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**Mouse-derived Cerebral Organoids as a tool for investigating
the role of *Foxg1* in forebrain development**

Jonothan Jay Marshall

Declaration	5
Glossary	6
Abstract	7
Lay abstract	9
Chapter 1 – Introduction	11
1.1 Reasons to study brain development	11
1.2 Telencephalic development	11
1.2.1 Cerebral Cortex	14
1.3 Forkhead box gene G1 (Foxg1)	17
1.3.1 Foxg1 in the dorsal telencephalon	18
1.3.2 Foxg1 in the ventral telencephalon	22
1.3.3 FOXG1 Syndrome	26
1.4 Cerebral Organoids	28
1.4.1 Current limitations	32
1.5 Thesis aims	33
Chapter 2 - Materials and Methods	35
2.1 Mouse embryonic cell lines	35
2.2 mESC culture	35
2.2.1 Passaging of cells	36
2.3 Dorsal telencephalic organoid protocol	36
2.4 Ventral telencephalic organoid protocol	40
2.5 Cumulative EdU labelling	40
2.6 Fixation and sectioning of organoid tissue	41
2.6.1 Fixation	41
2.6.2 Cryoprotection and Block Mounting	42
2.6.3 Sectioning	43
2.7 Immunohistochemistry	43
2.8 Light Microscopy	47
2.9 Image Analysis	47
2.9.1 Randomised grid quantification method	47
2.10 Data analysis and graph plotting	48
Chapter 3 - Development of a strategy for unbiased quantification of cerebral organoids	49
3.1 Introduction	49
3.2 Criteria for a robust and unbiased counting strategy	51
3.2.1 Removing unconscious bias from the selection of ROIs	51
3.2.2 Adaptability to the inherent variability of organoids	52
3.2.3 Taking the whole organoid into account	52

3.3 Experimental Design	53
3.3.1 Description of cell lines used	55
3.4 Methods to quantitate cell types in cerebral organoids	60
3.4.1 Concentric rings intensity profiles provide an imperfect snapshot of organoid differentiation	60
Discussion points	61
3.5 Randomised grid technique	62
3.5.1 Foxg1 homozygous mutant organoids show a significant decrease in size compared to other cell lines	69
Discussion points	69
3.5.2 Organoids derived from all cell lines show similar levels of Tbr2 expression	72
Discussion points	73
3.5.3 Organoid centre is not a reliable indicator of SVZ-like tissue in organoids of all three cell lines	77
Discussion points	78
3.5.4 Distance to the nearest outside edge is not a reliable indicator of SVZ-like tissue in organoids of all three cell lines	81
Discussion points	81
Section 3.5.5 Ventricular edges provide a potential candidate for delineating ROI landmarks	85
Discussion points	86
Chapter 4 – Investigation of Foxg1^{-/-} ventral telencephalic defects in organoids	88
4.1 Introduction	88
4.2 Chapter aims	89
4.3 Experimental design	90
4.2 Results	95
4.2.1 addition of SAG significantly increases proportion of total cells adopting a ventral telencephalic fate, regardless of timing of addition.	95
4.2.2 More cells in conditions V1 and V2 which become telencephalon are ventrally patterned	99
4.2.3 Proportion of cells adopting a telencephalic fate does not significantly differ with the addition of SAG	103
4.2.4 The addition of SAG leads to more non-telencephalic regions of the organoid becoming Olig2 positive	107
4.2.5 Proportions of marker expression are unchanged between days 10 and 12 but organoid tissue loses organisation	111
4.2.6 mESC derived organoids appear to recapitulate the ventral defect seen in Foxg1 ^{-/-} mice	116
4.3 Discussion	122
4.3.1 Efficiency of ventral telencephalic differentiation	122
4.3.2 A majority of cells fail to adopt a telencephalic identity across conditions	124
4.3.3 Organoids appear to recapitulate ventral defects seen in Foxg1 ^{-/-} mice	126
Chapter 5 – Investigation of cell cycle defects in Foxg1^{-/-} cerebral organoids	128
5.1 Introduction	128
5.2 Experimental Design	132
5.3 Results	134
5.3.1 Visualisation of variability seen at timepoints.	134
5.3.2 Foxg1 ^{-/-} mutant organoids show an elongation in cell cycle length	135

5.4 Discussion	141
Chapter 6 - Discussion	144
6.1 Organoids as a model of investigating Foxg1	144
6.1.1 Future uses of organoids to study Foxg1	146
6.2 Variability of organoids remains a limitation	148
6.3 A case for mouse cerebral organoids	149
6.4 Potential Future Work	150

Declaration

I declare that this thesis is an original report of my research, has been written by me and has not been submitted for any previous degree. Any collaborative contributions have been indicated clearly and acknowledged.

Jonothon Marshall
26/05/21

Glossary

BMP – bone morphogen protein
BP – basal progenitors
BrdU - Bromodeoxyuridine
CDK – cyclin dependent kinase
CDKN – cyclin dependent kinase inhibitor
CH – cortical hem
CP – cortical plate
D – dorsal
DT – dorsal telencephalon
EdU - 5-ethynyl-2'-deoxyuridine
ESC – embryonic stem cell
FBD – Forkhead binding domain
FGF – fibroblast growth factor
FIJI – Fiji Is Just Image J – (image analysis software)
Foxg1 – forkhead box G1
FOXO – forkhead box O
FUCCI - fluorescence ubiquitination cell cycle indicator
GBD – Groucho binding domain
GFP – green fluorescent protein
GMEM – Glasgow minimal essential medium
hiPSC – human induced pluripotent stem cells
iPSC – induced pluripotent stem cells
IWP2 - Inhibitor of WNT Production-2
JBD – JARID1B-binding domain
mESC – mouse embryonic stem cells
MZ – marginal zone
N-Cad – N-cadherin
NE - neuroepithelium
Nkx2.1 – NK2 homeobox1
Olig1 – oligodendrocyte transcription factor 1
Olig2 – oligodendrocyte transcription factor 2
oRGC – outer radial glia cells
PSC – Pluripotent stem cell
RGC – radial glial cells
RFP – red fluorescent protein
Shh – sonic hedgehog signalling
SVZ – subventricular zone
Tbr2 – T-box brain protein 2
Tel – telencephalon
TLE – transducing like element
V1 – ventral differentiation protocol 1
V2 – ventral differentiation protocol 2

VZ – ventricular zone

WT - wildtype

Abstract

The formation of the mammalian forebrain is a highly complex process requiring the precise interplay of numerous factors. One such factor is the gene *Foxg1* which begins to be expressed at the very earliest stages of telencephalic development, it has been linked to numerous processes including controlling cell cycle kinetics and patterning of the ventral telencephalon. The aim of this thesis is to use mouse embryonic stem cells to create cerebral organoids with *wildtype*, *Foxg1*^{-/-} and *Foxg1*^{+/-} genotypes.

The results showed that progenitors in *Foxg1*^{-/-} cerebral organoids had a slight but statistically insignificant premature elongation of the cell cycle. For wildtype the cell cycle at day 7 of the protocol was estimated at 22.3 hours, for the null mutant cells cycle was calculated as 26.2 hours. However, important to note the values for cell cycle were not the same as *in vivo*, highlighting the differences between the two systems. Furthermore, when investigating the requirement for *Foxg1* in the ventral telencephalon this was impeded by the fact cerebral organoids appeared to default to a cerebral cortical fate. On average 7.8%, SEM = ± 4.4% of telencephalic tissue within the cerebral organoids under the original protocol conditions became ventral telencephalon. However, they could be directed to adopt a more ventral telencephalic fate by activation of the *sonic hedgehog* pathway. This led to an average of 33.8%, SEM = ± 6.5% of telencephalic tissue in these organoids adopting

a ventral telencephalic fate. When *Foxg1*^{-/-} stem cells were put through this ventral protocol they failed to form ventral telencephalon, as seen *in vivo*.

These results highlight that overall cerebral organoids successfully recapitulate some key aspects of the *Foxg1*^{-/-} phenotype, such as the lack of ventral telencephalic tissue being formed. However, they also show some important differences between organoids and *in vivo*, including the differences in cell cycle lengths which did not show the level of differentiation in organoids as would be expected *in vivo*. This suggests that the kinetics of cells and the phenotypes seen in organoids may well be different to *in vivo* and as such this should be taken into account for future experiments.

Lay abstract

The mammalian brain is one of the most complex biological structures we know. In order for this to develop in the embryo from a small number of cells requires lots of different factors working perfectly together. Sometimes this process can go wrong leading to neurodevelopmental disorders. By understanding how the brain should develop it could help us to understand these disorders more. One of these factors is a gene called *Foxg1*, this gene is found expressed in the frontal part of the brain, the forebrain. Specifically, in a subregion called the telencephalon. The telencephalon can be further subdivided, into the dorsal and ventral telencephalon, each with their own architecture and function. *Foxg1* appears to be important in both of these regions but how it has its effects appears to differ between the regions. In the dorsal telencephalon it seems to affect the length of the cell cycle, the time taken for a cell to divide, by lengthening it. This then leads to a smaller dorsal telencephalon. Meanwhile it appears that *Foxg1* is required by cells to allow them to become ventral telencephalon, so when *Foxg1* is absent no ventral telencephalon is formed. There is still much about these processes we don't know, and a new technology called cerebral organoids may allow us to investigate this in ways we weren't able to before. Cerebral organoids are 3-dimensional structures formed from pluripotent stem cells. Pluripotent stem cells are special as they have the ability to become any type of cell which makes up an organism. By gathering these pluripotent stem cells together, you can direct them to adopt the fate of a

certain organ, they then self-organise and create tissue which mimics a lot of aspects of the real organ. However, we still don't know how well they mimic all the fine detail of brain development, so this thesis will look at whether cerebral organoids derived from mouse stem cells show the same defects that were found in mice with no *Foxg1*. This will then help us to know if cerebral organoids are a good model for studying this gene. Here we show that on the whole the cerebral organoids do mimic the defects well. The same patterns, including a complete lack of ventral telencephalon and an increase in the cell cycle in the dorsal telencephalon are witnessed in cerebral organoids made from cells lacking *Foxg1*. However, the exact values for these changes aren't the same as seen in real life. This suggest that organoids mimic the patterns and trends but there are differences in the exact mechanisms. This is very useful as it shows us some of the limitations of the system which is something for researchers to bear in mind when using cerebral organoids in future. Cerebral organoids provide a potentially powerful tool, complementing and improving upon existing ways of investigating brain development.

Chapter 1 – Introduction

1.1 Reasons to study brain development

The process of embryonic mammalian brain development is highly complex and involves a multitude of different components working in harmony. The adult mouse brain, albeit much smaller than a human brain, still contains around 70 million neurons (Herculano-Houzel *et al.*, 2006). This process is vulnerable to dysregulation which can lead to neurodevelopmental disorders in humans, however, much of our understanding comes from work in other model systems including mouse (Tan & Shi, 2013).

1.2 Telencephalic development

Once the area that will give rise to the nervous system is patterned, the neural plate folds into a singular neural tube. This creates the primordial ventricle in its centre and an apico-basal polarised epithelium is found around this ventricle (Lancaster & Knoblich, 2014; Suzuki & Vanderhaeghen, 2015). It is from the most anterior end of this tube that the forebrain arises. The cells at this anterior region seem to adopt an anterior forebrain fate by default and that it is the caudalising effect of certain morphogens (such as retinoic acid and *Wnts*) which overcomes this

(Chambers et al., 2009; Gaspard et al., 2008; Kawasaki et al., 2000; Pera et al., 2004; Vallier et al., 2004; Watanabe et al., 2005).

The forebrain subdivides into the telencephalon and diencephalon. The telencephalon is the area of focus for this thesis. The telencephalon can be distinguished from the diencephalon by the expression of the gene *Foxg1* which is found throughout the telencephalon but completely absent from the diencephalon (Danesin & Houart, 2012; Yu et al., 2004). The telencephalon further subdivides into the dorsal and ventral telencephalon (Figure 1.4). The cerebral cortex arises from the dorsal telencephalon, while the ventral telencephalon forms the ganglionic eminences, pre-optic area (POA) and parts of the amygdala.

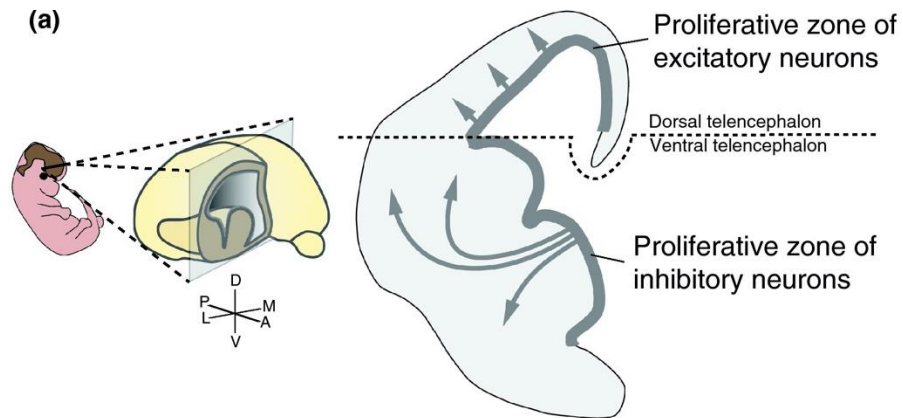


Figure 1.1 Schematic showing the telencephalon around E11.5, at this stage the telencephalon has further subdivided into dorsal and ventral with excitatory and inhibitory neurons being born in each region respectively. (Tyson and Anderson, 2011, Bandler et al, 2017)

1.2.1 Cerebral Cortex

The cerebral cortex is the seat of higher order cognitive abilities in mammals this is due to successful formation of cortical circuitry. In adult mice brains this consists of six cortical layers, the neurons found within each layer appear to have layer-specific expression patterns (Stiles & Jernigan, 2010). In mice the neurogenesis starts around E10.5 and lasts eight days (Figure 1.2). Prior to this the neuroepithelial cells (NEs) have been dividing symmetrically and increasing the progenitor pool. At the onset of neurogenesis these NEs become radial glial cells (RGCs) these cells are also progenitors but are characterised by the expression of the gene *Pax6*, similar to NEs, however they also have apical and basal processes which span the full width of the cortex (Britz et al., 2006; Levitt & Rakic, 1980). The RGCs and their precursor NEs reside on the ventricular edge, in an area termed the ventricular zone (VZ). These RGCs can divide symmetrically or asymmetrically and can give rise to both progenitors and neurons. When division occurs asymmetrically this can give rise to a new progenitor type called basal progenitors (BPs) or intermediate progenitor cells (IPCs). These lack the extended processes of RGCs and are located more basally, inhabiting a new layer referred to as the subventricular zone (SVZ). These predominantly form neurons via asymmetric division (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004).

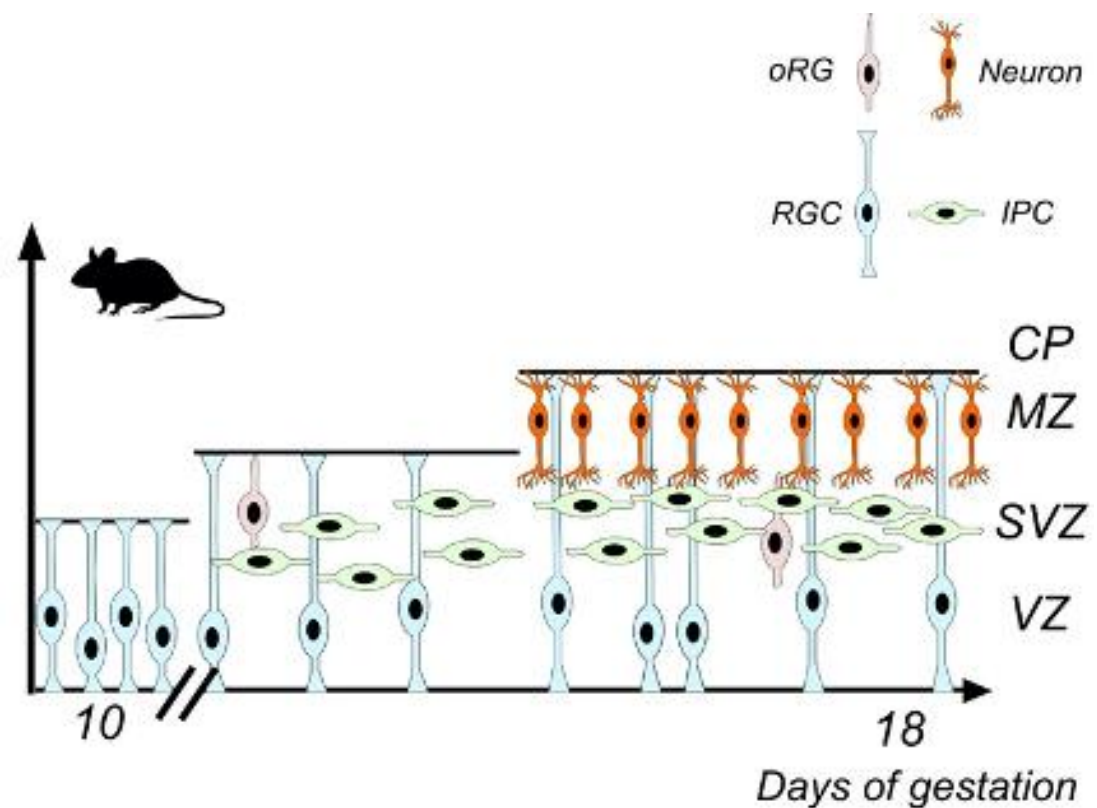


Figure 1.2. Schematic showing a simplified timeline of early murine cortical development. Starting at 10 days of gestation with a monolayer of neural stem cells which become radial glial cells (RGC) and the foundation of inside-out model of mammalian development with the development of a further proliferative hub in the subventricular zone. Newborn neurons migrate tangentially through the marginal zone to occupy their position in the cortical plate. NB outer radial glia cells are present in mice cortexes but to a much lesser extent than in humans. VZ – ventricular zone, SVZ – subventricular zone, MZ – marginal zone, CP – cortical plate, RGC – radial glial cell, oRG – outer radial glial cell, IPC – intermediate progenitor cell. Figure taken from Marshall and Mason, 2019.

The first cortical layer to form is layer 1, formed of Cajal-Retzius cells which have migrated in from regions including the cortical hem. Subsequent layers are created following an inside-out model of development, with the innermost (deep) layers forming first (Angevine & Sidman, 1961; Berry & Rogers, 1965; Gaspard & Vanderhaeghen, 2010; McConnell, 1995; Rakic, 1974; Tan & Shi, 2013). New-born neurons migrate to the correct layer using the extended processes of the RGCs (Levitt & Rakic, 1980). Once the neurons occupy their correct layers then the wiring of the cortical circuitry can commence (Gelman & Marín, 2010).

This circuitry is dependent on two neuronal subtypes; glutamatergic excitatory neurons and GABAergic inhibitory interneurons. The ratio of these cell types shows a dominance of glutamatergic cells (~80%) with most of the remainder composed of GABAergic cells (Wonders & Anderson, 2006). While both ultimately reside in the cerebral cortex, they arise in distinct regions. Glutamatergic neurons are born in the proliferative region of the dorsal telencephalon, whereas GABAergic interneurons arise in the ganglionic eminences of the ventral telencephalon. The medial ganglionic eminence produces the majority of cortical interneurons (Wonders & Anderson, 2006), which migrate tangentially through the dorsal telencephalon until at the correct position. They then begin migrating radially until they reside in the correct layer. Whereas the excitatory neurons are formed from the VZ and SVZ of the dorsal telencephalon and migrate radially.

1.3 Forkhead box gene G1 (*Foxg1*)

Foxg1 is one of the 44 forkhead box family genes that have been identified (Hannenhalli & Kaestner, 2009) and is the only member of that family's G subclass (Golson & Kaestner, 2016). It was previously referred to as *Brain-factor1* due to its presence in the rat telencephalon (Tao & Lai, 1992). Its importance in mammalian telencephalic development was highlighted by the severe hypoplasia and mortality at birth seen in homozygous *Foxg1* mice mutants (Xuan et al., 1995). This has made it a focus of interest for many years and subsequently many other phenotypes have been linked to *Foxg1*. *Foxg1* expression is first seen at around E.8.5 in the region that will give rise to the telencephalon, its expression is believed to be induced by *FGF8* signalling originating from the anterior neural ridge (Lagutin, 2003; Suda et al., 1997). It is seen throughout the dorsal and ventral telencephalon and its absence has been linked to hypoplasia in the dorsal telencephalon caused by defects to cell proliferation (Hanashima et al., 2002; Martynoga et al., 2005) as well as loss of ventral telencephalic patterning (Hanashima et al., 2007; Martynoga et al., 2005). In mice, it consists of a single exon located on chromosome 12 (Kumamoto and Hanashima, 2017). It's major domain is a forkhead binding domain (FBD) with a Groucho-binding domain (GBD) and JARID1B-binding domain (JBD) also present. This thesis aims to assess the ability of cerebral organoids to recapitulate these phenotypes.

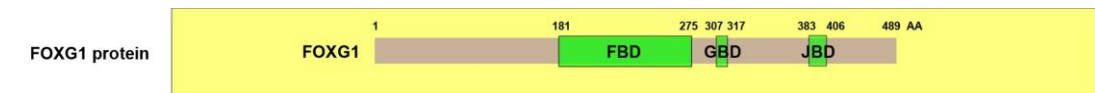


Figure 1.4 Domains of the Foxg1 protein in mice. 489 amino acids long with three main domains. a forkhead binding domain (FBD) with a Groucho-binding domain (GBD) and JARID1B-binding domain (JBD). Image adapted from (Kumamoto and Hanishame, 2017)

1.3.1 Foxg1 in the dorsal telencephalon

In the absence of *Foxg1* there is a large degree of hypoplasia seen in the cortex in homozygous mutants (Figure 1.3) (Xuan et al., 1995). Even in heterozygous mice there is significantly reduced cerebral hemisphere volumes, however variation has been seen between different mutant lines and genetic background appears to also play a role (Eagleson et al., 2007). This reduction in size may be caused by a premature elongation of the cell cycle in these mutants, thus progenitors exit the cell cycle and differentiate prematurely leaving a depleted proliferative population (Hanashima et al., 2002; Xuan et al., 1995). This elongation of the cell cycle predominantly affects phase G1 and becomes most pronounced from E11.5 onwards in homozygous mutants (Martynoga et al., 2005). Interestingly, in heterozygous mice the cerebral hemisphere volume did not show significant variance to wildtype until E15.5 (Eagleson et al., 2007). This premature

differentiation then leads to a marked reduction of the intermediate progenitors in the SVZ (Siegenthaler et al., 2008).

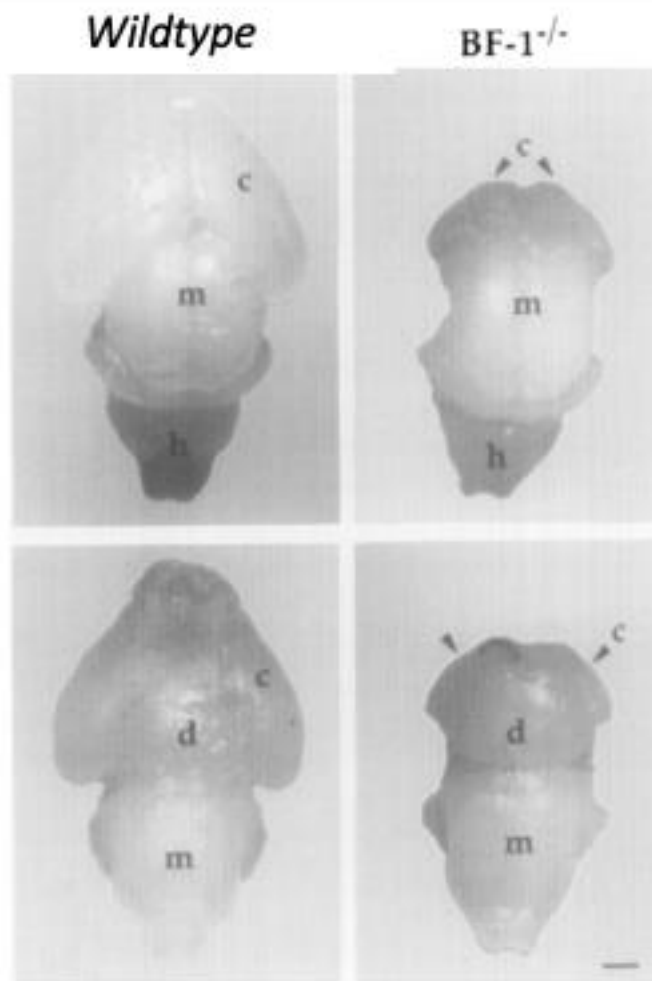


Figure 1.5. Figure showing greatly reduced cerebral hemispheres in *Foxg1*^{-/-} from hypoplasia, Martynoga et al., (2005) showed that premature elongation of the cell cycle in radial glial cells may lead to this defect. Image taken from newborn littermate pups (Xuan et al. 1995)

Like the other members of the Forkhead box family, *Foxg1* functions as a transcription factor. It was believed to have repressor activity due to WD40 repeats at the C terminus which bound to groucho/transducing-like enhancers (TLE) to form a co-repressor complex (Marçal et al., 2005; Yao et al., 2001). This activity has been linked with increased proliferation in the mammalian cortex. *Foxg1* appears to antagonise FOXO/SMAD complexes in progenitor cells which in turn reduces the level of cyclin-dependent kinase inhibitor 1a (CDKN1A) thus leading to less cells exiting the cell cycle and differentiating (Seoane et al., 2004; Vezzali et al., 2016). When *Foxg1* was underexpressed it showed increased levels of CDKN1A protein, suggesting a possible mechanism for premature differentiation in homozygous mutants, with CDKN1A promoting cell cycle exit (Marçal et al., 2005). However, the transcriptional activity does not appear to be the sole mechanism. When an otherwise normal *Foxg1* protein is introduced with a mutated DNA binding site, the cell cycle defects are rescued but premature differentiation still occurs (Dou et al., 2000; Hanashima et al., 2002). The exact role of *Foxg1* in cell cycle control is yet to be elucidated but an organoid model could well provide novel insights.

1.3.2 *Foxg1* in the ventral telencephalon

In the absence of *Foxg1* the ventral telencephalon fails to form (Figure 1.4) (Martynoga et al., 2005; Xuan et al., 1995). Originally it was suggested that its absence was explained by proliferative defects and just failed to grow similar to the dorsal telencephalon. However, chimeras formed of wildtype ventral telencephalon with homozygous mutant cells incorporated showed that these cells were unable to adopt a ventral telencephalic fate in a cell-autonomous manner (Martynoga et al., 2005). The same requirement for *Foxg1* is not in dorsal telencephalic patterning, in the absence of *Foxg1* the dorsal telencephalon still forms but shows hypoplasia (Manuel et al., 2011). Therefore, *Foxg1* seems to have region specific effects within the telencephalon.



Figure 1.6. Data from Xuan et al 1995 showing the loss of ventral telencephalic tissue and the ganglionic eminences in *Foxg1*^{-/-} mutants. Stainin-h shows presence of

Foxg1 in the telencephalon. Slices are taken at E12.5 with panels A and B rostrally located becoming more caudal by panels G and H. ge – ganglionic eminences

Foxg1 appears to be involved in ventral patterning, in part, via interaction with the morphogen *Sonic Hedgehog (Shh)*, this morphogen has been shown to have ventralising effects in multiple tissues including eye, telencephalon and spinal cord (Lupo et al., 2006). *Shh* is expressed in the preoptic area (POA) located between the telencephalon and hypothalamus (Wilson & Rubenstein, 2000). The complete absence of *Shh* leads to greatly diminished ganglionic eminences and the dorsal telencephalon expands ventrally (Corbin et al., 2003; Fuccillo et al., 2004) similar to *Foxg1* mutation. Conversely, the overexpression of *Shh* leads to ectopic expression of ventral telencephalic genes such as *Nkx2.1* and *Gsh2* in the dorsal telencephalon (Corbin et al., 2003). The boundary between the dorsal and ventral telencephalon, the pallium-subpallium boundary (PSB) expresses genes characteristic of both dorsal and ventral telencephalon such as *Pax6* and *Gsx2*, with *Pax6* expressed on the pallial side and *Gsx2* on the subpallial side. Interestingly when *Pax6* is deleted an expansion of the ventral telencephalon is seen, whereas the opposite is true in *Gsh2* null mutations (Carney et al., 2009; Hirata et al., 2002; Yun et al., 2001).

