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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Optimization of ex vivo human brain models to study remyelination and neuroregenerative therapies

Rida Chuhan

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
dr. Melissa SCHEPERS

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Prof. Dr. Zaal KOKAIA

MENTOR :
Mr. Yu-Ping SHEN
Dr. Sara PALMA-TORTOSA

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2025

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Optimization of *ex vivo* human brain models to study remyelination and neuroregenerative therapies*

Rida Chuhan[1,2], Yu-Ping Shen[1], Sara Palma-Tortosa[1], Raquel Martinez-Curiel[1] and Zaal Kokaia[1]

[1]Laboratory of Stem Cells and Restorative Neurology, Lund Stem Cell Center, Lund University, 22184 Lund, Sweden
[2]Faculty of Medicine and Life Sciences, Biomedical Research Institute, Hasselt University, Campus Diepenbeek, Agoralaan Gebouw C - B-3590 Diepenbeek, Belgium

*Running title: *Optimizing Human Brain Models for Regeneration*

To whom correspondence should be addressed: Zaal Kokaia, Tel: +46 70 536 59 17; Email: zaal.kokaia@med.lu.se

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ABSTRACT

Disorders affecting the central nervous system, such as stroke or multiple sclerosis (MS), cause irreversible loss of different neural cells (e.g. neurons and oligodendrocytes), resulting in devastating neurological deficits. However, current approved treatments do not restore damaged human brain tissue. This translational gap is due mainly to the limited relevance of animal models to the complexity of the human brain. In this study, we utilized adult human cortical tissue (hACtx) from epileptic patients undergoing surgical resection to develop two clinically relevant *ex vivo* models. First, we aimed to create an *ex vivo* human demyelination model using the demyelinating agent lysolecithin to explore potential therapies for MS. Second, we investigated the possibility of using induced pluripotent stem cell-derived dorsal forebrain organoids (dOGs) as a source for cell transplantation in human tissue. For this purpose, dOGs were transplanted onto hACtx organotypic slices, and their integration was subsequently assessed using immunohistochemistry. In the demyelination model, 1 mg/mL lysolecithin caused excessive glial activation, while 0.5 mg/mL produced better outcomes. Further testing of lower concentrations is necessary to optimize demyelination with minimal effect on neural loss. In the transplanted dOGs, we observed early axonal outgrowth by day 8, with more extensive projections by week 2 and 4, suggesting survival, differentiation, and structural integration into adult human tissue. Functional synapse formation still needs to be investigated. These models provide promising platforms for studying regenerative therapies in a human-relevant context and may bridge the translational gap between experimental data and clinical application.

INTRODUCTION

Central nervous system (CNS) disorders impose a significantly growing burden on worldwide health, as they appear to be the leading cause of global suffering (1,2). These patients often require social and economic support because of their physical, cognitive, and psychosocial limitations (2). The CNS, consisting of the brain and spinal cord, plays an important role in regulating the body's vital functions (3,4). It receives and processes sensory information after which it sends an electrical signal through the spinal cord to generate appropriate motor responses (4). More specifically the brain is responsible for coordinating movement, sensation, emotions, communication, thought processing and memory (5). There are numerous CNS-disorders, including Alzheimer's disease, Parkinson's disease, brain tumors, epilepsy, brain injury, stroke, and multiple sclerosis (MS) (1,4,3). These disorders are often accompanied by structural and functional changes in the brain, leading to a wide range of neurological symptoms such as memory loss, impaired motor function, cognitive decline, and sensory disturbances (1,2).

MS is a chronic autoimmune disease of the CNS characterized by inflammation, demyelination, and neurodegeneration (6). The incidence of MS keeps increasing worldwide,

together with its socioeconomic impact on young adults. The disease results from an aberrant immune response targeting myelin sheaths, leading to disrupted nerve signal transmission and neurological impairments (7). Although the exact cause of MS remains unclear, genetic and environmental factors, including Epstein-Barr virus infection and vitamin D deficiency, have been linked with the disease susceptibility (6,8). MS is a heterogeneous neurological disorder classified into distinct subtypes based on disease progression (9). Clinically isolated syndrome (CIS) is a single demyelinating episode with potential progression to MS. Next, relapsing-remitting MS (RRMS), the most common form, involves relapses and remissions, often advancing to secondary progressive MS (SPMS), marked by continuous decline of neurological function. Lastly, primary progressive MS (PPMS) worsens gradually from onset without relapses.

Current treatments for MS primarily focus on the autoimmune component of the disorder by reducing relapses, slowing disease progression, and managing symptoms (10,11). However, they do not address the root cause of the neurological deficits: demyelination. Several therapeutic approaches have been tested in animal models of MS, predominantly in rodents (12). Although no animal model fully recapitulates the complexity of MS, several rodent models have been developed to mimic distinct pathological features of the disease. The most widely used model is Experimental Autoimmune Encephalomyelitis (EAE), which explores the neuroinflammatory processes (13). EAE can be induced either actively, through immunization with CNS peptides, or passively, via the adoptive transfer of encephalitogenic T cells. In contrast, toxin-induced demyelination models focus on the degenerative aspects of MS (14). The cuprizone model, which involves dietary administration of a copper chelator, selectively induces oligodendrocyte death and demyelination, offering insights into mechanisms of oligodendrocyte loss, oligodendrocyte precursor cell (OPC) recruitment, and remyelination (15). Although its exact mechanism is unclear, cuprizone is believed to disrupt cellular metabolism. Another demyelination model uses focal injections of lysophosphatidylcholine (LPC), a compound toxic to myelinating oligodendrocytes but sparing axons (16). LPC disrupts myelin structure by binding to myelin proteins, leading to vesiculation and subsequent phagocytic clearance.

Despite the valuable insights these rodent models have provided and their contribution to the development of promising therapeutic strategies, a significant translational gap remains due to the complexity and distinct characteristics of the human brain. To bridge this gap, researchers have developed a variety of *in vitro* human cell-based systems, ranging from simple monolayers to complex 3D structures (17,18). For example, primary cell cultures, including oligodendrocytes, astrocytes, and neurons, offer biologically relevant models providing insight into remyelination mechanisms (18). However, they are limited by the need for embryonic tissue, short culture lifespans (typically around 10 days) and inability to mimic the complex structures of the human brain. In contrast, human induced pluripotent stem cells (hiPSCs) allow the generation of various CNS-celtypes, provide more sustainability, large-scale experimentation, and are promising for modeling patient-specific testing (19). Nonetheless, there remains a lack of structural organization and essential components—such as the immune system—that are integral to the human brain. To address these limitations, 3D models like spheroids and organoids have been developed, offering improved cytoarchitecture and cell-cell interactions that better mimic the human brain (20). Yet, despite their complexity, they still fall short of fully recapitulating the immune–CNS interplay and the mature architecture seen in vivo. Overall, while these in vitro models represent an increasing biological relevance and complexity, a significant gap remains regarding their ability to accurately mimic the adult human brain. There is a clear need for a human model that incorporates the brain's cellular composition, spatial organization, and immune interactions, which are essential for modeling complex neurological diseases such as MS.

This critical need for a human-relevant model is not unique to MS, but represents a broader challenge in CNS-disorder research. A major hurdle in treating CNS diseases lies in the limited capacity of current therapies that promote neuro-regeneration. Most available treatments focus on symptom management or halting disease progression, but fail to address

the core challenge of restoring lost neuronal connections. This limitation is largely due to the brain's structural complexity and the lack of appropriate human models to accurately predict the effects of regenerative therapies on the human brain.

