Ex vivo and In Vitro Models of OPC Maturation and Myelination for Drug Discovery

T. Modebadze, **B. Hall,** S. Giachetti, M. Barbour, **E. Malavasi**.
Concept Life Sciences, Edinburgh, United Kingdom

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1. Introduction

Oligodendrocyte precursor cells (OPCs) are a self-renewing cell population that gives rise to mature oligodendrocytes, the myelin-producing cells of the central nervous system. An intact myelin sheath is essential for fast and efficient signal transmission along axons and provides physical and trophic support to neurons.

OPC dysfunction and myelin loss can lead to severe neurological conditions, such as multiple sclerosis, and are increasingly proposed as major drivers of a broader spectrum of neurodegenerative diseases, including Alzheimer's disease. Drug development efforts are thus being directed towards the restoration of normal OPC function, creating the need for reliable *in vitro* models to assess candidate therapeutics that aim to promote remyelination.

The aim of the current study was to validate and characterise an organotypic brain slice model of myelination and assess the model's responsiveness to a known pro-myelinating therapeutic. In addition, we aimed to validate a rat OPC isolation method and further optimise conditions to quantify OPC maturation *in vitro.*

Taken together, these models provide a clear picture of the impact of candidate therapeutics on OPC proliferation, maturation and myelination.

2. Temporal upregulation of myelination genes

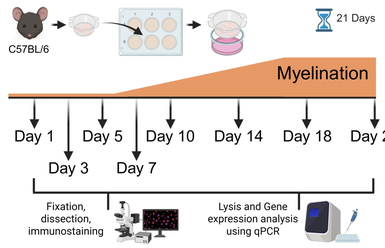

Figure 1: Methods. Using a vibratome, coronal forebrain slices from intact whole brains, of approximately 350 µm thickness were generated. Slices were plated onto culture inserts in 6-well plates in culture medium, with 2 slices per insert. Slices were maintained in an incubator set to 37 °C and 7.5 % CO_2, with medium changes occurring every 2- 3 days. Slices were maintained in culture for up to 21 days. At selected timepoints, slices were either lysed using RLT buffer (Qiagen) supplemented with BME or fixed for immunostaining. From lysates, RNA was isolated and following cDNA synthesis, the expression of target genes was determined by qPCR. Fixed slices were immunostained for neurofilament heavy chain (NFH) and Myelin basic protein (MBP), with images acquired using a 40x objective on a Leica Thunder Microscope.

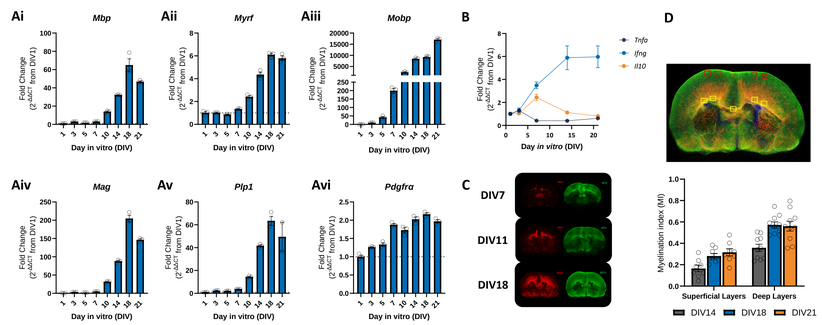

Figure 2: Results. (A-B) Fold Change values for *Mbp* **(Ai)**, *Myrf* **(Aii)**, *Mobp* **(Aiii)**, *Mag* **(Aiv)**, *Plp1* **(Av)**, *Pdgfrα* **(Avi)**, *Tnfα* , *Il10,* and *Ifng* **(B)** were calculated via the $2^{-\Delta\Delta Ct}$ method relative to Day 1. Data presented as mean ± SEM of up to 3 technical replicates. **(C)** Progression of *ex-vivo* myelination in organotypic brain slices. Exemplar images of MBP and NFH immunostaining at DIV7, DIV11, and DIV18 demonstrating myelin development in culture. **(D)** Myelination index (MI) was calculated as the proportion of NFH-positive axons co-localised with MBP. Spatial (laminar) and temporal differences in cortical myelination of mouse organotypic brain slices. The representative image indicates approximate regions of interest quantified within each organotypic slice. Data represented as mean ± SEM of n = 8-10 brain slices.

