

Investigating gene expression differences in acute and organotypic mouse hippocampal brain slices as models for studying the brain

by

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KNDSAG001

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN



In fulfillment of the requirements for the degree
MMedSci degree with specialisation in Neuroscience (Physiology)

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January 2025

Mandela Rhodes Scholarship and the University of Cape Town are acknowledged for financial support of this project.

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Acknowledgments

I want to start by thanking God because I have done this thesis through Christ who strengthens me (Philippians 4:13). Haha, see the citation I did there.

Many thanks to my beautiful family, church, and friends for their consistent love and support. I would not have been able to do this without my mother's delicious meal and a warm home to come back. I also want to thank my siblings for telling me to "do my work" every time I was slacking off. A sincere thank you to my greatest pastor and father in the entire world, pastor Moses Kyenda, for always encouraging me, praying for me, and believing in me.

Of course, this thesis would literally not be possible without my wonderful, kind, and outstanding supervisor. A heartfelt thank you to monsieur Joe for being a great mentor, teaching me how to do great science, believing in me as a scientist, and always being available for my crazy rants. We both know that I am, indeed, your favourite student and it's okay because you are my favourite supervisor!

Thank you, Teresa, for guiding me through this project and availing yourself when I was stuck with my code. Thank you to Josh, my lab uncle Thijs, and Roxanne, for always making small chats with me in the lab and bearing with my heater 24/7. Special thanks to the rest of the Raimondo and Dangarembizi lab for being such a wholesome lab family! You have all contributed to the wonderful experiences and moments I had throughout this degree, which made the excruciating writing period bearable.

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List of Abbreviations

Symbol	Description
aCSF	Artificial cerebrospinal fluid
Acutes	Acute brain slices
AMPA	A-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BBB	Blood-brain barrier
CA	Cornu Ammonis
CNS	Central Nervous System
DE	Differential expression
DEGs	Differentially expressed genes
ECM	Extracellular matrix
ELISAs	Enzyme-linked immunosorbent assay
Fc	Fragment crystallizable
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GO	Gene Ontology
IL6	Interleukin-6
IQR	Interquartile range
Lamp5	Lysosomal Associated Membrane Protein Family Member 5
LPS	Lipopolysaccharide
Mg ²⁺	Magnesium ions
NCC	Neurocysticercosis
NMDA	N-methyl-D-aspartate

OBSCs	Organotypic brain slice cultures
OPCs	Oligodendrocytes precursor cells
P	Postnatal day
PCA	Principal component analysis
Pvalb	Parvalbumin
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ScRNA-seq	Single-cell RNA sequencing
SD	Standard deviation
SEM	Standard error of the mean
SLE	Seizure-like event
Sncg	Synuclein gamma
SnRNA-seq	Single nucleus RNA sequencing
SRP	Signal recognition particle
Sst	Somatostatin
TNF- α	Tumor necrosis factor- α
UMAP	Uniform manifold approximation and projection
UMIs	Unique molecular identifier
VLMCs	Vascular leptomeningeal cells
Vip	Vasoactive intestinal peptide

Abstract

Organotypic brain slice cultures (OBSCs) are widely used to study neural circuit function, but their molecular and cellular composition relative to acute brain slices remains poorly characterized. Here, using single-nucleus RNA sequencing, I performed a comprehensive comparison of gene expression between OBSCs and acute brain slices prepared from postnatal day 7 mouse hippocampus. I identified significant differences in cellular composition, with OBSCs showing an overrepresentation of glial cells and a relative underrepresentation of neuronal populations. Notably, oligodendrocytes were almost exclusively found in OBSCs, while dentate progenitor cells were predominantly present in acute slices, reflecting ongoing developmental processes in culture. Differential gene expression analysis revealed over 4200 unique differentially expressed genes across major cell types, with excitatory neurons and astrocytes showing the most substantial transcriptional changes. Gene ontology analysis demonstrated upregulation of pathways involved in neuronal development and cell signalling alongside downregulation of axon guidance pathways in OBSCs. Specific cell-type analysis revealed distinct adaptations, including altered inflammatory responses in microglia and astrocytes as well as modified synaptic signalling in dentate gyrus cells. These findings provide crucial insights into how brain slice cultures adapt to ex vivo conditions and highlight important considerations for their use as experimental models in neuroscience research.

Chapter 1: Introduction

1.1. Brain slice preparations as an *in vitro* model to study the brain

Brain slice preparations are powerful *in vitro* models used to study the brain, alongside organoids and other artificial tissue constructs (Shuler & Hickman, 2014). These models have been instrumental in exploring both disease mechanisms and physiological functions of the central nervous system (CNS) (Gogolla et al., 2006; Holopainen, 2005; Stoppini et al., 1991). There are two primary types of brain slice preparations used to study the CNS: acute brain slices and organotypic brain slice cultures (OBSCs). Acute brain slices are prepared and used on the same day of dissection, allowing researchers to study short-term neuronal activity in the tissue. In contrast, OBSCs are maintained in culture for extended periods, enabling the exploration of long-term processes like synaptic plasticity, neurodegeneration, and the development of neural circuits over time (De Simoni et al., 2003a; Noraberg et al., 2005). Both models can be prepared from various brain regions, including the hippocampus (Liu et al., 2017), cortex (Croft et al., 2017) and hypothalamus (Sieg et al., 1998).

1.2. General advantages and disadvantages of brain slice models

There are several advantages to using brain slice preparations. Brain slices preserve the basic architecture and cellular diversity of the brain region from which they are derived, maintaining the structural integrity of neuronal networks and glial cells. This preservation allows for the study of neuronal connectivity, synaptic plasticity, and cellular responses in a controlled environment that mirrors many aspects of the *in vivo* brain (Humpel, 2015a). This is an improvement over primary dissociated cell cultures (another type of *in vitro* model), which limit the number and quality of cell-cell interactions that can be studied (Banker & Cowan, 1977; Sahu et al., 2019). Importantly whilst the interactions between resident brain cells such as astrocytes, microglia, and neurons can be

observed, this happens in isolation from peripheral immune cells or any other factors that can be derived from an intact vascular system.

The stability and optical access afforded by brain slice models greatly expand and facilitate the number of techniques that can be employed to study cellular and molecular processes at a high resolution (Humpel, 2015b). The extended time in culture makes the slices available for experiments that require advanced genetic techniques such as optogenetics (Borges-Merjane et al., 2020; Tønnesen et al., 2009). This also supports investigations into processes requiring longer observation periods, like synaptic plasticity and changes in gene expression (Butler-Ryan & Wood, 2021; Harwell & Coleman, 2016). In addition, brain slice models afford excellent control over the composition of the extracellular fluid, much more so than *in vivo*, which opens up important avenues for scientific investigation of biophysical processes, and also improves the quality of pharmacological manipulations (Annis et al., 1990; Kim et al., 2013a; Kleinberger-Doron & Schramm, 1990; Legradi et al., 2011; Stoppini et al., 1991b; Su et al., 2011). A further major advantage is that brain slice models often require fewer animals compared to *in vivo* studies, aligning with the 3Rs principle which was developed by William Russel and Rex Burch (Russell et al., 1959): Replacement, Reduction, and Refinement. Brain slice models (in particular OBSCs) can reduce the overall number of animals needed while still providing critical insights into brain function (Hubrecht & Carter, 2019). Lastly, another advantage of OBSCs is that they allow co-culturing with slices from other brain regions or specific cell types (Duport et al., 1998; Zimmer & Gähwiler, 1987), making it possible to study their interactions. Additionally, drugs and compounds that would not naturally cross the BBB can be applied directly onto the brain slice and their effects can be tested.

Brain slice preparations, while widely adopted in neuroscience research, face several key limitations when compared to studying the living brain. The process of creating these slices introduces multiple compromises: the tissue undergoes oxygen deprivation during preparation, tissue experiences ischaemia due to lack of blood supply, neural connections are physically cut off at the slice boundaries, and the cells may suffer damage from both mechanical forces and changes in fluid balance during the cutting process (Raimondo et al., 2017). Additionally, since they are isolated from the brain, these preparations lack the natural blood flow and pressure systems found in living tissue. Although researchers attempt to mimic these physiological conditions with

artificial solutions, this remains an imperfect substitute. These preparation-induced stressors mean that depending on the specifics of the brain slice preparation, different cell types are undergoing differential responses to the injury and will be susceptible to different rates of progressive cell death. Understanding these changes at a cell-type-specific level is important for our understanding of brain slice models and is a major goal of my project.

1.3. The hippocampus: an important region for brain slice preparation

The hippocampus is a particularly important region from which brain slice preparations can be made. This is because of its architecture, which repeats along the ventral dorsal axis meaning that slices prepared at 90 degrees to this axis retain neurons with much of their dendritic trees and axons intact (Per et al., 2009). This makes hippocampal brain slices ideal for studying neural circuitry and synaptic function (Per et al., 2009). Furthermore, dysfunction of the hippocampus is implicated in various neurological disorders, including Alzheimer's disease (Josephs et al., 2017), epilepsy (French et al., 1993), and schizophrenia, making it a critical target for neurophysiological research.