These phenotypes are reminiscent of those shown in *Foxg1* null mutants with almost a complete loss of the ventral telencephalon. Furthermore, *Shh* expression ventrally was lost in *Foxg1* mutants (Godbole et al., 2018; Kumamoto & Hanashima, 2017; Martynoga et al., 2005; Xuan et al., 1995). This implies that both *Foxg1* and *Shh* are required for successful patterning of the ventral telencephalon and its

delineation from the dorsal telencephalon. For instance, the cortical hem (CH) expresses *Wnts* and *BMPs* which appear to confer dorsal telencephalic fate. This region was significantly expanded in *Foxg1* mutants (Hanashima et al., 2004; Muzio, 2005). It was later discerned that *Foxg1* operates upstream of *Lhx2* and regulates the formation of the cortical hem (Godbole et al., 2018; Liu et al., 2018).

The exact mechanism by which *Foxg1* and *Shh* interact to pattern the ventral telencephalon is still unknown, a potential model is outlined in (Danesin & Houart, 2012). As previously mentioned, *Fgf8* initiates the expression of *Foxg1* from the anterior neural ridge. Meanwhile *Shh* is expressed independently in the POA. Evidence suggests that *Fgf8* and *Shh* form a positive feedback loop. The gene *Gli3* is a key component, it can occur in two forms; activator and repressor, depending on whether there are high or low levels of *Shh* respectively. This relates to its role as a transcription factor. It is suggested that Gli3A (activator) found in the ventral telencephalon activates *Foxg1* and the subsequent ventral telencephalic patterning. The model suggested by Houart also relates to the levels of *Foxg1* found, implying that while *Foxg1* is found both dorsally and ventrally it occurs in higher levels ventrally and this is partly how it enacts different effects.

1.3.3 *FOXG1 Syndrome*

In humans, mutation of one allele of *FOXG1* has been linked with a severe form of autistic spectrum disorder (ASD) termed *FOXG1 syndrome* (Wong et al., 2019).

Originally classified as a variant of Rett syndrome due to similarity of some symptoms such as epilepsy, microcephaly and intellectual disability. However, Rett syndrome, caused usually by mutations in *MECP2*, affects only females and shows regression from six to eighteen months, neither is true with *FOXG1 syndrome* and so is currently considered an entirely separate disorder (Candelo *et al.*, 2020).

FOXG1 syndrome has been characterised by its variability of symptoms, however, some phenotypes including hypoplasia and corpus callosum malformation appear consistent between cases (Cargnin et al., 2018) with almost 84% of suspected *FOXG1 syndrome* patients in one study showing corpus callosum defects (Pringsheim et al., 2019).

In one cohort of 83 patients, 54 different genetic variants were identified 20 frameshift, 17 missense, 15 nonsense, and 2 in-frame variants. Of these the frameshift and nonsense appeared throughout the gene, with missense mutations appearing clustered in the forkhead domain. In terms of severity of phenotypes, those mutations which truncated the protein appeared to correspond to the worse phenotypes (Mitter et al., 2018).

Anatomical abnormalities in human patients could also be seen, to a slightly lesser degree, in *Foxg1* heterozygote mice (Pringsheim et al., 2019). Suggesting that mice can still be successfully used to model aspects of the disorder.

1.4 Cerebral Organoids

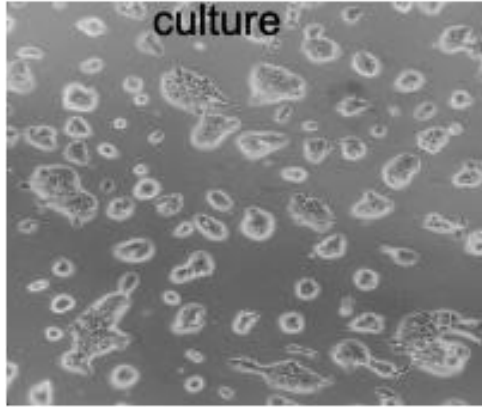
The ability to understand the fundamental mechanisms of the brain would allow greater insight into the causes of neurodevelopmental disorders. However, due to many legal and ethical concerns, the use of human brain tissue is difficult to access and use for research purposes. This has led to a number of model systems being developed, which each allow an aspect of development to be investigated. The majority of work done regarding *Foxg1* derives from mouse *in vivo* models.

Although chicks, rats and zebrafish have also given valuable insights into the role of *Foxg1* (Danesin & Houart, 2012). However, even with the mouse model there are limitations including some species specific differences in cortical development (Mason & Price, 2016). Another impediment is that any *in vivo* model still has accessibility issues. This was originally overcome with *in vitro* culturing of brain tissue. These were done with tissues from a wide variety of different species and each recapitulated key spatial and temporal aspects of *in vivo* counterparts (Bolz et al., 1990; Choi & Lapham, 1974; Hansen et al., 2010, 2013; Kim, 1976; Molnár & Blakemore, 1991; Price & Lotto, 1996).

Work to re-programme adult somatic cells into induced pluripotent stem cells (iPSCs) through use of the Yamanaka factors allowed stem cell lines to be derived directly from patients with neurodevelopmental disorders for study, providing a potential isogenic *in vitro* model of these disorders (Takahashi & Yamanaka, 2006).

The advent of current cerebral organoids started with work on mouse embryonic stem cells from (Eiraku et al., 2008). The suspension of mESC aggregates in culture medium with laminin (a key component of the extracellular matrix) showed self-organisation into structures reminiscent of the developing murine cortex (Figure 1.5). More recent work showed the manipulation of the *Shh* pathway lead to cerebral organoids of more ventral telencephalic identity (Danjo et al., 2011; Nasu et al., 2012). It was ultimately Lancaster et al., (2013) who combined the use of iPSCs and the 3D organoid culture system to create human cerebral organoids which mimicked key aspects of human cortical development, as well as recapitulating species specific differences with the mouse (Lancaster & Knoblich, 2014; Marshall & Mason, 2019).

mESC
culture

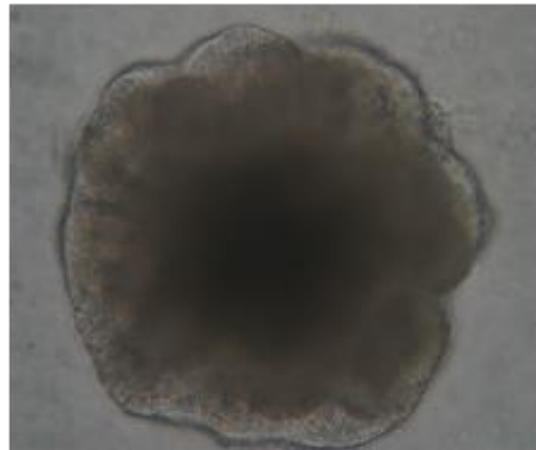


Day 0



5,000 mESCs IWP-2

Day 3



Day 7



Figure 1.7. Figure showing key stages of organoid development. First panel shows image of mouse embryonic stem cells in GMEM/F12 media. On day 0 of the protocol 5000 mESCs are aggregated into a low adhesion 96-well plate with the Wnt inhibitor IWP-2. 24 hours Matrigel is added. By day 3 the formation of neuroepithelial vesicles can be seen. On day 7 neural process can be seen branching from the organoid through the Matrigel.

1.4.1 Current limitations

These cerebral organoids are still in their infancy and therefore require further optimization. The lack of a vasculature inhibits the absorption of nutrients leading to the formation of a necrotic core, thus limiting the life span of these cultures. However, multiple attempts have been made to overcome this limitation. one such attempt is the use of bioreactors, in which the culture medium is in constant motion, this then maintains a large concentration gradient of nutrients so driving diffusion (Qian et al., 2016). Another promising avenue are air-liquid interface organoids (Giandomenico et al., 2019), this involves sectioning of organoids and exposing the centre to allow direct diffusion of nutrients. Further work to prolong the life span of organoids will allow their use to study the later stages of neurodevelopment.

Another key limitation of organoid protocols is the inherent variability which can be seen within batches but most notably between batches (Qian *et al.*, 2019). This can make reliable quantification of phenotypes in organoids difficult. This thesis aims to develop quantification strategies which takes this limitation into account.

Finally, this thesis focuses on the use of mouse cerebral organoids but for the study of some human specific disorder, human cerebral organoids would be more appropriate. At present the time line to create mouse cerebral organoids can take up to 10 days, whereas human cerebral organoids can take several months to

develop (Danjo et al., 2011; Lancaster et al., 2013b; Nasu et al., 2012; Paşca et al., 2015). This highlights the relevance of mouse cerebral organoids, the ability to rapidly optimise and adapt protocols, as well as for use with questions which do not have human specific answers (Marshall & Mason, 2019).

1.5 Thesis aims

Organoids have great potential for studying brain development and associated disorders. However, there is still no consensus on what they can and cannot be used for. A fuller understanding of what aspects of brain development they correctly recapitulate is needed.

This thesis aims to look at how successfully mouse cerebral organoids recapitulate key phenotypes from *Foxg1*^{-/-} mice. While there are still many questions regarding the role that *Foxg1* plays in telencephalic development there is already a large body of data regarding some phenotypes. The two phenotypes looked at in this thesis are;

- The hypoplasia in the dorsal telencephalon caused by premature elongation of the cell cycle
- The inability of *Foxg1*^{-/-} cells to adopt a ventral telencephalic fate

The first results chapter of this thesis looked at quantification methods in organoids. Due to only a part of organoid tissue adopting a desired fate it becomes

necessary to identify regions of interest. How these regions are distinguished may be open to bias. Several quantification strategies were assessed to find the most suitable and accurate for cerebral organoids.

The second results chapter begins by optimising existing protocols to steer organoids towards a ventral telencephalic fate. Once this is achieved, chimeric organoids derived from *Foxg1*^{+/+} and *Foxg1*^{-/-} cells were put through this protocol to see if the mutant cells showed the same *in vivo* patterning defects.

The final results chapter consists of a cumulative EdU experiment used to estimate cell cycle lengths in *Foxg1*^{+/+} and *Foxg1*^{-/-} dorsal telencephalic organoids. This was used to assess how accurately organoids mimic the cell kinetic phenotypes seen *in vivo*.

Chapter 2 - Materials and Methods

2.1 Mouse embryonic cell lines

Throughout this thesis 3 cell lines were used: a *Foxg1*^{+/+} line called *E14Tg2a* (Hooper et al., 1987), a *Foxg1*^{+/-} line called *Foxg1::Venus* (Eiraku et al., 2008) and a *Foxg1*^{-/-} line called *Foxg1*^{LacZ/Cre} (Manuel et al., 2011). A thorough description of all three cell lines and their genetics can be found in Section 3.2.

2.2 mESC culture

Mouse embryonic stem cells were cultured as previously described (Watanabe et al., 2005). Cells were grown in a T-25 flask (Falcon) in 10 ml of LIF/serum medium. The medium consisted of Glasgow's MEM (ThermoFisher) and was supplemented with a 1:500 dilution of Leukaemia inhibitory factor (LIF) grown in lab from plasmid lipofected into HEK 293 cells, 10% fetal bovine serum (ThermoFisher), 1 mM Pyruvate (ThermoFisher), 2 mM GlutaMax (ThermoFisher), 0.1 mM non-essential amino acids (NEAA) (ThermoFisher) and 0.1 mM β -mercaptoethanol (ThermoFisher). Flasks were stored in an incubator at 37°C and 5% CO₂.

2.2.1 Passaging of cells

Cells were passaged every two to three days. Old medium was aspirated out and three rounds of washing with 1x PBS were performed, 5 ml of PBS was added each time and swirled around the flask and then aspirated out. Two fresh T-25 flasks (Falcon) were coated with 1 ml 0.1% gelatin for at least 15 minutes. Gelatin was then aspirated out. 1 ml Tryple (ThermoFisher) was added to old T-25 flask and placed in an incubator at 37°C for 1 minute. After this 9 ml of the LIF/serum medium were added to the flask to quench the Tryple. The stem cell solution was triturated slowly for a minute to get a single-cell suspension. The original cell suspension was diluted 1:10 and 1:20 into the two new flasks with a total volume of 10ml in each flask.

2.3 Dorsal telencephalic organoid protocol

The protocol used was previously described (Eiraku et al., 2008). Confluent T25 flasks (~75%) were used for the organoid protocol. On day zero, medium was aspirated out of flask. 5mls 1x PBS (ThermoFisher) was used to wash cells and wash repeated twice. 1ml Tryple (ThermoFisher) was added to flask and left for 1 minute in a 37°C incubator until cells detached from plastic. 9 ml of LIF/Serum media was added to quench Tryple. All 10 ml of cell solution was transferred to a centrifuge tube and spun at 1000 rpm for 5 minutes. Stem cells formed a pellet and the

supernatant was removed. 10 ml of KSR Media were added. KSR Media consisted of GMEM (ThermoFisher) supplemented with 10% Knockout Serum Replacement (KSR; Invitrogen), 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, 0.1 mM 2-Mercaptoethanol. The pellet was resuspended with pipetting, the tube was then centrifuged again at 1000 rpm for 5 minutes. The supernatant was removed and a fresh 10 ml of KSR media added. This was to remove as much LIF/Serum media as possible. Pellet was resuspended to get a single cell suspension. The concentration of cells in solution was measured with the Countess II Automated Cell Counter (ThermoFisher). Once the concentration of the stock solution was known, the required concentration was calculated with the following equation;

$$C_1V_1 = C_2V_2$$

Where C represents the concentration and V represents the volume, subscript one denotes the original stock solution and subscript two denotes the final solution used for the protocol.

A final concentration of 50,000 cells/ml is required, then 100µl of this stock is placed into each well of a PrimeSurface 96 U-well plate (AlphaLabs), to give 5000 cells per well. From the cell counter we knew C_1 , C_2 would always be 50,000 cells/ml and V_2 was determined by how many wells were being seeded. It was calculated with the following equation;

$$V_2 = 100 \times (n + 2)$$

Where n is the number of wells to be used, the two additional wells worth of solution is to compensate for any lost while pipetting. From this the equation is rearranged to find the value for V_1 . This quantity of cells would be transferred to a fresh tube and topped up with fresh KSR media until at the same volume as V_2 . Finally, 2.5 μ M IWP-2 (SigmaAldrich) was added to the solution. 100 μ l solution was added to each well of the 96 well plate which was then stored in an incubator at 37°C (Figure 2.1).

The next day (day one), Matrigel (Corning) was added to each well at a concentration of 2 μ g/ml. The Matrigel was kept on ice and taken out at least one hour before use to allow slow thawing, to prevent premature formation of a gel. Pipette tips used were kept in a -20°C freezer prior to use. Plates were returned to the incubator after Matrigel addition.

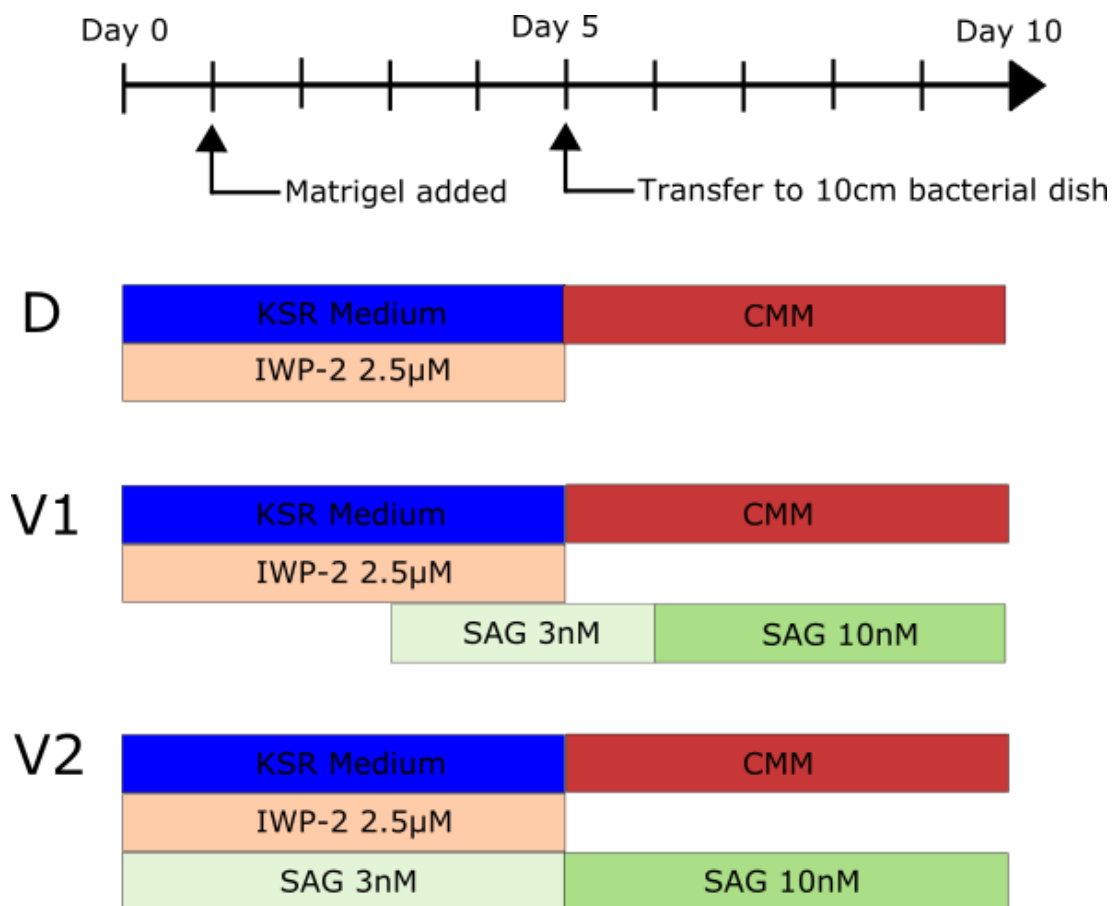


Figure 2.1 – schematic showing media changes during dorsal and ventral telencephalic organoid protocols from day 0 in vivo. Condition D is dorsal protocol described in section 2.3 and conditions V1 and V2 are described in section 2.4.

On day five, organoids were transferred to 10 cm bacterial grade dishes (ThermoFisher) and KSR medium was replaced with cortical maturation medium (CMM). CMM consisted of DMEM/F12 (Sigma Aldrich) supplemented with 1% Glutamax (ThermoFisher) and 1% 100x N2 (ThermoFisher). Each dish contained a maximum of 12 organoids in 8 ml medium, 4ml of this medium was replaced every 2 days. Organoids were maintained in this solution until collection.

2.4 Ventral telencephalic organoid protocol

The overall protocol for the generation of ventral telencephalic organoids was the same as the dorsal, except for the addition of Smoothed agonist (SAG) (Enzo) (Danjo et al., 2011).

Condition V1

Dorsal protocol was carried out and 3nM of SAG was added on day three, with this being increased to 10 nM at day six.

Condition V2

Dorsal protocol was carried out and 3 nM SAG was added on day zero, with this being increased to 10 nM at day five.

2.5 Cumulative EdU labelling

Cumulative EdU labelling was carried out as described for BrdU labelling (Nowakowski et al., 1989) but partially adapted for organoids as opposed to *in vivo*. The Click-iT EdU kit (ThermoFisher) was used. EdU was added to the bacterial grade dishes at day 7 to a final concentration of 10 μ M in medium, this was timepoint 0 hours. Several organoids were taken out of the bacterial dish and fixed at time

points 2, 4, 6, 8, 10, 12, 14 and 24 hours. Organoids were processed and sectioned as described in section 2.6.

Staining for the EdU took place prior to immunohistochemistry as described in the Click-IT EdU kit protocol. The sole exception was that no tissue permeabilisation was performed as this was unnecessary on sectioned tissue. Immunohistochemistry was then carried out the same as described and stainings were done for Sox2 and DAPI.

2.6 Fixation and sectioning of organoid tissue

2.6.1 Fixation

Organoids were fixed in a solution of 4% paraformaldehyde in PBS at room temperature for 30 minutes on a shaking platform. After 30 minutes 4% paraformaldehyde was aspirated from wells and replaced with fresh PBS and returned to shaking platform. This washing process was repeated 2 further times. Organoids were then either moved straight to the cryoprotection stage or stored in PBS at 4°C overnight.

2.6.2 Cryoprotection and Block Mounting

After fixation organoids underwent cryoprotection. PBS was aspirated out of wells and replaced with a 30% sucrose solution in PBS. This was stored overnight at 4°C on a shaking platform.

The 30% sucrose solution was replaced with a 50:50 mix of 30% sucrose and OCT compound. The plates were returned to 4°C on a shaking platform for at least one hour. Organoids were removed from wells with the use of Pasteur pipette, the end was cut off to allow a wider opening for organoids to pass through. Organoids were transferred to a truncated square tissue mould. All excess solution was removed to allow all organoids to rest against the bottom of the mould. This was to ensure all organoids were on the same plane of sectioning. Moulds were placed over dry ice and the 50:50 mix of 30% sucrose and OCT compound slowly added until full and frozen. Blocks were stored at -80°C prior to sectioning.

2.6.3 Sectioning

Organoids were sectioned via cryostat (Leica CM1800) to a thickness of 10 µm.

Tissue slices were placed onto ThermoFisher SuperFrost slides. Slides were air dried for 2 hours and then stored at -20°C for at least 12 hours prior to immunohistochemistry.

2.7 Immunohistochemistry

Slides boxes were removed from -20°C at least one hour prior to staining and left unopened to prevent condensation. Slides were then placed in a Coplin jar with 1x PBS and washed on a shaking platform for 20 minutes, this was repeated two further times.

Some antibodies used required antigen retrieval, for these sections slides were submerged in 10 mM sodium citrate (pH6) for 5 minutes at room temperature.

Slides were transferred to a microwave and placed on high setting (900W) for two rounds of 5 minutes and two further rounds at medium setting. After each 5-minute heating the liquid level was refilled to the original level with double distilled water to ensure consistent concentration of sodium citrate. After antigen retrieval slides were left in sodium citrate at room temperature until cool.

Tissue sections were then drawn around with a hydrophobic pen (Vector Laboratories ImmEdge Pen). A blocking solution of 20% donkey serum in 1x PBS was added to the slides which were placed in a humidified chamber and left for 2 hours at room temperature.

A 10% donkey serum in PBS solution was made, into which required concentrations of primary antibodies were added (a full list of antibodies and concentrations can be found in Table 2.1). 200µl of the 10% donkey serum + primary antibodies solution was added to each slide. Slides were returned to a humidified chamber and stored at 4°C overnight.

The next day slides underwent three more rounds of 20-minute agitated washing in 1x PBS. A 10% donkey serum in PBS solution was made and secondary antibodies were added, all secondary antibodies used were at a concentration of 1:200. Slides were placed back in a humidified chamber and 200µl of the 10% donkey serum + secondary antibodies solution was added to each slide. This was left at room temperature for 2 hours. Slides were returned to a Coplin jar with 1x PBS. 1ul of DAPI nuclear stain was added to 1 ml of 1x PBS and vortexed until DAPI was well dispersed. This 1:1000 DAPI solution was added to the Coplin jar which was placed on a rocking platform for 5 minutes. After this the PBS was discarded and three rounds of 20-minute washes in 1x PBS were carried out. Any excess solution was removed from slides prior to coverslips being mounted. Two drops of Vectashield hard set (Vector Labs) were added to each slide and a coverslip placed on at an

angle to ensure no air bubbles became trapped on the tissue. Coverslips were sealed with nail varnish. Slides were then stored at -20°C prior to imaging.

Marker	Manufacturer	Species	Conc.	AR (Y/N)	Cat. No.
Sox2	Abcam	Rabbit	1:500	N	92494
Tbr2	Abcam	Rabbit	1:500	Y	216870
Nkx2.1	Abcam	Rabbit	1:300	N	227652
Olig2	Millipore	Rabbit	1:400	Y	AB9610- AF555
Tuj1	Biolegend	Mouse	1:500	N	A488- 435L
N-Cadherin	BD Transduction Labs	Mouse	1:500	N	610920
GFP	Abcam	Chick	1:1000	N	13970
β- galactosidase	Invitrogen	Mouse	1:300	N	MA1-152

Table 2.1 – Table of primary antibodies used throughout the thesis, including; species, used concentration, catalogue number and whether antigen retrieval (AR) was required.

2.8 Light Microscopy

Images were taken using a Leica DM5500B epifluorescence microscope with a DFC360FX camera.

2.9 Image Analysis

Images were imported into the software programme FIJI where analysis and quantification was carried out. Whenever the brightness and contrast of images was adjusted to improve clarity this was applied across the entire image.

2.9.1 Randomised grid quantification method

The scale bars added to images by the LeicaX software were used to set the scale of images in FIJI. This was used to overlay a 5000 μm^2 grid on the image using the “Grid” plug-in. Not all grid squares contained organoid tissue, those that did were numbered starting in the top left corner and working left to right and top to bottom. The RANDBETWEEN function of Microsoft Excel was used to randomly generate a sample of squares for counting. The “Channel Tool” was used to split the RGB image into 3 separate channels which could be toggled through. The “Cell Counter” plug-in was used to count cells within the randomly selected squares.

2.10 Data analysis and graph plotting

Collection and recording of raw data were done through Microsoft Excel. Statistical analysis was also done via Excel. The graphing of data was done via the use of RStudio.

Chapter 3 - Development of a strategy for unbiased quantification of cerebral organoids

3.1 Introduction

Cerebral organoid research has used these 3D self-organising systems to investigate multiple areas of development and disease (Di Lullo & Kriegstein, 2017; Lancaster & Knoblich, 2014; Marshall & Mason, 2019). However, the novel nature of the field has meant that standardised methods of quantification have not been developed. Many papers rely on identifying regions of interest (ROIs) which will subsequently be quantified. Organoid protocols use pluripotent stem cells (PSCs), often in the presence of minimal external interference (Eiraku et al., 2008; Madeline A. Lancaster et al., 2013a; Paşca et al., 2015). The protocols depend on the self-organising abilities of the differentiated daughter cells themselves. The non-homogenous levels of pluripotency markers seen within PSC cultures highlight the variable states these cells can occupy (Karwacki-Neisius et al., 2013). This can subsequently impact the efficiency of *in vitro* differentiation and while protocols are efficient other cell types are almost always present too (Velasco et al., 2019). The greatest obstacle in quantifying organoids is this inherent variability. As shown in Figure 3.1, a typical organoid may contain a number of regions with expression patterns reminiscent of *in vivo* cortical tissue (shown inside green circles). However, in the same tissue there are regions which have unknown identity. The organoids shown in the figure highlight the differences seen within organoid cultures. Therefore, to reliably use organoids to investigate biological effects the correct

tissue must be identified and quantified. Such a process usually involves using the expression of genes found in an *in vivo* region as a delineating marker. The structures found in organoids, while reminiscent of *in vivo* structures, do not fully maintain the shapes or organisation seen *in vivo* (Nasu et al., 2012). This can lead to the absence of sharp boundaries between regions.

The sole use of gene markers to determine an ROI usually relies on a researcher deciding the region of interest for themselves. The lack of defined borders and variability in gene expression opens the drawing of ROIs up to subjective choices by the researcher. This can include unconscious bias into the quantification process. This problem is not addressed in the literature, with papers describing what markers were used to determine the ROI but not what measures were taken in cases which required subjective judgement. While important to use tissue which is appropriate to the question asked, it is also necessary to use all relevant tissue present within the organoid and not just the most aesthetic stretches of tissue grown.

3.2 Criteria for a robust and unbiased counting strategy

This chapter aims to address this deficit and to develop a counting strategy which meets a list of criteria:

- Removes unconscious bias from the selection of ROIs
- Adaptable to the inherent variability of organoids
- Takes the whole organoid into consideration
- Can robustly and accurately quantify the feature studied

3.2.1 Removing unconscious bias from the selection of ROIs

For this criterion to be successfully met, an appropriate strategy must be able to select ROIs without any subjective judgement on the part of the researcher. The approach taken in this thesis is to use landmarks already present in the organoids and use these as a point of reference for the ROIs. The distance of cortical tissue away from these landmarks was measured. The aim was to establish whether there was a strong correlation between cortical tissue and any of these landmarks. If so, then is the distance between cortical tissue and landmarks constant across organoids. If this is the case, then the landmarks and this perimeter could be used to identify ROIs without any subjective interference.

As aforementioned, organoids lack clean internal boundaries and vary in marker expression levels. Any ROI identified by a strategy using landmarks as opposed to

gene expression would likely contain non-desired cell types. These cell types may provide noise into the data but does provide a more accurate account of what is going on within the organoid by taking all tissue into consideration.

3.2.2 Adaptability to the inherent variability of organoids

Different cell lines, or batches of the same cell line, can vary greatly in structure and cellular composition. For this criterion to be met then any strategy developed must be suitable to be used regardless of this variability. Therefore, it should be trialled on organoids produced from multiple cell lines and batches.