3. Region-specific treatment effects on gene expression

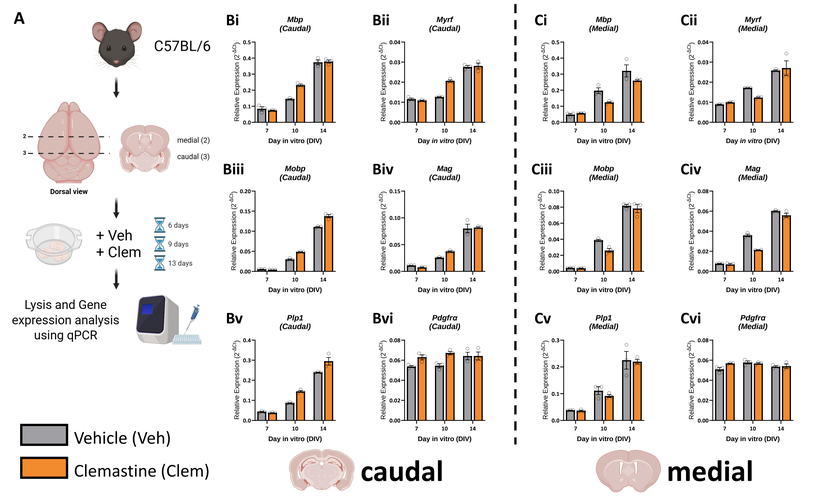

Figure 3: (A) Schematic of the experimental procedure. **(B-C)** Intact whole brains from a single mouse litter were dissected and processed as described in Figure 1. Slices were plated into culture inserts, with up to 2 slices per insert (2 caudal **(Bi-Bvi)** or 2 medial **(Ci-Cvi)** from different animals as technical replicates). From day 1, slices were treated with either Vehicle or Clemastine. Slices were maintained in an incubator set to 37 °C and 7.5 % CO_2, with medium changes occurring every 3 days. Slices were maintained in culture until Day 7, 10 or 14. At each timepoint, slices were lysed and processed as described in Figure 2. Using qPCR, the expression of *Mbp* **(Bi, Ci)**, *Myrf* **(Bii, Cii)**, *Mobp* **(Biii, Ciii)**, *Mag* **(Biv, Civ)**, *Plp1* **(Bv, Cv)**, and *Pdgfra* **(Bvi, Cvi)** were determined. Relative expression values were calculated via the $2^{-\Delta\Delta Ct}$ method relative to housekeeping controls. Data presented as mean ± SEM of up to n=3 technical replicates.

4. Region-specific treatment effects on myelination

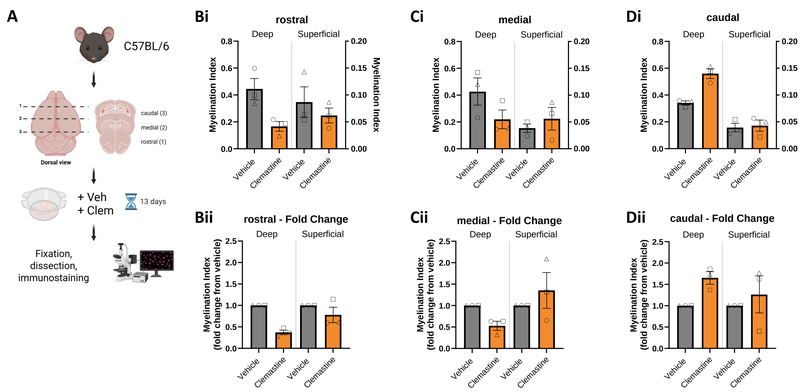

Figure 4: (A) Schematic of the experimental procedure. **(B-D)** Intact whole brains from a single mouse litter were processed, and slices collected from the rostral **(B)**, medial **(C)**, and caudal **(D)** regions of the brain and plated onto culture inserts in slice culture medium. From day 1, slices were treated with either Vehicle (Veh) or Clemastine (Clem), with medium changes occurring every 3 days. After 13 days of treatment, slices were fixed using 4 % paraformaldehyde (PFA) and immunostained with antibodies raised against NFH and MBP, along with relevant AlexaFluor-linked secondary antibodies. Nuclei were counterstained with DAPI. Z-stacks were acquired using a 40X objective on a Leica Thunder Microscope and analysed using Arivis Vision 4D image analysis software. The average myelination index (percentage of NFH signal colocalised with MBP) per region of interest in deep and superficial cortical layers was obtained for rostral **(Bi)**, medial **(Ci)**, and caudal **(Di)** slices. Fold change effects from vehicle treatment were also determined for each region **(Bii, Cii, and Dii)**. Data presented as mean ± SEM of 3 biological replicates.