The hippocampus comprises several regions: the dentate gyrus (DG), involved in new episodic memory formation and adult neurogenesis (Jacoby et al., 2000; Kempermann et al., 2015; S. Liu et al., 2003; Surget & Belzung, 2022; Van Praag et al., 2002); the cornu ammonis (CA) regions (CA1, CA2, and CA3), involved in memory processing and spatial navigation (Gilbert & Brushfield, 2009; Lee et al., 2004); and the subiculum, which relays processed information to other brain areas (Groenewegen et al., 1987; Nakai et al., 2024; Namura et al., 1994; O'mara et al., 2001; Umaba et al., 2021; Witter et al., 1990). The preservation of these structures in hippocampal slices is useful for studying the synaptic connectivity and functional architecture of this brain region. Ultimately, insights gained studying hippocampal brain slices can be useful for understanding memory formation, spatial navigation, and the pathology underlying various neurological diseases.

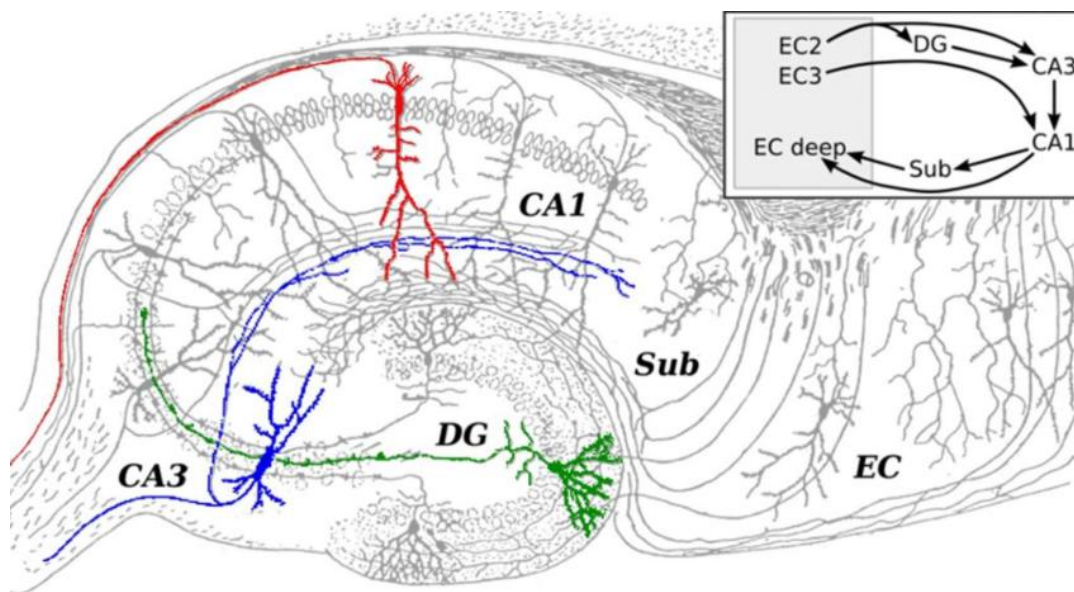


Figure 1.1) Hippocampus. Diagram showing the structure and circuitry of the rodent hippocampus depicted by Ramon y Cajal, 1911 page 753 (Adapted from (Sayegh, 2022)).

1.4. Brain slice preparations

1.4.1. The acute brain slice preparation

For decades, acute brain slices have been used extensively in neuroscience research (Bachelard, 1971). These slices, typically ranging from 200 to 600 μm in thickness, can be derived from various parts of the brain of both young and adult mice (Papouin & Haydon, 2018). Preparing these slices involves a short incubation period, usually no longer than an hour, to recover from the slicing injury (Papouin et al., 2017). Due to the mechanical stability that they provide, acute brain slices are typically used for electrophysiological experiments including local field potential recordings or whole cell patch clamp recordings. For example, acute brain slices have enabled key discoveries including the characterization of long-term potentiation, a cellular model of learning and memory (Teyler, 1999; Wilson & Tonegawa, 1997). Compared to cultured brain slices the neurons and synapses are thought to more closely resemble their state in the intact brain given the lack of time for culture-dependent changes to occur (De Simoni & Yu, 2006; Eguchi et al., 2020). However, acute brain slices have limitations. Mechanical damage from slicing can induce stress responses in neurons (Goldberg & Choi, 1993), including excitotoxicity

(Hartley et al., 1993). Even after the recovery period, these slices maintain a relatively short viability of just a few hours post-preparation, limiting their application in long-duration studies.

Recent technological advances have improved acute brain slice preparation. Advancements in tissue-slicing technologies, such as compresstomes and vibratomes (Paletzki & Gerfen, 2019), have increased the accuracy and reproducibility of slice preparation. Additionally, the development of protective slicing solutions, like N-methyl-D-glucamine (NMDG), has improved the preservation of healthy neuronal populations, even in aged mice (Ting et al., 2018).

1.4.2. Organotypic brain slice cultures

The development of OBSCs traces back to early experiments by Crain (1966), who demonstrated that these cultures could maintain bioelectric activities in mouse cerebral explants and replicate the neural activity seen *in situ*. The roller-tube and interface methods are the two primary techniques for culturing organotypic brain slices. In the roller-tube method, slices are attached to a glass coverslip and rotated in a tube containing culture medium, providing intermittent exposure to both medium and air (Gähwiler, 1981). The interface method, on the other hand, involves culturing slices on a porous membrane at the interface between culture medium and air, allowing for continuous nutrient diffusion while maintaining an air-exposed upper surface (Gähwiler et al., 1997). Whilst the roller-tube method generates thinner slices with better optical conditions, the interface method is more commonly utilised as it is best for maintaining the three-dimensional structure of the slices (De Simoni & Yu, 2006). OBSCs offer several advantages over acute brain slices, particularly for long-term studies of neural function and pathology (Stoppini et al., 1991). Their extended viability allows for prolonged experimental manipulation and observation, making them particularly useful for studying the long-term effects of various treatments or the progression of neurological conditions (Croft et al., 2019).

Synaptic plasticity

OBSCs have been a valuable tool for investigating plasticity in neural circuits. Their utility stems from several key advantages. First, pyramidal neurons in hippocampal OBSCs maintain normal synaptic transmission (De Simoni et al., 2003b; Debanne et al., 1995) and exhibit diverse forms of plasticity, including both long-term potentiation and depression (Bonhoeffer et al., 1989; Debanne et al., 1994; Emptage et al., 2001a; Muller et al., 1996). Additionally, these neurons are highly accessible for experimental manipulation – they can be readily penetrated with sharp electrodes, loaded with various dyes (including calcium-sensitive fluorophores), and genetically modified through biolistic or AAV-mediated transfection techniques. This accessibility has enabled sophisticated optical investigations of synaptic transmission across multiple domains, from studying calcium dynamics in dendritic spines (Emptage et al., 2001) to observing spine structural changes (De Roo & Ribic, 2017), including, directly visualizing the ionic forces that drive synaptic transmission (Selfe et al., 2024). Furthermore, the extended viability of OBSCs makes them particularly well-suited for investigating slower developmental processes, such as axon guidance (Galimberti et al., 2006), myelination (Miron et al., 2013), and the maturation of inhibitory circuits (Hasam-Henderson et al., 2018).

Neuron-glia interactions, neuroinflammation and neuroinfections

Given the presence of all resident brain cells in OBSCs, these *in situ* preparations are particularly valuable for studying glial cell types, and neuroglial interactions including in the context of neuroinflammation and neuroinfections. Whilst immunocompetent glial cells such as microglia and astrocytes are known to become active and respond to the slicing trauma in OBSCs prepared from neonatal tissue. They have also been shown to return to a resting, quiescent state over the course of a week (Delbridge et al., 2020b; Huuskonen et al., 2005). Additionally, glial process dynamics can be observed *in situ* (Benediktsson et al., 2005). The role of microglial and astrocytic responses to neuroinflammatory and neuroinfectious stimuli has also been studied including how these cell types might contribute to neuronal dysfunction and cytotoxicity (Bernardino et al., 2008; Papageorgiou et al., 2016). A particular advantage of OBSCs is their ability to

sustain long-term exposure to infectious agents. This enables experimental studies with various pathogens, including bacteria (Gianinazzi et al., 2004; Schmidt et al., 2001), fungi (Awala et al., 2023), and viruses (Rashidi et al., 2024). While acute brain slices typically remain viable for only several hours, OBSCs can maintain tissue integrity for extended periods, allowing researchers to observe host-pathogen interactions and disease progression over days to weeks (Bak et al., 2023). Recently, an interesting study by (Merighi et al., 2024) showed that OBSCs made from postnatal day 3 mouse spinal cord can be kept for up to 90 days in culture with a specialised growth medium.