3.2.3 Taking the whole organoid into account

The nature of pluripotent stem cells means the likelihood of achieving one hundred percent cortical-like tissue in an organoid is low (Velasco et al., 2019). The presence of different cell types in organoids is well established, especially in protocols such as the one used in this thesis which involves minimal external interference. These alternate cell types may well dampen the effects seen from an investigation and will be irrelevant to study for most researchers. However, considering that this thesis also looks at optimisations of the organoid protocols it is therefore important to be able to look at the organoid in its entirety. This can provide a snapshot of the relative health and differentiation efficiencies of organoids produced from different protocols, helping to better assess them.

3.3 Experimental Design

The cell lines chosen for this experiment were done so to investigate Foxg1. Homozygous wild-type (*E14Tg2a*), homozygous mutant (*Foxg1^{LacZ/Cre}*) and heterozygous lines (*Foxg1::Venus*) were used. The *E14Tg2a* line was chosen as it is a widely used line with its characteristics well described in the literature. Meanwhile, the presence of a Foxg1 reporter in the *Foxg1::Venus* line was used to identify potential regions of telencephalic identity. *Foxg1^{LacZ/Cre}* is the same genotype as the mutant mice which have well characterised mutant phenotype, this makes it a good line to use to compare how effectively organoids recapitulate *in vivo* phenotypes. These lines are non-isogenic and this requires caution when drawing biological conclusions. A detailed description of these lines and known differences follows later in this chapter (section 3.2.1). However, for the purposes of this chapter these differences are insightful. A good validation of any strategy would be its successful use on three variable cell lines.

For this experiment organoids were grown from all 3 cell lines and collected at day 8. These organoids were sectioned and processed as described in the Methods section. Organoids were double stained via immunohistochemistry for N-cadherin and Tbr2 proteins.

N-Cadherin is a transmembrane protein involved in cell-cell contact. In the developing mouse brain this protein can be found accumulated on the ventricular edge of the cortex, marking the apical edge of the neuroepithelium. This staining was used to identify the ventricular edge which is one of the landmarks investigated (Hatta et al., 1988; Nakagawa & Takeichi, 1995; Radice et al., 1997).

Tbr2 is expressed in intermediate (basal) progenitors in the murine developing cortex which are present in the SVZ (Hevner, 2019). In a previously published study mouse organoids showed clear lamination between neural and proliferative populations at early stages, but this broke down as development progressed (Nasu et al., 2012). At this early stage of development, the start of neuronal production is underway (Dwyer et al., 2016; Medina & Abellán, 2009). Here Tbr2 is used as a marker of the outer (basal) edge of the mouse proliferative zone. Considering that *Foxg1* is implicated in controlling the cell cycle (M. N. Manuel et al., 2011; Martynoga et al., 2005) the aim was to create a technique which would create ROIs encompassing progenitors.

3.3.1 Description of cell lines used

In this chapter, three existing cell lines were used, the aim was to grow cerebral organoids from all three lines. The non-isogenic nature of these lines may suggest they would behave differently in organoid cultures, however, no previous comparison of these cell lines has the extent of these differences is unknown. These cell lines were:

E14Tg2a

This line contains two functional alleles of Foxg1. The cell line was originally developed to show how using retroviral sequences which incorporated with the host DNA was a potential way of genetic manipulation (Hooper et al., 1987). In this case hypoxanthine guanine phosphoribosyl transferase (HPRT) deficiency was introduced into Ola/129 mouse blastocysts. This is the only genetic modification present within these lines. The cells retain their pluripotency and can produce all three germ layers if incorporated into a mouse blastocyst. This means that the more passages the line undergoes the more likely spontaneous differentiation is to occur. To counteract this, cells were thawed from liquid nitrogen cell store at a passage of between 11-15. Once cells were scaled up then new flasks were banked for future use. Cells were not used after passage 30 to ensure a pluripotent stem cell population.

Foxg1::Venus

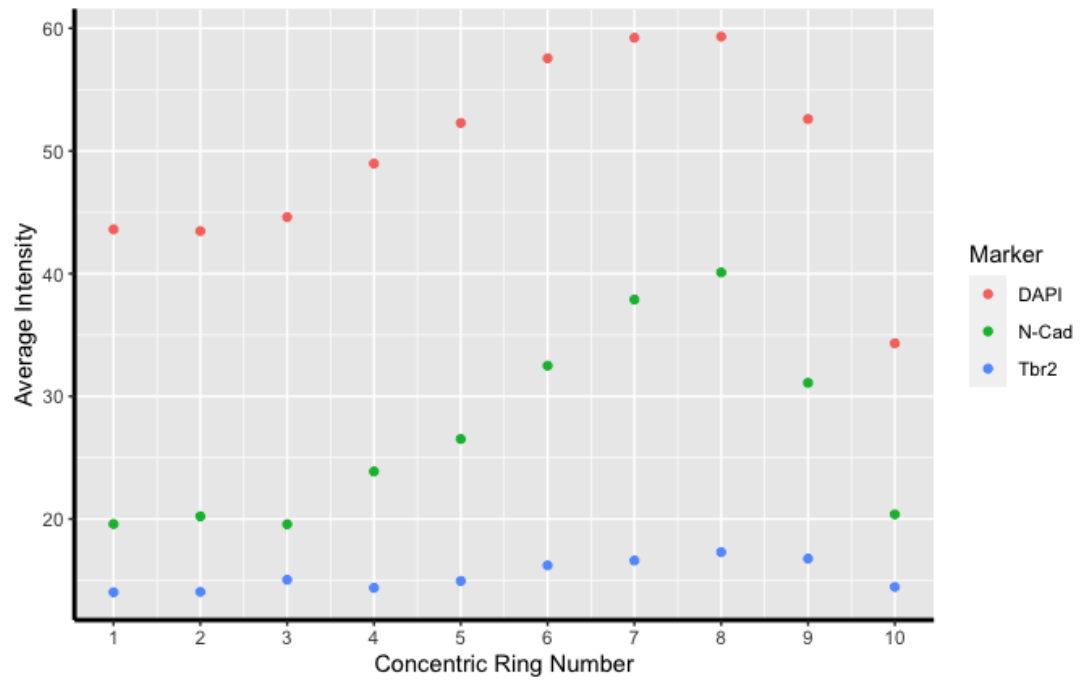
This line was originally created as a reporter line where a Venus GFP cassette was added in-frame in the *Foxg1* exon (Eiraku et al., 2008). This made it dependent on the *Foxg1* promoter while removing one allele of *Foxg1*. The parental line was the EB3 embryonic cell line which itself was derived from the Ola/129 mice. The EB3 line was created by introduction of an Ires-*bsd* knockout vector which disrupted one copy of *Oct3/4. Blasticidin-S Deaminase (bsd)* is a gene conferring resistance to the drug Blasticidin, used in the selection of mammalian lines. These cell lines have been reported as growing well *in vitro* and show efficient differentiation. Other *Oct4* heterozygous lines have shown higher levels of pluripotency after multiple passages as compared to wild-types (Karwacki-Neisius et al., 2013). This may then suggest that *Foxg1::venus* cells used in these experiments, which underwent the same protocol as the other cell lines, may also be less prone to spontaneous differentiation.

Foxg1^{LacZ/Cre}

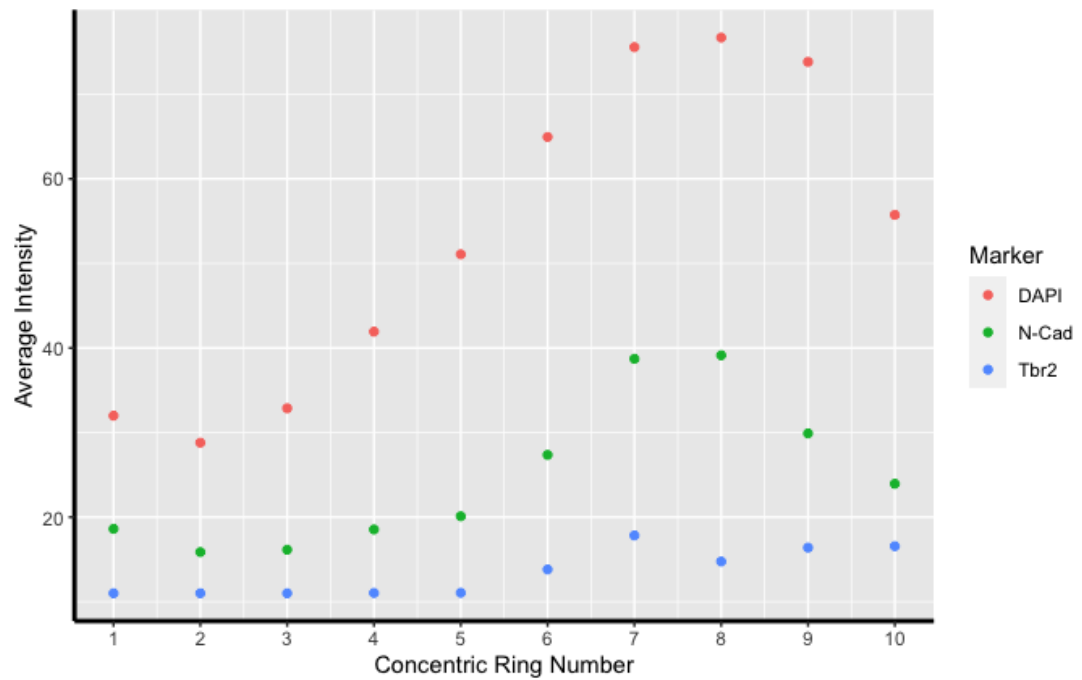
This cell line was originally created *in vivo* to create chimeric mice to investigate the cell-autonomous effects of *Foxg1* (Manuel et al., 2011). 129Sv:*Foxg1^{+/-LacZ}* female mice were mated with *Foxg1^{+/-cre}* lines to create *Foxg1^{LacZ/Cre}* blastocysts which were used for a series of experiments. The mutant mice were subsequently backcrossed onto a 129/Sv background, however it must be noted that these lines can be very variable when assessing data (Manuel et al., 2010). From blastocysts of these mice a stem cell line was derived. Mice of this genotype were found to be unviable

postnatally and showed severe microcephaly (Eagleson et al., 2007). Unusually, the presence of *Cre* in the *Foxg1* heterozygous line seemed to cause behavioural changes in the mice (Crawley et al., 1997; Wahlsten, 1982), as well as causing ectopic *Cre* activation and effects (Hébert & McConnell, 2000). The presence of *LacZ* also provides a marker for *Foxg1* expression when tissue is stained with Beta-galactosidase.

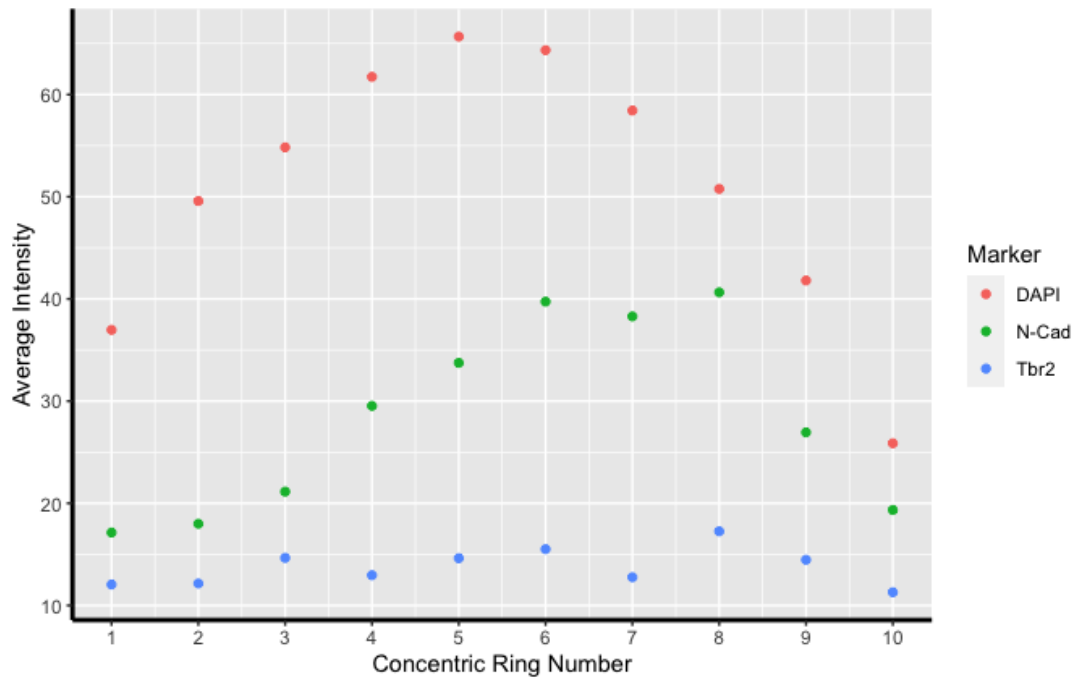
A



B



C



D

E

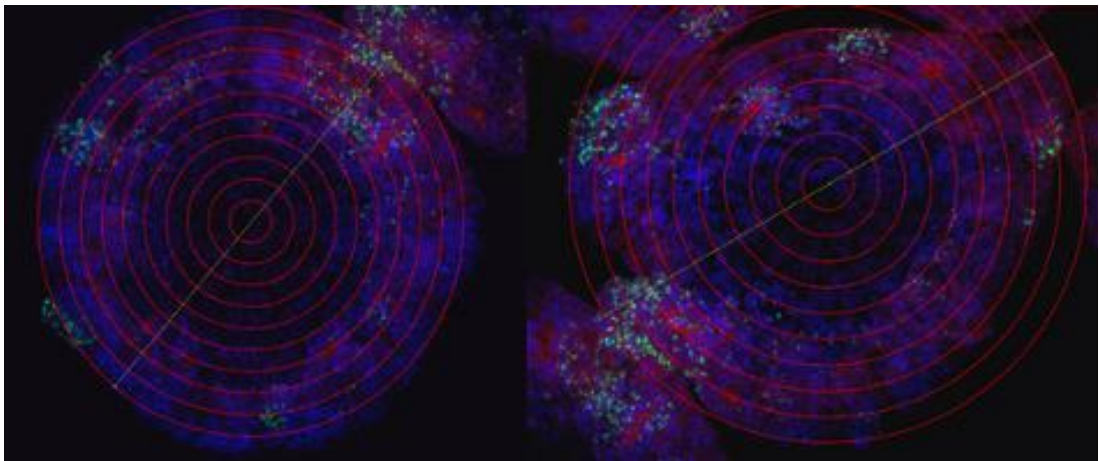


Figure 3.1 Concentric ring intensity profile. FIJI software created ten equidistant concentric rings radiating from the centre which we overlaid on organoids. Mean intensity profiles were taken along the perimeter of the rings. (A) Plot showing mean intensity profiles for three wildtype organoids from the same batch in

relation to concentric rings. Ring one corresponds to centre of organoid and radiates out to ring ten. (B,C) plots of two individual organoids (organoids 1 and 2), showing variation in mean intensities. Mean intensity is plotted against actual distances (μm). (D,E) Panel shows organoids 1 and 2 from the batch with overlaid concentric rings. Left of the panel shows an almost perfectly spherical section, right-hand of panel shows the more common irregular shaped organoids.

3.4 Methods to quantitate cell types in cerebral organoids

Once organoids from the three cell types were cultured, sectioned and processed, work began on trialling quantification methods. The criteria listed in section 3.1 were applied to each method to assess their effectiveness.

3.4.1 Concentric rings intensity profiles provide an imperfect snapshot of organoid differentiation

The first counting strategy trialled was concentric rings intensity profiles. FIJI software was used to measure the longest diameter of the organoid. After this FIJI software placed 10 equidistant concentric circles on the organoid with the furthestmost circle from the centre touching the furthest edge of the organoid. FIJI's intensity profile tool was used to calculate the average intensity along the circumference of each circle for three channels were used, each corresponding to a

different staining (blue=DAPI, red=Sox2, green=Tbr2). Figure 3.2 panel A shows cumulative results from this. The graphs show that in the centre of the organoid there is minimal levels of staining across all organoids. As the circles move away from the centre the intensity profiles increase until they plateau and subsequently decline again. This same pattern is seen across all 3 organoids trialled, two of which are individually graphed (figure 3.2 panels B and C)

Discussion points

A likely explanation for the lack of staining in the centre of the organoid is the presence of a necrotic core. The lack of a vasculature means that nutrients and waste products must travel via diffusion in and out of the organoid. As the organoid grows then the centre is starved of nutrients and undergoes cell death. This then explains why as you move outwards the gene expression increases. The healthier tissue is present in proximity to the outside edge and therefore to the nutrients present in the media. However, the outer circles show a dip in expression levels for some organoids which seems inconsistent with the idea that the healthiest tissue is located at the outer edge of the organoids. This is probably due to the irregular shape of the organoids. Organoids are rarely perfectly spherical; this means that for the outer circles they are now covering areas outside of the organoids and therefore show no gene expression (figure 3.2 panel E).

Overall, this method provides an interesting snapshot into the overall expression pattern of organoids and therefore meets the final criterion of investigating the whole organoid. It also does not require any subjective judgements on the part of the researcher. However, the information gathered condenses what is a highly intricate tissue into something overly simple. Furthermore, it relies on organoids being spherical which suggests it would not be able to deal with the variability of organoids. Therefore, while it may well be usefully in certain applications, for the aim of this thesis – to use an unbiased approach which extracts as much information as possible. It does not meet these criteria and it was not investigated further.

3.5 Randomised grid technique

The concentric circles approach lost vital information such as the true levels of gene expression and information on where cortical tissue appears within the organoid. This was addressed in the next approach. For this a grid was placed over the top of the organoids and random squares were then picked for counting. Initially, different sized grids were trialled (2500, 5000 and 10,000 μm^2) the latter was dismissed due to the large area of the organoid covered by each square and it covered both cortical and non-cortical regions. The 2500 μm^2 cells would instead provide very specific locational information, but the low numbers of cells present in

each square would mean a large number of cells would need to be counted to ensure biological effects could be detected. Ultimately the 5000 μm^2 grid was chosen as it was small enough to only cover regions of a single identity but was large enough to contain enough cells to make the counting process efficient. On average there was around 100 cells per square at 5000 μm^2 , as opposed to around 10-15 cells and ~300 cells for the 2500 and 10,000 μm^2 grid respectively. While the smallest grid provided the most precise locational information, the time-consuming process of randomly picking squares with the sheer quantity of squares to be counted made it unrealistic for a large investigation. A description of the randomised grid technique can be found in the Methods section 2.9.1.

A list of metrics to be measured was made:

a. Cross-sectional area (CSA) of the middle section of an organoid

This measurement was taken due to the microcephaly defect seen in *Foxg1* mutant mice; the CSA was to be used as a rudimentary gauge of organoid size to see if they showed this phenotype. While the shape of the organoid is irregular, they do all possess a relatively spherical shape. Therefore, it was assumed that the middle section of organoid would be the widest part of the organoid. The middle section was determined by the number of sections an organoid produced when sectioned on a cryostat (i.e. for an organoid which generated thirty sections, the fifteenth was used as the middle section).

- b. Percentage of Tbr2 positive cells in relation to DAPI stained nuclei in each square

Tbr2 is found expressed in intermediate (basal) progenitors and was used as a marker of SVZ-like tissue present in the organoids. The levels of Tbr2 positive cells throughout the organoid acts as an indicator for the levels of cortical differentiation within an organoid.

- c. Location of SVZ-like tissue in relation to the centre of organoid

Previous papers have described a necrotic core present in the centre of organoids apparently due to the lack of a vasculature. The distance from organoid centre to a ROI was measured to see if levels of cortical differentiation changed in relation to this necrotic core.

- d. Location of SVZ-like tissue in relation to the nearest outer edge

The inverse logic was applied when deciding to measure distance to the outer edge. If cortical tissue diminished close to the centre it would be hypothesised it would be more abundant closer to the outer edge and so the nutrient-rich media.

- e. Location of SVZ-like tissue in relation to the nearest ventricular edge
(defined by N-cadherin staining)

This landmark is one which is generated by the self-organising ability of organoids, it creates a fluid-filled lumen of which the edge becomes a ventricular edge, this then becomes the apical side of the neuroepithelium and the foundation for cortical growth. This would suggest there should be a strong correlation between the presence of a ventricular edge and SVZ-like tissue, however, what is not known

is if the SVZ-like tissue would always be located the same distance from the ventricular edge. If so this would provide an unbiased way of delineating ROIs.

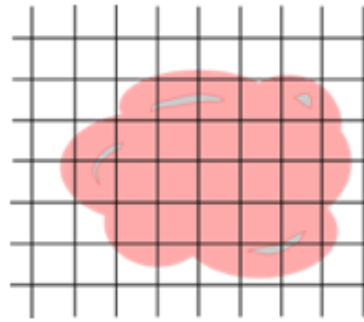
The quantification method in was carried out as followed (also illustrated in figure 3.2)

- A. Organoids underwent processing as described in methods section
- B. FIJI software was used to overlay a grid of defined sample square size (2500µm, 5000µm or 10000µm)
- C. Samples squares containing organoid tissue were numbered
- D. Random number generator was used to select squares to be counted – for each section 20% of the total squares occupied were selected and counted
- E. In each square the total number of cells (DAPI stained) were counted as was the number of intermediate progenitors (Tbr2) – this was used to calculate the proportion of intermediate progenitors in a square
- F. The centre of the organoid was determined by using FIJI to locate the organoid's centre of gravity. The distance from this centre was then measured to the centre of the sample square being counted
- G. The distance was measured from the centre of the sample square to both the nearest outside edge and to the nearest stretch of ventricular edge (marked with N-Cadherin). In the schematic shown both of these landmarks are shown in same sample square for convenience, in practice these landmarks could be several sample squares away.

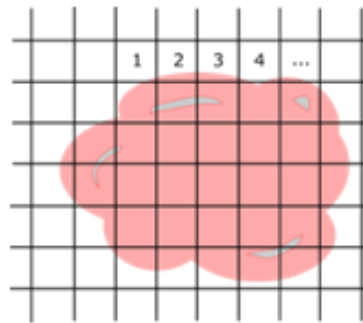
A



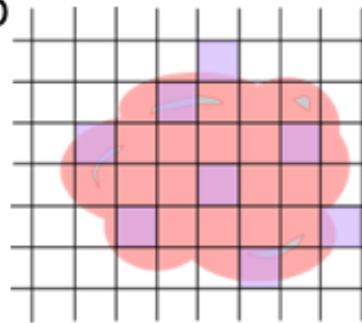
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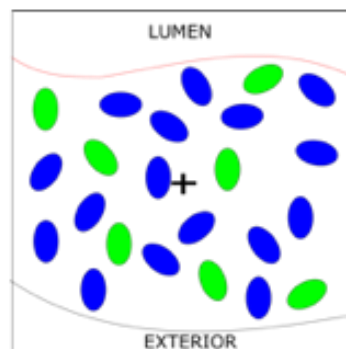
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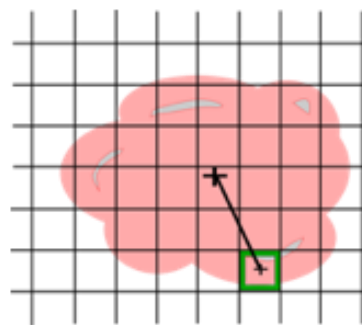
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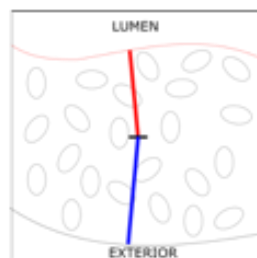
E



F



G



- Distance from centre of sample square to ventricular edge
- Distance from centre of sample square to outer edge

Figure 3.2 Schematic showing randomised grid quantification technique. B.FIJI software was used to overlay a grid of defined sample square size (2500µm, 5000µm or 10000µm) C. Samples squares containing organoid tissue were numbered D. Random number generator was used to select squares to be counted – for each section 20% of the total squares occupied were selected and counted E. In each square the total number of cells (DAPI stained) were counted as was the number of intermediate progenitors (Tbr2) – this was used to calculate the proportion of intermediate progenitors in a square F. The centre of the organoid was determined by using FIJI to locate the organoid's centre of gravity. The distance from this centre was then measured to the centre of the sample square being counted G. The distance was measured from the centre of the sample square to both the nearest outside edge and to the nearest stretch of ventricular edge (marked with N-Cadherin). In the schematic shown both of these landmarks are shown in same sample square for convenience, in practice these landmarks could be several sample squares away.

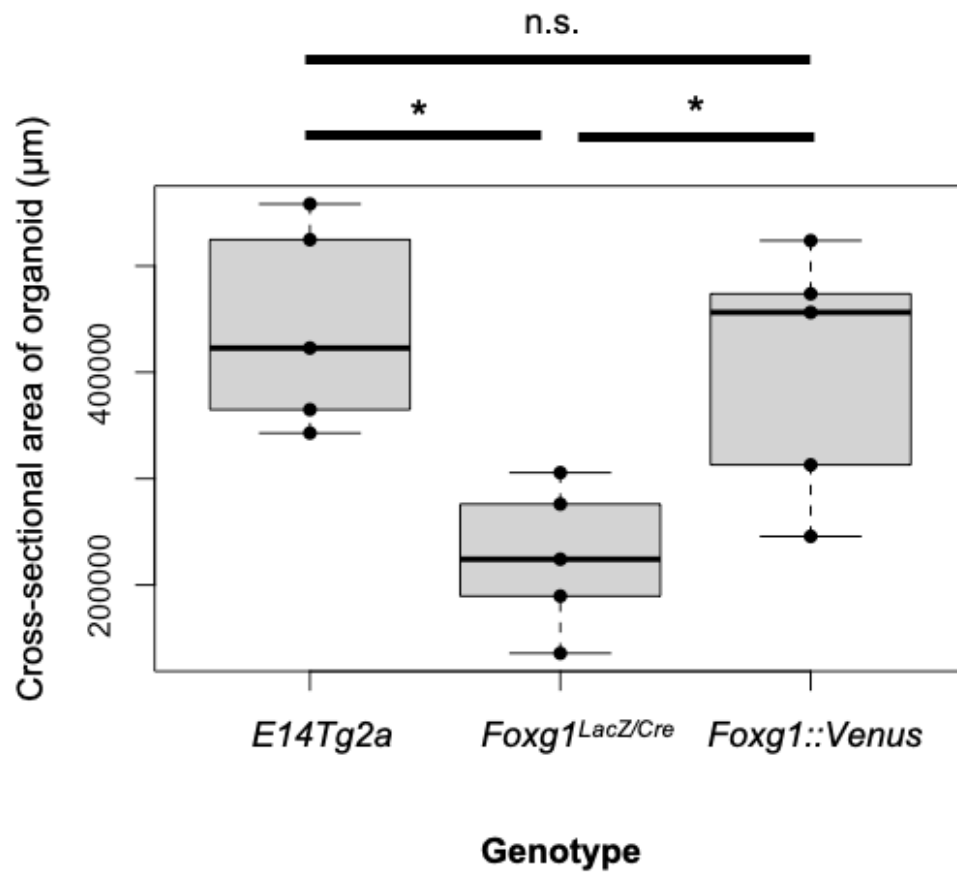


Figure 3.3 Comparison of cross-sectional area across genotypes. Middle section (described in section 3.3.2) was used to calculate cross-sectional area (CSA) in FIJI software. N= 5 organoids across 3 batches. Student t-tests were performed to investigate differences between genotypes.

