacts</td>
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<td>Cell number</td>
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<tr>
<td>Cells coverage</td>
</tr>
<tr>
<td>Cell complexity</td>
</tr>
<tr>
<td>Differentiation/Myelination</td>
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</tbody>
</table>
5.3.1. Synaptic proteins in relation with OPCs are not altered in the APP/PS1 mouse model of Alzheimer’s disease

Previous studies have shown a loss of spinal density in neurons after 3 months in APP/PS1 mouse model of AD, being more evident in areas close to the Aβ plaques (Bittner et al. 2012; Merino-Serrais et al. 2011). Alterations in spinal morphology in the CA1 has also been reported, with shorter spine necks and small heads in the APP/PS1 mouse model (Merino-Serrais et al. 2011). A small head spine implies reduced post-synaptic areas and lower number of vesicles in the pre-synaptic terminal (Harris and Stevens, 1989). PSD-95 expression determines the ratio of excitatory to inhibitory synapses by regulating the amount of pre-synaptic proteins such as vGAT and vGluT1 (Prange et al., 2004). Previous studies show controversial results for the expression of PSD-95, being variable in brains from AD patients and in mouse models of AD. Thus, in AD patients PSD-95 expression is increased in some areas of the brain while in mouse models there is a decrease in this protein. The pattern of expression of PSD-95 can also change from dendrites to soma in neurons (reviewed in Savioz et al. 2014). Hence, a study by Mitew and collaborators showed the differences in expression of different synaptic proteins in preclinical AD, end-stage AD and a transgenic mouse model (APP/PS1 specifically) and they found no differences in vGluT1 compared to controls in preclinical AD while there was a decrease in vGluT1 in the periphery of Aβ plaques and this was more evident in the center of the plaque. In the case of end-stage AD samples there was a decrease in vGluT1 overall, contrary to the observations in the mouse model, in which vGluT1 increased in areas without plaques, but decreased in the periphery and centre of the plaques. For vGAT, there were no changes in density in any of the three models in areas without plaques or in the plaque periphery, but there was a drastic reduction of vGAT density in the centre of the plaque (Mitew et al., 2013). In another study, Kiss and collaborators found that the expression of the post-synaptic protein Gephyrin in the CA1 and DG was increased in
early stages of AD, in 3 months old APP/PS1 mouse while it was decreased in late stages of AD in 12 months old APP/PS1 mouse when compared to controls. Another interesting finding was that Gephyrin was colocalized with the amyloid plaques unlike what it has been shown for other synaptic proteins in previous studies (Kiss et al., 2016). My results show no significant differences in the density of vGAT, Gephyrin, vGluT1 and PSD-95 in relation with OPCs in the CA1 region of 9 months and 14 months old APP/PS1 mouse, although vGluT1 appeared decreased, there was a high variation in all the synaptic proteins measured within the same group. Considering previous findings reported in the literature, the variation in my data could be due to the proximity of OPCs to Aβ plaques and this could be addressed by further studies to relate OPC synaptic contacts to plaque proximity.

5.3.2. OPC numbers and myelin density decrease in the APP/PS1 mouse model of AD

My results show a decline in the number of OPCs in the CA1 of the 9 months and 14mo APP/PS1 mouse although this is statistically significant only at 9 months, and the same tendency was observed in the other areas of the hippocampus studied. The number of sister cells was not changed at 9 months, but there was a significant reduction in OPCs sister cells in the 14mo. In the case of immature GPR17+ oligodendrocytes, the results suggest an increase in their number at 9 months in APP/PS1 (statistically significant in the Hilus and CA3), whereas in the 14 months APP/PS1 mouse there is a decrease in the number of GPR17+ cells in the CA1, but an increase in the rest of the areas analysed, being significant in the Hilus. Similarly, there was an increment in MBP immunostaining, taken as a measurement of the overall extent of myelination, in the 9 months APP/PS1 mouse in all the areas analysed, and this was statistically significant in the DG. In contrast, there was an evident loss of myelin at 14mo in APP/PS1 which was statistically
significant in the CA1. The possibility that OPC differentiation and myelination are augmented at 9 months in APP/PS1 is consistent with previous studies by Wu and colleagues that showed that the MBP density is increased in the 2 months APP/PS1 mouse compared to control (Wu et al., 2017). In addition, Behrendt and colleagues found an increase in OPCs and in differentiated oligodendrocytes at 6 months, as well as in Olig2+ cells in the APP/PS1 mouse compared to control, which could indicate an increase in proliferation and differentiation in this model of AD (Behrendt et al. 2013). This possible early promotion of OPC differentiation and myelination in APP/PS1 requires further analysis, but at 14 months the results consistently indicate a decrease in OPCs, sister cells, the pool of GPR17+ immature oligodendrocytes and the extent of myelination. This loss of oligodendrocyte lineage cells and myelin has been reported in human AD samples and appears to accelerate age-dependent loss in AD that may be related to Aβ plaques (Behrendt et al. 2013).

5.3.3. OPCs morphology changes in the APP/PS1 mouse model of AD

My findings show that both the area occupied by OPCs and their overall coverage were significantly decreased in the APP/PS1 mouse compared to control at 14 months, but not at 9 months. Analysis of OPC morphology showed that at 9 months there was a decrease in the length of processes, number of terminals and ramifications in APP/PS1, indicating a loss of morphological complexity of OPCs. The opposite was observed at 14 months, with increased length of processes of high order but not in the lower orders, and an increase in the number of end points and ramification points, which indicates higher complexity of OPCs in the APP/PS1 mouse at this age. A previous study has shown changes in OPCs morphology in tissue from AD patients, in which OPCs have been reported to become less complex in the presence of amyloid-β plaques (Nielsen et al.
2013). The changes at 14 months are suggestive of the injury response of OPCs, as reported in other pathologies, characterized by cellular hypertrophy and increased NG2 expression (Levine 1994; Jones et al., 2002; Hampton et al., 2004) and may be related to plaque load (Nielsen et al. 2013) that could act as an injury. The change in OPCs morphology could also be related to changes in the synaptic activity in the APP/PS1 mouse which would correlate with the results of chapter 4. In this study, the analysis of OPCs morphology and synaptic markers density were performed in OPCs distributed randomly (to avoid bias) in the area of the hippocampus studied (CA1) rather than choosing OPCs that were at certain distance, closer or further, from amyloid plaques. This approach could have contributed to the variability of the data generated. It would be interesting to study OPCs morphology and synaptic markers in relation with the proximity of these cells to amyloid plaques in the future.