Recently, many researchers have been exploring cell transplantation, described as the exogenous delivery of healthy newly generated cells, as a therapy for CNS-disorders, such as stroke or brain injury (21,22). Several animal studies show that cells transplanted to the brain not only survive, but also lead to functional improvement. For example, hiPSCs are widely used for regenerative purposes because of their capacity to proliferate and differentiate into a variety of functionally active cells (23). These hiPSC are generated *in vitro* from patients' somatic cells by introducing specific reprogramming factors. Previously, our research team conducted a study where they transplanted hiPSC-derived cortical neurons into the stroke-injured cortex of adult rats (24). This experiment yielded positive results, including improved neurological deficits and the establishment of both afferent and efferent morphological and functional connections with host cortical neurons. Following this, they investigated whether these hiPSC-derived cortical neurons could also integrate into adult human brain slices (25). For example, figure 1 demonstrates how the grafted hiPSC-derived cortical neurons (green) establish afferent synapses with adult human cortical neurons (brown). These findings revealed that the stem cells not only survived on the human brain slices but also differentiated and integrated into the adult human cortical neural circuits, forming functional synaptic connections and demonstrating potential for neuronal replacement therapies. A recent study by S. Cao *et al.* transplanted human cerebral organoids into the infarct zone of mice subjected to stroke (26). Organoids are mini-clusters of cells grown in a defined three-dimensional (3D) environment *in vitro*, where they self-organize and differentiate into functional cell types, mimicking the structure and function of an organ *in vivo* (27). As a result, it was found that the grafted organoids survived well in the infarcted core, differentiated into neurons and repaired infarcted tissue (28). They also sent axons to distant brain regions, integrated into the host neural circuit and thereby eliminated the sensorimotor defects of stroke mice. In contrast, it was found that transplantation of dissociated single cells from organoids failed to repair the infarcted tissue (26). This research demonstrated that hiPSC-derived cortical organoids have the potential to repair the stroke-lesioned brain in mice. Another study by Z. Wang *et al*. demonstrated that transplantation of human cerebral organoids into a rat model of traumatic brain injury (TBI) led to significant improvements in neurological motor function (28). This highlighted the regenerative potential of organoids in restoring lost brain function and supports their application in models of brain injury and repair. In general, when comparing organoid transplantation with regular cell transplantation, it appears that organoids offer some superior benefits (29). They provide diverse cell types, a larger cell number, better survival of the graft and enhanced axonal growth. While cortical organoids have shown promising results in repairing stroke-induced lesions in murine models, it remains unclear whether these findings can be translated to the human brain.

To address this limitation, the aim of this paper is to overcome the translational gap between rodent and human brain physiology by developing and utilizing *ex vivo* human brain models. To achieve this, we will first establish an *ex vivo* human demyelination model by applying the demyelinating agents cuprizone and lysolecithin to *ex vivo* human cortical slices obtained from epilepsy patients undergoing surgical resection. This human-relevant model offers a unique platform to evaluate potential therapies for MS within a biologically and clinically meaningful context, thereby advancing translational research and bridging the gap between preclinical findings and clinical application. In parallel, we will investigate the survival, differentiation, and functional integration of transplanted cortical organoids in *ex vivo* human brain slices. This will be assessed using immunofluorescence to evaluate structural integration. Through this approach, we aim to gain critical insights into the therapeutic potential of organoid transplantation for brain repair in human patients. Together, these models will provide promising platforms for studying regenerative therapies in a human-relevant context and may help bridge the gap between experimental findings and clinical application.

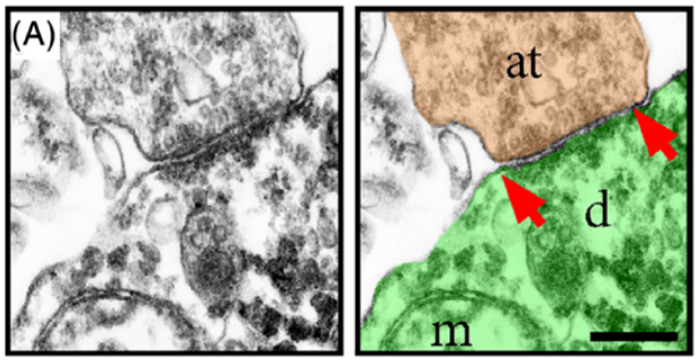


Fig. 1 - Grafted hiPSC-derived cortical neurons establish afferent synapses with adult human cortical neurons in organotypic cultures. Representative immuno-electron microscopy (iEM) images showing asymmetric synapses with continuous postsynaptic densities (red arrowheads) in grafted GFP+ (d, green) connected with host presynaptic axon terminals (at, brown) at 4 weeks (A) after ex vivo transplantation. m, mitochondria. Scale bar = 0.2 µm (19)

EXPERIMENTAL PROCEDURES

GFP-hiPSC culture - hiPSCs (iPS Core Facility) were cultured, passaged, cryopreserved and thawed according to the manufacturer's protocol provided by StemCell™ technologies (30), using the mTeSR™ Plus medium (StemCell™ technologies, United States). Briefly, culture plates were coated with recombinant human Laminin-521 (Gibco™, ThermoFisher Scientific, United States), and 200,000–300,000 cells were seeded per well of a 6-well plate for optimal growth. Passaging was performed when cells reached 70–90% confluency. hiPSCs were previously transduced with a PGK-GFP lentiviral vector (Addgene, United States, #19070) and GFP-positive cells were subsequently sorted using a BD FACSMelody cell sorter.

Human pluripotent stem cell-derived dorsal forebrain organoids formation –

dOGs Were generated using the STEMdiff™ Dorsal and Ventral Forebrain Organoid Differentiation Kits (STEMCELL™ Technologies) over a period of 41 days. Following differentiation, organoids were maintained in culture using the STEMdiff™ Neural Organoid Maintenance Kit. Briefly, on day 0, GFP-hiPSCs were seeded into AggreWell™800 plates using the Seeding Medium (supplementary, table 1). From day 1 to day 6, partial medium changes were performed daily using Forebrain Organoid Formation Medium (supplementary, table 2). On day 6, the formed embryoid bodies (EBs) were transferred to a suspension culture plate, and full medium changes with Forebrain Expansion Medium (supplementary, table 3) were carried out every other day from day 6 to day 25. From day 25 to day 41, organoids were cultured with Forebrain Organoid Differentiation Medium (supplementary, table 4), with full medium changes every other day. Upon reaching maturity at day 41, organoids were maintained using Forebrain Organoid Maintenance Medium (supplementary, table 5), with full medium changes every 2–3 days.

Organotypic cultures of human adult cortical tissue (hACtx) slices - Organotypic cultures of human hACtx were obtained from epileptic patients undergoing elective temporal lobe surgery, during which a part of the healthy neocortical tissue is removed to access the affected hippocampal area. This procedure is approved by the Regional Ethical Committee, Lund, Sweden (ethical permit number 2021-07006-01) and informed consent was obtained from all patients. The maintenance protocol for hACtx slices was previously optimized by our research group (31). Briefly, slices were maintained in optimized hACtx medium (supplementary, table 6), with medium changes every 2–3 days. BDNF (50 ng/mL, Peprotech, United States) and NT3 (50 ng/mL, Peprotech) were freshly added prior to each medium change. Note that the medium was equilibrated in the incubator at 37 °C and 5% CO2 for at least 2 h before each medium change.