3.5.1 Foxg1 homozygous mutant organoids show a significant decrease in size compared to other cell lines

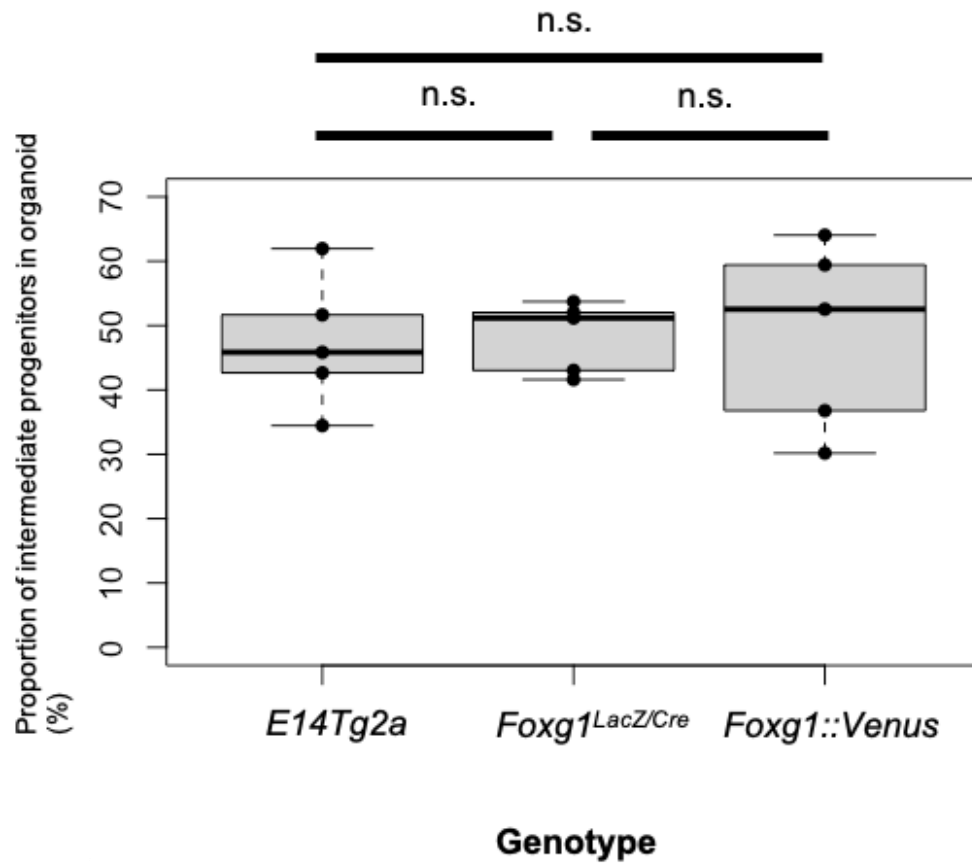
The cross-sectional area (CSA) of organoids grown from each of the three ES cell lines was measured using FIJI software. The perimeter of the middle section of each organoid was determined, this was assumed to be the midpoint and therefore widest point. While not an exact measure of organoid volume, for this thesis it is used as an indicator of overall organoid size. The data shows that *E14Tg2a* and *Foxg1::Venus* lines generated organoids of similar CSA (*E14Tg2a* mean CSA = 442706µm SEM = ± 42724; *Foxg1::Venus* mean CSA = 402528µm, SEM = ± 52882), use of a student t-test showed no significant variability (P=0.57). *Foxg1^{LacZ/Cre}* mutant line showed a smaller mean CSA (226258µm SEM = ± 30265). This was statistically compared with the other two lines and was found to be significantly different to *E14Tg2a* (P=0.03) and *Foxg1::Venus* (P=0.019).

Discussion points

The presence of severe cortical microcephaly in *Foxg1* deficient mice is well characterised in the literature. It could be expected that *Foxg1* deficient organoids would also show this same phenotype. The *in vivo* phenotype has been linked with cell cycle defects and premature differentiation seen. The organoids used in this

experiment were fixed at day 8, the initial starting culture was kept in culture at a stage comparable to E3.5. A rough estimation would mean that the organoids used were comparable to E11.5 embryos. At this stage microcephaly and cell cycle defects can already be detected (Martynoga et al., 2005)

This data provides a validation of the organoid system and could suggest that they are able to recapitulate the microcephaly phenotype seen *in vivo*. However, the CSA measurement takes into consideration all cell types present within the section, including those of non-telencephalic fate which do not express *Foxg1*. A more robust way of identifying microcephaly in organoids would be to quantify the volume of cortical tissue throughout the organoid, possibly using 3D imaging techniques such as BABB or iDISCO (Azaripour et al., 2016), this could be used to stain for a number of markers including those used in this chapter.



Foxg1::Venus Day 7

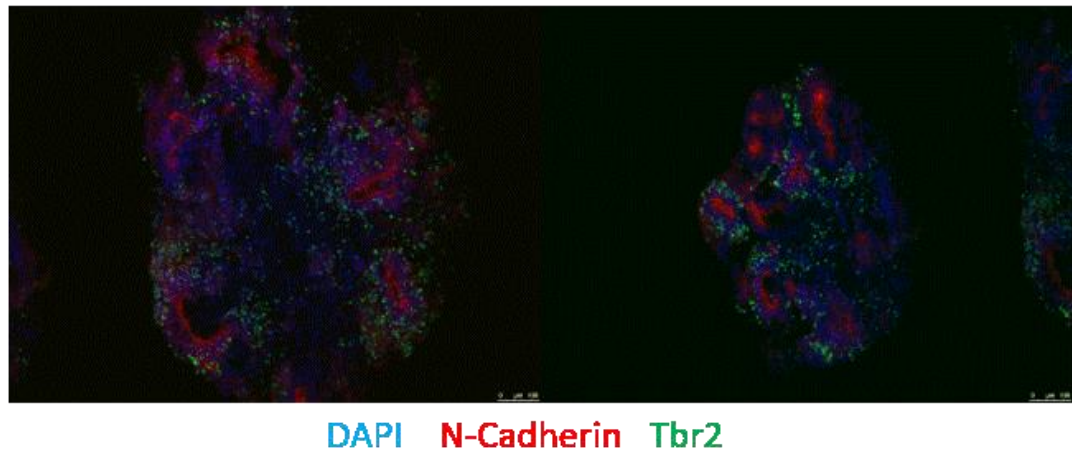


Figure 3.4 Comparison of average *Tbr2* expression levels between genotypes. Random sampling grid was used to count 20% of total occupied squares. The proportion of intermediate progenitors was calculated for each sampling square.

The values for sampling squares from the same organoid were averaged to provide the proportion of intermediate progenitors present across a whole organoid. The boxplot shows these averages. Images show representative organoids with N-cadherin (red) and Tbr2 (green) staining alongside DAPI (Blue). N= 5 organoids across 3 batches. Student t-tests were performed to investigate differences between genotypes.

3.5.2 Organoids derived from all cell lines show similar levels of Tbr2 expression

Tbr2 is a gene found in intermediate progenitors in the mouse developing cortex, found in the subventricular zone (Hevner, 2019). These intermediate progenitors are a second proliferative hub in the developing cortex (Section 1.2.1). In this experiment the *Tbr2* staining was used as a marker for this layer. In a previously published paper, mouse organoids at early stages followed the traditional inside-out growth pattern with laminations. However, at later stages this begins to break down and once neurons begin to appear the outer laminations are not formed (Nasu et al., 2012). It is unknown if this is consistent across all organoids. In organoids grown here at day 8 show clear lamination between *Tbr2* SVZ and *Sox2* Positive VZ.

20% of squares which contained tissue were randomly selected and counted. For each sampling square the proportion of intermediate progenitors were calculated as follows:

$$\left(\frac{\text{intermediate progenitors (Tbr2 – positive)}}{\text{All cells (DAPI staining)}} \right) \times 100$$

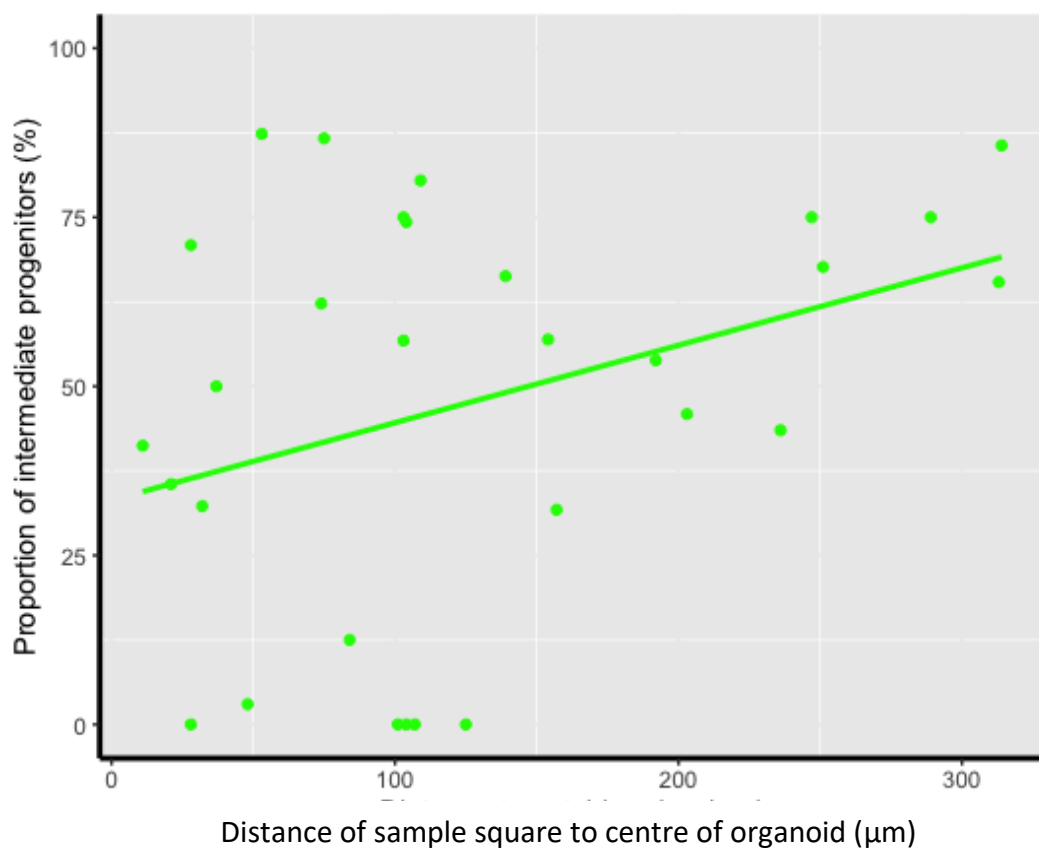
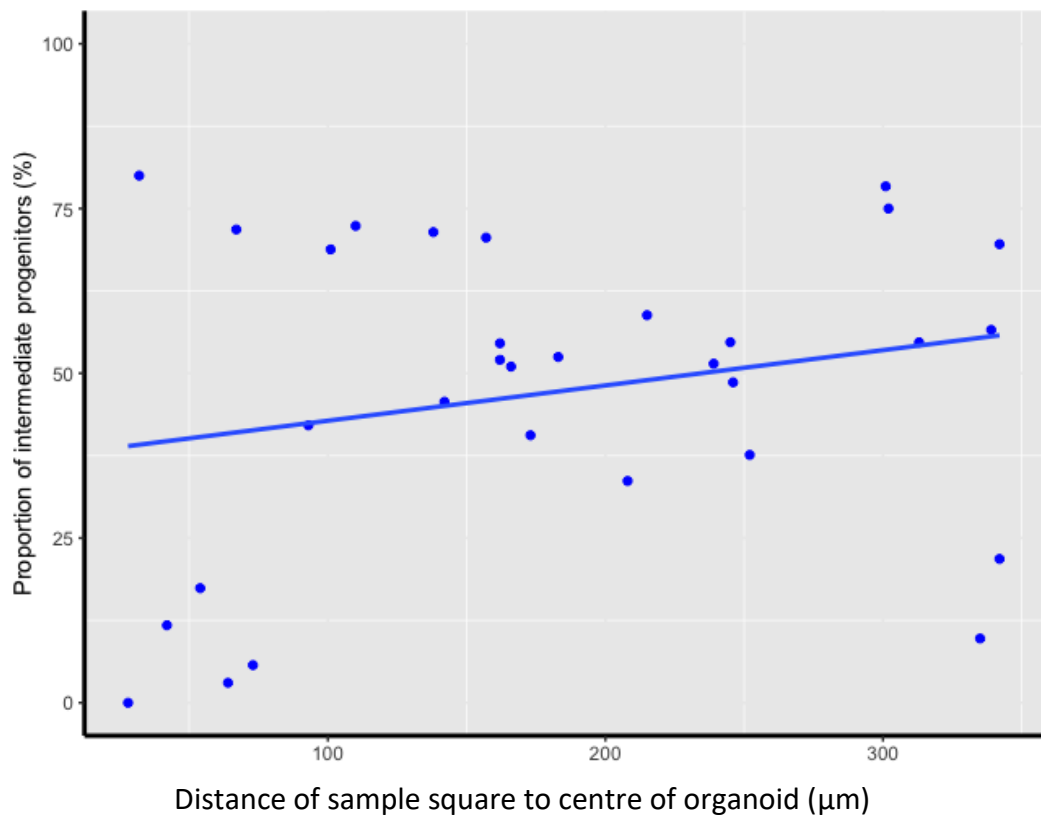
Once this was calculated the average proportion of intermediate progenitor for an organoid was calculated by averaging the values obtained from sample squares in the same organoid. Fig 3.4 shows these averages. All cell lines show variability in the levels of *Tbr2* seen, with *E14Tg2a* and *Foxg1::Venus* showing large ranges ~25-30%. However, *Foxg1^{LacZ/Cre}* organoids showed a much tighter range of means, all falling within a 10% range. Despite these differences in behaviour the overall mean for each genotype was surprisingly close. *E14Tg2a* – 47.3%, *Foxg1::Venus* – 48.6%, *Foxg1^{LacZ/Cre}* – 48.3%. This large degree of similarity between organoids cell lines meant that there was no statistical difference seen between any of the lines tested.

Discussion points

The mutant line shows a much tighter range in its data than either of the other two lines. This could well be linked back to the mutant organoids being considerably smaller than the other cell lines. This would mean that the diffusion limit of nutrients would have less of an effect on its growth. The smaller size would allow

the centre of the organoid to receive the required nutrients and hypothetically may a smaller necrotic core, these measurements were not taken in this thesis. The mutant organoids would be composed of more healthy tissue as a proportion of the whole organoid compared to the other cell lines. The random sampling grid would then be more likely to land on such healthy tissue. While the larger ranges seen in *E14Tg2a* and *Foxg1::Venus* could be attributed to more necrotic core squares being sampled.

The close proximity of the ratios together is very surprising. When you look at the range of *Tbr2* expression in individual squares (later figures) the different cell lines do not seem to behave similarly at all. Also, an average *Tbr2* proportion in the 40s seems high as well. As for the first point it could be an artefact of the random sampling. *Foxg1^{LacZ/Cre}* may show less range because of the likelihood of randomly selecting a outer square, but this is also true to a lesser extent for the other lines. Because squares to count are generated randomly and because more squares are located on the periphery it could be that the method shows a preference for counting cortical tissue rather than necrotic core. This could also explain why the levels of *Tbr2* expression were so high. This could also be because at the comparable *in vivo* stage it is towards the start of differentiation with high levels of proliferation still occurring.



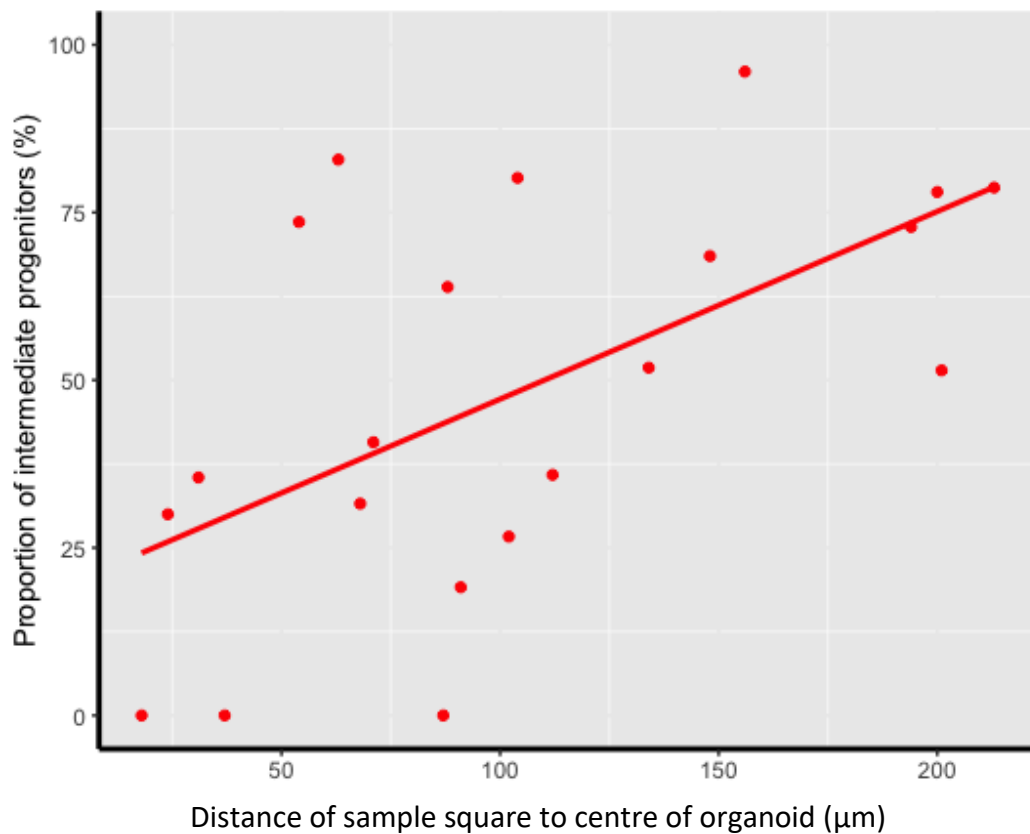


Figure 3.5 Distance from centre of organoid to closest SVZ-like tissue

Tbr2 positive cells were used as a marker for SVZ-like tissue. The proportion of intermediate progenitors for each sample square was plotted individually in relation to the distance from the centre of the sample square to the centre of the organoid (described in more detail 3.5). Top panel – *E14Tg2a*, middle panel - *Foxg1::Venus* and bottom panel - *Foxg1^{LacZ/Cre}*. All organoids were counted using a random sampling grid of 5000μm² and 20% of squares were counted. N= 5 organoids from 3 independently grown batches. Line of best fit was calculated using Excel software.

3.5.3 Organoid centre is not a reliable indicator of SVZ-like tissue in organoids of all three cell lines

Due to the irregular shapes of the organoids the centre of the organoid had to be ascertained. This was originally done by drawing lines between the widest points of the organoid and the point of intersection determined to approximate the centre. However, this was deemed to involve potential unconscious bias. Therefore, the perimeter of the organoid was selected using FIJI software, which was then used to computationally determine the centre of gravity of the irregular shape. Then the distance from this to each counted square was measured. To ensure consistency, the distance measured was always from centre of gravity to the centre of the square counted. The centre of the squares was determined by the intercept of diagonal lines drawn between corners.

To retain the positional data the values of individual squares were plotted and used to calculate a Pearson correlation coefficient. All three cell lines show a trend towards a positive correlation. Once Pearson correlation coefficients were calculated a P-value was also created to assess whether the correlation seen was statistically significant.

The wild-type line *E14Tg2a* showed a weak positive correlation with no significance seen (correlation coefficient = 0.2279, $P = 0.2176$). This was mimicked in *Foxg1::Venus* line (correlation coefficient = 0.3437, $P = 0.6291$). However,

Foxg1^{LacZ/Cre} showed a strong positive correlation which was statistically significant (correlation coefficient = 0.5793, P = 0.006).

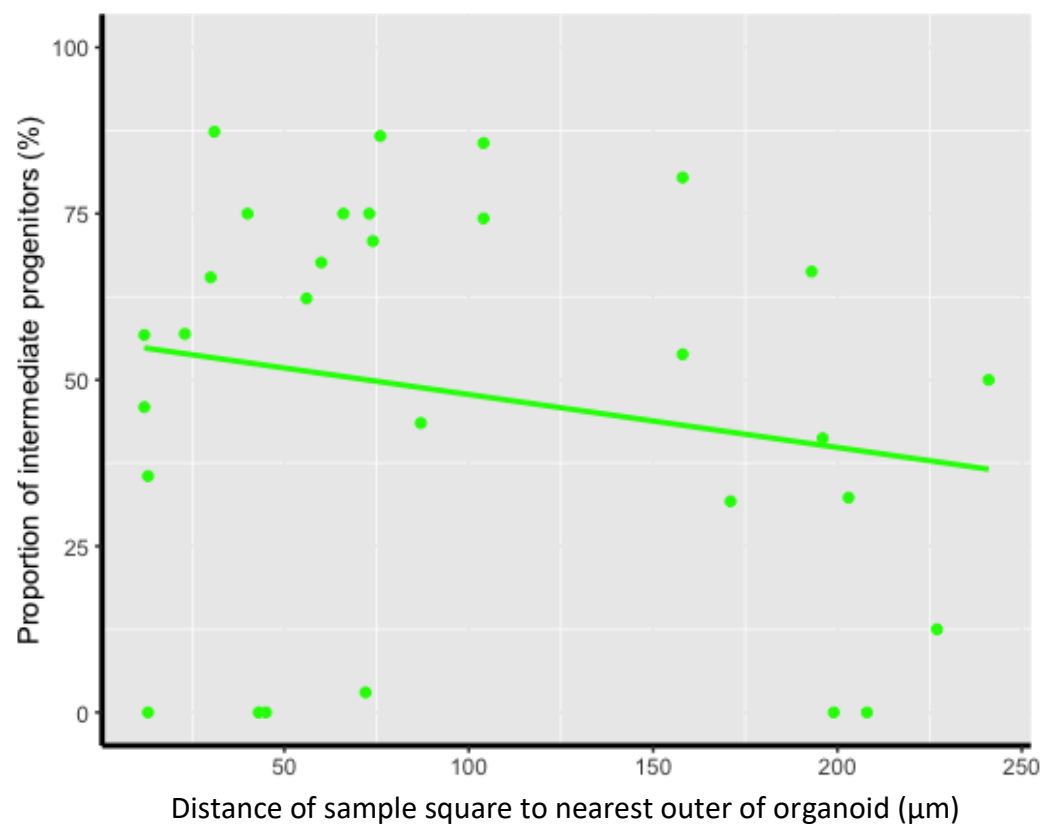
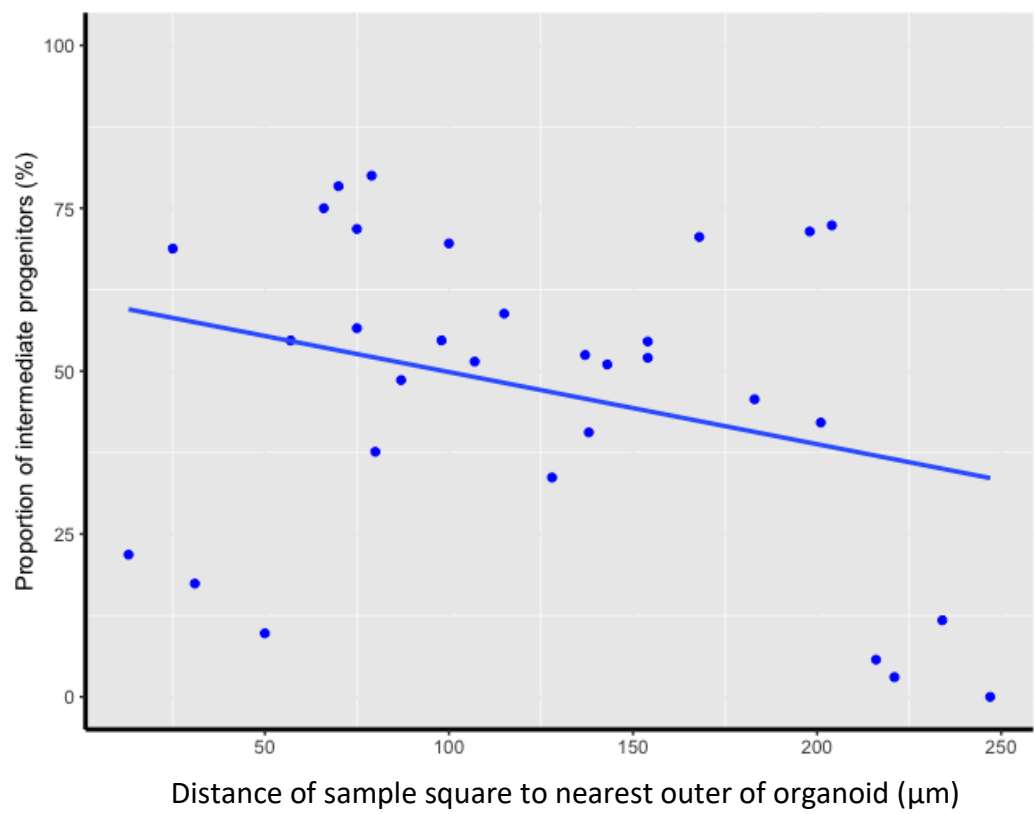
Discussion points

The positive correlation is to be expected as the diffusion gradient seen within organoids limits the nutrients available in the centre. Suggesting that tissue present further away from the centre, and by extension closer to the nutrients present in the media. The proportion of healthy tissue increases and so does the presence of cortical structures.

Conversely this could also be linked with cell death signals produced by cells in the necrotic core which could be actively promoting cell death in this region. Cell death has been shown to occur in mouse organoids but in these the presence of coordinated cell death was not investigated.

The fact that *Foxg1*^{LacZ/Cre} showed a significant correlation is surprising. These are the smallest organoids so the effects of diffusion should be lowest. However, the data suggests that how close the tissue is to the centre plays a huge role.

Regardless of the mutant results, these are not replicated in the other cell lines which implies that it is not a suitable landmark to use across all three.



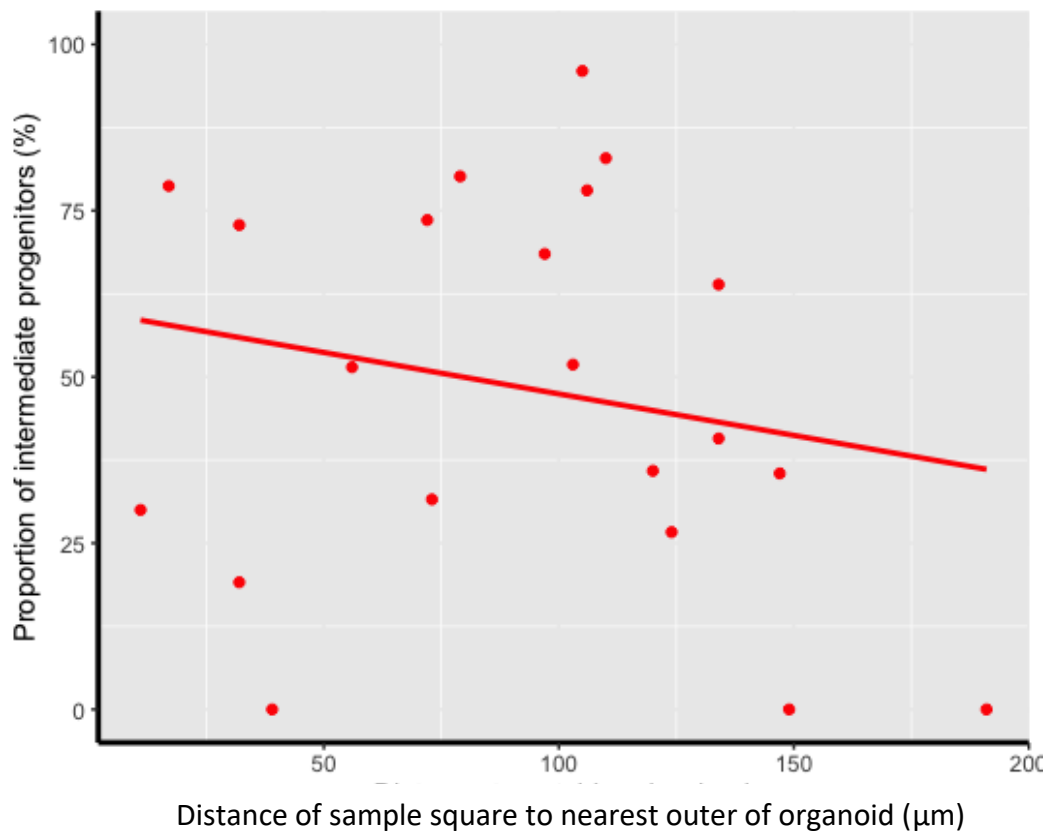


Figure 3.6 Distance from SVZ-like to closest outer edge of organoid

Tbr2 positive cells were used as a marker for SVZ-like tissue. The proportion of intermediate progenitors for each sample square was plotted individually in relation to the distance from the centre of the sample square to closest outer edge of organoid (described in more detail 3.5). Top panel – *E14Tg2a*, middle panel – *Foxg1::Venus* and bottom panel – *Foxg1^{LacZ/Cre}*. All organoids were counted using a random sampling grid of 5000μm² and 20% of squares were counted. N= 5 organoids from 3 independently grown batches. Line of best fit was calculated using Excel software.