5.4. Summary and Conclusions

Overall, there are two key results of this chapter. First, in the 9mo APP/PS1 mouse, the decrease in the number of OPCs, increase in the number of GPR17+ cells and a trend towards an increase in myelin density that could indicate promotion of the processes of differentiation and myelination at an early stage of APP/PS1. OPC connectivity was unaltered at this age and synaptic input may play a role in promoting OPC differentiation at these early stages of AD pathology. Secondly, in the 14mo APP/PS1 mouse, there was a striking decrease in OPC number and density, as well as reductions in the numbers of sister cells and GPR17+ cells, which are the direct progeny of OPCs, and a loss of myelin. These changes are associated with OPC shrinkage and increased branching consistent with a loss of synaptic signalling and an injury response in OPCs related to plaque load.
Chapter 6

Oligodendrogenesis in the ageing brain
6.1. Introduction and Aims

OPCs persist throughout adult life and are responsible for the life-long generation of OLs that are responsible for the myelination of new connections formed through new life experiences and replacement of myelin lost through natural ‘wear and tear’ and damage (Rivers et al. 2008; Dimou et al. 2008; Psachoulia et al. 2009; Zhu et al. 2011; Young et al. 2013). However, there is evidence that oligodendrocytes and myelin are lost in the ageing brain, resulting in white matter shrinkage and a loss of cognition (Bartzokis et al. 2003; Chen et al., 2011; Sim et al., 2002). Chapter 5 supports the possibility that there is myelin loss in the ageing brain and indicated this may be related to changes in OPCs and their capacity to differentiate into OLs. To address this directly, I have used the Pdgfra-CreERT2:Rosa26R-YFP mouse, in which expression of the reporter protein YFP is driven by Pdgfra and induced by tamoxifen injection, enabling fate-mapping of OPCs and their differentiation into myelinating OLs (Rivers et al. 2008; Psachoulia et al. 2009; Young et al., 2013). The aim of this chapter was to compare OPC differentiation into OLs at 3 and 18 months of age, using the Pdgfra-CreERT2:Rosa26R-YFP mouse and immunostained for the transcription factor Olig2, which is essential for OL specification and is expressed throughout the lineage (Dimou et al. 2008; Zhu et al. 2012), NG2 to identify OPCs, GPR17 for immature OLs, and APC/CC1 and MBP for mature OLs and myelin (Stallcup, 1981; Nishiyama et al. 1991; Nishiyama et al. 1996; Chen et al. 2009; Boda et al. 2011; Viganò et al. 2016).
6.2. Results

6.2.1. Oligodendrocyte lineage cells in ageing

To study the dynamics of OPCs in normal ageing, 3 months and 18 months old Pdgfra-CreERT2:Rosa26R-YFP mice of both sexes were injected with tamoxifen and immunolabelled to identify Rosa26R-YFP+ cells and to distinguish stages of oligodendrocyte differentiation, by using Olig2 (total number of cells of the OL lineage), NG2 (total number of OPCs), GPR17 (immature OLs) and APC (differentiated OLs), together with MBP (myelin). The total number of OL lineage cells was identified by Olig2 immunolabelling and decreased by half in the white matter of the corpus callosum between 3 months and 18 months, from 43.38±4.769 at 3 months to 24.67±2.38 at 18 months (Figure 6.1Aiii; **p<0.01; Unpaired t-test), whereas their number was increased in the grey matter of the cortex from 36.86±4.606 to 50.67±2.141 (Fig. 6.1Biii, *p<0.05; Unpaired t-test). Cell counts of YFP+ cells was used to identify OPCs and their progeny generated over the 15 days following tamoxifen-induced recombination, and their numbers were unaltered with age in the CC, whereas they were significantly increased in the 18 months cortex, compared to 3 months. There was similar variation in the hippocampus, where Olig2+ OLs were almost doubled in the 18 months CA3 region, from 23.25±6.005 to 51.89±1.285 (Fig. 6.2Bv, p<0.01; Unpaired t-test), and the latter was reflected by a significant increase in YFP+ cells, indicating increased generation of OL lineage cells (table 1). No other marked changes were observed in the other hippocampal regions, although there were small increases in the number of Olig2+ cells in the Hilus and YFP+ cells in the CA1 and DG (Table 6.1). The results indicate increased generation of OL lineage cells in the grey matter cortex and CA3 regions, whereas there is a loss of Olig2+ cells in the ageing white matter of the corpus callosum and this was not compensated for by increased production of new OLs.
Figure 6.1. Cells of the oligodendrocyte lineage in the CC and Cortex of an adult and an aged Pdgfra-CreER:Rosa26R-YFP mouse. (Ai-Aii, Bi-Bii) Olig2+ (red; red arrows) and GFP+ (green) cells in the CC (Ai-Aii) and the Cortex (Bi-Bii) of a 3 months (Ai, Bi) and an 18 months (Aii, Bii) Pdgfra-CreER:Rosa26R-YFP mouse. (Aiii, Biii) Quantification (cells per FOV) of total Olig2+ cells in the CC (Aiii) and Cortex (Biii) of a 3 months compared to an 18 months Pdgfra-CreER:Rosa26R-YFP mouse. (Aiv, Biv) Quantification (cells per FOV) of total GFP+ cells in the CC (Aiv) and Cortex (Biv) of a 3 months compared to an 18 months Pdgfra-CreER:Rosa26R-YFP mouse. (Av, Bv) Quantification (cells per FOV) of GFP+Olig2+ cells in the CC (Av) and Cortex (Bv) of a 3 months compared to an 18 months Pdgfra-CreER:Rosa26R-YFP mouse. *p<0.05; **p<0.01; Unpaired t-test. FOV 100 µm x 100 µm for CC and 200 µm x200 µm for Cortex. Scale bars= 50 µm. White arrows point to double immunolabelled cells.
Figure 6.2. Cells of the oligodendrocyte lineage in the CA1 and CA3 of an adult and an aged Pdgfra-CreER<sup>T2</sup> :Rosa26R-YFP mouse. (Ai-Aii) Olig2<sup>+</sup> (red; red arrows) and GFP<sup>+</sup> (green) cells in the CA1 (Ai-Aii) and the CA3 (Bi-Bii) of a 3 months (Ai, Bi) and an 18 months (Aii, Bii) Pdgfra-CreER<sup>T2</sup> :Rosa26R-YFP mouse. (Aiii, Bi) Quantification (cells per FOV) of total Olig2<sup>+</sup> cells in the CA1 (Aiii) and CA3 (Biii) of a 3 months compared to an 18 months Pdgfra-CreER<sup>T2</sup> :Rosa26R-YFP mouse. (Aiv, Biv) Quantification (cells per FOV) of total GFP<sup>+</sup> cells in the CA1 (Aiv) and CA3 (Bv) of a 3 months compared to an 18 months Pdgfra-CreER<sup>T2</sup> :Rosa26R-YFP mouse. (Av, Bv) Quantification (cells per FOV) of GFP<sup>+</sup> Olig2<sup>+</sup> cells in the CA1 (Av) and CA3 (Bv) of a 3 months compared to an 18 months Pdgfra-CreER<sup>T2</sup> :Rosa26R-YFP mouse. *p<0.05; **p<0.01; Unpaired t-test. FOV 200 µm x200 µm. Scale bars= 50 µm. White arrows point to double immunolabelled cells.
Table 6.1. Quantification of Olig2\(^+\) cells in various regions of the brain of the adult and aged Pdgfra-CreER\(^{T2}\):Rosa26R-YFP mouse