Application of OBSCs: Neurodegenerative disorders

Relatedly, OBSCs also serve as valuable models for studying neurodegenerative diseases including Alzheimer's (Mewes et al., 2012), Parkinson's (Stahl et al., 2009), and Huntington's diseases (Murphy & Messer, 2001; Smith et al., 2001). Their extended viability enables long-term observation of complex neuroglial interactions and progressive pathological changes that characterize these disorders. This temporal advantage allows researchers to monitor the gradual accumulation of toxic proteins, altered cellular functions, and the subsequent inflammatory responses that develop over days to weeks (Mccarthy et al., 2021; Tackenberg et al., 2013).

Alzheimer's disease

In Alzheimer's disease research, OBSCs have been instrumental in investigating synaptic dysfunction due to beta-amyloid (A β) toxicity and tau phosphorylation (Frezza et al., 2009; Mewes et al., 2012b; Suh et al., 2008). These slices have been used to investigate the role of nerve growth factor (NGF) in sustaining cholinergic neurons, particularly those from the basal nucleus of Meynert, demonstrating that NGF is essential for their survival and highlighting its potential as a neuroprotective treatment (Gähwiler et al., 1990; Humpel & Weis, 2002; Weis et al., 2001). OBSCs have also enabled studies on beta-amyloid toxicity, showing its role in apoptotic cell death (Allen et al., 1995), oxidative stress (Alberdi et al., 2010; Clapp-Lilly et al., 2001), pro-inflammatory responses (Akiyama et al., 2000; Harris-White et al., 1998), and intracellular signalling pathways

(Nassif et al., 2007). Furthermore, they have provided insights into tau hyperphosphorylation (Johansson et al., 2006) and beta-amyloid “plaque-like” deposits when OBSCs are cultured under specific conditions with apolipoprotein E4 (Marksteiner & Humpel, 2007). Thus serving as a potent model for exploring the cellular and molecular pathology of AD and testing therapeutic interventions.

Parkinson's disease

OBSCs have been instrumental in studying Parkinson's disease mechanisms through various approaches. Early studies established co-cultures of cortex-striatum and mesencephalon-striatum to examine the cortico-striatal and nigro-striatal pathways affected in this disease (Ostergaard et al., 1990; Plenz & Kitai, 1996). Researchers developed models focusing on the substantia nigra to study dopaminergic cell bodies (Jaeger et al., 1989; Schatz et al., 1999), which enabled the investigation of neurotoxins like rotenone that induce nigro-striatal pathway degeneration (Daviaud et al., 2013; Testa et al., 2005; Ullrich & Humpel, 2009). More sophisticated models emerged, such as triple slice cultures combining cortex-striatum-mesencephalon (Plenz & Kitai, 1996), which maintained the morphological and electrophysiological characteristics of *in vivo* preparations. These models allowed comparison of different dopaminergic neurotoxins (MPP+, rotenone, and 6-OHDA) and their effects (Betarbet et al., 2002; Perese et al., 1989; Perier et al., 2003; Stahl et al., 2009). Advanced techniques including sagittal slicing with specific angles were developed to study mechanical degradation of the nigro-striatal pathway (Daviaud et al., 2014). Studies of OBSCs have also revealed that genetically modified neural stem cells can thrive and form neural networks within damaged striatal tissue, as demonstrated through experiments with Wnt5a-enhanced cells (Tønnesen et al., 2011). This model's relevance was further strengthened when researchers successfully cultured embryonic stem cell derivatives in brain slices treated with 6-OHDA, showing they could mature into functioning dopamine-producing neurons (Kearns et al., 2006). Notably, OBSCs prepared from adult rodent tissue exhibit an inherent progressive loss of neuronal viability, which, while potentially limiting for some applications, actually provides an advantageous model for studying neurodegenerative processes (Kapoor et al., 1988). This spontaneous neurodegeneration mimics aspects of age-related neuronal loss and can be used to evaluate potential neuroprotective

interventions or to study the temporal dynamics of neuroinflammatory responses in the context of neuronal death.

Huntington's disease

OBSCs have made significant contributions to Huntington's disease research by providing models to mimic key aspects of the disease. Early OBSC models employed neurotoxins such as kainic acid (KA) (Matyja, 1986), quinolinic acid (QA) (Beal et al., 1991; Whetsell Jr. & Schwarcz, 1989), and 3-nitropropionic acid (3-NPA) (Storgaard et al., 2000) to replicate striatal neurodegeneration observed in postmortem tissue or *in vivo*. These studies revealed mechanisms of excitotoxicity and neuronal loss, including changes in neuronal markers and activity of cortico-striatal pathways. OBSCs can be used in Huntington's disease research in two ways: first, by preparing organotypic slices directly from transgenic mice that carry the mutant genes (Johnson et al., 2006; Murphy & Messer, 2004; Ortiz et al., 2011), and second, by using slices from normal rodents and introducing the Huntington's-disease-associated genetic material through transfection (Reinhart et al., 2011). For example, a significant breakthrough came with (Reinhart et al., 2011) who developed acute transfection of rat cortico-striatal slices with human pathological huntington gene constructs, enabling successful screening of neuroprotective and anti-inflammatory drugs. Additionally, coronal OBSCs can mimic the disease by mechanically transecting the GABAergic (medium spiny neuron) MSN pathway, allowing the study of striatal degeneration and mRNA expression in grafted cells without using neurotoxins (André et al., 2018). Recently, one study has demonstrated that pathology of the Huntington's disease can be modelled in rat sagittal cortico-striatal OBSCs using two approaches: AMPA-induced excitotoxicity and QA-induced striatal lesions, successfully replicating patterns of cell death which is characteristic of Huntington's disease (McCaughey-Chapman & Connor, 2022).

Epilepsy and seizures

OBSCs have been pivotally important in research into seizure mechanisms of epilepsy (Heinemann & Staley, 2014; Oblasov et al., 2024). They enable rapid drug screening,

identification of therapeutic targets, and exploration of pharmacoresistance mechanisms (Albus et al., 2008), such as reduced GABAergic efficacy during chronic seizures. For instance, hippocampal slices have been crucial in exploring the role of GABAergic inhibition in seizure control (Absalom et al., 2022; Burman et al., 2019; Miles & Wong, 1987). A study by Ellender et al. (2014) using hippocampal brain slices, showed that during the clonic phase of seizures, GABAergic inputs from parvalbumin interneurons become excitatory, sustaining seizure activity instead of inhibiting it. Further investigation has revealed that modulations of GABAergic transmission can either reduce or exacerbate seizure activity *in vitro*, depending on intracellular chloride levels (Raimondo et al., 2017). Moreover, a key feature of these cultures is the spontaneous development of seizure-like events (SLEs) over weeks *in vitro* (Berdichevsky et al., 2012; J. Liu et al., 2017). This temporal progression mirrors aspects of the latent period observed clinically between brain injury and the onset of seizures. The slice preparation process itself induces deafferentation – this means a loss of sensory input when the connections with the afferent nerve fibers are severed - which triggers compensatory but aberrant axonal sprouting (Zimmer & Gähwiler, 1987). These structural reorganizations parallel those observed in post-traumatic epilepsy patients (Hunt et al., 2009). Studies utilizing OBSCs have been particularly valuable in elucidating the role of the mTOR signalling pathway in epileptogenesis (Berdichevsky et al., 2013). The extended culture duration allows researchers to manipulate this pathway at different time points, revealing its crucial involvement in both the structural reorganization of neural circuits and the development of hyperexcitability.

Limitations of OBSCs

However, OBSCs also have limitations. While they preserve many aspects of *in vivo* neural architecture and function, they develop in isolation from systemic factors such as the blood-brain barrier, immune system interactions, and the endocrine system. This isolation can lead to differences in cellular and gene expression, which may not fully represent the *in vivo* state (Holopainen, 2005). For instance, a review by Lossi et al. (2009) described that the cellular composition and synaptic organization in OBSCs can change over weeks in culture, potentially diverging from the *in vivo* state. This highlights the

importance of carefully considering the culture duration when interpreting results from OBSC experiments.

Ultimately, OBSCs and acute brain slices are both valuable experimental models. Each has distinct characteristics that make it suitable for different types of studies. Understanding the differences between these two models is crucial for selecting the appropriate experimental approach. To better understand the complex cellular and molecular differences between these models advanced new techniques such as snRNA-seq have emerged as powerful tools for high-resolution comparative analysis of brain tissue.

1.5. A technology to analyse data from brain slices: single nucleus RNA sequencing

Single-nucleus RNA sequencing (snRNA-seq) has emerged as a powerful tool for analysing gene expression in brain tissue at unprecedented resolution (Lake et al., 2016). This technique enables researchers to capture the transcriptomic profiles of individual nuclei, offering insights into cellular heterogeneity and gene expression patterns within complex brain tissues (Grindberg et al., 2013). By applying snRNA-seq to both OBSCs and acute brain slices, we can gain a deeper understanding of how these models differ at the cellular and molecular levels, and how these differences might impact their use in various experimental contexts.