Cuprizone and lysolecithin treatment – To develop human demyelination models, hACtx slices were treated with either cuprizone (Sigma-Aldrich, United States) or lysolecithin (Sigma-Aldrich). Based on the literature,

cuprizone was applied at concentrations of 500 µM and 1 mM, with an incubation period of 24 hours (32). Cuprizone solutions were prepared in 50% H_2O and 50% ethanol. For lysolecithin treatment, concentrations of 0.5 mg/mL and 1 mg/mL were used, with an incubation period of 16–17 hours (33,34). Following treatment, media were replaced with fresh hACtx medium for 24 hours before tissue fixation with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for at least 4 hours.

Dorsal Forebrain Organoid (dOG) transplantation to human adult cortical tissue (hACtx) slices - After 41 days of differentiation, dOGs reached maturation and were transplanted onto hACtx slices, which were previously maintained in culture for 1–2 weeks after surgery. In addition to organoids transplanted at 41 days. Transplantation was facilitated using Matrigel (Corning, United States, #356234) to stabilize the organoids on the tissue surface. Organoids were transferred using pipette tips with manually cut edges to accommodate their size. Following transplantation, slices were maintained in optimized hACtx medium for an additional 2–4 weeks.

Immunohistochemical stainings - Transplanted and demyelinated hACtx slices were stained following the same protocol. After fixation with 4% PFA (≥4 h or overnight), slices were washed with KPBS (supplementary, table 7) and DAPI (1:1000) was applied in permeabilization solution (supplementary, table 8) for 2 h at RT before antigen retrieval (if required). For antigen retrieval, slices were incubated in sodium citrate buffer (ThermoFisher Scientific) at 65 °C for 2 h. Slices were then permeabilized at 4 °C (≥5 h or overnight) after which blocking solution (supplementary, table 9) with 10% normal donkey serum (NDS) (Merck-Millipore, Germany) was added for at least 5 h or overnight. Primary antibodies were applied for 48 h and secondary antibodies for 24 h in blocking solution with 10% NDS. Washes were performed before and after secondary antibody incubation with blocking solution. If not yet applied, DAPI staining was performed at this stage. Slices were then washed with KPBS, mounted on microscope slides (Epredia, China) coated with Gelatin (Sigma-Aldrich), dried, rinsed with Milli-Q, and covered with DABCO (Sigma-Aldrich) and coverslips (24 mm x 60 mm; thickness 1.5; Marienfeld Superior, Germany). Imaging was done with the confocal microscope (Zeiss, Germany, LSM 780) and scanner microscope (Olympus, germany, VS120 S6).

RESULTS

hACtx quality depends on culture duration post-surgical handling - We obtained hACtx tissue on three separate occasions from three different epilepsy surgery patients. In the first two cases, the quality of the tissue was suboptimal (Supplementary Figures S1 and S2). The quality of hACtx depends on factors such as culture duration, post-surgical handling, the nature of the surgery, proximity to the epileptic focus, and whether the patient previously underwent electrode therapy. Several factors likely contributed to this reduced viability, such as improper preservation immediately post-resection and culture durations. Following the initial unsuccessful attempt, we aimed to assess the condition of the tissue immediately after surgical removal and to evaluate how culture duration impacts tissue integrity. As shown in Supplementary Figure S1, 3 days in culture showed a noticeable decline in myelin signal compared to acutely processed tissue. Despite the compromised starting condition, we concluded that the hACtx tissue remained suitable for experimentation, as treated samples could still be compared to matched controls. In contrast, the third sample we received was of markedly higher quality, as confirmed by staining of acute tissue (Figure 2).

Lysolecithin treatment of hACtx induces effective demyelination - To develop a human *in vitro* demyelination model, we treated hACtx slices with demyelinating agents - cuprizone and lysolecithin. While most existing research uses *in vivo* demyelination models, few studies have explored demyelination *in vitro* settings. Guided by the limited available literature, we selected two initial concentrations for each agent: 500 µM and 1 mM for cuprizone, and 0.5 mg/mL and 1 mg/mL for lysolecithin. Figure 3A shows stainings of hACtx slices treated with 0.5 and 1 mg/mL lysolecithin, compared to untreated controls. Confocal imaging (Figure 3B) revealed a clear reduction in MBP signal following treatment, indicating effective demyelination. This observation was supported by quantitative analysis (Figure 3E), which confirmed a dose-dependent decrease in MBP intensity. As our aim was to selectively

demyelinate without inducing broader toxicity, we also assessed overall cell viability and neuronal preservation using DAPI and NeuN stainings. Overall cell counts remained stable (Figure 3C and 3D) suggesting that lysolecithin-induced demyelination did not cause significant neuronal loss.

High concentrated lysolecithin treatment of hACtx induces astrocyte activation without major effects on microglia or oligodendrocytes - To assess whether lysolecithin treatment affects the surrounding cell types, we performed immunostaining for microglia (IBA1), mature oligodendrocytes (CC1), and astrocytes (GFAP) (Figure 4A). No substantial changes were observed in the number or morphology of microglia or oligodendrocytes across treatment conditions. In contrast, GFAP staining revealed an increase in astrocyte signal intensity following lysolecithin treatment, suggesting glial activation (Figure 4B). This observation was further supported by quantitative analysis and threshold-based measurement of GFAP signal (Figures 4C and 4D).

In vitro generation of dOGs from GFP-hiPSCs - To investigate the integration of transplanted organoids in hACtx slices, we first generated dOGs from GFP-expressing hiPSCs, as detailed in the *Materials and Methods* section. Figure 5 illustrates the successful formation of dOGs from day 2 to day 41, with consistent GFP fluorescence confirming the identity and viability of the developing organoids.

Transplanted dOGs survive and integrate into hACtx forming axonal projecions - Following the generation of mature dOGs, we transplanted them onto organotypic slices of hACtx derived from epileptic patients undergoing surgical resection. The cortical slices were maintained in culture for 2–3 weeks prior to transplantation. As shown in Figure 6A, early indications of axonal outgrowth from the transplanted dOGs were observed on day 8 post-transplantation, becoming more prominent by two weeks. To further assess the integration and projection capacity of the transplanted organoids, we performed immunohistochemical staining on the cocultures at 2- and 4-weeks post-transplantation (Figures 6B and 6C). Staining for GFP, NeuN, and β-III-tubulin revealed the presence of axon-like projections extending from the GFP+ organoid into the host hACtx, suggesting structural integration and neuronal differentiation.