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>SL</th>
<th>Hilus</th>
<th>DG</th>
<th>CA3</th>
<th>CC</th>
<th>Cortex</th>
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<tbody>
<tr>
<td>3mo</td>
<td>18mo</td>
<td>3mo</td>
<td>18mo</td>
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</tr>
<tr>
<td>13.5 ± 1.18</td>
<td>11 ± 0.986</td>
<td>8.375 ± 0.9437</td>
<td>10.25 ± 0.7734</td>
<td>11.63 ± 1.117</td>
<td>15.22 ± 1.188</td>
<td>27.75 ± 2.403</td>
<td>28.22 ± 1.985</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>*p&lt;0.05</td>
<td>NS</td>
<td>***p&lt;0.001</td>
<td>**p&lt;0.01</td>
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Table 6.2. Quantification of GFP\(^+\) cells in various regions of the brain of the adult and aged Pdgfra-CreER\(^{T2}\):Rosa26R-YFP mouse

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>SL</th>
<th>Hilus</th>
<th>DG</th>
<th>CA3</th>
<th>CC</th>
<th>Cortex</th>
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<tr>
<td>3mo</td>
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<tr>
<td>2.625 ± 0.5957</td>
<td>4.167 ± 0.5426</td>
<td>1.75 ± 0.25</td>
<td>2 ± 0.3651</td>
<td>2.857 ± 0.4592</td>
<td>3.333 ± 0.5578</td>
<td>3.667 ± 1.085</td>
<td>4.333 ± 0.8433</td>
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<td>NS</td>
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<td>NS</td>
<td>*p&lt;0.05</td>
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Table 6.3. Quantification of GFP\(^+\)Olig2\(^+\) cells in various regions of the brain of the adult and aged Pdgfra-CreER\(^{T2}\):Rosa26R-YFP mouse

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>SL</th>
<th>Hilus</th>
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<tr>
<td>3mo</td>
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<td>18mo</td>
<td>3mo</td>
<td>18mo</td>
</tr>
<tr>
<td>GFP(^+)Olig2(^+)</td>
<td>3.125 ± 0.6685</td>
<td>5.333 ± 0.6236</td>
<td>2.375 ± 0.4199</td>
<td>2.25 ± 0.368</td>
<td>2.375 ± 0.4199</td>
<td>2.556 ± 0.294</td>
<td>3.25 ± 0.4532</td>
</tr>
<tr>
<td>*p&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>*p&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>**p&lt;0.01</td>
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</table>
6.2.2. Fate-mapping of YFP+ OPCs in normal ageing

Once induced in OPCs in the Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse, cells retain expression of the YFP reporter, which enables the extent of OPC differentiation to be measured by double immunostaining for GFP and the markers for the different stages of OL differentiation, NG2, GPR17 or APC. Notably, at 18 months there was a significant decrease in the number of NG2\textsuperscript{+} OPCs in both the CC (Fig. 6.6 Ai, Aiv; Fig. 6.7 A; \textit{***p}<0.001; Unpaired t-test; Table 6.4) and Cortex (Table 6.4; \textit{*p}<0.05; Unpaired t-test), as well as the SL of the hippocampus (Table 6.4; \textit{*p}<0.05; Unpaired t-test), although no significant changes were found in the CA1 (Fig. 6.3 Ai, Aiv; Fig. 6.4 A, Table 6.4), Hilus, DG or CA3 (Table 6.4). In contrast, the number of GPR17\textsuperscript{+} immature oligodendrocytes were significantly decreased in Hilus, DG and CA3 (Table 6.4; \textit{*p}<0.05, \textit{*p}<0.05, \textit{**p}<0.01, respectively; Unpaired t-test), whereas no significant differences were found in the CA1 (Fig. 6.3 Aii, Av; Fig. 4 A), SL (Table 6.4) and Cortex (Table 6.4). In the CC there was also a significant decrease of GPR17\textsuperscript{+} cells (Fig. 6.6 Aii, Av; Fig. 6.7 A; Table 4). Finally, there was a significant decrease of mature APC\textsuperscript{+} OLs in the CC (Fig. 6.6 Aiii, Avi; Fig. 6.7 A, \textit{***p}<0.001; Unpaired t-test; Table 6.4) and the CA1 (Fig. 6.3 Aiii, Avi; Fig. 4 A, \textit{***p}<0.001; Unpaired t-test; Table 6.4), but not in the other areas analysed. To find out the distribution of cells of the OL lineage in each stage of differentiation at 3 months and 18 months, I calculated the percentage of cells of each stage out of the total number of oligodendrocyte lineage cells counted in each area. According to this calculation, more than 50\% of the cells of the OL lineage are mainly mature OLs in most of the areas in both ages (Fig. 6.4B, Fig. 6.7B; Table 6.4), reaching 80\% in highly myelinated areas such as the CC (Fig. 6.7 B; Table 6.4). In the case of OPCs, these are around 20-40\% of the total number of cells in the areas analysed (Fig. 6.4 B; Table 6.4), being lower in the CC where the OPCs represent around 15\% of the...
total number of cells and this percentage drops to 13% at 18 months (Fig. 6.7 B; Table 6.4). Immature OLs represent the lowest percentage of cells in the OL lineage, being around 2-15% in the areas analysed and this percentage decreases overall in the 18 months mice (Fig. 6.4 B; Fig. 6.7 B; Table 6.4). Overall, the data indicate a decline in differentiated OLs in the ageing brain and, notably, this was very marked in white matter of the CC, where there was also a loss of OPCs and the pool of GPR17+ immature OLs.