3.5.4 Distance to the nearest outside edge is not a reliable indicator of SVZ-like tissue in organoids of all three cell lines

This criterion involved the distance to the outside edge also being counted. The hypothesis would be that due to the diffusion limit of the organoids that the healthy tissue would be found on the outside edge of the organoids. The inverse of what was investigated in the previous section. The preliminary data from the concentric circle intensity profiles adds support to this. However, it was unknown whether all organoids across different cell lines would show the same effect and have healthy tissue present within the same distance from the perimeter.

An overall negative correlation is shown in the data (Fig 6). However, none of these correlations show statistical significance. *E14Tg2a* organoids show a correlation of -0.3051 (P = 0.0952). The heterozygous *Foxg1::Venus* line shows a weak correlation of -0.1991 (P = 0.2917). Finally, the mutant *Foxg1^{LacZ/Cre}* line shows a correlation of -0.2040 (P = 0.3763).

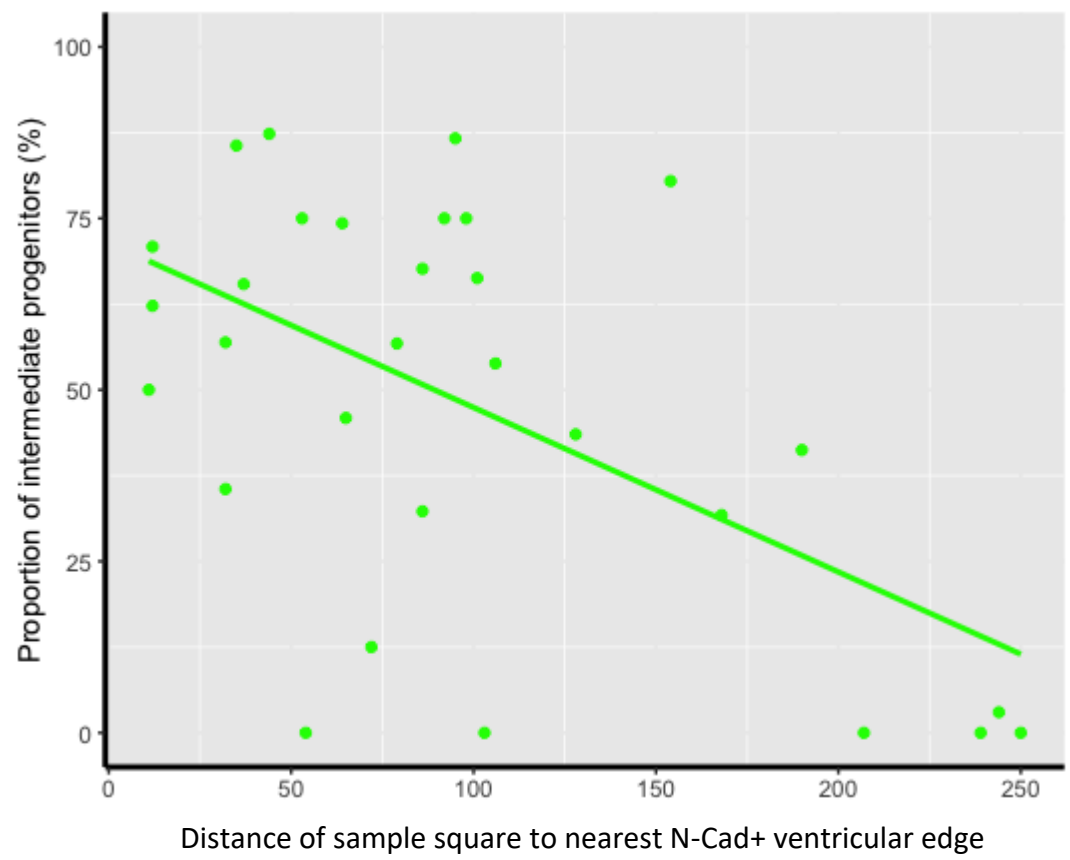
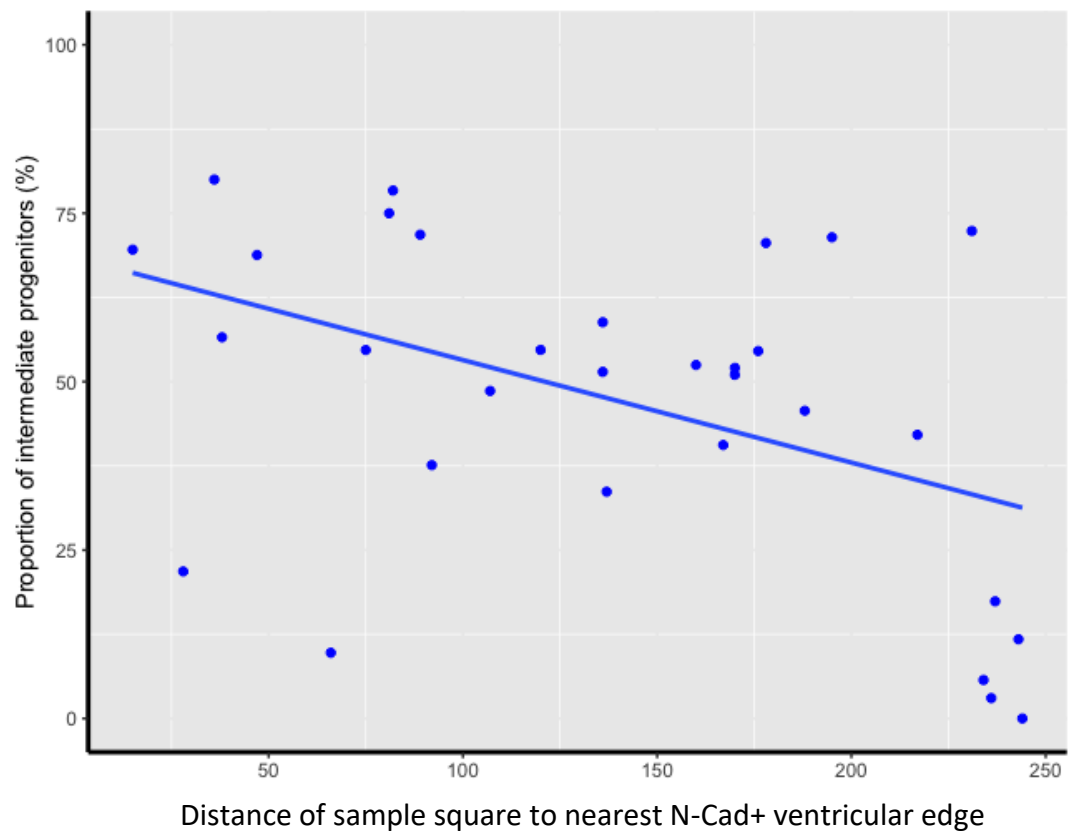
Discussion points

The data here, while implying that the diffusion gradient plays a role in the presence of cortical tissue, does not provide a strong enough correlation.

Therefore, the perimeter of the organoid is not a good landmark with which to draw ROIs for cortical tissue. This could be due to variation in organoid size. The absolute distance being used here may not provide a reliable metric. Alternatively,

a relative distance, corresponding to the size of each organoid may be appropriate.

Also, for all these criteria it was calculated by looking at a 2D cross section of a 3D object with no overall polarisation of structures. It is possible that the nearest landmark for each square was in fact in the Z dimension.



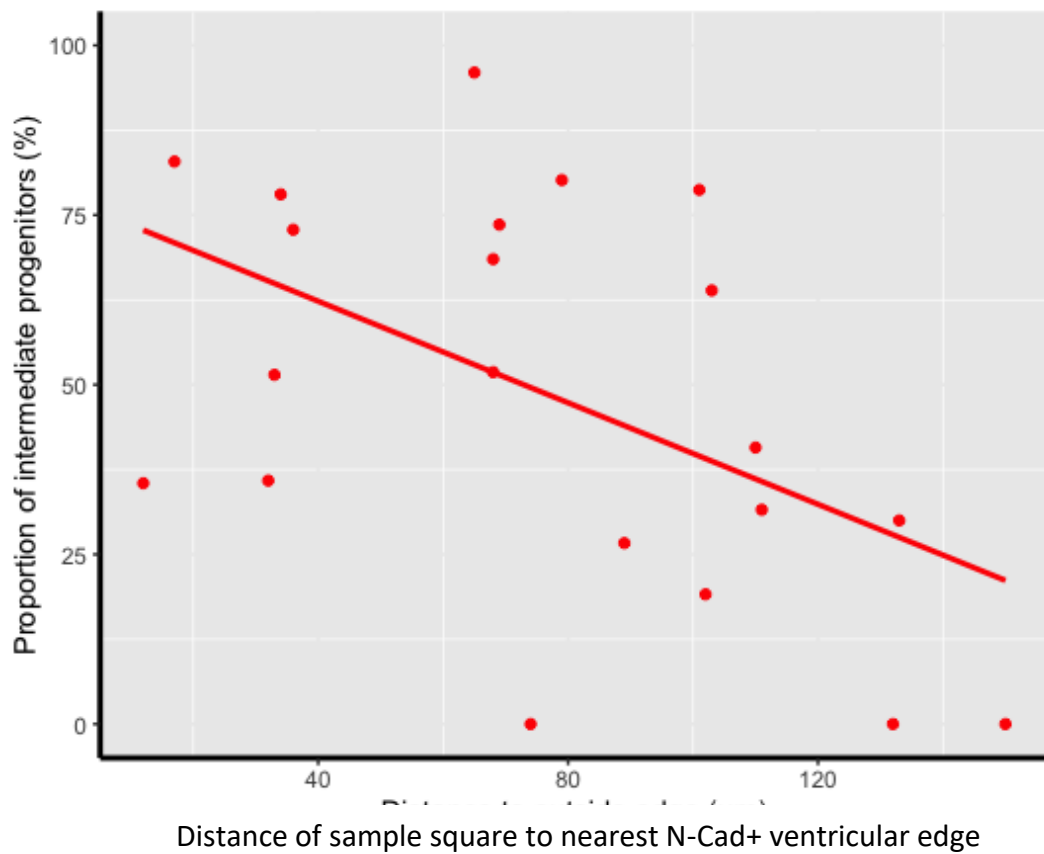


Figure 3.7 Distance from SVZ-like to nearest ventricular edge in organoid

Tbr2 positive cells were used as a marker for SVZ-like tissue, N-Cadherin used to mark ventricular edge. The proportion of intermediate progenitors for each sample square was plotted individually in relation to the distance from the centre of the sample square to nearest ventricular edge in organoid (described in more detail 3.5). Top panel – *E14Tg2a*, middle panel - *Foxg1::Venus* and bottom panel - *Foxg1^{LacZ/Cre}*. All organoids were counted using a random sampling grid of 5000 μm^2 and 20% of squares were counted. N= 5 organoids from 3 independently grown batches. Line of best fit was calculated using Excel software.

Section 3.5.5 Ventricular edges provide a potential candidate for delineating ROI landmarks

Across all 3 cell lines a negative correlation was observed which showed statistical significance. *E14Tg2a* ($r = 0.4587$, $P = 0.009$), *Foxg1::Venus* ($r = -0.5634$, $P = 0.001$) and *Foxg1^{LacZ/Cre}* ($r = -0.5010$, $P = 0.0210$). This then suggests that the use of N-cadherin to stain the ventricular edge is in fact a strong contender for a reliable landmark.

The line of best fit was plotted on each of the graphs. From this the equation of the line was calculated and rearranged to allow the x value to be determined. The process was to substitute the y value with 50 and solve the equation. This would give the distance from the VE within which sample squares were more likely to contain more than 50% of cells being Tbr2 positive. Such a radius would not encompass all proliferative tissue within an organoid but it would give an insight into the relative sizes of cortical tissue produced by different cell lines. This was carried out for each cell line.

Equation of a linear graph is $y=mx+c$, with m corresponding to the gradient of the line and c as the y intercept. For *E14Tg2a* the equation of the line was $y= -0.1522x + 68.43$, substituting y for 50 provides an x value of 121 μ m.

Foxg1::Venus heterozygous line showed an equation of $y = -0.2389x + 74.01$, substituting y for 50 provides an x value of 89 μ m.

Foxg1^{LacZ/Cre} line showed an equation of $y = -0.2741x + 77.27$, substituting y for 50 provides an x value of 73 μ m.

Discussion points

The correlation between the VE and presence of SVZ-like tissue was the only landmark which showed significance. This is perhaps unsurprising as the ventricular edge is the foundation of cortical proliferation. While all showed significance, when these correlations were converted to distances variation is seen between the cell lines. In *E14Tg2a* the distance from the VE to where squares were more likely to contain more than 50% Tbr2 cells was 121 μ m, larger than the 86 μ m and 73 μ m seen in *Foxg1::Venus* and *Foxg1*^{LacZ/Cre}. The fact that the mutant line showed the smallest distance is perhaps linked to the overall reduction in size of the organoids from this line. The size of cortical tissue present is most likely linked to the overall size of the organoid. However, this does not explain the difference between wild-type and heterozygous lines as there is large differences in the distance seen but no corresponding difference in CSA.

Overall, these results show that the VE is a good structure to identify cortical tissue, and its presence marked by N-cadherin should provide the basis for ROI. However, the same measurements cannot be used for different organoids, the variability of size within such cultures and cell lines prohibits this. To circumvent this each cell line used should undergo a similar unbiased evaluation as described here. This will

provide information on the growth and differentiation of such lines and would inform the development of an unbiased counting strategy.

Chapter 4 – Investigation of *Foxg1*^{-/-} ventral telencephalic defects in organoids

4.1 Introduction

Foxg1 null mutant mice are non-viable after birth and show severe neurodevelopmental defects, including complete lack of the ganglionic eminences in the ventral telencephalon (Xuan et al., 1995). In mice, all cortical GABAergic inhibitory neurons derive from the ganglionic eminences, with an estimated 80% deriving from the medial ganglionic eminence (MGE) alone (Hu et al., 2017; G. Miyoshi et al., 2007). The correct formation of cortical circuitry requires the successful balancing of both excitatory glutamatergic and inhibitory GABAergic neuronal populations. An imbalance in the proportions of these neuronal types has been linked to phenotypes seen in a number of ASD (Casanova et al., 2003; Rubenstein, 2010), including in iPSC derived organoids with altered levels of *FOXG1* (Mariani et al., 2015).

Previous work has suggested that the lack of ventral telencephalic tissue in null mutant mice is due to a failure of ventral telencephalic progenitor cells to proliferate (Xuan et al., 1995). Later work using chimeras highlighted that cells fail to adopt a ventral telencephalic fate entirely in a cell-autonomous manner (Manuel et al., 2011; Martynoga et al., 2005). The exact mechanism of how *Foxg1* coordinates the adoption of ventral telencephalic fate is unknown. Investigations

have shown that *Foxg1* interacts with both the *Wnt* and *Shh* pathways, which are linked to the patterning of the dorsal and ventral telencephalon respectively (Danesin & Houart, 2012). It is then thought that cells with *Foxg1* which are exposed to *Shh* would adopt a ventral fate. A full description of the current literature on this process can be found in the introduction chapter (Chapter 1.2.1).

4.2 Chapter aims

Cerebral organoids formed in the presence of few extrinsic signals appear to preferentially adopt a dorsal telencephalic fate (Eiraku et al., 2008; Madeline A. Lancaster et al., 2013a; Nasu et al., 2012).

This chapter sought to adapt the original Sasai protocol used in order to generate organoids with a larger proportion of ventral telencephalic tissue. Once organoids had been generated with sufficient ventral telencephalic tissue this protocol was then used with *Foxg1*^{-/-} mESCs to investigate whether the *in vivo* phenotypes are recapitulated in organoids.

4.3 Experimental design

Two cell lines were used throughout this chapter, *Foxg1::Venus* and *Foxg1^{LacZ/Cre}*.

Both cell lines were chosen as they contain reporters for *Foxg1* expression. Initially, *Foxg1::Venus* was used to optimise the ventral telencephalic protocol. This was done because of the high levels of dorsal telencephalic differentiation seen in this cell line in Chapter 3. The protocol described by (Danjo et al., 2011) this was an adaptation of their original protocol which was used in Chapter 3. In this protocol the addition of Smoothened agonist (SAG) was used, which activates the sonic hedgehog pathway. The paper suggests that higher levels of SAG added would generate MGE tissue, whereas lower levels would see the production of LGE (Danjo et al., 2011). This is based on the anatomy of the developing brain, the MGE is in closer proximity to the ventral floor plate where *Shh* is expressed (Xu et al., 2005). Implying the cells forming the MGE would be exposed to higher levels of *Shh*.

Two different variations of the ventral protocol were trialled, the first (referred to as V1) followed the Sasai protocol. 3nm of SAG was added on Day 3 of the protocol with the concentration increased to 10 nM on Day 6. The second protocol (referred to as V2) used the same levels of SAG but 3nm was included from Day 0 which increased to 10 nM from Day 5. This second protocol was designed to investigate whether the earlier activation of the *Shh* pathway would lead to an increase in ventral telencephalon present. The original protocol which generates organoids of

mainly dorsal fate (referred to as condition D) was used as a control. A full description of the protocols used can be found in the Methods section 2.3 and 2.4.

Organoids were fixed, cryoprotected, sectioned and stained as previously described. Quantification was carried out using the grid method described in Chapter 3 with adjustments to make it applicable to the questions of this chapter.

Organoids were fixed at day ten and twelve of the protocol, this is due to literature suggesting that while much of dorsal and ventral cortical development occurs in parallel, interneuron neurogenesis lasts longer than excitatory neurogenesis (Fairén et al., 1986; Marín & Müller, 2014).

Areas of telencephalic-like tissue were identified through *Foxg1* expression, using a *anti-venus* antibody. This was due to the need for antigen retrieval which caused the venus protein to lose its fluorescence. So, for the purposes of this Chapter *Foxg1* is assumed to be exclusively marking telencephalon. Organoids from all three conditions were stained for *Olig2* which is expressed both dorsally and ventrally in both developmental and mature brain stages throughout the central nervous system. It is also found in high expression levels in oligodendrocyte progenitors which can be found in the ventral telencephalon (Meijer et al., 2012). In the telencephalon *Olig2* expressing cells are exclusively found in the ventral telencephalon (Lu et al., 2000; G. Miyoshi et al., 2007; Petryniak et al., 2007). Therefore, the co-expression of *Foxg1* and *Olig2* was used to identify ventral

telencephalon. This measure would underestimate the true levels of ventral telencephalon found in these organoids as *Olig2* positive progenitor cells are only a subset of the ventral telencephalon (Danjo et al., 2011). This is due to there being no known specific marker of the entire ventral telencephalon. However, this is still a useful gauge of the protocol's effectiveness.

The images were overlaid with a 5000 μm^2 grid (Figure 4.1). The middle section of the organoid was quantified (description of how middle section was determined can be found in Chapter 3.5). 20% of occupied squares were randomly selected for quantification. A number of metrics were counted for each square:

- Total number of cells present (determined by DAPI staining)
- Number of *Foxg1*+ cells present. The ratio of *Foxg1*:DAPI was used to determine the proportion of cells adopting a telencephalic fate
- Number of cells double positive for *Olig2* and *Foxg1*. The ratio of these cells was compared to the total number of cells to determine what proportion of cells adopted a ventral telencephalic fate. It was also compared to the proportion of *Foxg1* positive cells, to determine how much of the telencephalic tissue present in the organoids became ventral.
- Number of cells expressing *Olig2* but not *Foxg1*. *Olig2* is found in multiple ventral regions throughout the nervous system. By looking at this measure, we could see if the overall fate of the organoid was being changed.

The *Foxg1::Venus* and *Foxg1^{LacZ/Cre}* lines were put through protocols D, V1 and V2.

Organoids were then co-stained with *Nkx2.1* and *Foxg1* to distinguish MGE-like areas. This was quantified to see if organoids recapitulated the phenotypes seen *in vivo*.

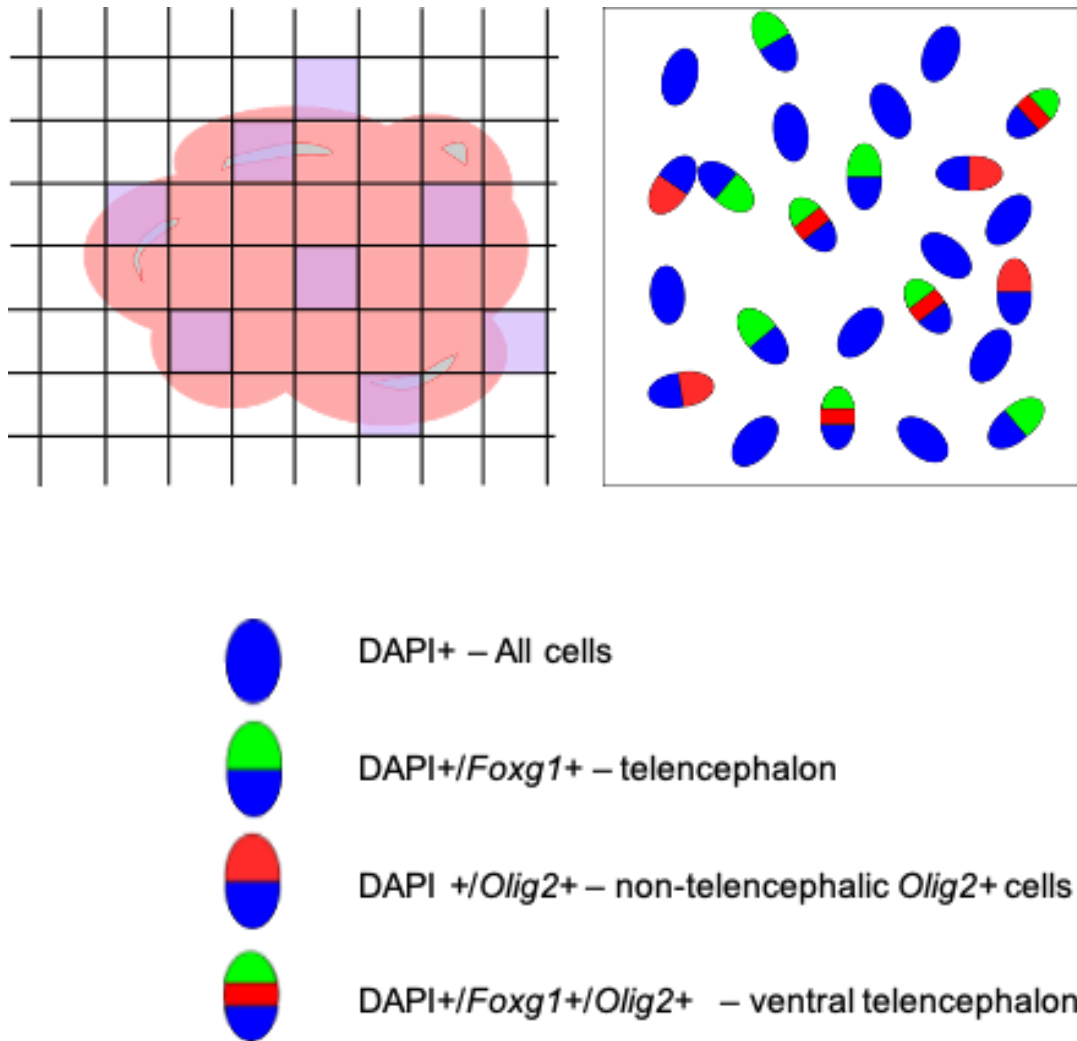


Figure 4.1 – Schematic showing quantification method used to test ventral telencephalon protocols. Middle sections of organoids were overlaid with a 5000 μm^2 grid. 20% of occupied squares were counted. All cells were counted by using the DAPI staining. Telencephalon was marked by the presence of Foxg1. The proportion of cells Olig2+/Foxg1- was also counted. Double positive cells were counted as ventral telencephalic progenitors.

4.2 Results

4.2.1 addition of SAG significantly increases proportion of total cells adopting a ventral telencephalic fate, regardless of timing of addition.

The proportion of cells from the entire organoid becoming ventral telencephalon was calculated by the following equation:

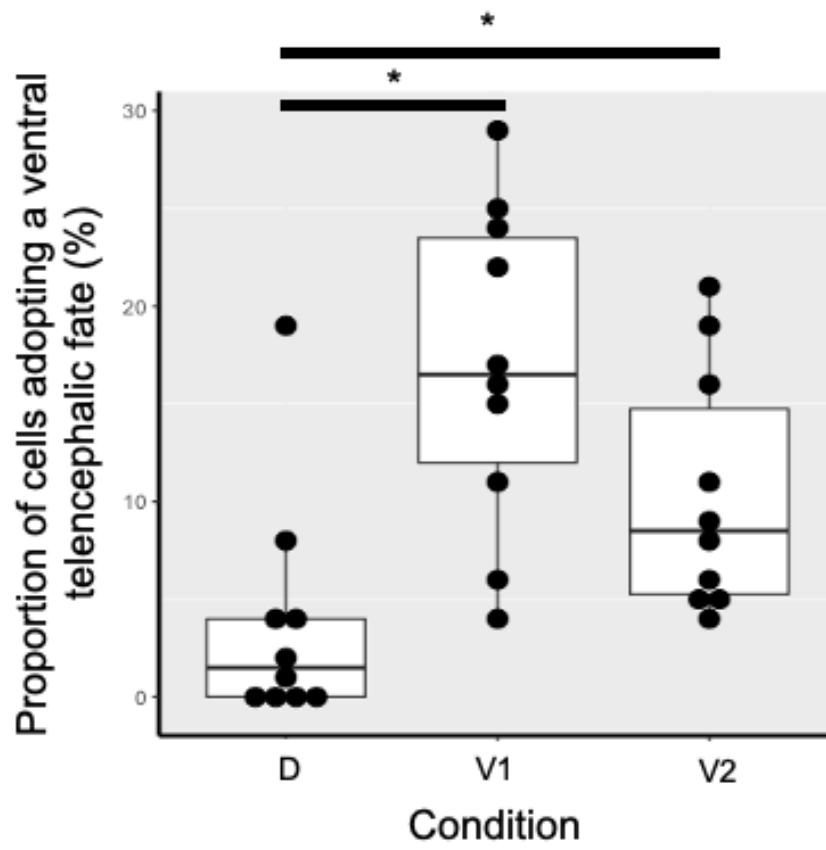
$$\frac{\text{Foxg1 and Olig2 double positive cells}}{\text{Total number of cells (DAPI)}} \times 100$$

Foxg1:Venus cell line was used to test all 3 conditions. *Foxg1:Venus* organoids exposed to condition D collected at day ten of the protocol showed low proportions of cells adopting ventral telencephalic identity (Figure 2, panel A) (average proportion of ventral telencephalic cells = 3.8%, SEM = $\pm 1.9\%$). Both conditions V1 and V2 showed increased levels of ventral telencephalic tissue (V1 – average prop. of ventral telencephalic cells = 16.9%, SEM = $\pm 2.6\%$. V2 - average prop. of ventral telencephalic cells = 10.4%, SEM = $\pm 2.0\%$). A non-paired, two-tailed student T-test was used to compare the averages of each condition. Conditions V1 and V2 both showed statistically significant increases in proportion of ventral telencephalic tissue when compared to condition D (condition D compared to condition V1 – P =

0.015. condition D compared to condition V2 – $P = 0.036$). However, no significant difference was seen between the averages of conditions V1 and V2 ($P = 0.074$).

This pattern was also observed in organoids collected at day twelve (Figure 2, panel B). Average proportion of ventral telencephalic cells in condition D – 2.6%, SEM = $\pm 0.7\%$. V1 – 15.1%, SEM = $\pm 2.4\%$. V2 – 13.3%, SEM = $\pm 2.2\%$. With statistical significance seen between conditions D and V1 ($P = 0.01$) and conditions D and V2 ($P = 0.0002$), but not between conditions V1 and V2 ($P = 0.54$).