1.5.1. Advantages and limitations of snRNA-seq in brain slice models

SnRNA-seq offers several key advantages when applied to brain slice models. The first advantage of snRNA-seq is its ability to profile the transcriptome of individual cells, allowing researchers to trace these transcripts back to their cell of origin using barcodes and computational methods (Ding et al., 2020). Using unique molecular identifiers (Kivioja et al., 2011) minimizes amplification bias, and ensures accurate quantification of each gene even at low abundance (Haque et al., 2017; Ziegenhain et al., 2017). In contrast, bulk RNA sequencing measures average gene expression from many cells

simultaneously, making it difficult to identify individual cell types and their specific contributions to the overall transcriptome (Li & Wang, 2021). Furthermore, unlike methods such as reverse transcription, *in situ* hybridization (Lein et al., 2006), and microarrays, snRNA-seq is unbiased and does not require prior knowledge of target genes and there is no need for probes (Okaty et al., 2011; Okoniewski & Miller, 2006; Z. Wang et al., 2009; Y. Zhang et al., 2014). This means that the technique can uncover a wide range of genes, including rare or novel ones, as well as new cell types that would otherwise remain undetected (Habib et al., 2016). Another significant advantage is the versatility of snRNA-seq, which can be used with a variety of tissue samples, including fresh, frozen, and fixed tissue, whereas whole cells are less resilient to thawing and mechanical stress when isolated from frozen tissue (Krishnaswami et al., 2016a; Lacar et al., 2016). This is particularly beneficial for human samples, which are often frozen (Krishnaswami et al., 2016). It is also important for my study, as I wanted to first freeze my tissue samples to allow for flexibility in the timing of the experiments.

An additional consideration is that single-cell RNA sequencing, scRNA-seq, (as opposed to snRNA-seq) uses enzymes to dissociate the tissue which can affect the integrity of RNA in neurons and works primarily on tissue from younger organisms (Darmanis et al., 2015; Grindberg et al., 2013b; Habib et al., 2017). Whilst snRNA-seq also employs tissue dissociation, it has a lower incidence of dissociation-induced transcriptional stress responses as it does not require suspension of single cells but rather nuclei (Grindberg et al., 2013). Nuclei are less susceptible to spurious gene activation during tissue dissection and isolation, resulting in improved reliability and fewer artifacts during the processing stage (Armand et al., 2021). This advantage makes snRNA-seq particularly suited for analyzing samples that are fragile or difficult to process, such as human brain tissues. The main limitation of snRNA-seq is its inability to sequence cytoplasmic RNA, as it retains only nuclear transcripts (Bakken et al., 2018). However, nuclear transcripts still provide a reliable proxy for gene expression within specific cells, maintaining the method's overall utility.

Therefore, I chose to use snRNA-seq for my study as this technique is useful in unravelling the cell-type-specific differential gene expression differences between acute brain slices and OBSCs. At the time of writing this thesis, there has not been a study that has employed snRNA-seq in OBSCs. Nonetheless, recent advancements have highlighted

the ability of snRNA-seq to provide detailed insights into brain slice models. For instance, Bormann et al. (2024) used snRNA-seq to analyze glial cell responses in a rodent model of ischemic stroke, identifying subtype-specific transcriptional signatures in oligodendrocyte precursor cells (OPCs) and astrocytes, as well as a shared immuno-glial crosstalk with myeloid cells that influences brain recovery mechanisms (Bormann et al., 2024). Similarly, Chen et al. (2022) used snRNA-seq to reveal the detrimental effects of prenatal e-cigarette exposure on brain development. Therefore, snRNA-seq offers the chance to fully characterize brain slice models and better inform how they might be used in a host of scientific contexts.

1.5.2. Technical challenges and considerations

While snRNA-seq offers numerous advantages, it also presents certain technical challenges, particularly when used with brain slice models. The process of isolating nuclei from brain slices can be complex. Maintaining the quality of nuclei during isolation is crucial for obtaining accurate and reproducible results. Advanced protocols and reagents have been developed to enhance nuclei preservation and improve RNA yield, but these methods must be carefully optimized for each tissue type and experimental condition (Krishnaswami et al., 2016).

Another challenge lies in the bioinformatics analysis of snRNA-seq data (Lähnemann et al., 2020). The complexity of brain tissues, with their diverse and tightly regulated cell populations, requires sophisticated computational tools (Chen et al., 2019) to accurately classify cell types and states that rely on mapping onto reference cell atlases (Hon et al., 2018; Shen et al., 2012). This is particularly important when analyzing brain slices, where the cellular context is critical for understanding the functional implications of gene expression changes. Recent advances in machine learning and computational biology have begun to address these challenges, improving the accuracy and resolution of cell-type identification and functional annotation in snRNA-seq studies (Lähnemann et al., 2020).

One big concern is RNA contamination in brain slices, particularly within the scope of snRNA-seq, which poses a significant challenge that must be carefully addressed during data processing. This contamination can originate from various sources, including

cytoplasmic RNA inadvertently introduced during nucleus isolation (Slyper et al., 2020), ambient RNA present in the tissue or reagents, and residual cellular debris (Caglayan et al., 2022). Additionally, the mechanical and chemical stresses involved in tissue dissociation and nuclear isolation can further contribute to RNA leakage, increasing the risk of contamination in sequencing data (Arceneaux et al., 2023). Mitigating RNA contamination is crucial for ensuring the accuracy and reliability of gene expression profiles derived from snRNA-seq data.

Since snRNA-seq is designed to capture only nuclear RNA, the presence of cytoplasmic RNA will distort the resulting gene expression data (Arceneaux et al., 2023). To address RNA contamination in snRNA-seq data, several strategies have been developed:

- Quality control and filtering: Implementing rigorous quality control measures, such as filtering out low-quality nuclei and reads that are not likely to be real cells, is crucial (Hong et al., 2022). For example, genes known to be cytoplasmic or mitochondrial are often excluded from downstream analyses to mitigate the effects of contamination (Zheng et al., 2017).
- Cell Ranger software: Developed by 10x Genomics, it filters out reads that do not align with the expected nuclear transcriptome (Zheng et al., 2017).
- EmptyDrops function in DropletUtils R-package: An algorithm that detects empty droplets in droplet-based single-cell RNA sequencing data, adapted for use in snRNA-seq to identify and discard droplets containing only ambient RNA (Lun et al., 2019).
- CellBender software: A tool that employs a Bayesian inference model to estimate the proportion of ambient RNA in each cell or nucleus, allowing for the subtraction of these contaminating reads from the final gene expression counts (Fleming et al., 2023).

These computational approaches have significantly improved the accuracy and reliability of snRNA-seq data by effectively mitigating RNA contamination, particularly in studies involving complex tissues like brain slices. While both acute and organotypic brain slices are widely used in the study of brain slice models, to date there has been no comprehensive, cell-type-specific transcriptomic comparisons between these two models. This gap limits our understanding of how culturing conditions affect gene expression across various cell types in the brain. Furthermore, although OBSCs are

known to undergo changes over time, the specific alterations in gene expression and cellular composition for each cell-type are not well characterized, particularly in comparison to acute brain slices.

1.6. Research aims and objectives

To address these gaps in the literature the overall aim of this thesis is to characterize cell-type-specific gene expression differences between OBSCs and acute brain slices.

Objectives:

- 1.1. Perform single-nucleus RNA sequencing on both OBSCs and acute brain slices to identify cell-type-specific gene expression profiles.
- 1.2. Quantify and compare the relative abundance of different cell types between OBSCs and acute brain slices.
- 1.3. Perform differential gene expression analysis for each cell-type for both OBSCs and acute brain slice conditions.
- 1.4. Conduct pathway enrichment analysis on differentially expressed genes for each cell-type.

By addressing these objectives, my research will significantly contribute to our understanding of the molecular differences between OBSCs and acute brain slices. For instance, identifying functional neuro-immune pathways could support the use of OBSCs in studies of long-term neuroinflammation or neurodegenerative disease progression. Conversely, if OBSCs show key deviations from acute brain slices, such as the absence of certain progenitor cell types or widespread downregulation of developmental pathways. In that case, it may limit their modelling of *in vivo*-like conditions. Ultimately, my research will be crucial for interpreting results from experiments using these models.

Chapter 2: Research Method

2.1. Animals

The mice were handled in accordance with the South African National Standard (SANS 10386:2008) and the Veterinary and Paraveterinary Professions Act (Act 19 of 1982), with approval from the UCT Research Animal Unit Ethics Committee (AEC 021/026). This study used C57BL/6 mice, housed in the animal facility under standard 12-hour light-dark cycles.

2.2. Hippocampal brain slice preparation

Brain slices were made from postnatal day 7 (P7) male or female C57BL/6 mice using the procedure described by Stoppini et al., (1991). Briefly, euthanasia was performed through cervical dislocation, followed by immediate decapitation to ensure minimal post-mortem delay. The brains were extracted from the skull and placed in cold dissection media, at 4 °C, to preserve tissue integrity. The dissection media consists of: Earles balanced salt solution (#E2888, Sigma-Aldrich, USA) with 6.1 g/l HEPES, 6.6 g/l glucose, and 5% 1M NaOH (all reagents were from Sigma Aldrich).