6.2.3. Oligodendrocyte precursor cells differentiation in ageing

To study the differentiation process in OPCs, slices were immunostained for GFP and several markers for different stages of differentiation in the oligodendrocyte lineage as explained above. In order to identify OPCs, cells co-immunolabeled for GFP and NG2 were counted in the 3 months and 18 months Pdgfra-CreERT2:Rosa26R-YFP mice 10 days after the treatment with tamoxifen was finished. There was an overall increase in the number of GFP+/NG2+ cells in the 18 months compared to the 3 months old mice (Fig. 6.5 A; Fig. 6.8 A; Table 6.5), but this was only statistically significant in the CA3 (Table 6.5; *p<0.05; Unpaired t-test). Immature OLs were identified by co-labelling of GFP and GPR17. There was an overall decrease of GFP+/GPR17+ cells in the 18 months mice in most of the brain regions studied, but this was only statistically significant in the CC (Fig. 6.6 Aii, Av; Fig. 6.8 A; Table 6.5; *p<0.05; Unpaired t-test). Contrary, there was a significant increase of GFP+/GPR17+ cells in the CA1 in the 18 months mice (Fig. 3 Aii, Av; Fig. 5 A; Table 5; *p<0.05; Unpaired t-test). Mature OLs were identified by double immunostaining with GFP and APC. An overall decrease of GFP+/APC+ cells was observed in the 18 months mice compared to the 3 months, being this decrease statistically significant in the CA1 (Fig. 6.3 Aiii, Avi; Fig. 6.5 A; Table 6.5; **p<0.01; Unpaired t-test), CC (Fig. 6.6 Aiii, Avi; Fig. 6.8 A; Table 6.5; **p<0.01; Unpaired t-test) and Cortex.
(Table 6.5; *p*<0.05; Unpaired t-test). To find out how many GFP+ cells were in each differentiation stage and know whether the differentiation rate at 3 months and 18 months were different, I calculated the percentages of GFP+/NG2+, GFP+/GPR17+ and GFP+/APC+ cells among the total number of GFP+ cells in each region studied for both groups of age. In general, OPCs (GFP+/NG2+ cells) represented the highest percentage of GFP+ cells, being around 50-60% of the total in the 3 months old and increasing to 70-90% in the 18 months mice (Fig. 6.5 B; Fig. 6.8 B; Table 6.5). Immature OLs (GFP+/GPR17+) represented around 17-30% of the total GFP+ cells in both ages and this percentage dropped to 10% in the CC of the 18 months mice (Fig. 6.8 B; Table 6.5). The proportion of mature OLs (GFP+/APC+) was lower than the rest of cells (between 5-25% of the total) in all the regions at 3 months and it was 0% at 18 months in almost all the regions, with exception of the SL and Hilus, in which the percentage of GFP+/APC+ was around 5% (Fig. 6.5 B; Fig. 6.8B; Table 6.5). In summary, in most of the regions analysed, most of GFP+ cells stay as OPCs (50-60% in the 3 months mice and 70-90% in the 18 months mice) and only a small group differentiate into immature OLs in both groups of age. It is interesting to notice that while at 3 months in all the areas of the brain there are a proportion of GFP+ cells that differentiated into mature OLs (GFP+/APC+) 10 days after the induction of the GFP reported by treatment with tamoxifen, at 18 months these GFP+/APC+ cells are absent in most areas analysed.
Figure 6.3. Cells of the oligodendrocyte lineage in the CA1 of an adult and an aged Pdgfra-CreER T2:Rosa26R-YFP mouse. (Ai-Av) Cells of the oligodendrocyte lineage in a 3 months (Ai-Aiii) and an 18 months (Aiv-Avi) Pdgfra-CreER T2:Rosa26R-YFP mouse. (Ai, Aiv) Cell of the oligodendrocyte lineage immunolabelled for NG2 (red; red arrows) and GFP (green) in the CA1 of a 3 months (Ai) and an 18 months (Aiv) Pdgfra-CreER T2:Rosa26R-YFP mouse. (Aii, Av) Cell of the oligodendrocyte lineage immunolabelled for GPR17 (red; red arrows) and GFP (green) in the CA1 of a 3 months (Aii) and an 18 months (Av) Pdgfra-CreER T2:Rosa26R-YFP mouse. (Aiii, Avi) Cell of the oligodendrocyte lineage immunolabelled for APC (red; red arrows) and GFP (green) in the CA1 of a 3 months (Aiii) and an 18 months (Avi) Pdgfra-Rosa mouse. Scale bars= 50 μm. White arrows point to double immunolabelled cells.
Figure 6.4. Quantification of cells of the oligodendrocyte lineage in different stages of differentiation in the CA1 of an adult and an aged Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP mouse. (A) Quantification (cells per FOV) of NG2<sup>+</sup>, GPR17<sup>+</sup> and APC<sup>+</sup> cells in the CA1 of a 3 months and an 18 months Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP mouse. (B) Percentage of cells in each differentiation stage (NG2<sup>+</sup> (blue), GPR17<sup>+</sup> (yellow) and APC<sup>+</sup> (red)) of the total number of cells quantified (in cells per FOV) in the CA1 of a 3 months and an 18 months Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP mouse. **p<0.001; Unpaired t-test. FOV (200 µm x200 µm).
Figure 6.5. Quantification of GFP+ cells of the oligodendrocyte lineage in different stages of differentiation in the CA1 of an adult and an aged Pdgfra- CreER$^{T2}$:Rosa26R-YFP mouse. (A) Quantification (cells per FOV) of GFP$^+$ NG2$^+$, GFP$^+$ GPR17$^+$ and GFP$^+$ APC$^+$ cells in the CA1 of a 3 months and an 18 months Pdgfra-CreER$^{T2}$:Rosa26R-YFP mouse. (B) Percentage of GFP$^+$ cells in each differentiation stage (NG2$^+$ (blue), GPR17$^+$ (yellow) and APC$^+$ (red)) of the total number of GFP$^+$ cells quantified (in cells per FOV) in the CA1 of a 3 months and an 18 months Pdgfra-CreER$^{T2}$:Rosa26R-YFP mouse. *p<0.05; **p<0.01; Unpaired t-test. FOV (200 µm x 200 µm).
Figure 6.6. Cells of the oligodendrocyte lineage in the CC of an adult and an aged Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. (Ai-Avi) Cells of the oligodendrocyte lineage in a 3 months (Ai-Aiii) and an 18 months (Aiv-Avi) Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. (Ai, Aiv) Cell of the oligodendrocyte lineage immunolabelled for NG2 (red; red arrows) and GFP (green) in the CC of a 3 months (Ai) and an 18 months (Aiv) Pdgfra-Rosa mouse. (Aii, Av) Cell of the oligodendrocyte lineage immunolabelled for GPR17 (red; red arrows) and GFP (green) in the CC of a 3 months (Aii) and an 18 months (Av) Pdgfra-Rosa mouse. (Aiii, Avi) Cell of the oligodendrocyte lineage immunolabelled for APC (red; red arrows) and GFP (green) in the CC of a 3 months (Aiii) and an 18 months (Avi) Pdgfra-Rosa mouse. Scale bars= 50 µm. White arrows point to double immunolabelled cells.
Figure 6.7. Quantification of cells of the oligodendrocyte lineage in different stages of differentiation in the CC of an adult and an aged Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. (A) Quantification (cells per FOV) of NG2\textsuperscript{+}, GPR17\textsuperscript{+} and APC\textsuperscript{+} cells in the CC of a 3 months and an 18 months Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. (B) Percentage of cells in each differentiation stage (NG2\textsuperscript{+} (blue), GPR17\textsuperscript{+} (yellow) and APC\textsuperscript{+} (red)) of the total number of cells quantified (in cells per FOV) in the CC of a 3 months and an 18 months Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. *p<0.05; ***p<0.001; Unpaired t-test. FOV (100 µm x 100 µm).
Figure 6.8. Quantification of GFP+ cells of the oligodendrocyte lineage in different stages of differentiation in the CC of an adult and an aged Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. (A) Quantification (cells per FOV) of GFP\textsuperscript{+}NG2\textsuperscript{+}, GFP\textsuperscript{+}GPR17\textsuperscript{+} and GFP\textsuperscript{+}APC\textsuperscript{+} cells in the CC of a 3 months and an 18 months Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. (B) Percentage of GFP\textsuperscript{+} cells in each differentiation stage (NG2\textsuperscript{+} (blue), GPR17\textsuperscript{+} (yellow) and APC\textsuperscript{+} (red)) of the total number of GFP\textsuperscript{+} cells quantified (in cells per FOV) in the CC of a 3 months and an 18 months Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mouse. *p<0.05; **p<0.01; Unpaired t-test. FOV (100 µm x 100 µm).
Table 6.4. Quantification of NG2$^+$, GPR17$^+$, and APC$^+$ cells in various regions of the brain of the adult and aged Pdgfra-CreER$^{T2}$::Rosa26R-YFP mouse