A



B

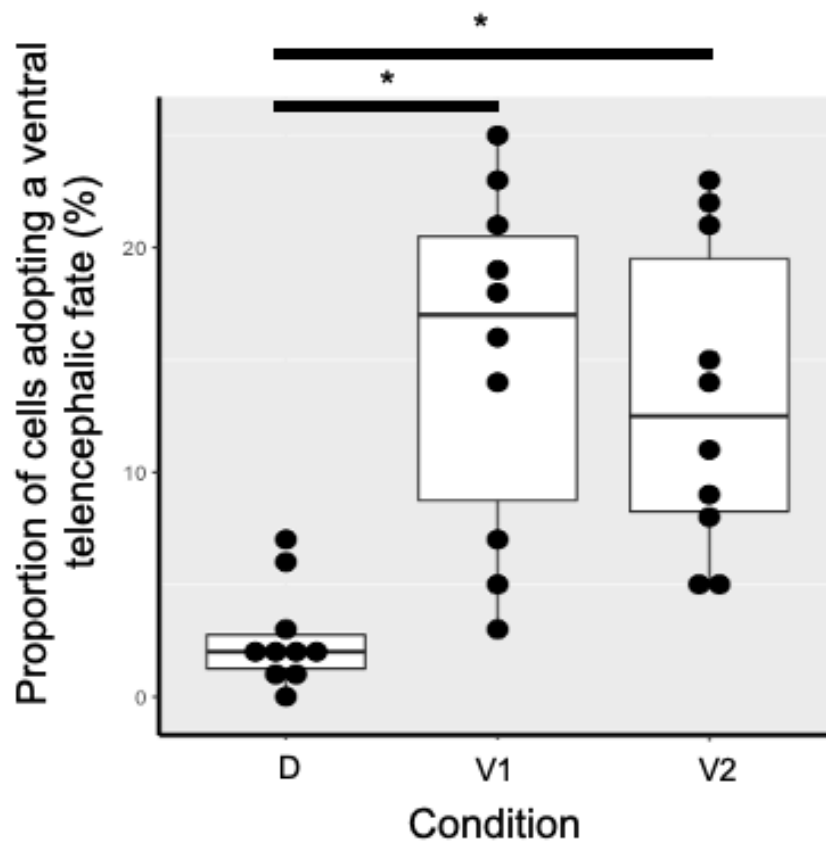


Figure 4.2. Comparison of the proportion of cells adopting a ventral telencephalic fate (%) across different protocol conditions. Plots show the proportion of all cells in the counted region which were double positive for Foxg1 and Olig2. Middle section (described in section 3.3.2) was used quantified using the randomised grid method described in Chapter 3. n = 10 organoids from 5 batches. One-way ANOVA was performed to investigate differences between conditions. (A) shows comparison between conditions at day ten of protocol. (B) shows comparison between conditions at day twelve of protocol. Asterisks show significance between variables ($P < 0.05$).

4.2.2 More cells in conditions V1 and V2 which become telencephalon are ventrally patterned

To further understand the changes in cell fate occurring it was investigated whether the proportion of cells adopting a ventral telencephalic identity compared to all cells becoming telencephalon was changing between conditions. To determine this the following equation was used:

$$\frac{\text{Foxg1 and Olig2 double positive cells}}{\text{Foxg1 positive cells}} \times 100$$

The results from this equation mirrored the results seen in section 4.2.1. At day ten a significant difference was found between condition D (7.8%, SEM = $\pm$ 4.4%) and condition V1 (33.8%, SEM = $\pm$ 6.5%) (P = 0.004). The same was seen between conditions D and V2 (19.2%, SEM = $\pm$ 3.0%) (P = 0.045). No significant difference was seen between conditions V1 and V2 (P = 0.06) (Figure 3, panel A)

At day twelve the same pattern was seen. D – 4.5%, SEM = $\pm 1.2\%$. V1 – 48.4%, SEM = $\pm 4.8\%$. V2 – 34.3%, SEM = $\pm 6.6\%$. With statistical significance seen between conditions D and V1 ($P = 0.0007$) and conditions D and V2 ($P = 0.0003$), but not between conditions V1 and V2 ($P = 0.9$) (Figure 3, panel B)

These results, in conjunction with those in section 4.2.1, show that organoids grown in the presence SAG show a higher proportion of cells adopting a ventral telencephalic fate as a fraction of all cells present as well as showing significant increases in the number of ventral telencephalic cells as a fraction of telencephalic cells present in the organoid. However, even with these significant increases the majority of the organoid continued to adopt an unknown fate. Even when looking exclusively at the telencephalic (Foxg1+) areas of the organoid, no condition was able to get more than 50% of cells to become ventral.

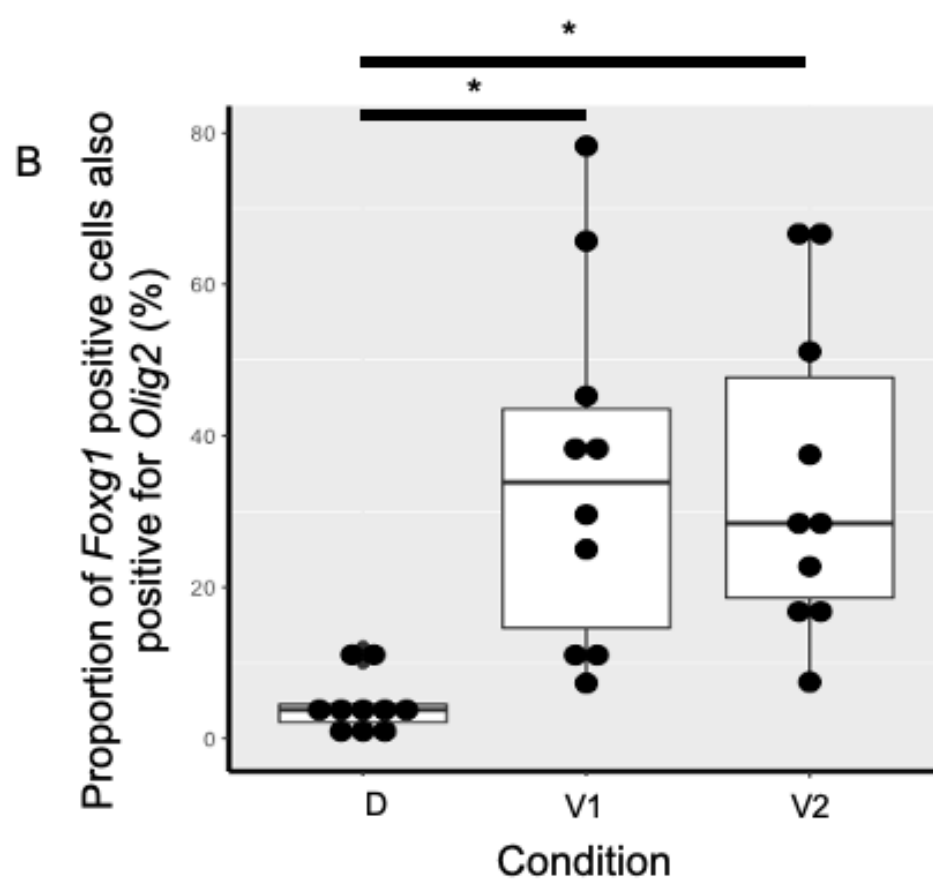
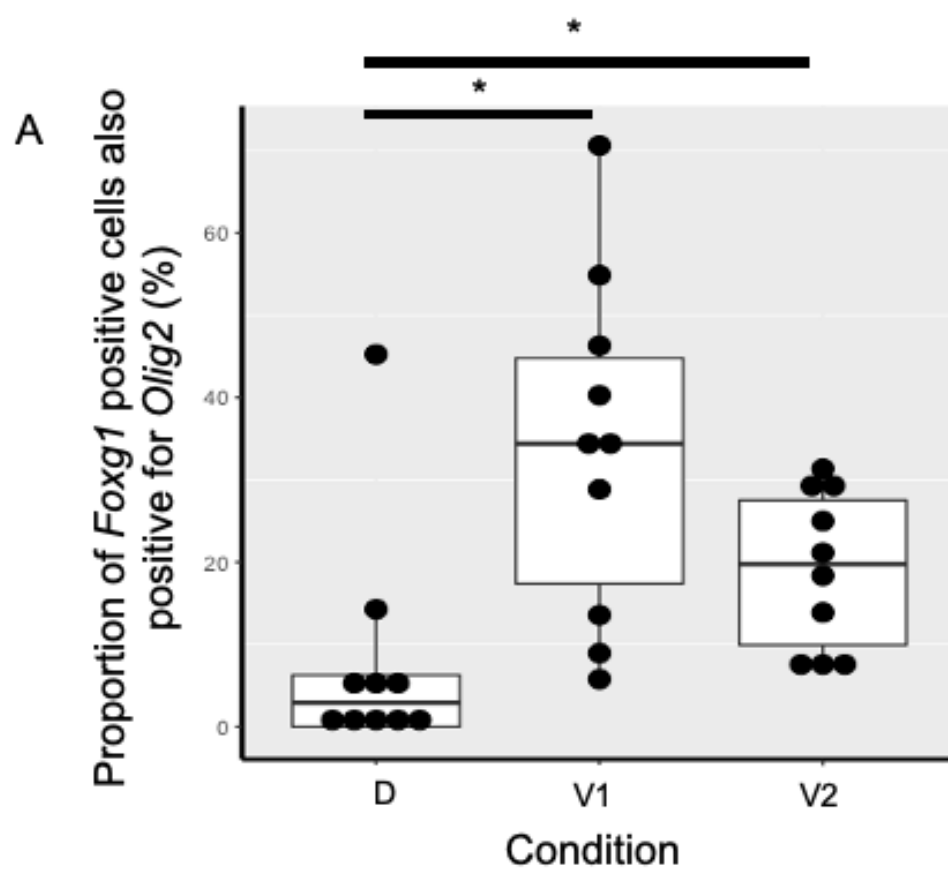


Figure 4.3. Comparison of the proportion of telencephalic cells adopting a ventral telencephalic fate (%) across different protocol conditions. Plots show the proportion of Foxg1 positive cells in the counted region which were double positive for Foxg1 and Olig2. Middle section (described in section 3.3.2) was quantified using the randomised grid method described in Chapter 3. n = 10 organoids from 5 batches. Student t-tests were performed to investigate differences between conditions. (A) shows comparison between conditions at day ten of protocol. (B) shows comparison between conditions at day twelve of protocol. Asterisks show significance between variables ($P < 0.05$).

4.2.3 Proportion of cells adopting a telencephalic fate does not significantly differ with the addition of SAG

The addition of SAG has been shown to increase the proportion of ventral telencephalon seen in the organoids. It was then investigated whether the proportion of the organoid adopting a forebrain identity was altered due to the changes in conditions. The proportion of cells adopting a telencephalic fate was calculated by the following equation:

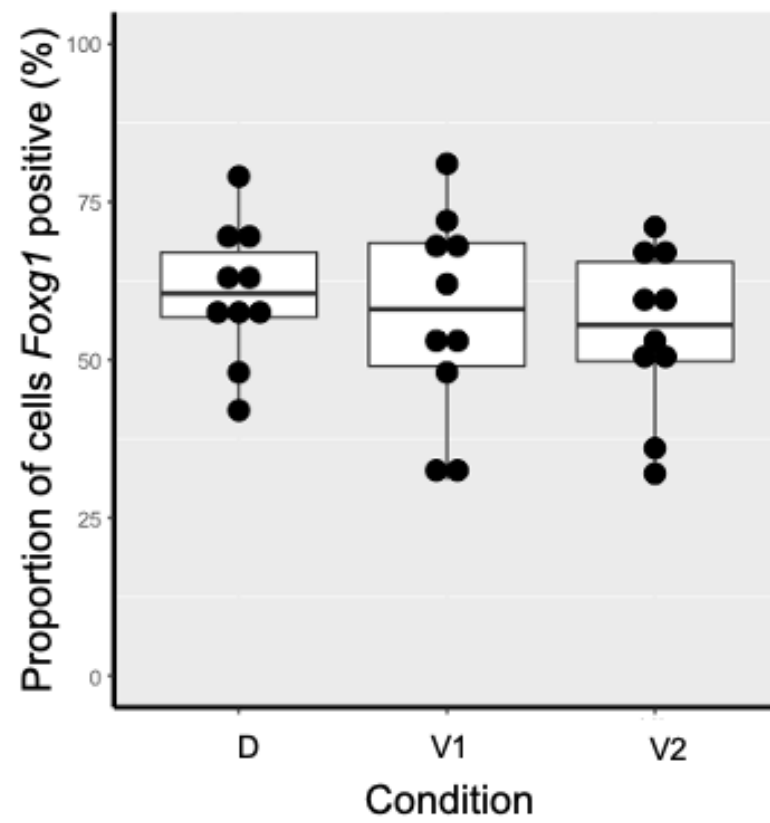
$$\frac{\text{Foxg1 – positive cells}}{\text{Total number of cells (DAPI)}} \times 100$$

At day ten all three conditions showed similar proportions of *Foxg1* expressing cells (Condition D – 60.8%, SEM = ± 3.4%, V1 – 57.0%, SEM = ± 5.2%, V2 – 54.6%, SEM ± 4.1%). With no statistically significant difference seen between any of the conditions – condition D to V1 (P = 0.54), D to V2 (P = 0.26), and V1 to V2 (P = 0.72)(Figure 4, panel A).

The same was also seen in organoids collected at day twelve (condition D – 56.9%, SEM = ± 3.1%, condition V1 – 48.4%, SEM = ± 4.8%, condition V2 – 44.4%, SEM = ±

5.4%). Again, no significant difference was found, condition D to V1 ($P = 0.15$), D to V2 ($P = 0.06$) and V1 to V2 ($P = 0.6$) (Figure 4, panel B).

A



B

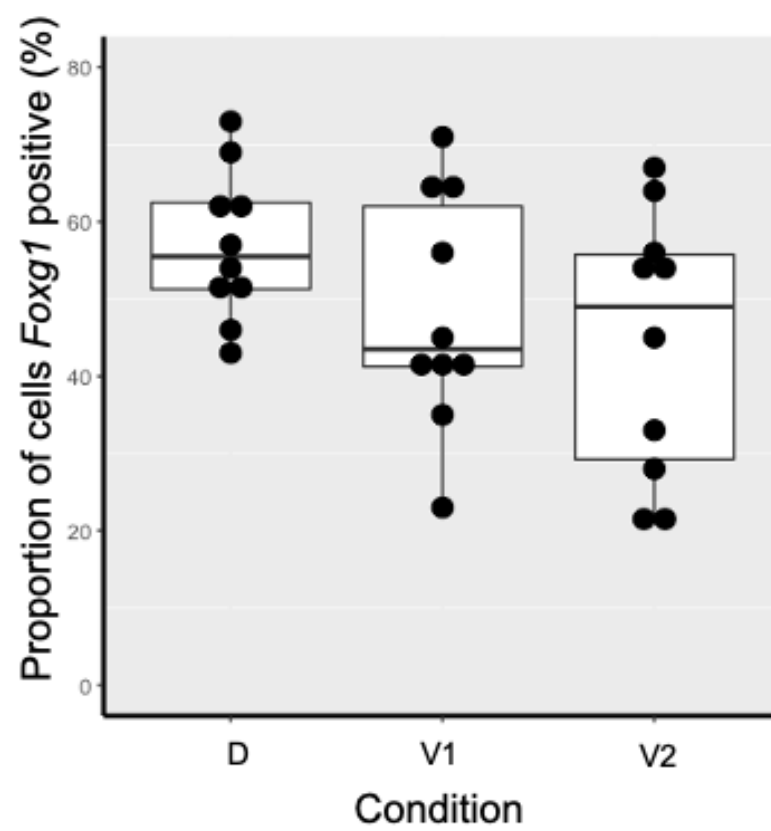


Figure 4.4. Comparison of the proportion of cells adopting a telencephalic fate (%) across different protocol conditions. Plots show the proportion of all cells in the counted region which were positive for Foxg1. Middle section (described in section 3.3.2) was used quantified using the randomised grid method described in Chapter 3. n = 10 organoids from 5 batches. Student t-tests were performed to investigate differences between conditions. (A) shows comparison between conditions at day ten of protocol. (B) shows comparison between conditions at day twelve of protocol. Asterisks show significance between variables ($P < 0.05$)

4.2.4 The addition of SAG leads to more non-telencephalic regions of the organoid becoming *Olig2* positive

The previous data showed that the addition of SAG led to a greater proportion of cells in the organoid becoming ventral telencephalon. Furthermore, the addition of SAG had no significant effect on the amount of *Foxg1* positive tissue present. However, *Foxg1* was never ubiquitously expressed in any condition. With some regions of the organoid failing to adopt a telencephalic fate at all. It was unknown whether the activation of the *Shh* pathway would also have a ventralising effect on these tissues. *Olig2* expression was quantified in regions which were non-telencephalic (*Foxg1*-negative).

The equation used to determine the proportion of non-telencephalic ventral tissue was:

$$\frac{\text{Olig2 – positive, Foxg1 – negative cells}}{\text{Total number of cells (DAPI)}} \times 100$$

The results showed that at both days ten and twelve, condition D showed low levels of such tissue (day ten – 2.8%, SEM = ± 1.1%. day twelve – 2%, SEM = ± 0.9%). The

addition of SAG in condition V1 saw an increase in the proportion of such cells (condition V1 day ten – 4.7%, SEM = ± 1.7 . day 12 – 5.3%, SEM = $\pm 0.9\%$), however, this was only significantly different to condition D at day twelve ($P = 0.02$).

Alternatively, condition V2 showed levels of *olig2*+/Foxg1- cells (day ten – 9.8%, SEM = $\pm 2.2\%$. day twelve – 5.3%, SEM = $\pm 0.7\%$). Which was significantly different to condition D at both day ten and twelve ($p = 0.01$ and $p = 0.009$ respectively). No significant difference was seen between conditions V1 and V2 at either time point.

This data highlights how the addition of SAG alters the fate of numerous cell types in the organoid, not just those adopting a telencephalic fate. Due to *Olig2* being found throughout the CNS (Meijer et al., 2012) it is difficult from this data to ascertain what identity this tissue is adopting.

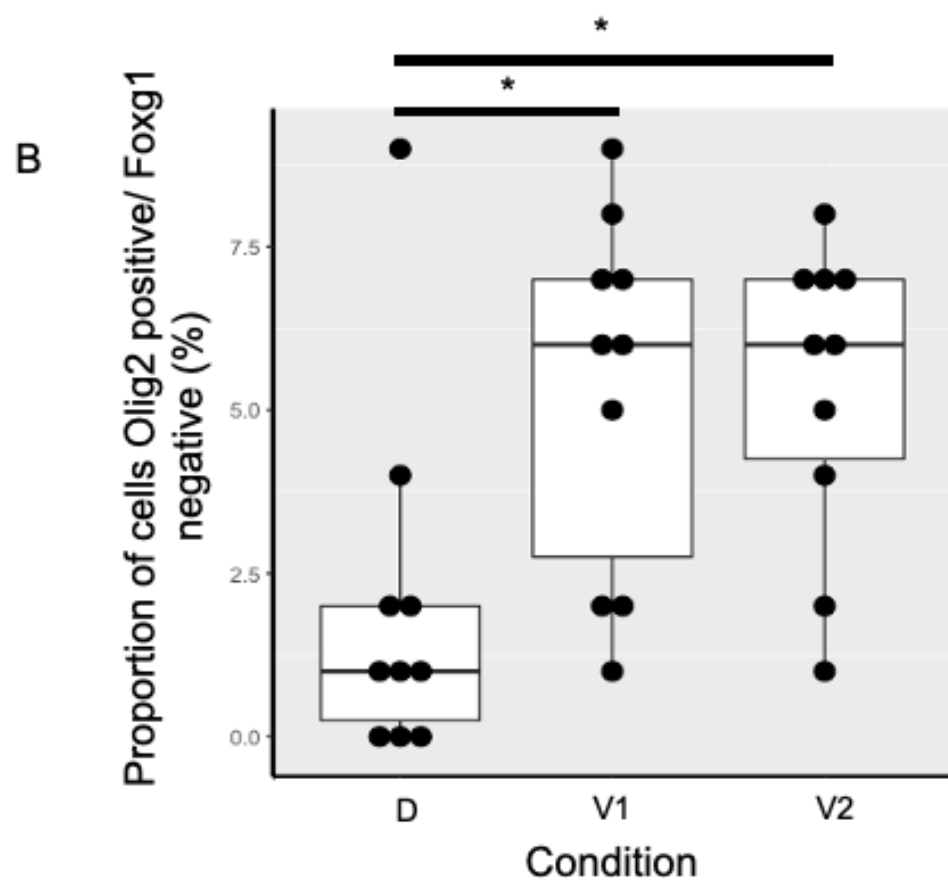
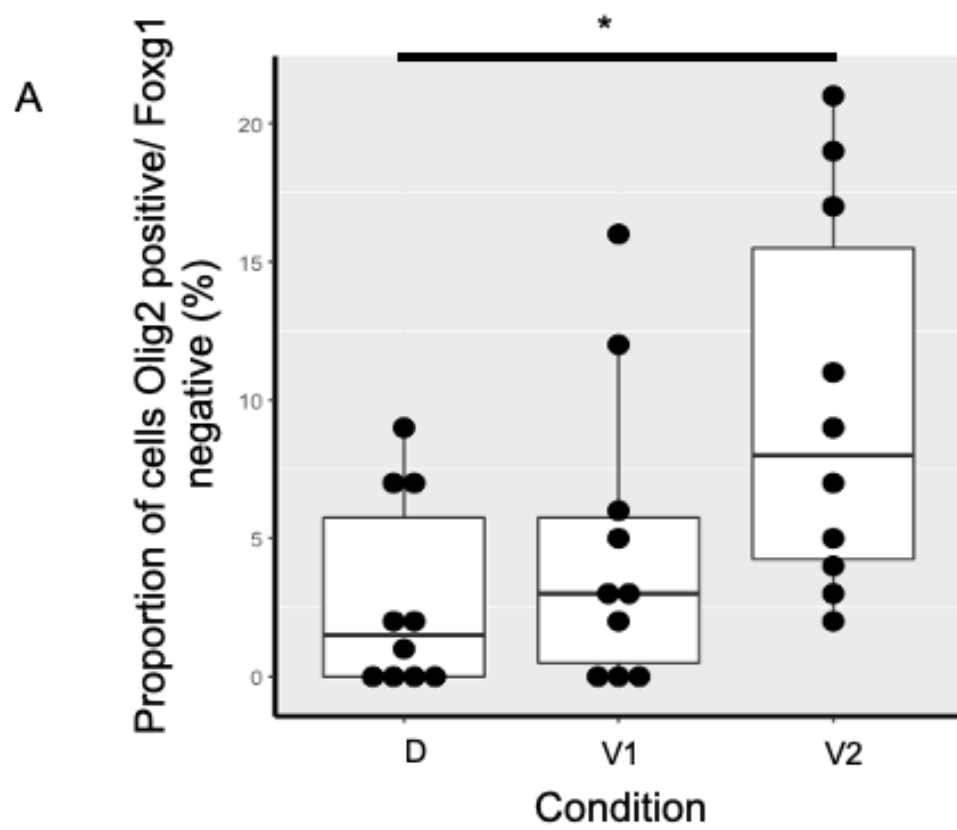


Figure 4.5. Comparison of the proportion of non-telencephalic cells adopting a ventral fate (%) across different protocol conditions. Plots show the proportion of Foxg1 negative cells in the counted region which were positive for Olig2. Middle section (described in section 3.3.2) was used quantified using the randomised grid method described in Chapter 3. n = 10 organoids from 5 batches. Student t-tests were performed to investigate differences between conditions. (A) shows comparison between conditions at day ten of protocol. (B) shows comparison between conditions at day twelve of protocol. Asterisks show significance between variables ($P < 0.05$).

4.2.5 Proportions of marker expression are unchanged between days 10 and 12 but organoid tissue loses organisation

Organoids have been shown to recapitulate key features of early neurodevelopment but begin to lose structure and organisation with time (Nasu et al., 2012). Therefore, it is preferable to study phenotypes in organoids at the earliest stage possible. This was an obstacle for studying ventral telencephalic defects as literature suggests that the ganglionic eminences develop later than the cortical regions. To decide the best day to collect the organoids we compared organoids from day ten and twelve. This was done to see if there was more ventral tissue present at day twelve, suggesting it could be more useful, or whether the loss of organisation by day twelve made day ten more appropriate.

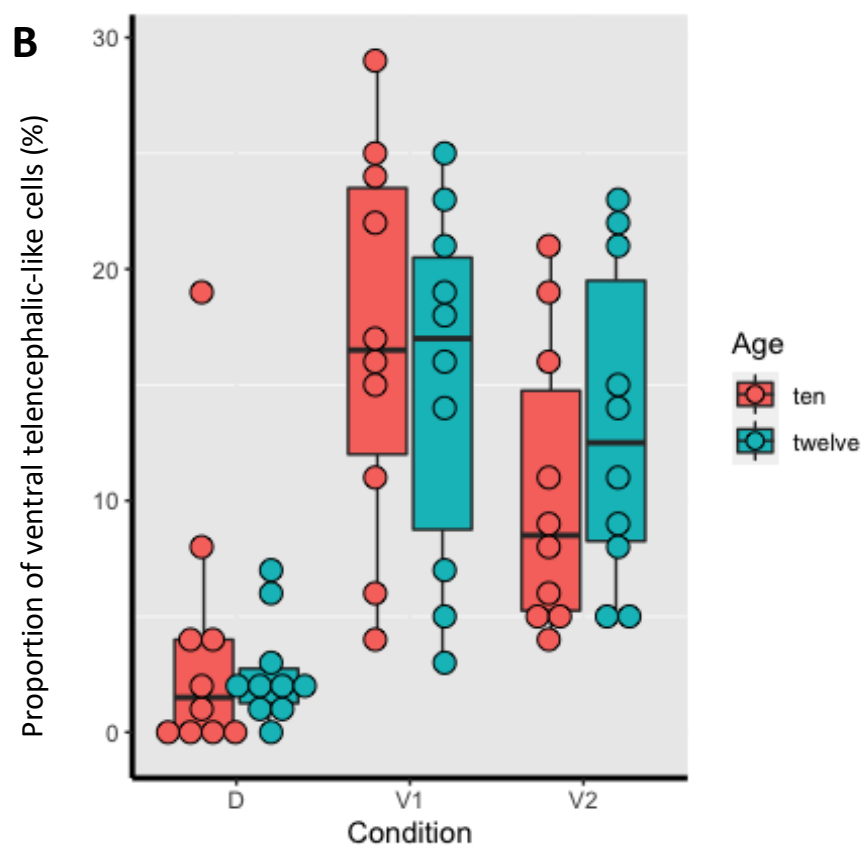
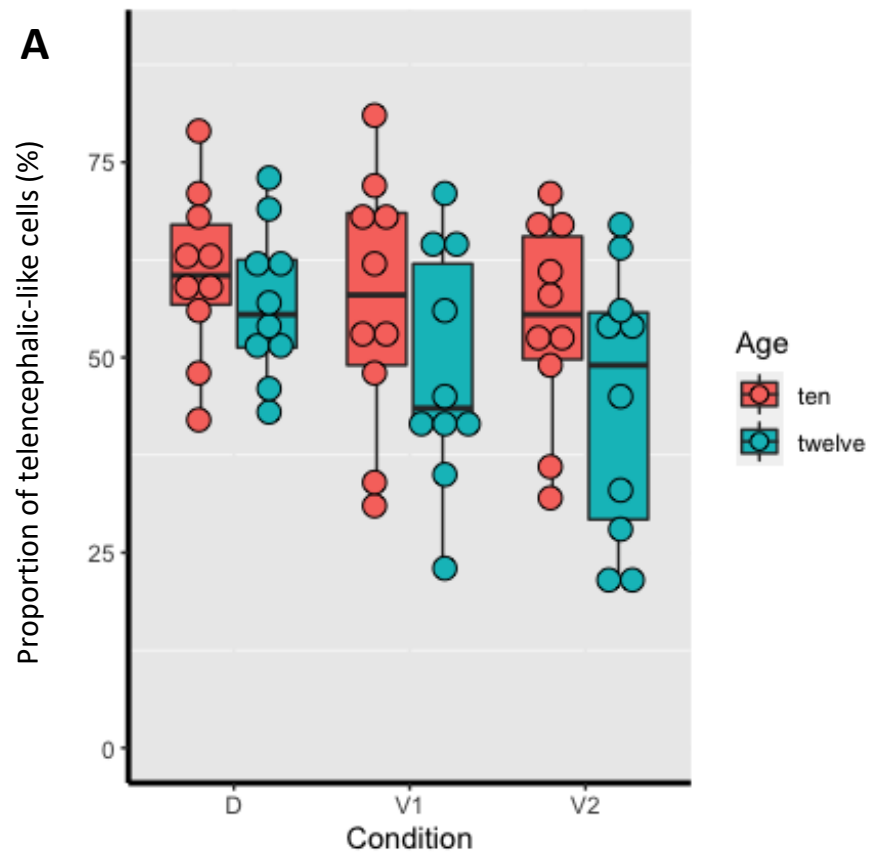
Three metrics which were previously investigated were compared between day ten and twelve. This included;

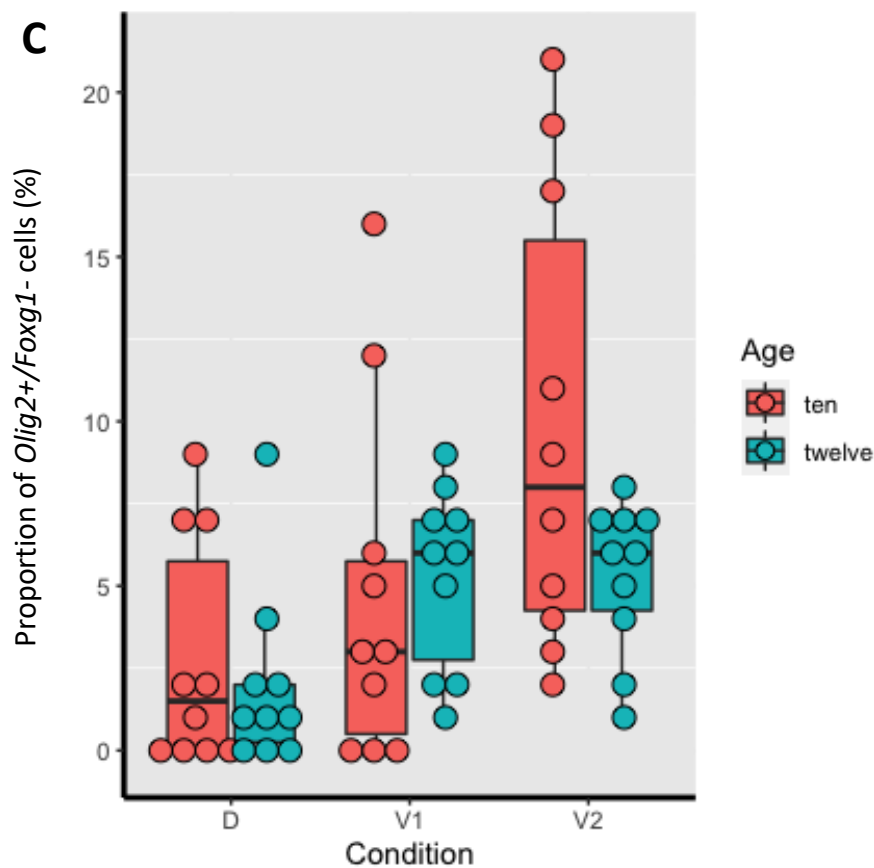
- The proportion of telencephalic cells in the whole organoid (section 4.2.3)
- The proportion of ventral telencephalic cells in the whole organoid (section 4.2.1)
- The proportion of *Olig2+*/*Foxg1*- cells in the whole organoid (section 4.2.4)

Figure 4.6 shows the comparison and no significant difference was found between any of the conditions at different ages. Suggesting that the proportions of each of the three cell types investigated is consistent between day ten and twelve.