In the dissection media, the brain was cut into two hemispheres, and the hippocampal region was dissected out from each hemisphere. The hippocampi were then cut into thin, 350 µm, slices using the McIlwain tissue chopper. From this point, some brain slices were frozen immediately at -80 °C (acute brain slices), and the other group was incubated (OBSCs) and remained for 7 days in culture before freezing.

In the OBSCs group, 6 slices were plated per well on Millicell-CM membrane inserts (pore size: 0.4 µm) (#Z354988, Sigma-Aldrich, USA) in a 6-well plate with each well containing 1.2 ml growth media. The slices were cultured in a humidified incubator maintained at 35–37 °C with 5% carbon dioxide (CO₂). The growth media consisted of 23% (vol/vol) Earles balanced salt solution (#E2888, Sigma-Aldrich, USA); 50% (vol/vol) minimum essential medium (#M2279, Sigma-Aldrich, USA); 25% (vol/vol) heat-inactivated horse serum (#H1138, Sigma-Aldrich, USA); 2% (vol/vol) B27 (#17504044, Invitrogen, Life Technologies, USA) and 6.2 g/l D-glucose.

2.3. Single-nucleus RNA sequencing

2.3.1. Dataset generation for single-nucleus RNA sequencing

SnRNA-seq datasets were generated using the 10X Genomics platform. Hippocampal brain slices from each group, OBSCs and acute brain slices, were pooled together and stored at -80 °C. Each sample within each treatment group consisted of approximately 36 hippocampal brain slices, pooled from multiple animals and brain slice preparations from different days. This was to ensure adequate sample size and to minimise a batch effect. In total, 3 biological replicates were generated per group.

Nuclear isolation was performed by homogenizing the frozen slices in a lysis buffer, followed by cell lysis and centrifugation. The nuclear pellet was then resuspended in nuclear suspension buffer. Nuclear viability was estimated using a 20 µl of a 1:1 mixture of nuclear suspension and trypan blue, loaded onto a haemocytometer, and the nuclei were counted under a microscope, with a target nuclear viability of over 5%. After centrifugation and filtering through a 40 µm strainer, viability was rechecked using trypan blue or DAPI stain. Samples were continually on ice throughout the experiment.

The sequencing process followed the Chromium Next GEM 3' Single Cell 3 Reagent Kits v3.1 protocol, where the instructed concentration of nuclear suspension stock was used to achieve the desired number of nuclei. For the early samples, the target number of nuclei per sample was set at 10,000 (OBSC samples), but for subsequent samples, the target was adjusted to 6,000 (acute brain slices) nuclei per sample to improve sequencing depth. Due to the different experiment runs, the OBSC samples were collected in the early samples batch while the acute brain slices were collected in later samples. Sample dilutions with nuclease-water were combined with Master Mix reagents (59.1% RT Reagent B, 7.5% Template Switch Oligo, 6.3% Reducing Agent B, 27.4% RT Enzyme C). Samples were loaded into row 1 of the 10X Genomics Single Cell 3' Chip G, alongside Gel Beads (row 2) and Partitioning Oil (row 3). A 50% Glycerol solution filled empty wells. Using the 10X Genomic chromium controller which uses a microfluidics system, nuclei were encapsulated in oil droplets containing barcoded beads, enabling RNA-to-cDNA conversion with incorporated barcodes. Once GEM generation and barcoding were complete, cDNA library preparation commenced. This process involved cDNA PCR amplification, selecting transcripts based on size, attaching adaptors for sequencing, and introducing sample-specific indices to enable demultiplexing (i.e.,

tracing back to the different original samples because all samples were pooled together) with Illumina. The final libraries, passing different quality control checks, were sequenced via the Illumina Novaseq 6000 S2 flow cell machine according to the recommended read length, and resulting raw base calls were demultiplexed to generate fastq files. These fastq files were later used for computational analysis.

2.3.2. Single-nucleus RNA sequencing bioinformatics analysis

All bioinformatics analyses for the snRNA-seq were conducted using the high-performance computing facilities provided by the University of Cape Town's Information and Communication Technology Services (ICTS) team.

To align the paired-end sequencing reads, I employed the 10x Genomics Cell Ranger 7.1.0 count pipeline (Zheng et al., 2017). This tool mapped the reads to the mouse reference transcriptome, specifically the refdata-gex-mm10-2020-A version. This reference data was obtained from the 10X Genomics downloads portal. In addition to mapping, the pipeline also performed data filtering, removing any barcodes that were empty. Throughout this process, I utilized the default settings provided by the pipeline, which notably included the incorporation of intronic regions in the analysis. This allowed for an unbiased view of the data – i.e., including genes that were mapped in the intronic regions and not used for translation.

Ambient RNA removal

To analyze ambient RNA contamination, I adapted the method described by Caglayan et al. (2022). Briefly, ambient RNA markers in the dataset were identified using emptyDrops (Lun et al., 2019), and ambient RNA was subsequently removed using CellBender version 0.3.0 (Fleming et al., 2023).

I processed the raw feature-barcode matrix from the Cellranger version 7.1.0 output of each sample. For instance, in OBSC sample 1, the raw feature-barcode matrix was loaded, and a Seurat (version 4.3.0) object was generated using the CreateSeuratObject function. The data was then reordered based on UMI counts (ncount_RNA) in descending order, ensuring that cells with the highest UMI counts appeared first. This was achieved by extracting the metadata, sorting it, and reconstructing a new Seurat object with the

sorted metadata. After reordering, the top 20 000 cells were selected. Noting that I only retained 6000 to 10 000 nuclei per sample, the 20 000-cut-off ensured that real cells and empty droplets were retained. This subset was saved as a new Seurat object. Firstly, I used the `nuclear_fraction_annotation()` function from the DropletUtils version 1.28.0 package to calculate the nuclear fraction of each cell (Caglayan et al., 2022). These ratios, representing the proportion of reads mapping to introns versus exons, were added to the Seurat object metadata and visualized against UMI counts. Since some newly retained barcodes did not exclusively reflect ambient RNAs (e.g., they could represent doublets or low-quality nuclei from various cell types), clustering was performed to identify clusters with a high proportion of newly retained barcodes that were distinct from annotated cell types for each dataset. Ambient clusters were identified using the `ambclusterfind()` function, which classified clusters enriched in empty droplets as ambient. An excess number of barcodes were retained to ensure the inclusion of ambient clusters. PCA was applied and clusters were identified based on the proportion of empty droplets in each cluster. Clusters with a high proportion of empty barcodes (above a calculated cutoff, 1.5 times the proportion of barcodes in the data classified as empty) or the largest cluster by size (number of cells) were classified as ambient clusters and annotated in the metadata as such. Ambient clusters were further divided into high-intronic and low-intronic groups using an intronic read ratio cutoff of 0.2 for low and 0.4 for high. Genes enriched or highly abundant in these groups were subsequently annotated as ambient RNA markers.

Based on the ambient RNA markers identified through the emptyDrops analysis, I next employed CellBender for systematic ambient RNA removal. Through iterative runs of CellBender and comparisons with the ambient genes list previously identified by emptyDrops, I tested various parameter combinations to optimize the removal process. The changes in parameters included adjustments to thresholds, FPR, epochs, learning rates, and expected cell numbers. While optimal parameters varied across datasets, I found that `--low-count-threshold 100` consistently performed well across all samples (see Supplementary Table 1). Performing well meant that the number of cells/nuclei found by CellBender was similar to the Cellranger output. This combined approach - using computational identification of ambient RNA clusters, marker gene analysis, and parameter optimization - successfully reduced ambient contamination and enabled more accurate downstream analysis of the snRNA-seq data.

Quality control

After the RNA removal, the dataset underwent processing using the standard Seurat pipeline (Hao et al., 2021), implemented in either R version 4.0.5 (R Core Team, 2021) or 4.2.0 (R Core Team, 2022). The initial quality control process was performed to filter out low-quality nuclei and potential artefacts, ensuring the integrity of the data. To enhance data quality, I applied filters to eliminate low-quality nuclei and empty droplets that contained no nuclei. The filtering criteria retained only barcodes that met the following conditions: over 500 unique molecular identifiers (UMIs), more than 250 expressed genes, a $\log_{10}(\text{genes per UMI})$ exceeding 0.8, and a mitochondrial ratio below 0.2 (Amezquita et al., 2020).