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>SL</th>
<th>Hilus</th>
<th>DG</th>
<th>CA3</th>
<th>CC</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3mo</td>
<td>18mo</td>
<td>3mo</td>
<td>18mo</td>
<td>3mo</td>
<td>18mo</td>
<td>3mo</td>
</tr>
<tr>
<td>NG2+</td>
<td>8.75 ± 1.161</td>
<td>7 ± 0.7303</td>
<td>6.125 ± 0.5489</td>
<td>3.6 ± 0.7483</td>
<td>7.143 ± 0.6701</td>
<td>7.333 ± 0.8433</td>
<td>13.17 ± 2.722</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>$p&lt;0.05$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>***$p&lt;0.001$</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td></td>
<td>29.97%</td>
<td>39.32%</td>
<td>39.38%</td>
<td>25.11%</td>
<td>37.29%</td>
<td>39.26%</td>
<td>38.84%</td>
</tr>
<tr>
<td>GPR17+</td>
<td>1.444 ± 0.4444</td>
<td>2.556 ± 0.3768</td>
<td>1.556 ± 0.2422</td>
<td>1.111 ± 0.2003</td>
<td>1.889 ± 0.2606</td>
<td>1.222 ± 0.147</td>
<td>2.111 ± 0.3889</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>*$p&lt;0.05$</td>
<td>*$p&lt;0.05$</td>
<td>**$p&lt;0.01$</td>
<td>*$p&lt;0.05$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.95%</td>
<td>14.35%</td>
<td>10%</td>
<td>7.75%</td>
<td>9.86%</td>
<td>6.54%</td>
<td>6.23%</td>
</tr>
<tr>
<td>APC+</td>
<td>19 ± 2.104</td>
<td>8.25 ± 1.25</td>
<td>7.875 ± 0.6928</td>
<td>9.625 ± 0.9808</td>
<td>10.13 ± 0.9149</td>
<td>10.13 ± 0.875</td>
<td>18.03 ± 1.133</td>
</tr>
<tr>
<td></td>
<td>***$p&lt;0.001$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>***$p&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>65.08%</td>
<td>46.33%</td>
<td>50.62%</td>
<td>67.14%</td>
<td>52.83%</td>
<td>54.20%</td>
<td>54.93%</td>
</tr>
</tbody>
</table>
Table 6.5. Quantification of GFP<sup>+</sup>NG2<sup>+</sup>, GFP<sup>+</sup>GPR17<sup>+</sup> and GFP<sup>+</sup>APC<sup>+</sup> cells in various regions of the brain of the adult and aged Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP mouse

<table>
<thead>
<tr>
<th></th>
<th>Cells of the oligodendrocyte lineage in various areas of the brain in the ageing Pdgfra-Rosa26 cre-lox mouse at different stages of differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA1</td>
</tr>
<tr>
<td><strong>GFP+ NG2</strong></td>
<td></td>
</tr>
<tr>
<td>3mo</td>
<td>2.625 ± 0.5957</td>
</tr>
<tr>
<td>18mo</td>
<td>NS</td>
</tr>
<tr>
<td><strong>GFP+ GPR17</strong></td>
<td></td>
</tr>
<tr>
<td>3mo</td>
<td>0.7778 ± 0.2222</td>
</tr>
<tr>
<td>18mo</td>
<td>*p&lt;0.05</td>
</tr>
<tr>
<td><strong>GFP+ APC</strong></td>
<td></td>
</tr>
<tr>
<td>3mo</td>
<td>1.125 ± 0.295</td>
</tr>
<tr>
<td>18mo</td>
<td>*p&lt;0.01</td>
</tr>
</tbody>
</table>

Legend: NS = Not significant

*Significant difference (p<0.05)
**Highly significant difference (p<0.01)
6.2.4. Myelination in ageing

In the previous sections of this chapter my results showed a loss of OPCs, immature and mature OLs in ageing in several areas of the brain analysed. To determine whether this loss of cells of the oligodendrocyte lineage in ageing was accompanied by myelin loss in the ageing brain, I measured MBP density in the CC and Cortex of 3 months and 18 months Pdgfra-CreER\textsuperscript{T2}:Rosa26R-YFP mice. In both areas of the brain, there is a trend towards a decrease in MBP density although this is not statistically significant (Fig. 6.9 Ai-Avi).
Figure 6.9. Changes in myelin in the CC and Cortex of an adult and an aged Pdgfra-CreER\textsuperscript{T2} : Rosa26R-YFP mouse. (Ai-Aii; Aiv-Avi) MBP\textsuperscript{+} (red) immunostaining in the CC (Ai, Aii) and the Cortex (Aiv, Av) of a 3 months (Ai, Aiv) and an 18 months (Aii, Av) Pdgfra-CreER\textsuperscript{T2} : Rosa26R-YFP mouse. (Aiii, Avi) Quantification of MBP\textsuperscript{+} density CC (Aiii) and the Cortex (Avi) of a 3 months compared to an 18 months Pdgfra-CreER\textsuperscript{T2} : Rosa26R-YFP mouse. ns=Not significant; Unpaired t-test. FOV (100 µm x 100 µm). Scale bars= 50 µm.
6.3. Discussion

As described above, OPCs produce myelinating OLs through a process of differentiation and this is important for the correct function of the brain. Previous studies using the Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP have shown that OPCs generate OLs in all regions of the adult brain even in 8 and 12 months old mice (Rivers et al. 2008; Young et al. 2013) and that those OLs produce myelin with shorter internodes (myelinated regions of axons placed between two nodes of Ranvier) but compact and functional (Young et al. 2013). They also show that the OLs generation by OPCs was slowed down with age and the same was reported for OPCs proliferation (Psachoulia et al. 2009).

To examine the dynamics of OPCs in adult and ageing brain, I used the Pdgfra-CreER<sup>T2</sup>:Rosa26R-YFP mouse which allows to follow the fate of OPCs by turning on permanently the expression of YFP reporter protein under the control of Pdgfra promotor by tamoxifen injection. I also analysed the differences in oligodendrocyte lineage cells number between adult and ageing brain. The key findings of this sections are that in the 18 months brains the number of cells of the oligodendrocyte lineage are decreased in various areas of the brain, in grey and white matter. Moreover, there seems to be a decrease in the formation of mature OLs in ageing, although these cells represent the highest percentage of cells within the OL lineage in the adult and ageing brain. Another element to consider is the loss of myelin in the ageing brain despite this not being statistically significant.