However, while the proportions of cells seen did not vary, a clear loss of organisation was seen. structures of neuroepithelium seen in Figure 4.7 (green staining) is surrounded by a distinct layer of *Tbr2*⁺ intermediate progenitors. Whereas, at day twelve there is still the presence of neuroepithelium, this is less abundant, and the majority of intermediate progenitors are not anchored to any discernible neuroepithelium.

Based on this data, day ten was chosen to go forward. The lack of any significant increase in ventral tissue by day twelve suggested that day ten of the protocol was adequate to generate ventral telencephalon. This coupled with the presence of clear, organised neuroepithelium made organoids from day 10 easier to quantify.





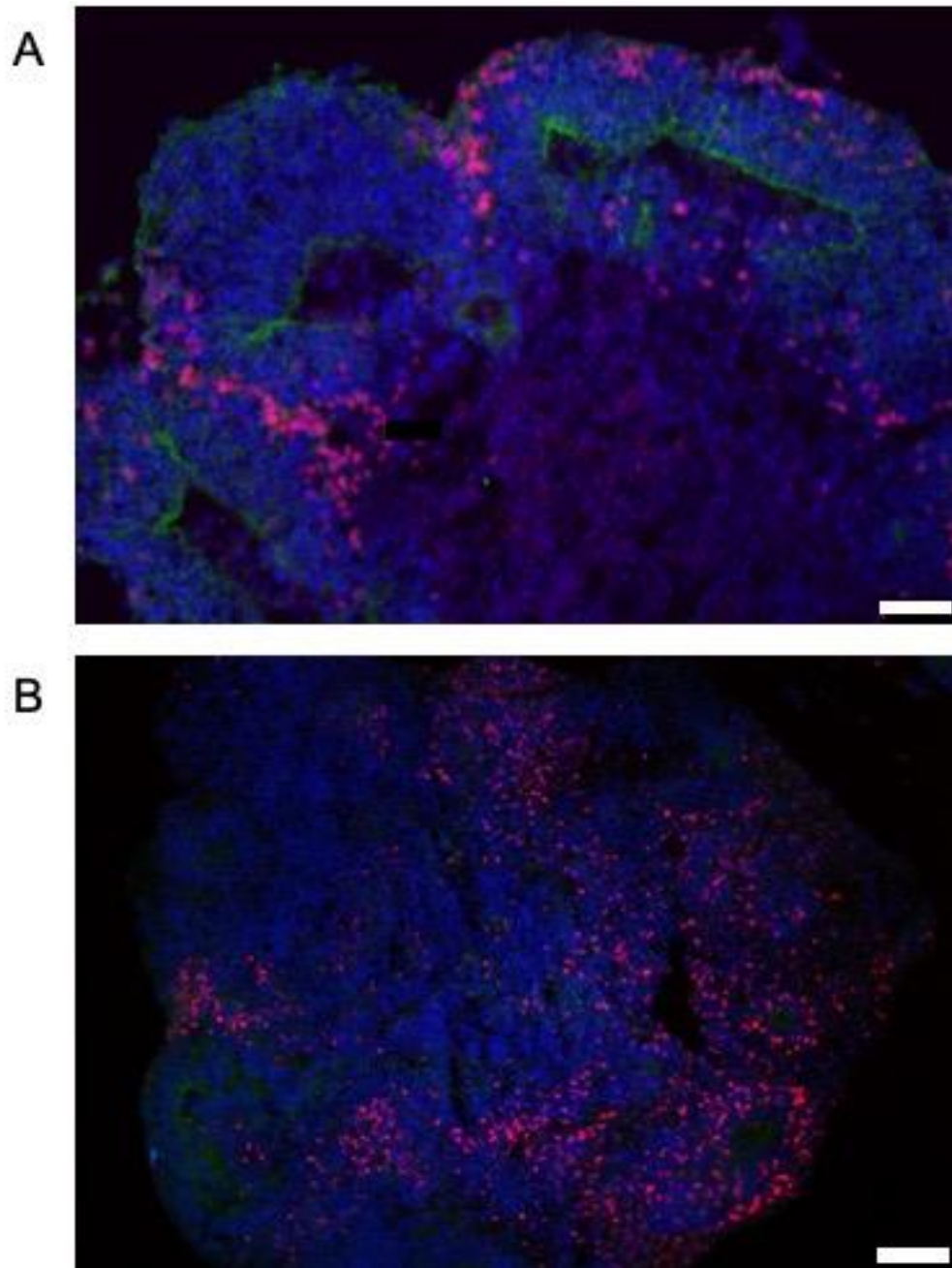


Figure 4.7. Images showing the loss of organization of E14Tg2a organoids at day twelve of the protocol. (A) Organoid grown in condition D at day 10, scale bar = 50

um. (B) Organoid in condition D at day twelve, scale bar = 100µm. Red staining = Tbr2, green staining = N-Cad.

4.2.6 mESC derived organoids appear to recapitulate the ventral defect seen in Foxg1^{-/-} mice

Previous data explains why day ten was chosen as the optimum time point. The data also shows no huge difference between the efficiency of conditions V1 and V2. For the final part of the chapter, condition V1 was chosen. While there was no significant difference between conditions V1 and V2 in levels of ventral telencephalic tissue, condition V1 did show higher amounts and so was taken forward.

Organoids were grown in condition V1 to day 10, from both the *Foxg1::Venus* and the *Foxg1^{LacZ/Cre}* lines. The hypothesis was that organoids derived from the mutant cell line would be unable to generate any tissue resembling ventral telencephalon.

Organoids were stained for *Foxg1* expression. In the *Foxg1::Venus* this was done via the Venus GFP marker, whereas, in the *Foxg1^{LacZ/Cre}* then sections were stained with anti-beta-galactosidase antibodies which recognise the product of the *LacZ* gene

produced under the control of the *Foxg1* promoter. Sections were then stained with an antibody for Nkx2.1. This marker was chosen as it is found abundantly in the proliferative zone of the MGE (Danjo et al., 2011), which has been shown to give rise to the majority of cortical interneurons (Hu et al., 2017; Xu et al., 2004).

Figure 4.8 shows organoids grown in this experiment. The *Foxg1::Venus* organoids contained a number of regions where *Foxg1* and *Nkx2.1* expression overlaps. Indicating regions which are likely to be MGE progenitors. However, in the *Foxg1^{LacZ/Cre}* organoids, no such regions were seen, suggesting that they are unable to form ventral telencephalon.

This was then quantified from three separate organoids and the results are shown in Figure 4.9. Across the three mutant organoids no cells were found to be positive for MGE progenitor markers (0%, SEM = $\pm 0\%$). Whereas, the *Foxg1::Venus* line showed larger amounts of such tissue (26.6%, SEM = $\pm 9.5\%$). Implying strongly that it does recapitulate the *in vivo* phenotypes (a low n number due to the COVID-19 pandemic meant statistical comparison was not possible).

Furthermore, the mutant organoids showed a decrease in the proportion of tissue becoming telencephalon (21.6%, SEM = $\pm 2.9\%$) compared to the *Foxg1::Venus* (44.3%, SEM = $\pm 6.4\%$).

This data suggests that *Foxg1* mutant organoids fail to form ventral telencephalon, as well as less of the organoid adopting a telencephalic fate overall.

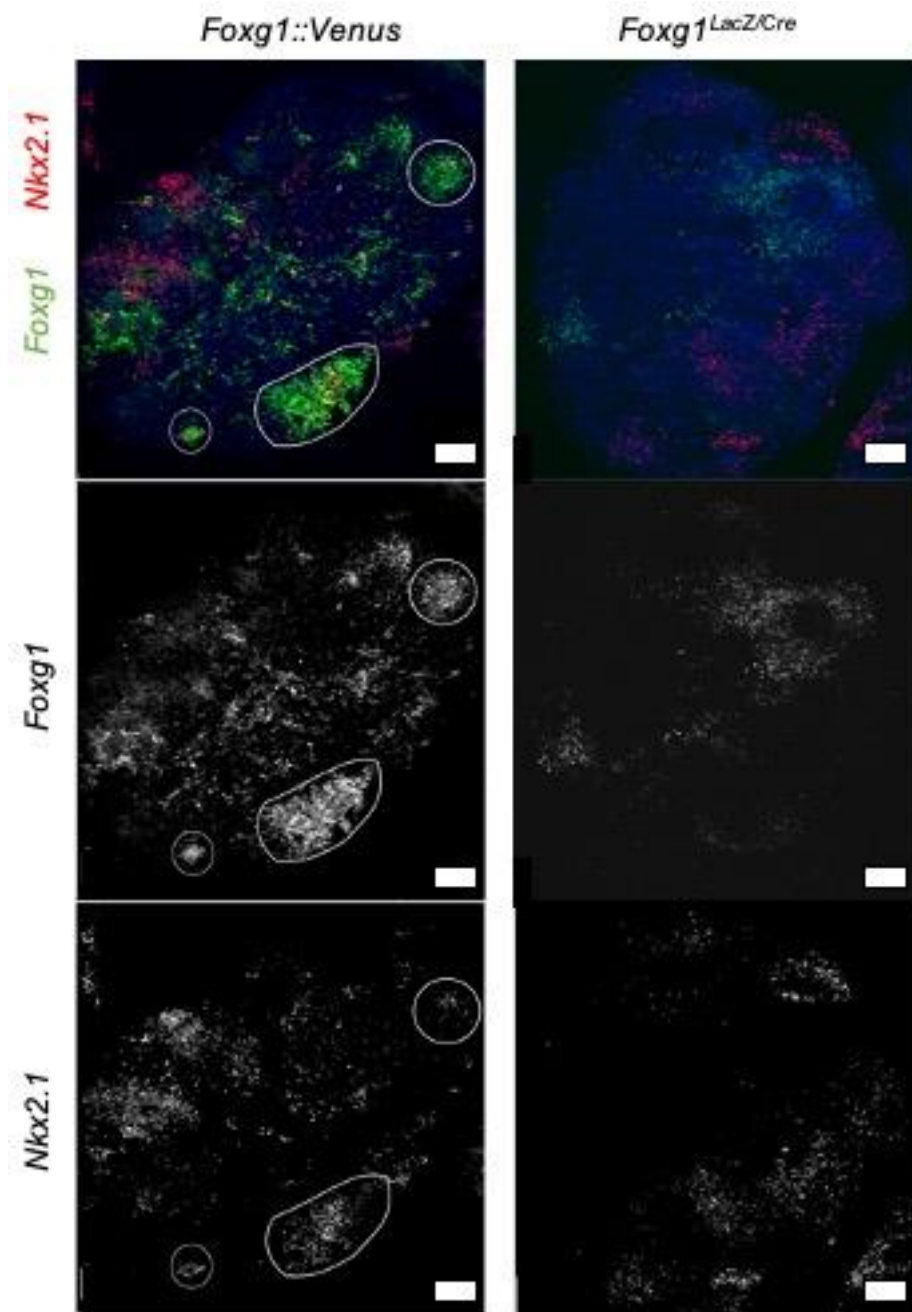
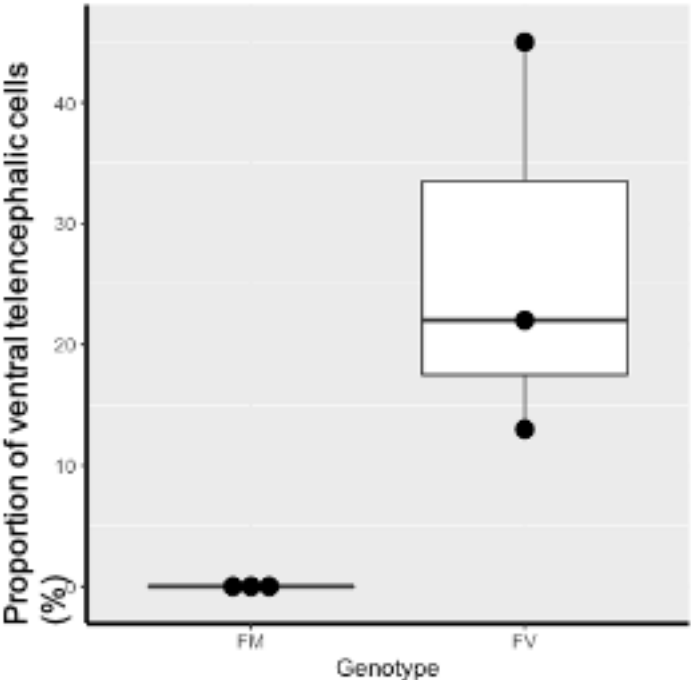


Figure 4.8. Comparison of ventral telencephalic tissue in organoids grown in condition V1 at day ten, derived from *Foxg1::Venus* and *Foxg1^{LacZ/Cre}* cell lines. In the *Foxg1::Venus* line an anti-venus antibody was used. In *Foxg1^{LacZ/Cre}* cell line ant-beta-galactosidase antibody was used. Both diluted in donkey serum at concentration of

1:500. Areas of co-staining can be seen in *Foxg1::Venus* to indicate presence of MGE progenitors. No such signs of overlap are seen in *Foxg1^{LacZ/Cre}*. Scale bars – 100µm

A



B

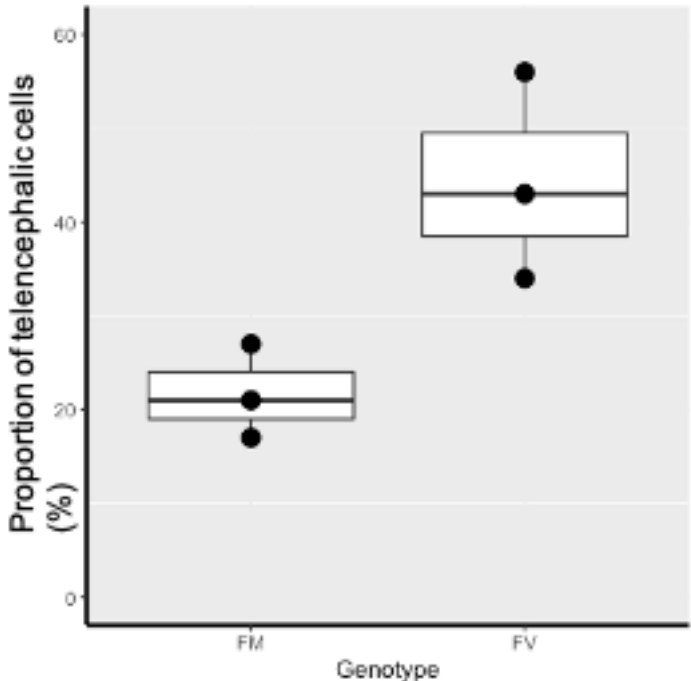


Figure 4.9. Comparison of organoids derived from Foxg1^{LacZ/Cre} and Foxg1::Venus mESCs. (A) a ventral telencephalic fate (Foxg1 and Nkx2.1 positive as a proportion of all cells). (B) A telencephalic fate (Foxg1 positive as a proportion of all cells). Middle section (described in section 3.3.2) was used quantified using the randomised grid method described in Chapter 3. n = 3 organoids from 2 batches. Student t-tests were performed to investigate differences between genotypes.

4.3 Discussion

4.3.1 Efficiency of ventral telencephalic differentiation

Organoids derived using protocols V1 and V2 showed significant increases in the proportion of ventral telencephalic cells when compared to either all cells or just telencephalic. But in both cases, this was never a majority of cells.

The protocols trialled in this chapter all involved the addition of SAG to the media. Due to the lack of a vasculature in organoids the only way for nutrients or signalling molecules to penetrate the organoids is via diffusion. There has been work done which looks at the factors which effect diffusion specifically in organoids (McMurtrey, 2016, 2017). Two of the main limitations are:

1. Concentration difference between areas
2. Distance over which diffusion occurs

As the organoids grow over time the diffusion distance inevitable increases. This will limit the diffusion of molecules to the centre of the organoid and has been linked to the presence of a necrotic core (Madeline A. Lancaster et al., 2013a). The organoids are grown in Matrigel, an extra-cellular matrix (ECM) solution. Containing

a variety of ECM proteins and growth factors which promotes self-organisation, polarisation and proliferation (Nasu et al., 2012). The variable nature of Matrigel has led some groups working on intestinal organoids to seek alternate ECM gels (Jee et al., 2019). This variable growth rate makes it difficult to predict the size of individual organoids within a batch, leading variation in diffusion between organoids of the same batch. Furthermore, the diffusion rate is impacted by the density of the ECM gel itself, with some 3D *in vivo* tissue grown in high density gels to receive 40% less oxygen than controls (Colom et al., 2014). These multiple factors could then limit the amount of SAG available to cells within the organoids, thus limiting the successful patterning.

In addition, in this protocol half the medium was refreshed with fresh SAG-containing medium every 2 days. However, it is unknown the rate at which the SAG was taken up by the cells. This makes it almost impossible to accurately know the concentration of the SAG in the media at any given time. Especially as the rate of diffusion and uptake will vary as the organoid grows. If the levels of SAG in the media drop this could also limit inward diffusion.

There are a number of ways these limits with diffusion could be circumvented. An obvious way is to vascularise organoids. This would provide a constant supply of nutrients and signalling molecules to deeper regions of the organoid. Multiple attempts have been made to do this in brain organoids but more work is still

required till it is consistently reliable (Cakir et al., 2019; Mansour et al., 2018; Pham et al., 2018).

Another way would be to increase the surface area of the organoid exposed to the media to allow greater rates of diffusion. It would work similarly to air-liquid interface organoids described by Lancaster (Giandomenico et al., 2019), where organoids are sectioned to expose the centre of the organoid.

In order to ensure the concentration of nutrients and signalling molecules remains constant in the media, microfluidic chambers could be employed. This could deliver precise levels of signalling molecules to organoids, this has proven promising in cancer research (Sontheimer-Phelps et al., 2019).

4.3.2 A majority of cells fail to adopt a telencephalic identity across conditions

Regardless of the addition of SAG, all conditions showed similar proportions of cells adopting a telencephalic identity. This is promising for the protocol optimisation as it suggests that the addition of SAG in these protocols still generates organoids with ample telencephalic tissue for quantification.

However, in all conditions there was never a majority of cells becoming telencephalic. This is not an obstacle to the questions that are asked in this chapter,

but it does suggest that there are still limitations in the organoid protocol which could be improved upon.

The protocol used involves the addition of IWP-2 at the start of the protocol, this is a *Wnt* inhibitor (Eiraku et al., 2008; Nasu et al., 2012). At early stages of development *Wnt* is believed to act as a caudalising factor (Nordström et al., 2002). So, inhibition of this would passively encourage organoids to adopt a forebrain identity. This may explain the variety of tissues present in cerebral organoids as the pluripotency of the mESCs allows cells to adopt multiple identity when there is no external signalling present. Furthermore, the IWP-2 may also face limits to diffusion. It is added to the medium when the mESCs are in single-cell dilution, allowing access to every cell. However, once an aggregate is formed in a low-adhesion well, the limitations of diffusion are relevant (McMurtrey, 2016, 2017).

The use of the *Foxg1::Venus* line may also impact the organoid's composition. Its *Oct4* heterozygosity led to increased levels of pluripotency marker expression. This led to less spontaneous differentiations, which in conjunction with the lack of diffusion could explain the variety of tissues seen. Further work could be done to assess what kinds of tissue can be found in those non-telencephalic areas, as well as investigating how that compares to organoids derived from different cell lines. Similar work using single-cell RNA sequencing (sc-RNA-seq) in human organoids has

begun to give glimpses into their composition but it is unknown whether this is the same in mouse cerebral organoids (Bhaduri et al., 2020; Pollen et al., 2019)

4.3.3 Organoids appear to recapitulate ventral defects seen in *Foxg1*^{-/-} mice

Cells lacking *Foxg1* fail to adopt a ventral telencephalic in the presence of *Shh*, in a cell-autonomous manner (M. N. Manuel et al., 2011; Martynoga et al., 2005; Xuan et al., 1995). This has been linked to a potential imbalance in the proportion of excitatory and inhibitory neurons seen in iPSC-derived organoids with variable levels of *FOXG1* (Mariani et al., 2015). If organoids were to be a useful tool to investigate *Foxg1* mutant defects, then they should recapitulate these phenotypes. The data in section 4.2.6 shows that there was no ventral telencephalic tissue present in organoids derived from the *Foxg1*^{LacZ/Cre} line. The low n number for this data set (due to time constraints from the COVID-19 pandemic) meant that statistical analysis was impossible. However, the stark contrast between genotypes is extremely promising. If further samples were quantified it would seem likely that it would continue to show the pattern that organoids do indeed recapitulate *Foxg1* *in vivo* phenotypes.

Interestingly the proportion of cells adopting a telencephalic fate is reduced in mutant organoids. Previous work showed that *Foxg1*^{-/-} cells which were used to form a chimera in a *Foxg1*^{+/+} animal, were able to integrate into the ventral

telencephalon but expressed dorsal, rather than ventral telencephalic markers (M. Manuel et al., 2010). It may then be expected that there would be more dorsal tissue present than in the *Foxg1::Venus* ventral organoids. A possible explanation to this decrease of overall telencephalic identity could be due to the proliferative defects seen in the dorsal telencephalon of mutants. The cortex shows severe microcephaly due to proliferative defects in progenitors (M. N. Manuel et al., 2011; Martynoga et al., 2005; Xuan et al., 1995). Potentially, cells are unable to form ventral telencephalon so adopt a dorsal fate, however, this leads to proliferative defects so by the time the organoids are collected there is a lower proportion of cells expressing telencephalic markers. To understand if this is the case then it would need to be investigated whether organoids also showed these proliferative defects, which is studied in Chapter 5.

Overall, this chapter has shown that organoids are adaptable and with the right media conditions can be directed to desired fates. It also shows that organoids seem able to recapitulate key aspects of early-stage neurodevelopmental defects.

Chapter 5 – Investigation of cell cycle defects in *Foxg1*^{-/-} cerebral organoids

5.1 Introduction

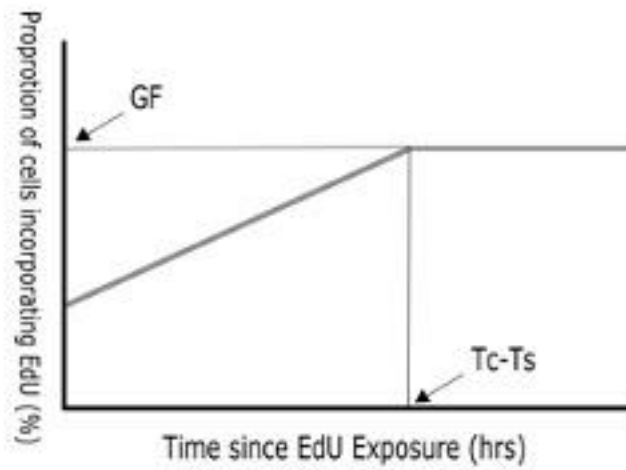
The previous chapter showed the ability of cerebral organoids to adopt a ventral telencephalic fate and that the ventral patterning defects seen in *Foxg1*^{-/-} mice *in vivo* (M. N. Manuel et al., 2011; Martynoga et al., 2005) appear to be witnessed in organoids. This chapter focussed on whether the proliferation defects found in the dorsal telencephalon of *Foxg1*^{-/-} mutant mice are also seen in organoids.

A balancing act takes place in cortical progenitors during development. They may undergo either differentiative or proliferative divisions, creating neurons and progenitors respectively (Tan & Shi, 2013). Too many differentiative divisions depletes the progenitor population, leading to reduced neuronal numbers overall (Dehay & Kennedy, 2007). As neurogenesis progresses there is a gradual elongation of the cell cycle. This coincides with a switch from mainly proliferative to differentiative divisions (Dehay & Kennedy, 2007). In particular, the cell cycle phase G1 has been implicated in this, with a noticeable increase in its length (Caviness et al., 1995). This same process has been suggested to occur in *Foxg1*^{-/-} mutants.

Analysis of cell cycle lengths showed a premature elongation of the cell cycle in mutants (this became significantly different at around E11 and the difference continued to increase at later time points) (M. N. Manuel et al., 2011). The main area of elongation appeared to be phase G1. It was suggested that this was the cause of hypoplasia seen in the mutant mice (Xuan et al., 1995), the premature elongation caused cortical progenitors to begin differentiation. Thus, causing an initial increase in neuron numbers but ultimately depleting the progenitor pool leading to lower numbers of neurons at the end of neurogenesis.

This chapter aimed to investigate whether this same elongation of cell cycle was witnessed in *Foxg1*^{LacZ/Cre} organoids.

A

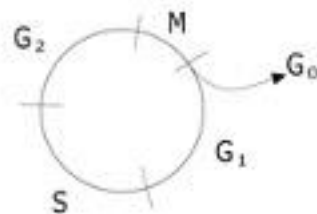


$$\text{Slope} = GF/Tc$$

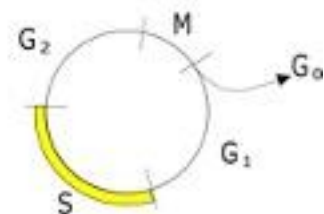
$$\text{y intercept} = GF \times (Ts/Tc)$$

B

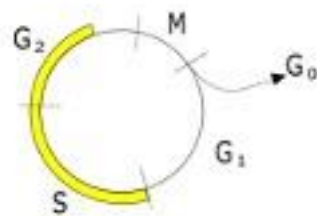
Prior to EdU exposure



EdU exposure



2hrs after EdU exposure



24hrs after EdU exposure

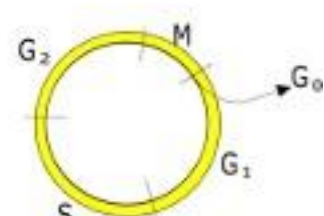


Figure 5.1 Panel A – A graphical explanation of the cumulative EdU experiment. The proportion of cells incorporating EdU gradually increases after initial exposure to EdU as more cells enter the S phase. The proportion increases until it reaches a plateau when all proliferating cells have been labelled, called the growth fraction (GF). The time point when GF is reached was calculated to be equal to the cell cycle length minus S phase length (T_c and T_s respectively). From these equations for both the y intercept and slope gradient were calculated. These equations are rearranged to give an equation to calculate T_c which is shown in section 5.2. Panel B – schematic showing the gradual accumulation of EdU by proliferating cells. Cells progress through the cell cycle in a clockwise direction. Yellow shading represents proportion of proliferating cells incorporating EdU. Prior to EdU exposure no staining is seen, at the point of exposure any cell in S phase would now incorporate EdU. 2 hours later, some cells in S phase would have entered G2 phase but retain EdU labelling and more cells would have entered S phase. By 24 hours it was hypothesized that all proliferating cells would have passed through S phase and incorporated EdU.