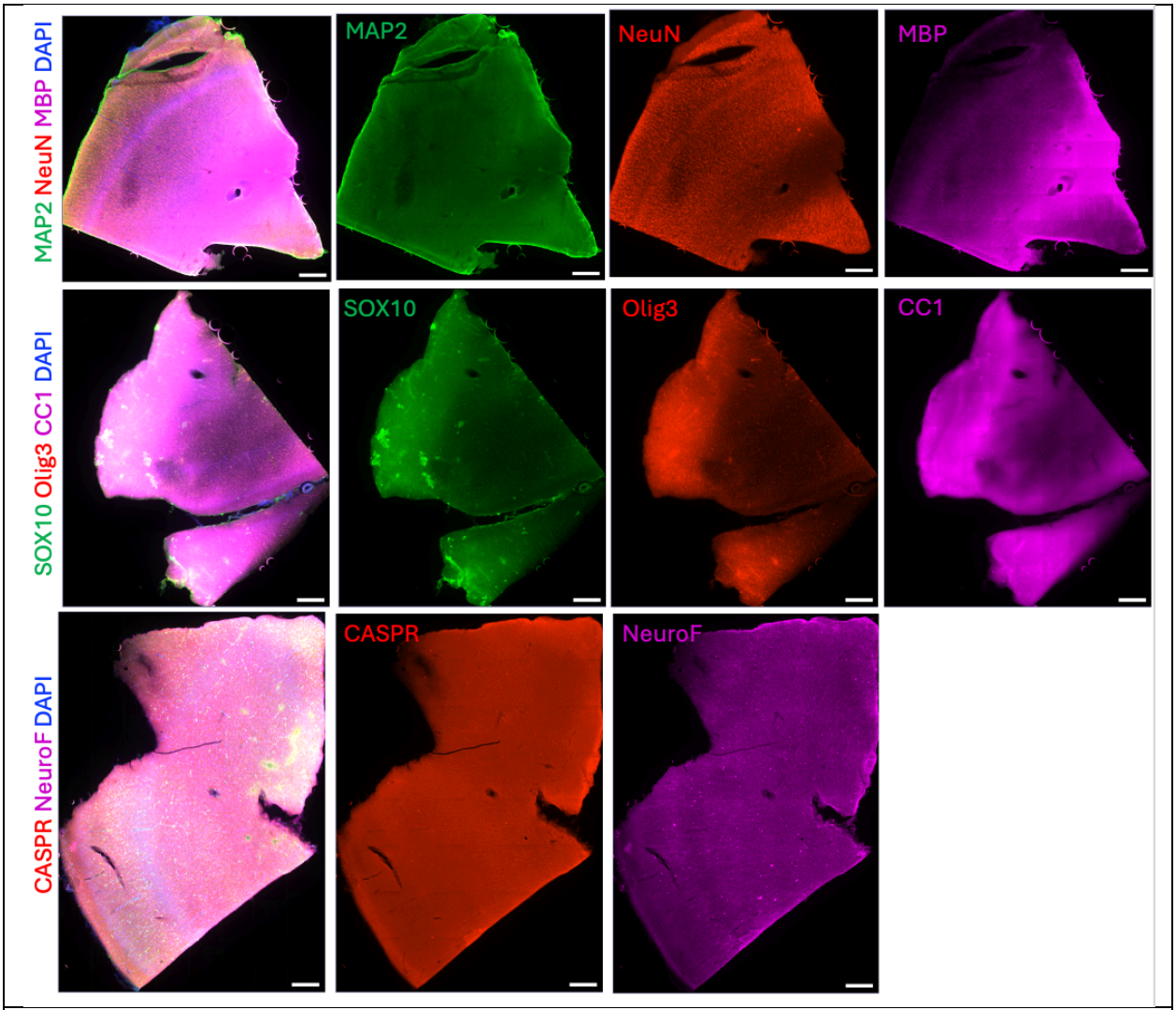
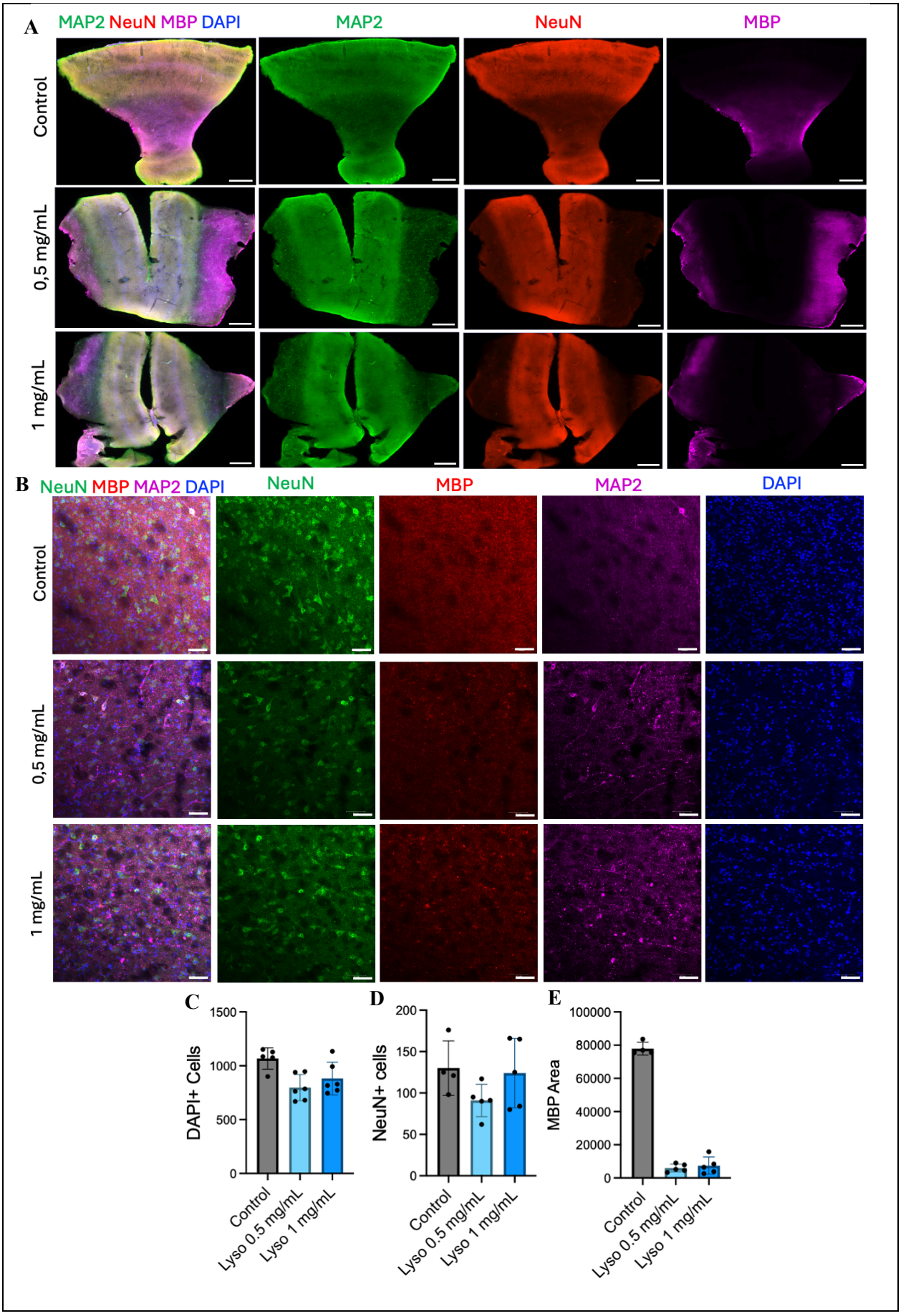


Fig. 2 - Acute human adult cortical tissue shows excellent quality conditions. Scanning images of IHC stainings for neuronal (MAP2, NeuN and MBP), oligo (SOX10 Olig2 CC1), paranodal (CASPR) and cytoskeletal (NeuroF) markers on hACtx tissue. Scale bar: 1 mm. hACtx: human adult cortical tissue. IHC: immunohistochemistry.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Lyso 0.5 mg/mL
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Lyso 0.5 mg/mL
Lyso 1 mg/mL
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MBP Area
100000
80000
60000
40000
20000
0
Control
Lyso 0.5 mg/mL
Lyso 1 mg/mL

Fig. 3 - Lysolecithin treatment of adult human cortical tissue induces effective demyelination. A+B) Scanning (A) and confocal (B) images of IHC stainings for neuronal (MAP2 and NeuN) and myelin (MBP) markers on hACTx. Tissue was either treated with 0,5 or 1 mg/mL lysolecithin, or left untreated (control). Scale bars: 2 mm (A) 50 um (B). **C+D+E)** Quantification of total cells (C), total neurons (D) and MBP intensity (E) for each subgroup (control, 0,5 and 1 mg/mL lysolecithin). Error bar = SD. hACtx: human adult cortical tissue. IHC: immunohistochemistry.