5. *In vitro* OPC maturation Assay

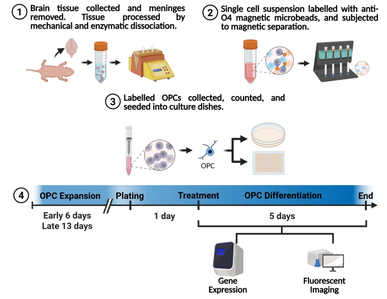

Figure 5: Methods. Brains were harvested from Sprague-Dawley rat litters (P6-8), meninges were removed, and the tissue was diced. The triturated tissue was then dissociated using a Neural Tissue Dissociation kit (P) (Miltenyi) **(1)** and magnetically separated using Anti-O4 microbeads (Miltenyi) **(2)**. The isolated $O4^+$ cells were then counted and seeded in PDL coated culture dishes in OPC medium supplemented with proliferative factors (Proliferation Medium) and maintained in an incubator for up to 7 days to allow for OPC Expansion **(3)**. Once complete, OPCs were detached and seeded into PDL-coated culture vessels in OPC proliferation medium for 1 day. Next, the proliferative factors were removed, and cell treatments applied. Cell cultures were then matured for 1-5 days. At various timepoints, Gene expression and fluorescent imaging was performed **(4)**.

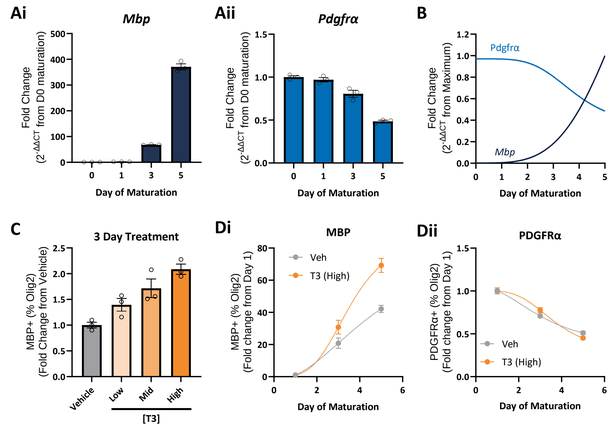

Figure 6: Results Expression of *Mbp* **(Ai)** and *Pdgfrα* **(Aii)** over a 5-day maturation timecourse was evaluated via qPCR, with fold change calculated via the $2^{-\Delta\Delta Ct}$ method relative to Day 0 controls. **(B)** Fold change responses from maximal were also calculated for each target. Data presented as mean ± SEM. n=3 technical replicates. **(C)** OPCs treated for 3 days with Vehicle or T3 (Low, Mid, High) were fixed and immunostained using antibodies raised against Olig2, MBP, or PDGFRα. Positive cell counts were quantified using a CellInSight High Content Imaging System. Fold change in expression of MBP, compared to Vehicle treatment, was then calculated. Data represented as the mean ± SEM of 3 technical replicates. **(D)** OPCs treated for up to 5 days with either Vehicle or T3 (High) were fixed and immunostained using antibodies raised against Olig2, MBP, or PDGFRα. Positive cell counts were quantified using a CellInSight High Content Imaging System. Fold changes in expression, compared to Day 1, for MBP **(Di)** and PDGFRα **(Dii)**, were then calculated. Data represented as the mean ± SEM of 3 technical replicates.

6. Summary and conclusions

- Expression of myelin-associated genes increases over time, with myelination proceeding from deep to superficial layers (Figure 1 and Figure 2). **Highlights a complex *Ex-vivo* brain slice model that exhibits progressive myelination, mirroring *in vivo* myelin development.**
- Through the quantification of gene expression (Figure 3) and Myelination (Figure 4), the effects of Clemastine treatment, a known remyelinating therapeutic, were shown to be region- and time-specific. **Demonstrates the requirement of models of varying complexity to provide robust, reliable results and allows customisation to specific experimental or therapeutic contexts.**
- Isolation of $O4^+$ OPCs generates cells that express key stage-specific markers, and respond to T3, a known promoter of OPC maturation. **Produced a reproducible protocol to isolate and expand OPCs from rat brain to serve as a responsive, stage-specific model to assess the impact of remyelinating therapeutics.**

All graphics were prepared on BioRender.com

Dr Benjamin Hall
Dr Elise Malavasi