5.2 Experimental Design

A cumulative EdU experiment was designed, based on a previously published cumulative BrdU experiment (Nowakowski *et al.*, 1989). In this experiment Click-iT EdU kits (ThermoFisher) were used rather than BrdU for simplicity of use and detection. Cerebral organoids were grown as described in Methods section 2.3 and derived from previous work (Eiraku *et al.*, 2008; Nasu *et al.*, 2012). EdU was added into a 10cm dish with day 7 organoids, organoids were then collected at 2-hour intervals until 14 hours, then left overnight and a final sample was collected at the 24-hour time point. Organoids were then stained and imaged to determine the labelling index – EdU cells/DAPI. A full description of the methodology can be found in Chapter 2.5.

At the 2-hour time point, any cell which had been in S phase at any point in the prior 2 hours would have incorporated EdU and would be stained. At later time points more cells would have gone through S phase so the proportion of cells which had incorporated EdU would continue to increase until it hit a plateau. This maximum would be when every proliferating cell had gone through S phase and been labelled with EdU (Figure 5.1 panel A). The speed at which cells within a specific tissue reached this plateau would be due to the cell cycle length, a longer cell cycle length would take longer for every proliferating cell to incorporate EdU. By determining the labelling indexes at various time points it is possible to plot a

graph (as seen in figure 5.1 panel B). The labelling index is calculated by the following equation;

$$\text{Labelling index (LI)} = \frac{\text{total number of EdU + cells}}{\text{total number of DAPI + cells}}$$

For this experiment it was assumed that the entire proliferating population would be labelled with EdU by 24 hours, this is because *in vivo* the cell cycles recorded were less than 24 hours.

Once the graph was plotted the cell cycle length (T_c) was calculated by the following;

$$T_c = \frac{GF}{\text{Gradient of line}}$$

Once T_c was calculated the length of S phase (T_s) could also be estimated;

$$T_s = T_c \times y \text{ intercept}$$

These equations provide an estimate, there are a number of assumptions made which may alter these values from the true values:

1. It assumes that all cells within a population have the same cell cycle length.

2. It assumes that cells are dividing asynchronously and are equally divided throughout the entire cell cycle.

5.3 Results

5.3.1 Visualisation of variability seen at timepoints.

Figure 5.3 indicates that there is a marginal increase in cell cycle length in the *Foxg1^{LacZ/Cre}*, however, this did not reach statistical significance. When the data values of each individual organoid are plotted (Figure 5.4) we now see the levels of variability within the experiment.

However, the overall trend of increasing labelling indexes is still seen. Furthermore, there do not appear to be many outliers (a potential wildtype outlier is noticeable at the 24-hour timepoint). This suggests that there is a certain degree of variability between organoids but not enough to obscure the overall phenotype. This is promising as it may just require larger sample sizes to see less noticeable phenotypes such as difference in cell cycle lengths between genotypes. Ultimately, the variability does not appear to be so much that it obscures any biological phenotypes present.

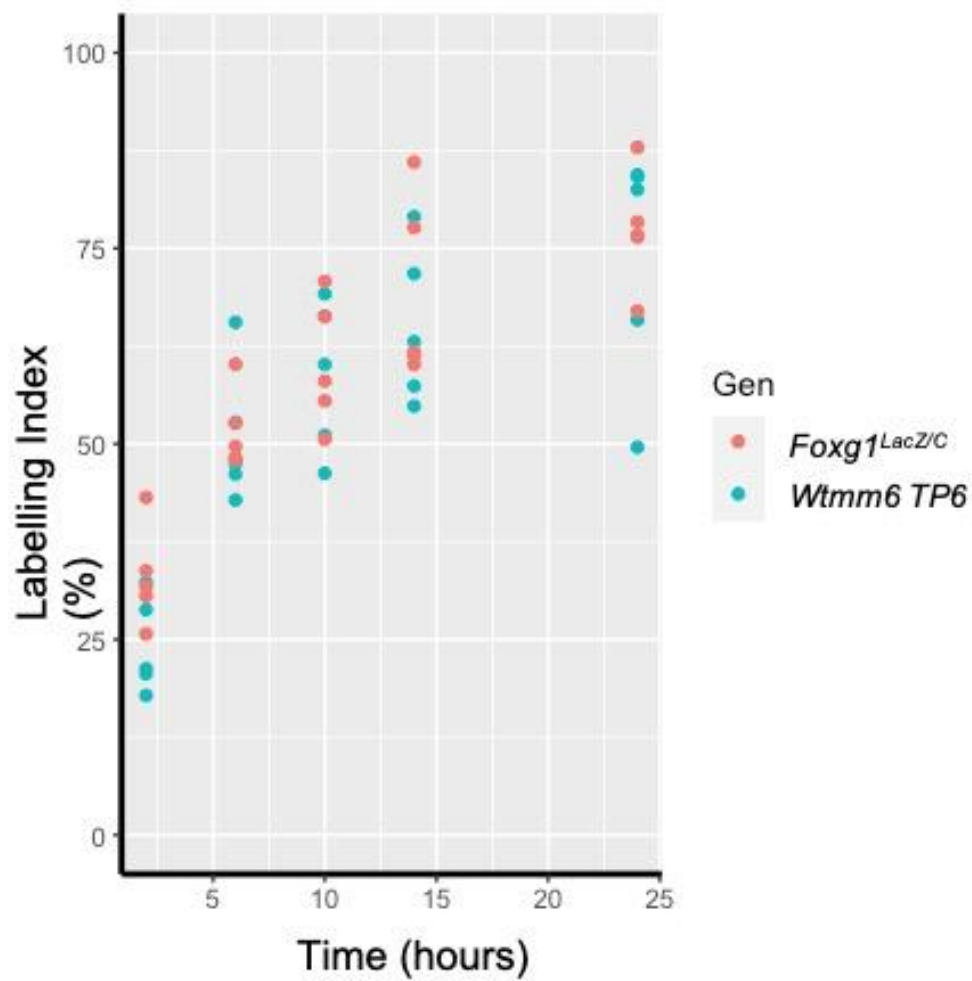


Figure 5.2 Graphical representation of the variability between organoids. Each data point corresponds to the labelling index calculated for a single organoid. $N = 5$ organoids from 3 separate batches per genotype for each timepoint.

5.3.2 *Foxg1*^{-/-} mutant organoids show an elongation in cell cycle length

Organoids were grown from both *Wtmm6 TP6* and *Foxg1^{LacZ/Cre}* lines and collected at 2,6,10,14 and 24 hours timepoints after the addition of EdU. The labelling indices

were calculated for each of these time points and the average for each time point and genotype calculated. The results are presented graphically in Figure 5.2.

To calculate T_c and T_s , the y intercept and gradient of graph must be known.

Microsoft Excel was used to determine the gradient of the linear phase of increase in labelling index, using all time points except 24 hours. The 24-hour time point was used as the growth fraction (GF). The gradient, GF and y-intercept were as follows:

Wtmm6 TP6 – gradient of incline = 3.27

y-intercept = 23.57 %

GF = 73.29 %

Foxg1^{LacZ/Cre} – gradient of incline = 2.94

y-intercept = 30.05 %

GF = 77.27 %

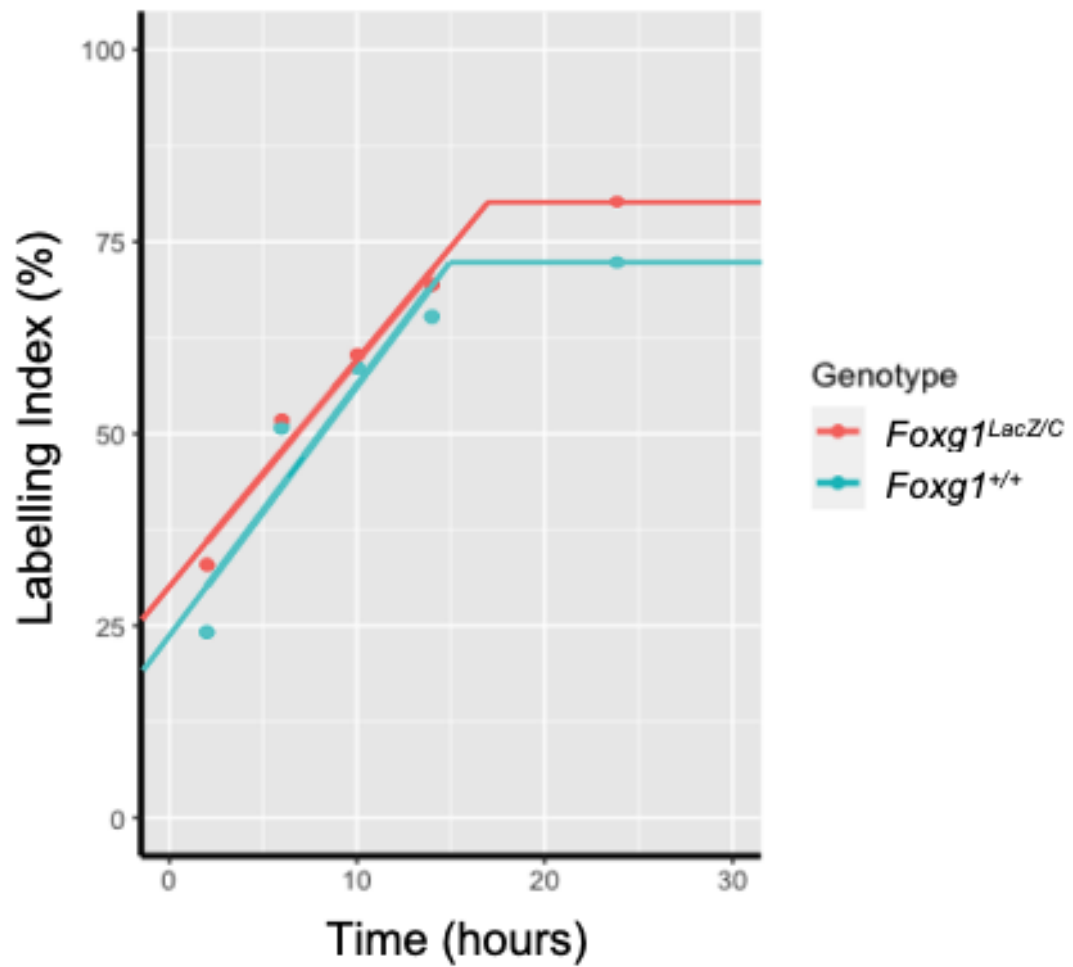


Figure 5.3. Results of EdU cumulative experiment. The labelling index (LI) was calculated for the 2,6,10,14 and 24-hour time points by calculating the proportion of EdU positive cells out of the entire population of cells (stained with DAPI). This was done for both wildtype and mutant genotypes. Data points shown are averages across 3 independent batches of organoids. It was assumed at the 24-hour time point all proliferating cells would have incorporated EdU. N = 5 organoids from each of 3 independent batches per data point.

The y-intercept provides an estimate value for the proportion of cells in S phase at the point of EdU exposure, this appeared to be slightly higher in the mutants.

The *Foxg1* mutant line also showed a shallower gradient. This is what would be expected if the *Foxg1* mutant organoids did have a longer cell cycle than wildtype.

These values were inputted into the equations found in section 5.2.

For the control line Tc was estimated to be 22.3 hours, and Ts at 5.3 hours.

Whereas the mutant line had a Tc of 26.2 hours and a Ts of 7.9 hours (Figure 5.3). A student t-test showed no statistically significant difference between the cell cycle lengths between genotypes ($p = 0.63$).

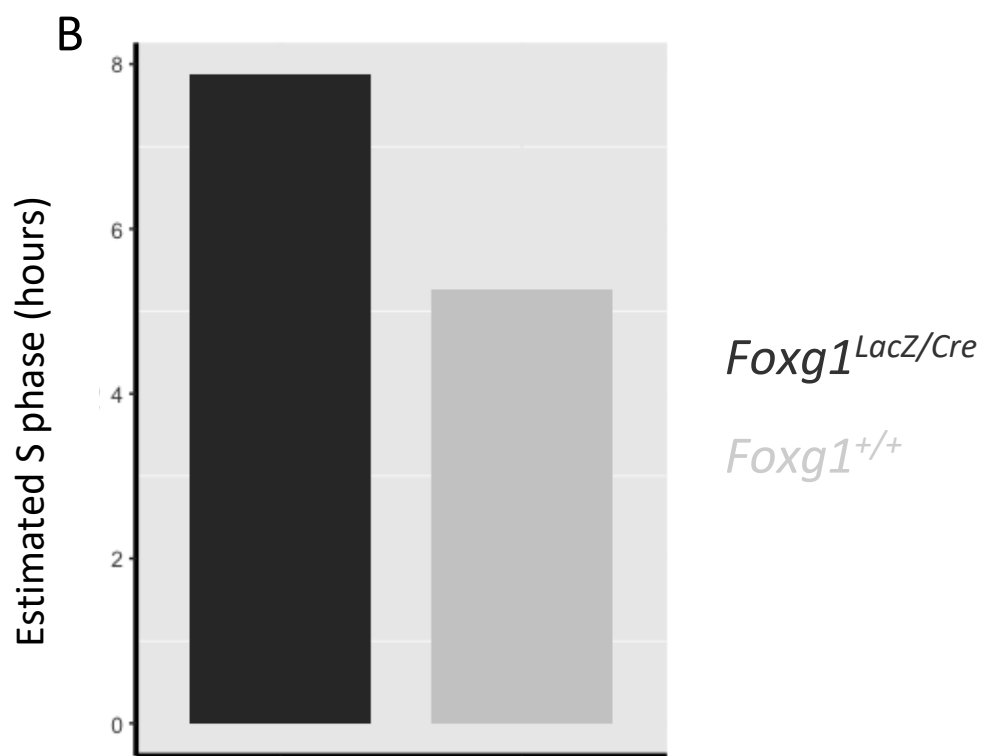
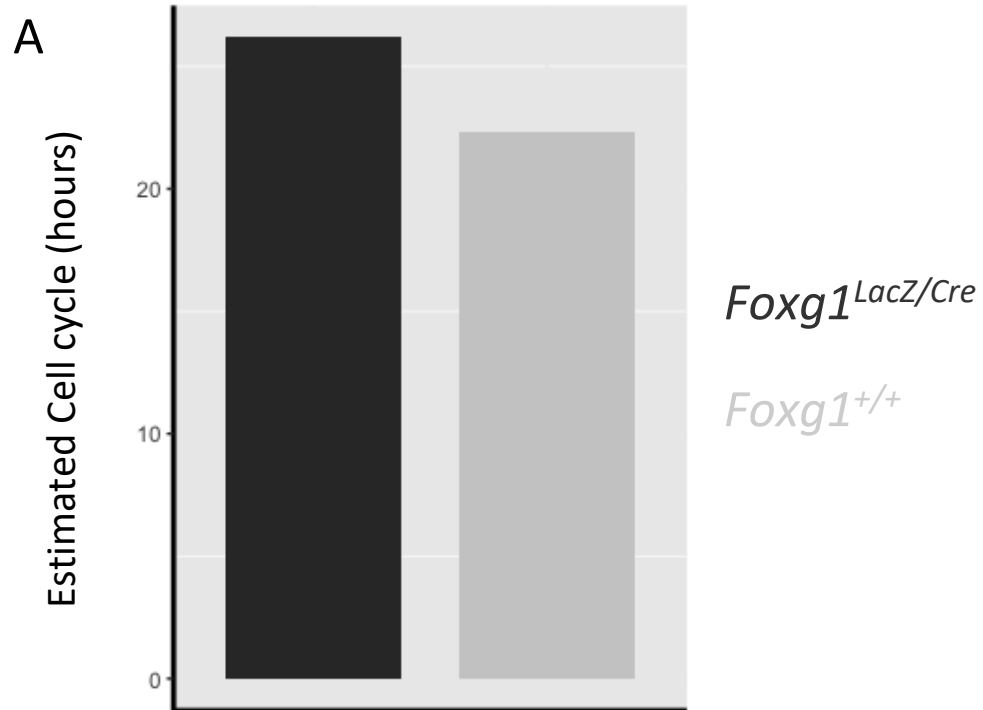


Figure 5.4 Estimated lengths of cell cycle and S phase. Panel A - cell cycle length estimations from mutant ($Foxg1^{LacZ/Cre}$) and $Foxg1^{+/+}$ (Wtmm6 TP6) genotypes. Panel B – S phase length estimations from mutant ($Foxg1^{LacZ/Cre}$) and wildtype (Wtmm6 TP6) genotypes. Both show an increase in length for mutant organoids but cell cycle length was not statistically significant ($P=0.63$). A student T-Test was used. $N = 5$ organoids across 3 independent batches per data point.

5.4 Discussion

While no statistical significance was seen in the data, it does suggest there may be a longer cell cycle in *Foxg1*^{-/-} mutant cells. The true extent of this phenotype may be obscured in organoids due to the variability which is seen in Figure 5.4. For this experiment organoids were collected at the timepoints 2, 4, 6, 8, 10, 12, 14 and 24 hours. However, due to time constraints from COVID19 not all time points could be processed and analysed. Instead the timepoints 2, 6, 10, 14 and 24 hours were prioritised to provide an overall view of the data. If the remaining timepoints were to be analysed and included in this dataset it may well lead to a significant difference seen in the cell cycle lengths.

The variability of organoids is well discussed in the literature (reviewed in Marshall and Mason, 2019). From the data we saw that variability between organoids, while significant, wasn't enough to completely hide largescale changes taking place in the organoids. For example, most organoids appear to show similar values for the labelling index across genotype at each timepoint. However, the variability did appear great enough to hide more minute changes such as that between genotypes.

Furthermore, in the original Martynoga paper significance was not seen between genotypes until E11 (Martynoga et al., 2005). The cell lines used here were harvested from blastocysts at E3.5 and then grown for 7 days during the organoid

protocol (Hooper et al., 1987; M. N. Manuel et al., 2011). This may imply that they would be analogous to an E10.5, although this is speculation and it is impossible to know what effect multiple rounds of passaging has had on the relative age of the stem cells. Regardless, using organoids of a later age may circumvent this and the phenotype may become more pronounced. In this experiment day 7 was used because of the high levels of organisation and structure present which deteriorates in the subsequent days.

Recent attempts to prolong the life of organoids (most done in human derived organoids) have included the use of 3D printed bioreactors (Qian et al., 2016). The continual movement maintains a strong nutrient gradient across the organoid potentially increasing life span. However, such equipment can be expensive and does not allow large scale studies to be carried out. An alternate approach is the Air Liquid interface organoids developed in the Lancaster Lab (Giandomenico et al., 2019). This overcomes the issue of nutrient diffusion by sectioning the organoid, exposing what would become a necrotic core to the nutrient-rich media. This is less expensive than the bioreactor approach but more labour intensive. Therefore, limiting the number of organoids that can be grown at once and the data presented in this chapter implies that a large number of organoids must be analysed for any given experiment. While none are perfect these techniques may well be applicable to mouse organoids, with further improvements it would allow this investigation to be reliably done at later timepoints. One of the potential advantages of trialling out

these improvements on mouse organoids is the rapid growth and relative cheapness (Marshall & Mason, 2019).

Chapter 6 - Discussion

6.1 Organoids as a model of investigating Foxg1

This thesis aimed to assess the ability of organoids to recapitulate key phenotypes of *Foxg1* mutations in mice forebrain development. There are numerous phenotypes which have been directly or indirectly associated with *Foxg1*, including changes in cellular morphology, suppressing early cortical fate, determining population size of Cajal-Retzius cells and of *Tbr2* expressing intermediate progenitors. However, for the sake of this thesis we chose to focus on just two of these phenotypes, ventral telencephalic patterning defects and premature elongation of cell cycle in the dorsal telencephalon. (Danesin & Houart, 2012; Dou et al., 2000; Fuccillo et al., 2004; Hanashima et al., 2004, 2007; Martynoga et al., 2005; Goichi Miyoshi & Fishell, 2012). Both of these phenotypes were chosen due to their large-scale effects on the developing brain, in the hopes the phenotypes would be visible in the variable organoids system.

The results shown go some way to validate cerebral organoids as a model for studying *Foxg1*. *Foxg1*^{-/-} organoids showed both a prematurely elongated cell cycle in the dorsal telencephalon and a complete inability to form ventral telencephalon when directed to adopt a ventral fate. The cell cycle lengths calculated in chapter 5 were different to those calculated *in vivo* (M. N. Manuel et al., 2011). However, work on human-derived organoids have shown that organoids spontaneously

activate stress pathways, affecting cellular specification and differentiation (Bhaduri et al., 2020). This coupled with the lack of a vasculature leading to a necrotic core (Madeline A. Lancaster et al., 2013b) perhaps partly explains why the exact cellular kinetics are not witnessed in organoids.

The protocols used here rely on the innate signalling and organisation of the embryonic stem cells with minimal interference (Danjo et al., 2011; Eiraku et al., 2008; Nasu et al., 2012). This then leads to large portions of the organoids failing to adopt any telencephalic identity at all, in chapter 4 no organoid was seen with more than 50% of cells expressing both *Foxg1* and *Olig2*, markers for the ventral telencephalon. Despite this, the fact that both phenotypes investigated could still be clearly seen is promising.

6.1.1 Future uses of organoids to study *Foxg1*

Foxg1 is expressed throughout the telencephalon, both dorsally and ventrally. One limitation of organoids is that it grows distinct regions in isolation. This is very different to what occurs *in vivo* with a constant interplay between different brain regions. One way that this can be replicated in organoids is by the use of assembloids. This involves patterning organoids separately for different regions then introducing them and allowing to fuse. These assembloids have already been done in human organoids for a number of different regions, including cortico-motor organoids and, crucially or for *Foxg1*, pallial-subpallial assembloids (Andersen et al., 2020; Sloan et al., 2018). This would then allow the full telencephalon to be studied. Considering that *Foxg1* simultaneously interacts with *Wnt* signals dorsally and *Shh* signals ventrally (Danesin & Houart, 2012) it would be highly useful to see how *Foxg1* behaves in a model with both present.

In addition to that, due to the self-organisation of organoids it was always presumed that endogenous signalling was present (Eiraku et al., 2008; Nasu et al., 2012) and work in human organoids showed that markers were present for some signalling centres (Renner et al., 2017). However, these signalling centres appeared randomly distributed and it is unknown if they produce the same morphogen gradients as seen *in vivo*. This is an issue when studying *Foxg1*, it has been implicated in repressing early cortical fates, such as Cajal-Retzius cells originating in the cortical hem, as well as limiting the size of the cortical hem (Danesin & Houart,

2012; Muzio, 2005). It has also been linked in restricting the size of the preoptic area, a producer of *Shh*, as well as acting downstream of *Shh* in ventral telencephalic patterning (Du et al., 2019; M. Manuel et al., 2010; Martynoga et al., 2005). So, in order to investigate these effects in organoids a thorough investigation of endogenous signalling centres must be done. Ideally this would take into account the 3-dimensional nature of the organoids. There are numerous clearing protocols, allowing 3D imaging of structures much larger than organoids (Muntifering et al., 2018). This in conjunction with staining for morphogen secreting centres will give us a better view of how organoids develop overall but also allow us to investigate the role of *Foxg1* in telencephalic patterning.

6.2 Variability of organoids remains a limitation

In chapter 5, the premature elongation of progenitor cell cycles was witnessed but failed to be statistically significant. In no doubt part to the variability shown between organoids in that chapter. As mentioned there are a number of ways the extreme environment organoids are grown in may affect their behaviour (Bhaduri et al., 2020; Madeline A. Lancaster et al., 2013b).

Recent work in human-derived organoids helps us to better understand the cellular variability seen. Single cell RNA sequencing (Sc-RNA-Seq) experiments have shown differences between the proportions of different cell types seen in organoids and *in vivo*. For instance a 45% reduction in cells expressing *HOPX*, expressed in outer radial glial cells (Bhaduri et al., 2020). Outer radial glial cells are not found in abundance in mouse *in vivo* (Mason & Price, 2016) but it seems likely that similar variations in cellular composition would be found in mouse organoids. Particularly given as some human organoid protocols have much greater levels of chemical and morphogen interference compared to mouse protocols (Nasu et al., 2012; Paşca et al., 2015). However, only similar SC-RNA-seq in mouse organoids would prove this.

6.3 A case for mouse cerebral organoids

A look at the body of work on cerebral organoids will show that the majority pertains to human cerebral organoids (Di Lullo & Kriegstein, 2017; M. A. Lancaster & Knoblich, 2014; Paşca et al., 2015). The use of iPSCs now allows cerebral organoids to be created from patients with neurodevelopmental disorders, in theory providing a more accurate genetic model of the disorders. However, work from this thesis has shown that while organoids recapitulate key aspects of development, the phenotypes are not exactly the same as seen *in vivo*. This comparison was only possible because of the wealth of literature *in vivo* mouse models (Danesin & Houart, 2012; M. Manuel et al., 2010; Martynoga et al., 2005; Xuan et al., 1995). The same breadth of data, for ethical and legal reasons, is not available for humans *in vivo*. It then becomes difficult to compare how similar phenotypes in human organoids are to real life development. Mouse organoids can therefore play a role as a bridge between what we have learnt *in vivo* and human organoids. In order to use organoids to their fullest potential a thorough knowledge of their strengths and limitations is required, including how accurately they recapitulate phenotypes. This would be difficult with human organoids alone but as shown in this thesis relatively easy with mouse organoids.

There is no doubt that there are aspects of human development and disease which can only be accurately studied in a human model (Marshall & Mason, 2019; Mason & Price, 2016). However, there are areas where mouse organoids do show

advantages. One such area is protocol optimisation. Current human protocols depend on highly specialised, costly reagents with timescales of several months (Madeline A. Lancaster et al., 2013b; Paşca et al., 2015). Mouse protocols, meanwhile, have much shorter timescales (one to two weeks) and cheaper reagents (Danjo et al., 2011; Nasu et al., 2012). In such a variable system as organoids, protocol optimisation can be highly time-consuming. Using mouse organoids could shorten the time required. Work has already shown that the same protocol (with modified time scales according to species) can generate cerebral tissue in both mouse and human organoids (Madeline A. Lancaster et al., 2013b). A quick screening of reagents and protocols in mice may narrow down the optimisation process, requiring less to be trialled in human organoids (Marshall & Mason, 2019).

Mouse cerebral organoids may not outperform human organoids but they can be used in a complementary manner.

6.4 Potential Future Work

The described experiments give an inconclusive glimpse into the cerebral organoids. However, the *in vitro* nature of organoids means that they are suitable for a variety of techniques.

The protocol used in this thesis to investigate cell cycle defects relied on a number of assumptions that we know to be false. For instance, cells in the same tissue do

not all have the cell cycle length. To get around this the cell cycle defects could be investigated using the FUCCI plasmid (Sladitschek et al, 2015). This plasmid uses GFP and RFPs alternating throughout the cell cycle. This coupled with live imaging, which has shown good promise in organoid types of all kinds (Rios and Clevers, 2018), would allow for the cell cycle of individual cell to be accurately recorded.. It would also although the difference between the cell cycle lengths in organoids and *in vivo* to be compared more thoroughly to assess the utility of organoids as a model system.

In regards to investigating the effects of *Foxg1* haploinsufficiency in the ventral telencephalon the methods described in this thesis are highly descriptive. There are a number of methods for ventral optimisation done in hiPSCs which could be adapted to mouse cerebral organoids or for future work to be done in hiPSC derived organoids (Pasca et al, 2015) to try and ensure greater differentiation efficiency. Once this has been achieved scRNA sequencing could be used to assess the genetic expression of all cells throughout the organoids (Kinugawa et al, 2020, Velasco et al, 2019). This would be able to show much stronger proof that those cells which are adopting a ventral fate (i.e. through expression of *Olig2* or *Nkx2.1*) are showing no *Foxg1* expression.

Importantly, in order to ensure that it is the lack of *Foxg1* leading to this phenotype it would be imperative that a rescue experiment is performed. This could take numerous forms. If patient derived organoids are used, then CRISPR could be

utilised to replace the dysfunctional allele and a comparison of phenotypes could be seen in the isogenic cell lines. The use of CRISPR with *FOXP1* has been shown in human cells (Croci et al. 2020) so should be transferable to mice.

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