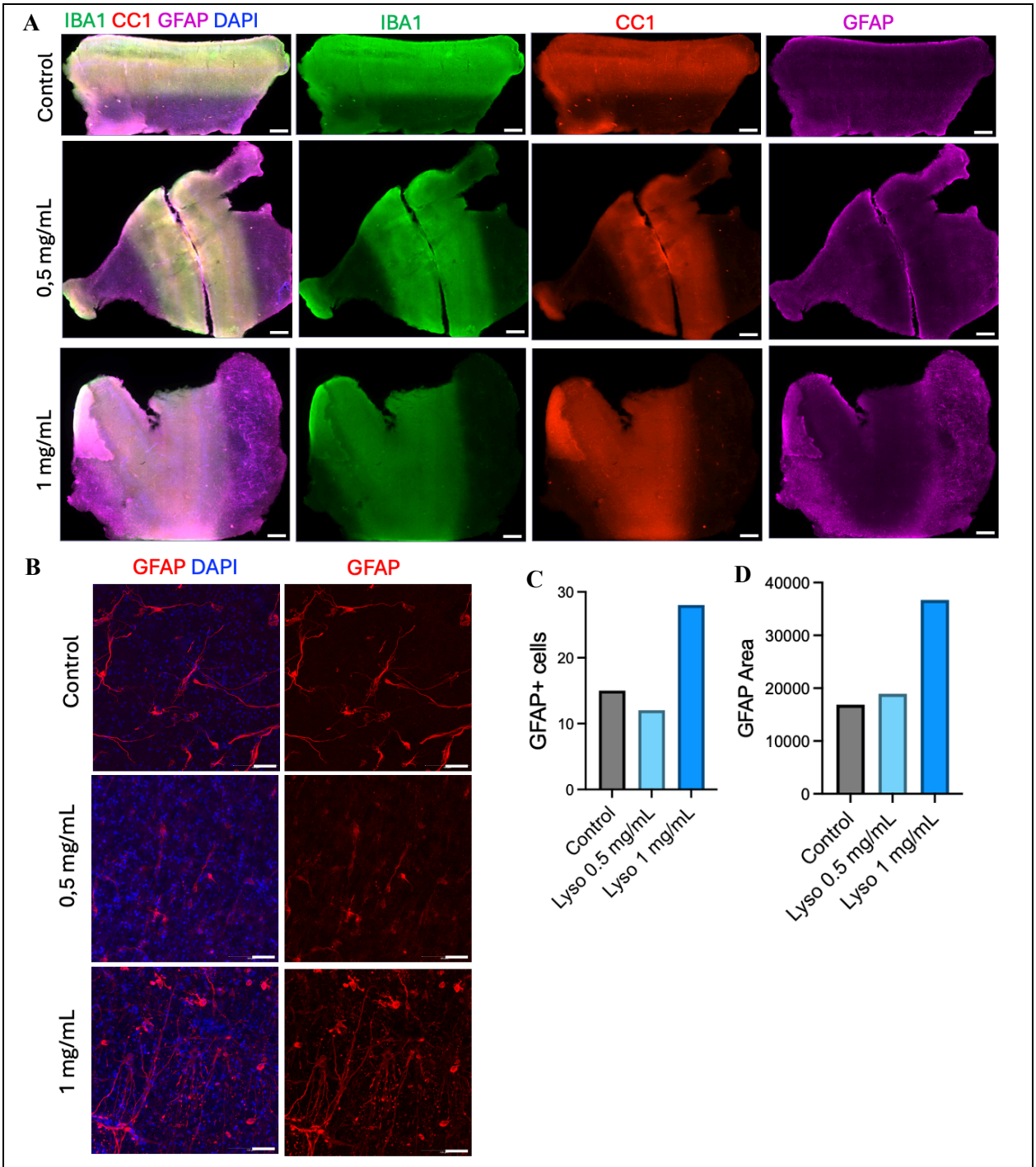


Fig. 4 - High concentrated lysolecithin treatment of adult human cortical tissue induces astrocyte activation. A+B) Scanning (A) and confocal (B) images of IHC stainings for microglia (IBA1), mature oligodendrocyte (CC1) and astrocyte (GFAP) markers on hACTx. Tissue was either treated with 0,5 or 1 mg/mL lysolecithin, or left untreated (control). Scale bars: 2 mm (A) 50 um (B). **C+D)** Quantification of total astrocytes (C) and GFAP intensity (D) for each subgroup (control, 0,5 and 1 mg/mL lysolecithin). hACtx: human adult cortical tissue. IHC: immunohistochemistry

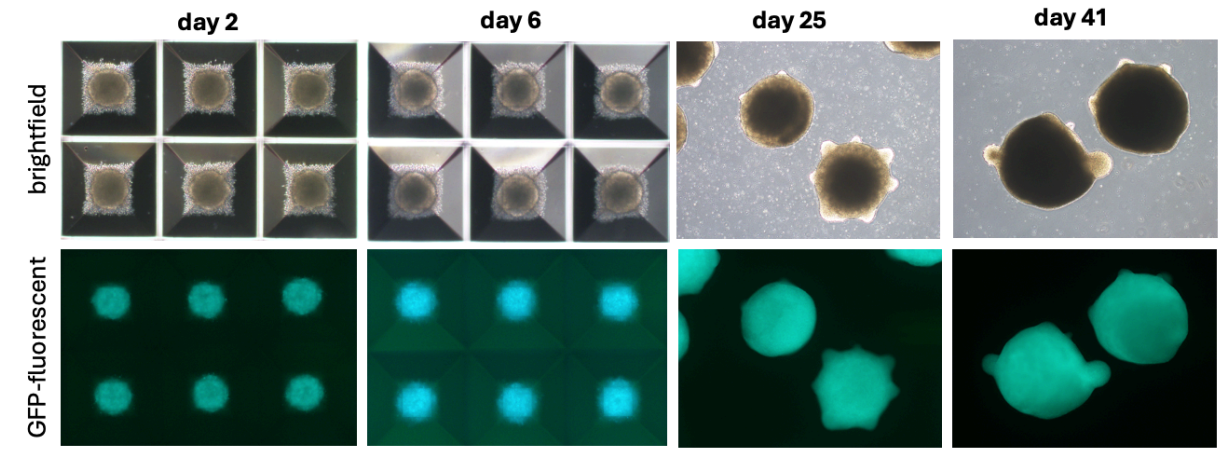
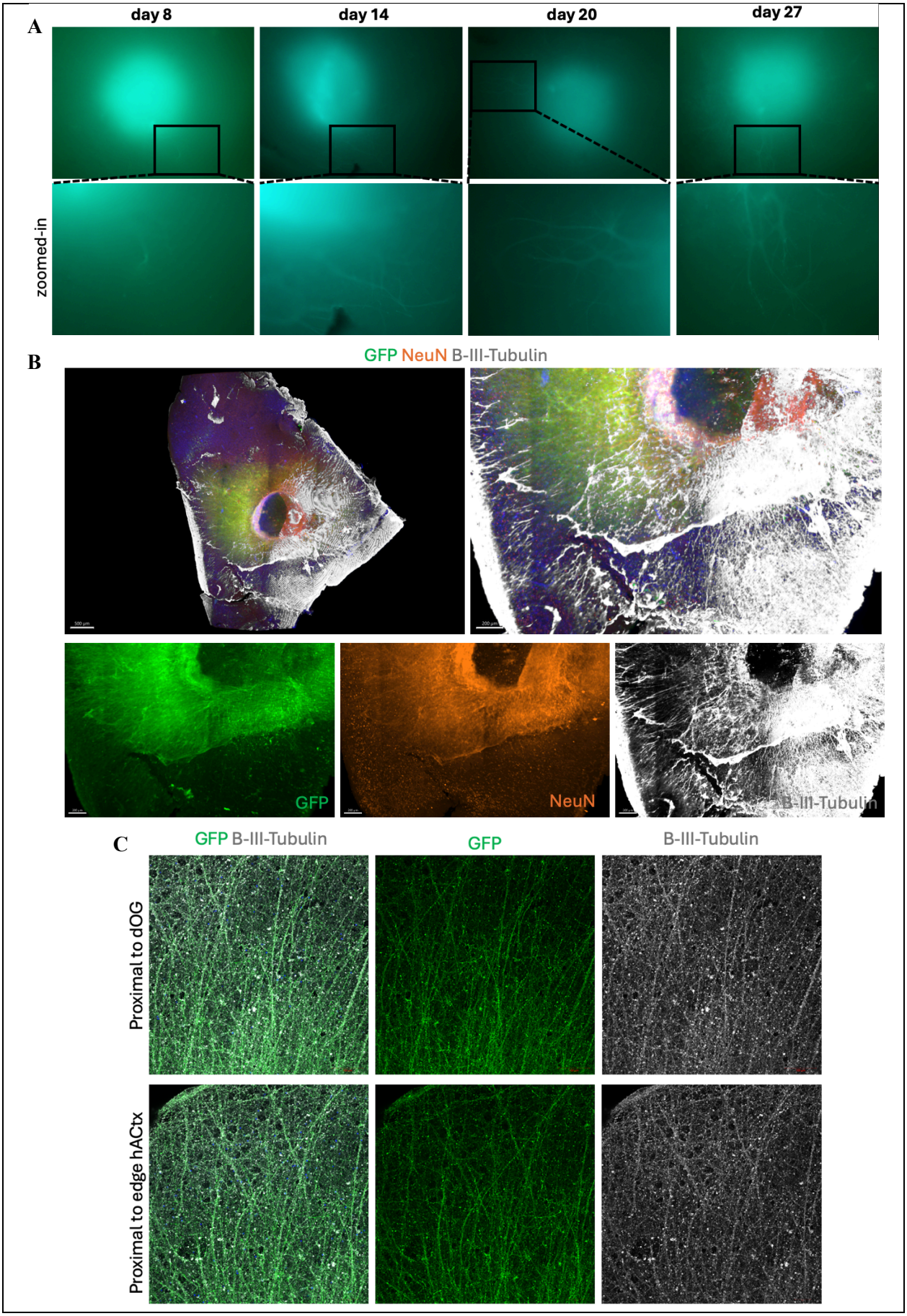