Secondly, droplets that had captured more than one nucleus (i.e., doublets) were removed. To identify doublets, I utilized two tools: DoubletFinder version 2.0.6 (McGinnis et al., 2019) and Scrublet version 0.2.3 (Wolock et al., 2019). These tools differ in their accuracy, with DoubletFinder being the most precise, while Scrublet has good specificity but lower sensitivity (Xi & Li, 2021). Both methods begin by generating artificial doublets through combining random pairs of observed transcriptomes - essentially adding together the gene expression profiles of two different cells to simulate what a doublet's profile would look like. These artificial doublets serve as a reference point for identifying real doublets in the dataset. After creating these simulated doublets, both tools use Principal Component Analysis (PCA) to reduce the dimensionality of the data and place both real and artificial cells in the same mathematical space. They use K-Nearest Neighbors (KNN) in the reduced space to calculate a doublet score by analyzing the proportion of artificial doublets among each cell's k-nearest neighbors. A key challenge both tools address is the detection of homotypic doublets (two cells of the same type) versus heterotypic doublets (two different cell types), as homotypic doublets are harder to identify since their transcriptional profiles closely resemble single cells of that type. The tools differ in their final classification approach: DoubletFinder requires users to specify an expected doublet rate (typically calculated as 0.8% per 1000 cells captured in the experiment) and uses this rate along with an optimized nearest-neighbor parameter (pK) to make its final classifications, while accounting for the expected homotypic doublet rate based on cell-type proportions in the sample. Scrublet, on the other hand, takes a more automated approach by analyzing the distribution of doublet scores across

all cells and fitting this to a model with two populations (singlets and doublets), allowing it to automatically determine the classification threshold without user input about expected doublet rates.

For DoubletFinder, I set the threshold based on an estimated 10% homotypic doublet rate. Scrublet's threshold was set at 0.3, corresponding to the separation point of peaks in a bimodal doublet score histogram generated from the data. To increase the probability of correctly identifying real doublets, the final set of doublets included all those identified by DoubletFinder or Scrublet (i.e., I took the union of their outputs). These identified doublets were then removed from the merged dataset of all 6 samples – 3 OBSC samples and 3 acute brain slice samples.

Sample integration and clustering

After quality control, the data was normalized using `SCTransform()` to adjust for sequencing depth variations across cells and to stabilize the variance. This prevents highly expressed genes from dominating the variation sources. For downstream integration, I focused on the top 2000 variable genes and regressed out the mitochondrial RNA ratio for each barcode, as only nuclear and not mitochondrial transcripts (from the cytoplasm) were expected in the data. Cell cycle phase testing revealed that the different cell cycle phases did not significantly contribute to dataset variation and so this was not regressed out.

Sample integration was performed using Seurat's `FindIntegrationAnchors()` function, which identified anchors between samples based on the top 2000 variable features and 30 dimensions. This was followed by `IntegrateData()` to align similar cells across all samples using a canonical correlation analysis. This integration step was crucial for ensuring consistent cell-type identification across samples.

PCA was then performed on the integrated dataset to reduce dimensionality while preserving key variation patterns. Based on an elbow plot, the top 30 principal components were selected for downstream analysis. Finally, I clustered the data using the `FindNeighbors()` and `FindClusters()` functions, which employed a graph-based approach to group cells with similar gene expression profiles. This created a KNN graph where nodes represent cells and edges connect cells with similar expression patterns.

The graph was then partitioned using the Louvain algorithm for community detection to identify distinct cell populations within the dataset. The clustering results were visualized using Uniform Manifold Approximation and Projection (UMAP), which showed clear separation between different cell types.

Cluster annotation and marker identification

Once the clusters were identified, the next step was to determine their corresponding cell types. Cluster annotation was performed using both automated tools and manual inspection, including SCSA (Cao et al., 2020), Allen Brain label transfer, and visualization of known marker genes through bubble and feature plots. Differentially expressed genes (DEGs) for each cluster were identified using the `FindAllMarkers()` function, with a resolution of 0.4, resulting in 28 clusters. SCSA, an automated annotation tool, was then used to compare these DEGs with known cell-type markers from databases such as CellMarker (Zhang et al., 2019), CancerSEA (Yuan et al., 2019), and a custom database from mousebrain.org (Zeisel et al., 2018). SCSA provided predicted cell-type annotations for each cluster along with confidence scores.

Manual inspection was conducted to supplement the automated approach by visualizing known cell-type-specific markers using feature and bubble plots and cross-referencing these with the DEGs identified by `FindAllMarkers()`. While automated and manual methods generally agreed, ambiguous clusters were also identified. Ambiguous clusters had overlapping marker expression for multiple cell types (Supplementary Figure 1). These clusters were subsequently removed from the dataset as they lacked clear cell identities. The annotations that I obtained at this stage are further referred to as user-defined.

Next, I performed label transfer using the Allen Mouse Brain atlas as a reference to fully validate my annotations. Members of our lab downloaded the Whole Cortex & Hippocampus - 10x Genomics (2020) with 10x-Smart-Seq Taxonomy (2021) from the Allen Brain database, filtering for hippocampal nuclei that expressed between 500 and 7500 genes. The processing steps that were initially used for my dataset, was also applied to the Allen reference data, including SCTransform for normalization and scaling. Using Seurat's `FindTransferAnchors()` function with SCT normalization, anchor nuclei between

the datasets were identified, projecting my dataset onto the Allen reference data PCA structure using the top 30 dimensions. TransferAnchors() then assigned labels to the nuclei in my dataset based on their anchored counterparts in the Allen dataset. The Allen annotations provided a higher resolution for the neuronal populations and showed distinct sub-classes.

After obtaining both user-defined and Allen annotations, a stringent filtering method was applied to create four types of cluster annotations: (a) coarse annotations, (b) nuanced annotations, (c) Allen mouse brain clusters, and (d) subclass annotations. The coarse annotations represented broad cell types without any subdivisions for excitatory and inhibitory neurons. These broad cell types were astrocytes, microglia, OPCs, oligodendrocytes, ependymal cells, endothelial cells, pericytes, excitatory neurons, dentate progenitors, dentate gyrus cells, cajal-Retzius cells, inhibitory neurons. In contrast, the nuanced annotations included these subdivisions, providing a more detailed classification of neuronal populations. Excitatory neurons had the following subdivisions: CA1, CA2/CA3, CA3, and Subiculum/Entorhinal neurons. While inhibitory neurons were subdivided into: Pvalb, Lamp5, Sst, Sncg, and Vip neurons.

The Allen mouse brain clusters and subclass annotations followed a similar structure to the coarse and nuanced annotations. However, these were based exclusively on cells identified through the Allen label transfer. This ensured that the Allen-derived annotations remained distinct from the user-defined methods.

For the user-defined coarse clusters, the approach emphasized agreement between all manual and automated annotation methods. This included retaining cell types that were not identified by the Allen label transfer if supported by other methods. For instance, the Allen label transfer did not have the 'ependymal cells' and 'OPCs' annotations while the other methods identified this cell-type in my dataset. In this case, I retained the annotations of 'ependymal cells' and 'OPCs' in my user-defined annotations. In the case of OPCs, a further criterion for retention in my user-defined annotations, was if they appeared in the Allen annotations as Oligos. At the same time, subdivisions for excitatory and inhibitory neurons were incorporated using the Allen subclass annotations. This combined strategy ensured both flexibility and consistency in the final annotations.

Differential expression analysis

Following cluster annotation, differential expression (DE) analysis was performed using a pseudobulk approach with DEseq2 version 1.36.0 (Love et al., 2014) and related tools like ashR version 2.2-47 (Stephens, 2016), tidyverse version 1.3.1, Matrix.utils version 0.9.8, pheatmap version 1.0.12, and ggplot2 version 3.3.3. Pairwise DE analysis was conducted between OBSCs and acute brain slices based on their coarse cluster annotations.

Adhering to DEseq2 guidelines for single-cell data, gene expression counts were extracted from the Seurat object, and metadata were used to define sample and cluster groupings. A pseudobulk matrix was created by aggregating counts across cells within each cluster-sample combination using the `aggregate.Matrix()` function from the Matrix.utils package. The resulting matrix contained genes as rows and cluster-sample pairs as columns. Metadata for samples and clusters was reformatted and joined to include information on experimental groups (OBSCs and acute brain slices), batches (the snRNA-seq experiment rounds), and sample identifiers.

Cluster-specific DE analyses were performed using DEseq2. For each cluster, a DEseqDataSet was created from the `DEseqDataSetFromMatrix()` function using the pseudobulk matrix and associated metadata. Gene expression counts were normalized internally by DEseq2, and rlog transformation was applied for visualization. Principal component analysis (PCA) plots were generated using the `plotpca()` function to evaluate sample clustering based on group, batch, and sample identifiers. PCA plots generated were color-coded by `group_id` (brain slice preparation), `sample_id` (the different samples), and `batch` (the snRNA-seq experiment rounds). The design used was `~group_id` as the primary condition of interest, without any batch corrections to allow for a more unbiased comparison between the two groups.

Differential expression was assessed using the Wald test, comparing specified experimental groups (e.g., OBSCs vs. acute brain slices). Log fold-change shrinkage was applied using the ashR method provided by the apegglm package version 1.30.0 to ensure reliable estimates. Genes were considered significantly differentially expressed if the adjusted p-value (`padj`) was <0.05 and $|\log_2 \text{fold change}| \geq 0.58$.