6.3.1. Cells of the oligodendrocyte lineage undergo major changes in ageing

In order to study changes in cells of the oligodendrocyte lineage in ageing I first analysed the overall number of cells immunostained for the oligodendrocyte lineage marker Olig2.
Interestingly, there was an almost 2-fold decrease in the number of Olig2$^+$ cells in the 18 months CC compared to the 3 months, which is consistent with previous experiments published by Doucette and colleagues that showed a reduction of 3-fold in Olig2 expression in the CC of 16 months mice compare to 2 months mice (Doucette et al. 2010). In contrast, my data show significant increase in the number of Olig2$^+$ cells in Cortex and CA3 in the ageing brain. To know whether these changes were due to a specific group of cells of the oligodendrocyte lineage in a particular stage of differentiation, I analysed the number of cells in each one of the stages (OPCs, immature OLs and mature OLs). In the case of OPCs, my analysis showed a significant decreased of NG2$^+$ cells in the SL, CC and Cortex of the 18 months mice compared to the 3 months and no significant changes in the rest of areas analysed. This is consistent with previous publications reporting that the cell cycle of NG2 cells is slower in ageing (Psachoulia et al. 2009) which could explain this reduction in OPCs number. There is also a significant reduction in the number of immature OLs, identified as GPR17$^+$ cells in the Hilus, DG, CA3 and CC of the 18 months mice. In a previous study, it has been shown that GPR17$^+$ cells decrease in number as the brain ages, both in grey and white matter, although this study only showed the development of GPR17$^+$ cells up to adult age and not ageing (Boda et al. 2011). The number of mature OLs, identified by APC was also decreased in the CA1 and the CC, being this finding consistent with previous studies in rat (Chen et al. 2011) and in the Pdgfra-CreERT$^{12}$:Rosa26R-YFP mouse where there were a 18% of mature OLs in the 8 months mice after 100 days of the Cre induction (Psachoulia et al. 2009). It is important to notice that in the case of the CC, one of the main myelin tracts in the brain, there is a decrease in OL lineage cells at all stages of differentiation which could be the cause of myelin loss in ageing. My data show a trend towards a loss of myelin in CC and Cortex of the 18 months brain, although this data is not statistically significant. Additionally, I calculated the percentage of oligodendrocyte lineage cells at each of the developmental
stages and I found that in the majority of the areas studied, most of the cells are mature OLs, followed by OPCs and being the proportion of OPCs lower in white matter tracts such as the CC. Interestingly, the percentage of GPR17+ cells (immature OLs) represent a very small proportion of the total number of OL lineage cells both in the adult and the ageing brain.

6.3.2. OPCs differentiation into mature OLs is slow down in ageing

To study the fate of OPCs in adult and ageing brain, I used 3 months and 18 months Pdgfra-CreERT2:Rosa26R-YFP to follow the dynamics of YFP+ cells. I analysed how many of the cells at each different stages of differentiation were co-labelled with YFP, which will indicate that their origin is an OPC in which the expression of YFP was induced by tamoxifen injection. After 10 days of the induction of the expression of YFP protein, I analysed the number of GFP+ cells and this was not significantly altered in most of the areas analysed, except in the CA3 where it was significantly increased at 18 months. The number of Olig2+/GFP+ cells was increased at 18 months in the CA1, DG and Cortex but it wasn’t altered in the rest of the areas analysed. The number NG2+GFP+ cells don’t seem to be altered in ageing in most of the areas analysed, except in the CA3 where it is increased. In the case of GFP+/GPR17+, there is an increase in the number of cells in the CA1 at 18 months, but a decrease in the CC at this same age. The number of GFP+/APC+ cells is decreased at 18 months in most of the brain regions. To further analyse these changes, I calculated the percentage of cells in each stage of differentiation of the total of GFP+ cells. After 10 days of the end of the tamoxifen injections, most of the GFP+ cells were OPCs (NG2+), some of them were GPR17+ cells (immature OLs) and very few were APC+ cells (mature OLs). In the 18 months mice, the proportion of GFP+/APC+ cells after 10 days were significantly decreased or non-existent compared to the 3 months mice,
which indicates that the process of differentiation is slowed down with ageing. This is consistent with previous findings by Psachoulia and colleagues and Zhu and colleagues (Psachoulia et al. 2009; Zhu et al. 2011) and might be related to the decrease in MBP at 18 months that my data shows, although it is not statistically significant. My findings indicate that in 10 days, a proportion of OPCs can differentiate into immature OLs in both adult and ageing brain but the differentiation step from immature OLs to mature OLs is decreased in the ageing process.

6.4. Summary and Conclusions

In summary, the results of this chapter support previous studies showing a decrease in cells of the oligodendrocyte lineage overall in ageing and a gradual decrease in the differentiation process with the addition of the demonstration that in the ageing brain, OPCs can differentiate in a period of time as short as 10 days into immature OLs but the further differentiation to myelinating OLs may need a longer period of time.
Chapter 7

General discussion
7.1. Summary of key findings

The overall aim of this thesis was to contribute to the current knowledge about synaptic signalling onto OPCs in normal ageing and in AD. There is abundant evidence of the existence of associations between neurons and OPCs (Wigley and Butt, 2009) at synapses, both glutamatergic (Bergles et al., 2000) and GABAergic (Lin and Bergles, 2004), as well as with nodes of Ranvier (Butt et al., 1999). However, it remained unclear what effects neuron-OPC signalling has on OPCs, with respect to their differentiation and myelination. The results in Chapter 3 support previous studies by showing the intimate relation of OPCs with the pre-synaptic proteins vGAT and vGluT1 and the post-synaptic proteins Gephyrin and PSD-95. The expression of potassium channels such as Kir4.1 was also confirmed in this chapter as well as the differentiation of OPCs into immature and mature OLs in the adult brain. This section also provided an insight into the varied morphology of OPCs and immature OLs in different areas of the brain, in addition to a detailed description of the distribution of the different cells of the oligodendrocyte lineage and myelin in the adult brain. Chapter 4 demonstrated for the first time that synaptic signalling regulates the number and morphology of OPCs, with the loss of synaptic signalling resulting in a halving of the OPC population and marked cell shrinkage. In addition, the results indicated that the loss of synaptic signalling results in dysregulation of myelination, although it appears to be increased and not decreased as proposed in my initial hypothesis. Notably, the results in Chapter 5 demonstrate an equivalent effect on OPCs in the APP/PS1 mouse model of AD, with a significant decrease in OPC numbers and cellular complexity at 9 months old, that occurs only at much later ages in controls, consistent with the loss of synaptic signalling that occurs in AD. In addition, there was an apparent increase in OPC differentiation and myelination in AD, similar to that observed following synaptic silencing. Finally, Chapter 6 provides further evidence that oligodendrocyte lineage cells decrease with natural aging, indicating the changes in AD
represent accelerated aging. A key finding is that ageing OPCs rapidly differentiate into immature GPR17+ OLs, but their subsequent differentiation into mature myelinating OLs is significantly retarded, resulting in a loss of myelin. Overall, this thesis adds new knowledge on the function of synaptic signalling in regulating OPC number and morphology and how these are implicated in the changes in OPCs in ageing and AD.