Fig. 5 - Succesful generation of dorsal forebrain organoids from GFP-hiPSCs. Brightfield (top) and GFP-fluorescent (bottom) images of dOGs in culture over time (day 2, 6, 25 and 41). dOG: dorsal forebrain organoid. GFP: green fluorescent protein. hiPSC: human induced pluripotent stem cells.


A
day 8
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zoomed-in
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GFP NeuN B-III-Tubulin
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NeuN
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C
GFP B-III-Tubulin
GFP
B-III-Tubulin
Proximal to dOG
Proximal to edge hACtx

Fig. 6 - Transplanted dorsal forebrain organoids form axonal projections towards host adult human cortical tissue. A) GFP-fluorescent images of dOG transplanted on hACtx in culture over time (day 8, 14, 20 and 27 after transplantation). **B)** IHC staining of dOG transplanted on hACtx (2 weeks) for organoid (GFP), neurons (NeuN) and neuronal microtubuli (B-III-Tubulin). Tissue subsequently underwent a clearing procedure to enhance staining visibility. Scale bars: 500 um and 200 um. **C)** Confocal images of dOG transplanted to hACtx (4 weeks), stained for organoid (GFP) and neuronal microtubuli (B-III-Tubulin). Scale bar = 100 um. dOG: dorsal forebrain organoid. hACtx: human adult cortical tissue. GFP: green fluorescent protein. IHC: immunohistochemistry.

DISCUSSION

In this study, we first aimed to optimize an *ex vivo* human demyelination model relevant for MS. While current experimental therapies for MS are commonly tested in rodent models, these often fail to translate effectively to the complex physiology of the human brain. To address this, we explored the use of demyelinating agents - cuprizone and lysolecithin - on *ex vivo* adult human cortical slices. However, initial experiments revealed that cuprizone treatment caused excessive tissue damage, likely due to the requirement of 50% ethanol for solubilization. As a result, cuprizone was excluded from further experiments, and subsequent demyelination was performed exclusively using lysolecithin. Based on the results shown in Figures 3 and 4, a lysolecithin concentration of 1 mg/mL appears to be too high, as it induces activation of glial cells. The goal of this model is to achieve effective demyelination while minimizing off-target effects on surrounding tissue. At present, a concentration of 0.5 mg/mL shows better outcomes, however further experiments with concentrations below 0.5 mg/mL is necessary to determine the optimal dose that balances maximal demyelination with minimal toxicity to surrounding neural tissue. Important to note, is that the improvement in quality of hACtx tissue seen in figure 2 was likely due to the fact that this time, one of our PhD students was present in the operating room and received the cortical tissue immediately after resection. This experience highlighted the critical importance of minimizing delays and ensuring proper handling between surgical resection and culture setup. Previous delays in tissue transfer likely contributed to the degradation observed in earlier samples, with uncertainty around how long the tissue had been stored, under what conditions, and in which medium. Although we consistently made efforts to reach the operating room as quickly as possible once notified, this finding highlights the need for improved coordination with the surgical team to ensure immediate and standardized tissue retrieval for optimal experimental outcomes. Therefore, the major limitation of working with human tissue is its limited availability and variability in quality, which can be influenced by multiple factors, as discussed in the *results* section. Another important consideration is the potential for spontaneous remyelination. In rodent models, natural remyelination has been reported to occur already at day 7 post-injection (35). This phenomenon has not yet been evaluated in our human *ex vivo* model. To assess whether spontaneous remyelination occurs under our conditions, future experiments should include extended incubation times beyond the current 48-hour post-treatment window. Comparing myelin integrity at both short and prolonged time points would help determine the extent of any endogenous repair mechanisms.