Enrichment analysis

For pathway analysis, Enrichr version 3.0 (Chen et al., 2013) was used to perform enrichment analysis on individual gene sets that were either all upregulated or downregulated for each cell-type. This step was critical to understanding the biological pathways associated with DEGs and how these pathways varied between cell types. By analyzing upregulated and downregulated genes separately, the analysis provided more precise insights into the specific functional processes enriched in each direction of gene regulation. Enrichr identifies pathway enrichment by calculating the overlap between the input gene list and predefined gene sets using Fisher's exact test (Xie et al., 2021). This statistical method evaluates whether the observed overlap is greater than expected by chance, providing a measure of significance for each pathway. Enrichr ranks pathways based on relevance and adjusts p-values using the Benjamini-Hochberg method to correct for multiple comparisons. Only pathways with an adjusted p-value (p_{adj}) < 0.05 were considered significantly enriched. This threshold ensured that the results were statistically robust while minimizing the risk of false positives. Error handling ensured that cell types without enriched pathways or upregulated genes were skipped, enabling uninterrupted execution.

The GO Biological Processes 2021 database was selected as the reference for enrichment analysis due to its comprehensive coverage of gene sets related to biological processes and this was the database that other studies in the lab have used. Comparing the DEG sets against this database allowed for the identification of pathways that were highly relevant to the biological context of the data.

Proportions calculations

To compare cellular composition between OBSCs and acute brain slices, I analyzed the proportions of 12 distinct cell types across six samples from the two brain slice preparation groups. Using the tidyverse version 1.3.1 and dplyr version 1.0.9 packages in R version 4.0.5, I transformed the data into long format with the `pivot_longer()` function. Proportions for each cell-type within each sample were calculated as the nuclei count of the cell-type divided by the total nuclei count in the respective sample (e.g., the proportion of astrocyte nuclei in the OBSC 1 sample). Samples were categorized as "acute brain slices" or "OBSCs" using a custom grouping function. I visualized these

proportions using ggplot2, creating stacked bar plots and calculating cumulative proportions.

To compare cell-type distributions between OBSCs and acute brain slice groups, I calculated average proportions for each group by summing the proportions of each cell-type across individual samples within a group and dividing by the number of samples in that group (e.g., summing astrocyte proportions across OBSC 1, OBSC 2, and OBSC 3, then dividing by 3). I used these average proportions to compute scaled proportions for each cell-type within the OBSC and acute groups, employing the `group_by()` and `summarise()` functions from dplyr. Visualizations of these scaled proportions and associated statistical results, such as p-values and adjusted p-values, were created using ggplot2. Custom colour palettes ensured consistency across plots. This approach provided a nuanced understanding of cell-type distribution differences between OBSCs and acute brain slices, accounting for both within-sample proportions and between-group comparisons.

Statistical analysis for proportions

Statistical analysis was performed to compare the proportions of cell types between OBSCs and acute brain slices. First, the Shapiro-Wilk normality test was applied to determine whether the data for each cell-type followed a normal distribution. For normally distributed cell types, Welch's t-test was used, while the Mann-Whitney U test was applied for non-normally distributed cell types. Welch's t-test was chosen due to unequal variances between the groups, as the OBSC group had higher counts per sample than the acute group.

To account for multiple comparisons, I applied Bonferroni (Henry, 2015) and Benjamini-Hochberg corrections (Haynes, 2013) using the `p.adjust()` function with the "bonferroni" and "BH" methods, respectively. Using both Bonferroni and Benjamini-Hochberg corrections balances stringency and sensitivity, with Bonferroni minimizing false positives and BH maximizing true positive detection. A significance threshold of $p < 0.05$ was used for all tests. A difference in proportions for each cell-type was considered significant if it was identified as significant by either of the two tests. This comprehensive statistical approach ensured robust and reliable results.

Chapter 3: Results

3.1. Utilising single nucleus RNA sequencing to compare gene expression in acute and organotypic brain slices

To quantify differences in gene expression between OBSCs and acute brain slices, I performed snRNA-seq on isolated hippocampal nuclei (Figure 3.1B). The nuclei were extracted from flash-frozen brain tissue following the method described by Guillaumet-Adkins et al. (2017). Sequencing libraries were then constructed using the 10x Genomics platform and sequenced using Illumina next-generation sequencing technology. These tools have been widely used to capture the complex gene expression profiles of brain tissues (Zheng et al., 2017).

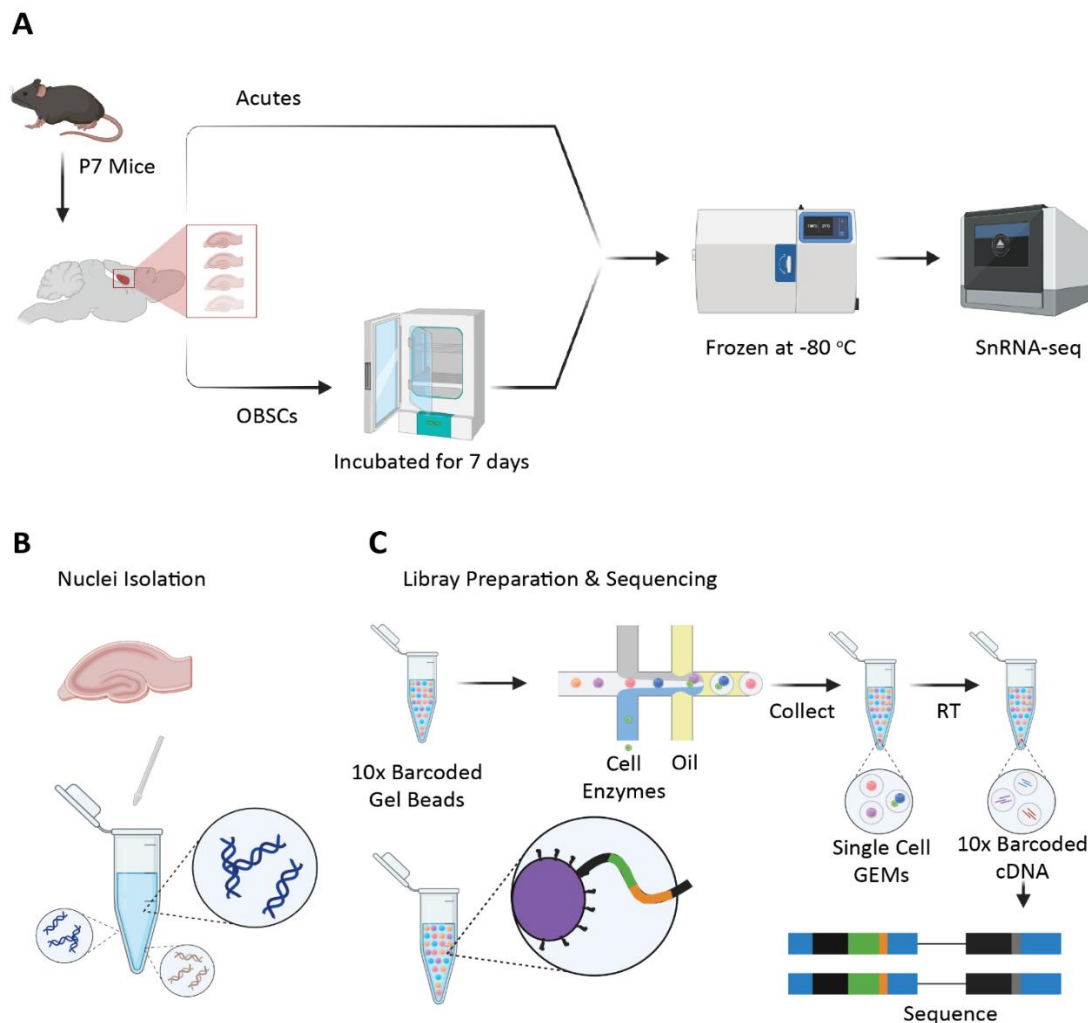


Figure 3.1) Brain slice preparation yielded healthy tissue for snRNA-seq. **A)** Hippocampal brain

slices were prepared from postnatal day 7 mice. After preparation, the acute brain slice group (Acutes) was frozen at -80°C. In contrast, the organotypic brain slice culture (OBSC) group was first cultured for 7 days before being frozen at -80°C. The slices were then further processed for snRNA-seq. **B)** Nuclei were isolated from the two brain slice preparation groups by homogenising the tissue in a lysis buffer. **C)** Sequencing library preparation was performed using the 10x Chromium Single Cell 3' Gene Expression library kit. This process included the generation of gel bead-in-emulsion (GEM), barcoding, and final library preparation. Sequencing was then conducted via Illumina. The raw sequencing data was processed using Cell Ranger version 7.1.0 software. Further computational analysis was performed in R using the Seurat (Hao et al., 2021) analysis pipeline.