7.2. Synaptic communication regulates OPC morphology

OPCs contact neuronal synapses (Wigley and Butt, 2009), and so it is not surprising their association with the pre- and post-synaptic proteins vGAT, vGluT1, Gephyrin and PSD-95 (Chapter 3). This association has been shown in previous studies in cortex, cerebellum, hippocampus and somatosensory cortex using IHC (for neurons and NG2 cells, GAD65 and vGAT) (Lin and Bergles, 2004; Wigley and Butt, 2009; Orduz et al., 2015), EM (Bergles et al., 2000), and for vGluT1 in CC following demyelinating injury (Sahel et al., 2015). The physiological importance of OPCs integration into these circuits is not fully understood, but it has been shown that OPCs respond electrically to glutamate signals with currents mediated by AMPA receptors (Bergles et al., 2000) and that blockade of either electrical activity in axons by TTX or AMPA receptors result in a reduction in the length of processes and branching in OPCs (Fannon et al., 2015). Moreover, OPCs have also been reported to respond electrically to GABA via GABA_A receptors, inducing a temporary inhibition of AMPA receptors that could regulate glutamatergic signalling onto OPCs (Lin and Bergles, 2004). This is supported by my findings in Chapter 4, which showed shrinkage of OPC processes and loss of complexity after synaptic inhibition by BoNT/A in the CA1 of the hippocampus. The target of BoNT/A is the SNARE protein SNAP25, a presynaptic protein present mainly in glutamatergic neuronal terminals (Verderio et al., 2004; Frassoni et al., 2005), therefore, the silencing of glutamate release
by BoNT/A could be responsible for the morphological changes in OPCs observed in the results of Chapter 4. Additionally, previous studies demonstrated the increase in the number of synaptic vesicles and their diameter after synaptic silencing by BoNT/A (Caleo et al., 2012). This is consistent with my findings in Chapter 4, which showed an increase in pre-synaptic proteins vGluT1 and vGAT. Surprisingly, the increase in both types of proteins contradicts the previous statement about the absence of SNAP25 in GABAergic terminals, since vGAT vesicles are also affected. This may be due to an indirect effect of the synaptic silencing on GABAergic neurons. Similarly, there was an increase in the post-synaptic protein PSD-95, which it is known to be essential for AMPA receptor clustering and for regulation of synaptic strength, as well as being regulated by the activity of neighbouring synapses (reviewed in Keith, 2008). The increase in PSD-95 after synaptic silencing by BoNT/A may be due to loss of activity from close neurons causing the upregulation of PSD-95. The increase in pre-synaptic and post-synaptic proteins does not relate to an increase in signalling onto OPCs. Moreover, there is the possibility that the increase in the vGAT:NG2, vGluT1:NG2 and PSD-95:NG2 indexes is due to the cell shrinkage, but at least in the case of PSD-95 there was an increase in the overall PSD-95 density (data not shown) so it is reasonable to assume the increase in synaptic connectivity is real. Overall, the results clearly demonstrate that OPCs have a high level of synaptic connectivity, both direct synaptic signalling and indirect spillover-type signalling.

7.3. Synaptic communication regulates OPCs number, proliferation and differentiation

In Chapter 4, I provided evidence of the decrease in OPCs number, with no major changes in number of sister cells, as well as an apparent increase in immature OLs and increase in myelination after synaptic silencing. This is consistent with the promotion of the processes of differentiation and myelination by synaptic silencing, but partially
contradicts previous studies that showed increased cell proliferation, increased cell differentiation and decreased myelination by blockade of AMPA receptors or neuronal electrical activity (Fannon et al., 2015). The latter study was performed in developmental animals, while my experiments were in adult mice and the reaction of OPCs to synapses is different (Vélez-Fort et al., 2010), which requires further study. On the other hand, it has been shown previously in another study that blockade of AMPA receptors by NBQX did not have any effect on the number of oligodendrocyte lineage cells or MBP density, but the blockade of GABAergic signalling by GABAzine increased the number of cells of the oligodendrocyte lineage, mainly OPCs and mature OLs, indicating that GABA decreases the number of OPCs and mature OLs via GABA\_A receptors (Hamilton et al., 2016). This could explain the decrease of OPCs in my results after synaptic silencing, since probably glutamatergic communication is impaired in my model, but GABAergic communication may be still active, although I also observed an increase of myelination, whereas GABA release has been shown to decrease the number of myelinated axons and the myelin thickness (Hamilton et al., 2016). My findings on synaptic silencing are also contradictory to a previous study that there is a decrease in myelination after inhibition of electrical signals or AMPA receptor blockade (Fannon et al., 2015). However, previous studies were primarily on the effects of glutamatergic and GABAergic signalling on developmental myelination, and my results indicate that OPCs in the adult brain are markedly altered following synaptic silencing and this is associated to increased myelination.
7.4. OPC dynamics in the ageing brain

Several studies using tamoxifen inducible Cre-lox mice have tried to elucidate the dynamics of OPCs in developmental and ageing brain. In Chapter 6, I used a Cre-lox tamoxifen inducible Pdgfra-CreER:Rosa26R-YFP transgenic mice (Psachoulia et al., 2009) to follow the fate of OPCs in adult and ageing brain. My results showed a decrease in oligodendrocyte lineage cells with age, as well as a block in their terminal differentiation. This is consistent with previous reports in which OPCs cell cycle was increase with age, as well as a slow down in the generation of mature OLs, that was 18% after 100 days of tamoxifen injection in 8 months old mice (Psachoulia et al., 2009).

Another study using NG2creER\textsuperscript{TM}BAC:ZEG double-transgenic mice, in which tamoxifen inducible CreER\textsuperscript{TM} recombinase is expressed specifically in NG2-expressing cells showed that the percentage of OLs generated from OPCs after 60 days of Cre recombination induction by tamoxifen decreased from 60% mature OLs generated at P2 to 30% mature OLs generated at P60 (2 months), indicating that the generation of OLs declines with age (Zhu et al., 2011). My results showed 25% of YFP\textsuperscript{+} cells in the 3 months old were also positive for APC in the CA1 while only 19% of them were positive for APC in the CC, indicating that they were mature OLs generated after the tamoxifen injection and in a period of 15 days following the first tamoxifen dose. This is consistent with a study by Richardson and colleagues that showed that 1/3 of the YFP\textsuperscript{+} cells in the optic nerve of 120 days (4 months) old mice were positive for APC 65 days after tamoxifen induction of the reporter (Young et al., 2013). The difference in the percentage of mature OLs generated may be due to the shorter period after tamoxifen induction in my experiment. Interestingly, no APC\textsuperscript{+} YFP\textsuperscript{+} cells were detected at 18 months old 15 days after tamoxifen in the CA1 or in the CC, although in both regions there were YFP\textsuperscript{*}GPR17\textsuperscript{*} cells (around 30\% and 10\%, respectively), which could mean that even though the OPCs are differentiating at 18 months old, this process it is not fast enough to
generate mature OLs in such a short period of time. This is also consistent with previous publications showing GPR17+ cells as a pool of adult progenitors, that differentiate slowly in normal conditions but increase their differentiation rate into mature OLs in case of injury (Viganò et al., 2016). Supporting this previous study, another one showed an increase in the production of mature OLs after 21 days of generating a demyelinating lesion in the CC of 13 months old mice (Crawford et al., 2016). Therefore, it would be interesting to test whether the same results occur in 18 months old animals and a demyelinating injury accelerate the production of mature OLs or, contrary because of ageing, the process of differentiation would remain slow.