The second aim of this study was to transplant dOGs, derived from GFP-hiPSC, onto hACtx tissue as a potential regenerative therapy for stroke-related lesions. The *in vitro* differentiation of hiPSCs into dOGs was successful and their transplantation onto hACtx slices revealed initial axonal outgrowths by day 8, with more extensive projections evident by two weeks post-transplantation. These findings suggest that cortical organoids can survive, differentiate, and extend into adult human brain tissue, indicating a capacity for structural integration. Whether these projections form functional synaptic connections remains to be determined in next phases of this study. Several complementary techniques can be employed to evaluate functional connections between transplanted dOGs and hACtx. Calcium imaging for example, is a widely used method in neuroscience for assessing neuronal activity and functional synaptic integration (36). In addition, electron microscopy provides high-resolution structural evidence of synapse formation and could be used to determine whether transplanted dOGs form afferent synapses onto hACtx neurons. This approach

has previously been applied by our research group to assess synaptic integration of hiPSC-derived cortical neurons transplanted into adult human cortical tissue, as described in the introduction of this thesis (Figure 1). Finally, whole-cell patch-clamp electrophysiology remains the golden standard for detecting neuronal excitability and synaptic function in brain tissue (37). By directly recording neuronal activity, this technique allows us to determine whether the transplanted organoids contribute to functional network activity and whether the regenerated region exhibits properties comparable to native cortical tissue. An important consideration in stem cell-based transplantation therapies, is the potential risk of tumorigenesis. This concern is particularly relevant given the future intention of applying cortical organoid transplantation *in vivo* human brain settings. Although our organoids have reached a maturation stage, they may retain pluripotent characteristics that allow for further differentiation and axonal overgrowth. As shown in the confocal images (Figure 6C), some projections extend across the full width of the host tissue. While such extensive outgrowth is promising *in vitro*, it raises concerns about uncontrolled growth or aberrant integration in a clinical setting. Therefore, it is important to highlight that this experiment is a proof-of-concept and the first attempt at transplanting cortical organoids onto adult human brain tissue. Further optimization and safety evaluation of the transplantation protocol are required before any clinical translation can be considered. From a therapeutic perspective, transplantation of cortical organoids alone may not be sufficient to restore complex brain architecture and function. A combinatorial approach involving additional supporting cell types may be necessary. For instance, our research group recently developed a rapid protocol for generating stem cell-derived oligodendrocytes (OLs), which could be cotransplanted with cortical organoids to promote remyelination and enhance functional integration (38). Furthermore, we have not yet investigated the vascularization status of the grafted region—an essential factor for long-term survival and functional maturation of transplanted tissue. Future studies should explore strategies to promote vascular integration alongside neuronal and glial cell transplantation.

CONCLUSION

This thesis presents the successful development and optimization of two novel ex vivo human brain models that advance our understanding of remyelination and neuroregeneration in a human-relevant context. First, we established an ex vivo demyelination model using lysolecithin-treated adult human cortical slices, identifying 0.5 mg/mL as the most promising concentration for inducing demyelination while preserving tissue integrity. Second, we demonstrated that hiPSC-derived dorsal forebrain organoids can survive, extend axons, and structurally integrate into adult human brain tissue following transplantation. While the functional integration and safety of such grafts require further investigation, these models represent a significant step forward in bridging the translational gap between preclinical studies and clinical application. Together, they provide valuable platforms for evaluating regenerative therapies in complex human neural environments and hold promise for the future development of personalized interventions for CNS disorders such as multiple sclerosis and stroke.

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Author contributions – Dr. Sara Palma-Tortosa, Yu-Ping Shen and Raquel Martinez-Curiel conceived and designed the research. Yu-Ping Shen and Rida Chuhan performed the experiments. Rida Chuhan performed the data analysis and wrote the paper.

SUPPLEMENTARY

Table 1: Seeding Medium

Product	Concentration	Supplier
STEMdiff™ Neural Organoid Basal Medium 1	1x	STEMCELL™ Technologies (cat. no. 08621)
Y-27632	1:100	STEMCELL™ Technologies (cat. no. 72302)

Use immediately

Table 2: Forebrain Organoid Formation Medium

Product	Concentration	Supplier
STEMdiff™ Neural Organoid Basal Medium 1	1x	STEMCELL™ Technologies (cat. no. 08621)

Store at 2-8°C for up to 3 weeks

Table 3: Forebrain Expansion Medium

Product	Concentration	Supplier
STEMdiff™ Neural Organoid Basal medium 2	1x	STEMCELL™ Technologies (cat. no. 08622)
STEMdiff™ Neural Organoid Supplement A	1:50	STEMCELL™ Technologies (cat. no. 08623)
STEMdiff™ Neural Organoid Supplement B	1:1000	STEMCELL™ Technologies (cat. no. 08624)

Store at 2-8°C for up to 3 weeks

Table 4: Forebrain Organoid Differentiation Medium

Product	Concentration	Supplier
STEMdiff™ Neural Organoid Basal medium 2	1x	STEMCELL™ Technologies (cat. no. 08622)
STEMdiff™ Neural Organoid Supplement A	1:50	STEMCELL™ Technologies (cat. no. 08623)
STEMdiff™ Neural Organoid Supplement C	1:1000	STEMCELL™ Technologies (cat. no. 08625)

Table 5: Forebrain Organoid Maintenance Medium

Product	Concentration	Supplier
STEMdiff™ Neural Organoid Basal medium 2	1x	STEMCELL™ Technologies (cat. no. 08622)
STEMdiff™ Neural Organoid Supplement A	1:50	STEMCELL™ Technologies (cat. no. 08623)

Store at 2-8°C for up to 3 weeks

Table 6: Human adult cortical tissue (hACTx) medium

Product	Concentration	Supplier
BrainPhys (Neural medium) without Phenol red	1x	StemCell technologies (cat. no. #05791)
B27	1:50	ThermoFisher Scientific (cat. no. 17504001)
AB/AM	1:50	Gibco (cat. no. 15240062)

Glutamax (L-Glutamine)	1:200	Gibco (cat. no. 35050061)
Gentamicin	1:1000	Gibco (cat. no. 15750037)

Store at 2-8°C for up to 3 weeks

Table 9: Potassium Phospate Buffer Saline (KPBS) (1x)

Product	Concentration	Supplier
Distilled water	1x	/
Potassium dihydrogen Phospate (KH2PO4)	0.2 g/L	Merk Millipore (cat. no. 104873)
Potassium phospate dibasic (K2HPO4)	1.15 g/L	Sigma Aldrich (cat. no. P3786)
Sodium chloride (NaCl)	8.0 g/L	Sigma Aldrich (cat. no. S3024)