7.5. OPCs in the APP/PS1 mouse model of Alzheimer's disease

As shown in Chapter 4, loss of synaptic communication causes OPCs shrinkage and loss of cell complexity. In Chapter 5, similar results were observed in OPCs in the APP/PS1 mouse model of AD at 9 months old. At this age, OPCs presented shorter processes, a smaller number of ramification points and lower number of end points that in the control mice. This may be due to synaptic impairment that presents in the APP/PS1 mouse model after 3 months (Bittner et al., 2012), supporting the findings in Chapter 4, although it is important to consider that the synaptic silencing caused by BoNT/A (Chapter 4) is an abrupt change compared to the synaptic loss in the APP/PS1 mouse model of AD, that happens during a longer period of time. Therefore, the results obtained in chapter 4 and chapter 5 can be comparable in terms of the effect of the lack of signalling onto OPCs, although it would be interesting to analyse the effect of a sustained silencing (for a period longer than 14 days) by BoNT/A in OPCs. Similar morphological changes were observed in vitro when NG2 cells were incubated with Aβ1-42 oligomer and fibril (Nielsen et al., 2013), raising the possibility that changes in OPCs morphology at 9 months old in the
APP/PS1 mouse could be due to the Aβ load in the brain. The number of OPCs was halved in the 9 months old APP/PS1 mouse, but interestingly, there was an increase in the number of immature OLs, indicating that the lost OPCs could have differentiated and perhaps the loss of complexity of the cells, the decrease in the OPCs number and the increase in immature OLs are associated to the process of differentiation. Conversely, in the 14 months old APP/PS1 mouse OPCs presented an increase in complexity with higher number of ramification points and end points, as well as longer processes. Interestingly, the number of OPCs did not decrease in the APP/PS1 mouse compared to the control but was at the levels of the 9 months APP/PS1, indicating that a premature ageing might have happened at 9 months old in the APP/PS1 mouse, but it stopped at 14 months old. On the other hand, the number of sister cells was halved in the 14 months old APP/PS1 mouse, as well as the number of immature OLs, which could be due to an impairment of the processes of proliferation and differentiation as a reaction to neurodegeneration. The loss of OLs lineage cells is consistent with previous studies that show loss of these cells in human samples of AD patients (Behrendt et al., 2013). Likewise, OPCs increase in complexity at 14 months old in the APP/PS1 mouse may be consistent with a hypertrophy caused by overreaction to damage due to Aβ plaques, synaptic impairment and neurodegeneration. This hypertrophy of OPCs has been shown previously in cases of brain injury (Levine, 1994). Furthermore, I found interesting to compare the morphology of OPCs in the process of normal ageing, comparing 9 months and 14 months old controls, and in the process of ageing within AD, comparing 9 month and 14 months old APP/PS1 and using the data that I had already available (see Fig. 7.1 and 7.2). Interestingly, no significant differences were found in OPCs morphology at 9 months old and 14 months old in the control mice as shown by Sholl analysis (Fig. 7.1 A-C). Contrary, in the APP/PS1 mice there were notable differences between the 9 months old and the 14 months old OPCs, with a significant increase in the number of end points, number of
nodes and length of processes in general and per branch order (Fig. 7.2 A-C). The results demonstrate that OPCs initially undergo premature cellular atrophy at 9 months in APP/PS1, which occurs later in normal ageing, whereas in the aged APP/PS1 brain OPCs undergo hypertrophy reminiscent of an injury response that may be related to pathology. The changes in OPCs are associated with decreased myelination, supporting the hypothesis that decreased regenerative capacity of OPCs underpins age-related myelin loss and this is exacerbated in AD.
Figure 7.1. Sholl analysis of NG2 cells does not show changes in cell morphology in normal ageing. (A) Number of end points of the processes. (B) Number of nodes (ramification points in the processes). (C) Processes length. (D) Length of processes of different branch orders. Two-way ANOVA followed by Sidak’s multiple comparisons test. n=3. 9 cells/group were analysed.

Figure 7.2. Sholl analysis of NG2 cells shows changes in cell morphology in ageing in the APP/PS1 mouse model of Alzheimer’s disease. (A) Number of end points of the processes. (B) Number of nodes (ramification points in the processes). (C) Processes length. (D) Length of processes of different branch orders. Two-way ANOVA followed by Sidak's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, p<0.0001. N=3. 9 cells/group were analysed.
7.6. Myelination in normal ageing and Alzheimer’s disease

Myelination of the brain it is essential for its correct function and previous studies have shown that exists and age-dependent loss of myelin and that this is accelerated in AD (Bartzokis et al., 2003; Bartzokis, 2004). In chapter 6, my results show a loss of MBP density in Cortex and CC of 18 months old mice compared to 3 months old controls. This loss of myelin was accompanied by a loss of cells of the oligodendrocyte lineage, including OPCs, immature OLs and mature OLs. This is consistent with an age-dependent impairment in the myelination process and it is supported by previous studies showing a decrease in MOG (Myelin oligodendrocyte glycoprotein) expression in 16 months old mice compared to 2 months old controls, as well as a decrease in oligodendrocyte lineage cells in the same mice (Doucette et al., 2010). The same findings were shown by my results of MBP density in the 14 months old APP/PS1 mouse, but this time the loss of myelin was significantly evident. As in normal ageing, it was accompanied by loss of OPCs, sister cells and immature OLs. This may be consistent with an acceleration of the ageing process in AD, since the changes found at 14 months old in the APP/PS1 mouse were more prominent than the changes found in normal ageing at 18 months old. Contrary, in the 9 months old APP/PS1 mouse there was no loss of myelin and the decrease in OPCs might have been balanced by an increase in immature OLs.
7.7. Conclusions

In conclusion, this PhD provides evidence of the association of OPCs with synaptic proteins, their differentiation potential in adult brain as well as in ageing brain, with a slow down in the process in the latter. Moreover, my results show evidence of a role of synaptic signalling in OPCs dynamics, morphology and differentiation. Furthermore, I provided evidence of age-related myelination impairment and myelin loss in APP/PS1 mouse model of AD. Lastly, my results show a relation between loss of cells of the oligodendrocyte lineage and myelin loss in normal ageing and in the APP/PS1 mouse model of AD, as well as the possibility of a hypertrophy of OPCs as a reaction to damage in the APP/PS1 mouse model. Together, my thesis provides evidence of the importance of OPCs dynamics for the correct function of the CNS both in normal ageing and in AD.


Seifi, M., Croteen, N. L., Van Der Want, J. J., Metzger, F., Swinney, J. D., May, P. J. and


Sun, W. and Dietrich, D. (2013) ‘Synaptic integration by NG2 cells.’, Frontiers in


Viganò, F., Schneider, S., Cimino, M., Bonfanti, E., Gelosa, P., Sironi, L., Abbracchio, 174


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<tr>
<td>PGRS Name:</td>
<td>Irene Chacon de la Rocha</td>
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<td>Department:</td>
<td>PHBM</td>
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<tr>
<td>First Supervisor:</td>
<td>Arthur Butt</td>
</tr>
<tr>
<td>Start Date: (or progression date for Prof Doc students)</td>
<td>10/2014</td>
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<td>Study Mode and Route:</td>
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Title of Thesis: Oligodendrocyte precursor cells in ageing and Alzheimer’s disease

Thesis Word Count: 38948 (excluding ancillary data)

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