Store at 2-8°C for up to 3 weeks

Table 7: Permeabilisation solution

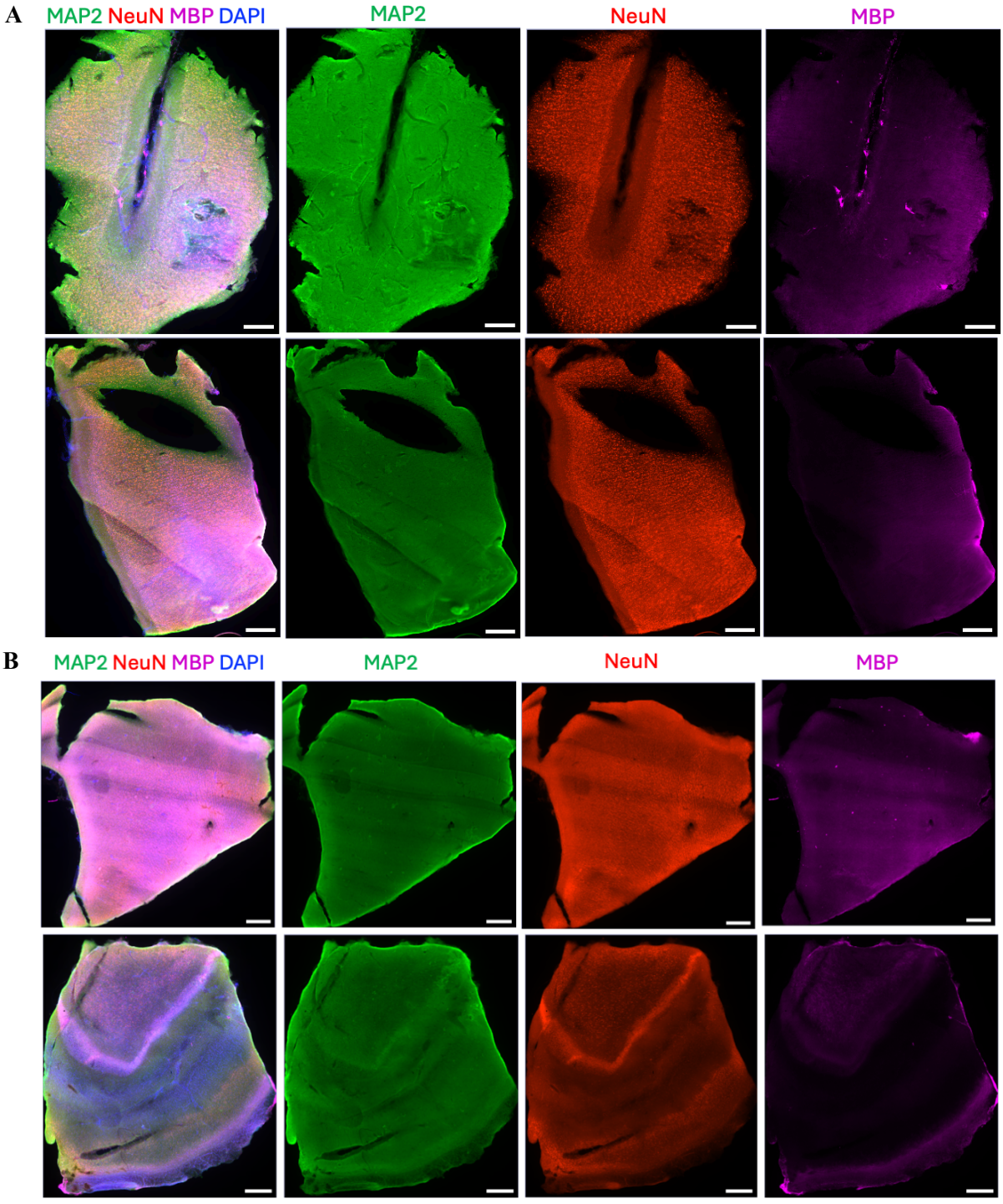
Product	Concentration	Supplier
Triton X-100	1%	ThermoFisher Scientific (cat. no. 327371000)
Bovine Serum Albumin (BSA)	0,02%	Jackson ImmunoReserach (cat. no. 001-000-162)
Potassium Phospate Buffer Saline (KPBS)	1x	See table 9

Store at 2-8°C for up to 3 weeks

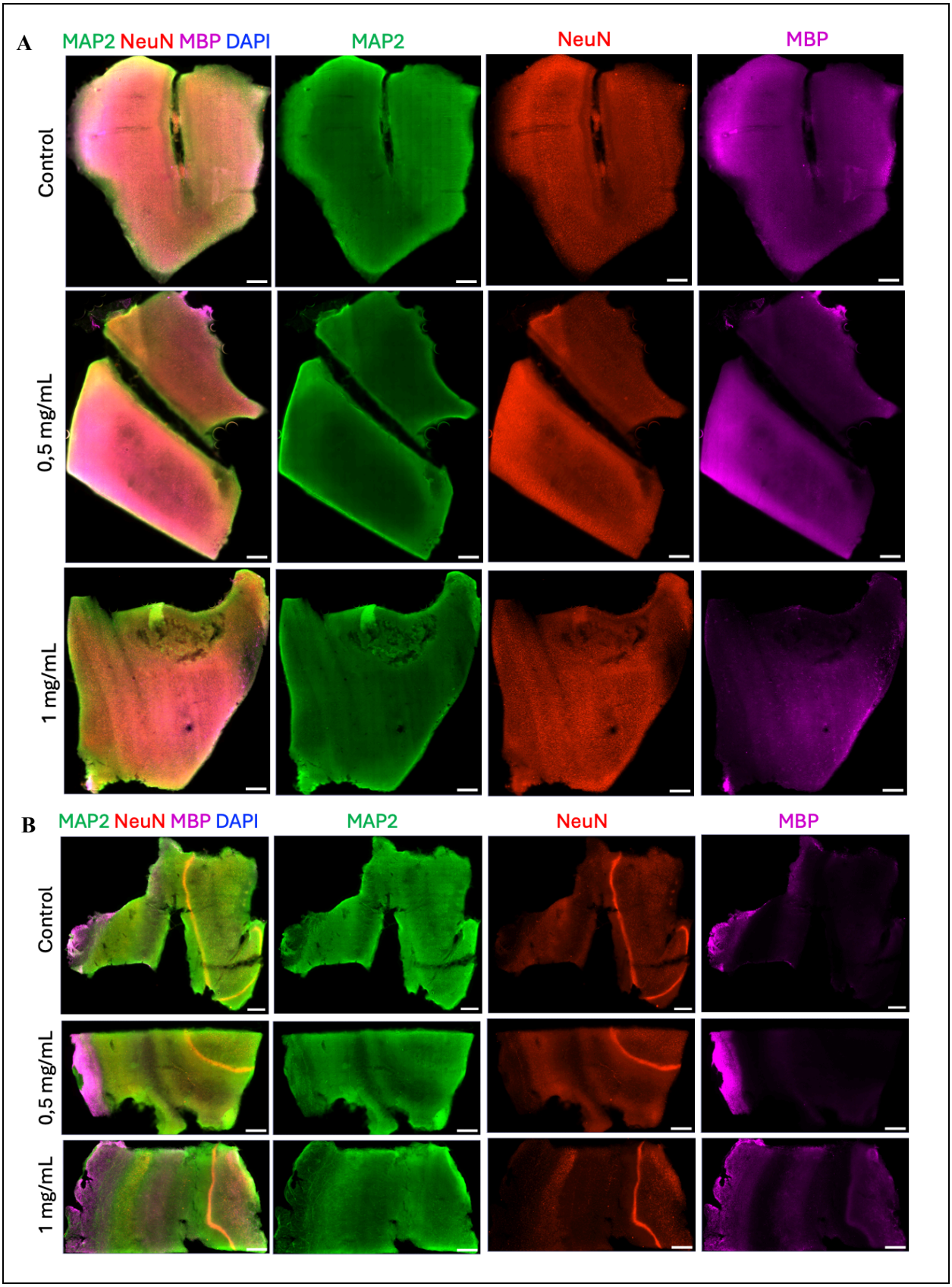
Table 8: Blocking solution

Product	Concentration	Supplier
T-KPBS	0,2%	See table 9
Bovine Serum Albumin (BSA)	1%	Jackson ImmunoReserach (cat. no. 001-000-162)
NaN3	0.05%	Sigma Aldrich (cat. no. 71289)

Store at 2-8°C for up to 3 weeks



Supplementary fig. 1 - Acute (A) vs 3 days in culture (B) shows effect of culturing on viability and quality conditions of hACtx. Scanning images of IHC stainings for neuronal (MAP2, NeuN and MBP). Scale bar: 1 mm. hACtx: human adult cortical tissue. IHC: immunohistochemistry.


A
MAP2 NeuN MBP DAPI
MAP2
NeuN
MBP
Control
0,5 mg/mL
1 mg/mL
B
MAP2 NeuN MBP DAPI
MAP2
NeuN
MBP
Control
0,5 mg/mL
1 mg/mL

Supplementary fig. 2 - Lysolecithin treatment of second round of hACtx. A+B) Scanning images of IHC stainings for neuronal (MAP2 and NeuN) and myelin (MBP) markers on hACTx, cortex **(A)** and hippocampus **(B)**.Tissue was either treated with 0,5 or 1 mg/mL lysolecithin, or left untreated (control). Scale bars: 1 mm. hACtx: human adult cortical tissue. IHC: immunohistochemistry