3.2 Pre-processing of the data using CellBender to remove ambient RNA contamination from the brain tissue

In snRNA-seq, downstream analysis typically assumes that each droplet captures RNA exclusively from one nucleus (or occasionally multiple nuclei in doublets or multiplets). However, this assumption is often challenged by ambient RNA contamination within the droplets. Such contamination can arise from non-nuclear RNA in the cytoplasm or from degraded nuclei. Consequently, two scenarios may occur: "empty droplets," where only ambient RNA is captured without a nucleus, and mixed-content droplets, where ambient RNA is captured alongside nuclear RNA from the enclosed nucleus or nuclei. Both scenarios can significantly confound downstream analysis, potentially leading to misinterpretation of the sequencing data.

To identify whether ambient RNA might be a problem in my dataset, I first examined elbow plots from my data, an example plot from an OBSC sample is shown in Figure 3.2A. Given the clear presence of an "empty droplet plateau," which indicates a high ambient RNA background signal, I used the emptyDrops function developed by Lun et al., (2019) which is also implemented in the DropletUtils package available from the Bioconductor project (Huber et al., 2015) to identify and characterize ambient RNA contamination in my samples (Figure 3.2B-D). I found that the ambient cluster reflecting empty droplets was characterised by low intronic read ratios (i.e., the ratio of introns to exons) compared to all other clusters in the samples (Cluster '0' in Figure 3.2D). Nuclear RNA typically has higher intronic content due to the presence of pre-mRNA. Therefore, the fact that the

ambient cluster '0' had such a low intronic read ratio indicates that my samples' source of ambient RNA contamination is predominantly cytoplasmic.

To correct for ambient RNA contamination in my data, I processed the raw output data from Cell Ranger version 7.1.0 (Zheng et al., 2017) using CellBender version 0.30 software, which has been shown to substantially reduce ambient RNA contamination in single-nucleus RNA datasets such as my own (Fleming et al., 2023). CellBender uses machine learning techniques to identify the RNA profile of empty droplets and remove this ambient noise signature from every droplet. Considerable settings optimization was required to achieve optimal performance (Supplementary Table 1). On average, CellBender removed 32.27% of the counts of non-empty droplets in OBSCs, compared to 21.10% in acute brain slices. This resulted in a more precise and reliable transcriptomic profile of the different nuclei. This improvement is evident in Figure 3.2F (with CellBender), where the distinct clustering of nuclei after sample integration is more pronounced and distinguishable than in Figure 3.2E (without CellBender). What is particularly evident is that ambient RNA correction removes cluster non-specific nuclei in the central area of the UMAP plot.

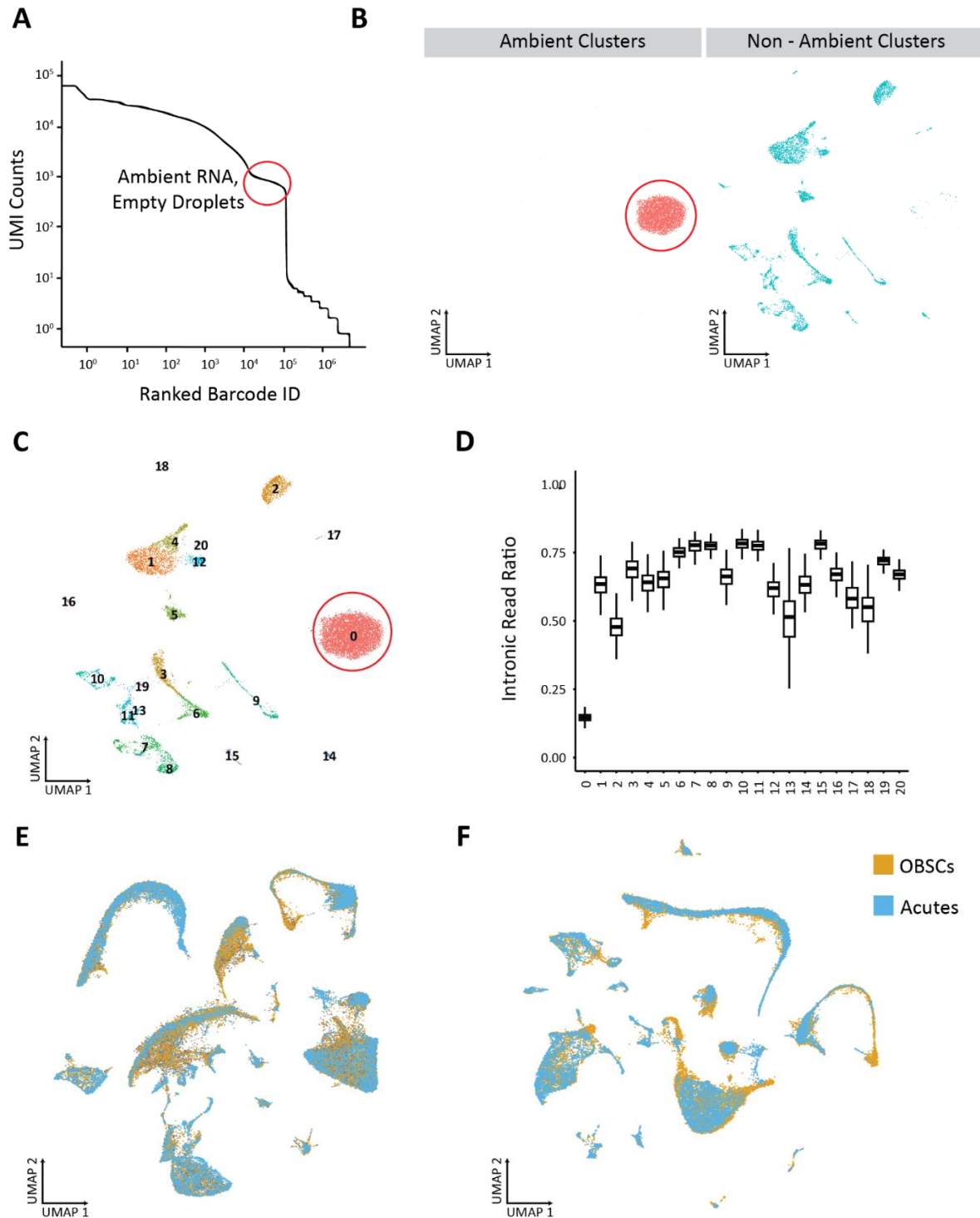


Figure 3.2) Ambient RNA characterization and removal using CellBender. **A)** An Elbow plot to visualize the distribution of UMI counts across barcodes or droplets from a single organotypic brain slice culture sample. Droplet barcodes of cells were ordered in descending order of their total UMI counts; at the upper left side of the UMI curve, there are droplets that contain actual cells and thus have very high UMI counts. On the other hand, the curve's plateau shows droplets containing only ambient RNA; these have between 700 and 1000 UMI counts, demonstrating

significant ambient RNA contamination in this representative sample. **B)** Uniform Manifold Approximation and Projection (UMAP) of a single OBSC sample with the ambient cluster in pink. **C)** Same UMAP with the clusters labelled using a K-nearest neighbor (KNN) technique. The ambient cluster is labelled '0'. **D)** Intronic read ratios of all clusters in 'C'. Cluster 0 has an intronic read ratio of 0.14, much lower than the rest of the clusters, which fall between 0.5 and 0.75. **E)** UMAP plot of integrated samples (3 OBSCs, 3 acute brain slices) without CellBender in the processing pipeline. OBSCs in orange and acute brain slices in blue. **F)** UMAP plot of the same integrated samples with CellBender.

3.3 Quality control and normalization in organotypic brain slice cultures and acute brain slices

Another potential source of artifacts in my data is the presence of doublets or multiplets — droplets that have captured two or more nuclei, resulting in a higher number of genes and UMI counts. I used two well-established doublet removal tools: Doublet Finder (mcginnis et al., 2019) and Scrublet (Wolock et al., 2019), on each of the six individual samples to remove a total of 5,540 doublets (11.17%) out of 49,592 droplets from the integrated dataset. The remaining droplets underwent further quality control steps — following the Seurat pipeline — to ensure that only high-quality droplets containing single nuclei were retained.

Since the goal was to isolate only nuclei during the process, without any cytoplasmic components, I first removed any cells with a mitochondrial ratio greater than 20%. The mitochondrial ratio measures the percentage of cell reads originating from mitochondrial genes. Next, I removed low-quality cells with fewer than 250 genes or a UMI count below 500 or above 35,000. Following this process, I retained a total of 33,922 cells (68.40%) out of the initial 49,592 droplets across all six samples (Figure 3.3A). The OBSC samples had a higher droplet count than the acute brain slices (Figure 3.3A). Once the data was cleaned, as expected, for all 6 samples, UMI counts were strongly (positively) correlated to the number of genes detected per droplet (Figure 3.3B). Further, normalization was performed using the SCTransform() function, which adjusts for technical variations in read depth.

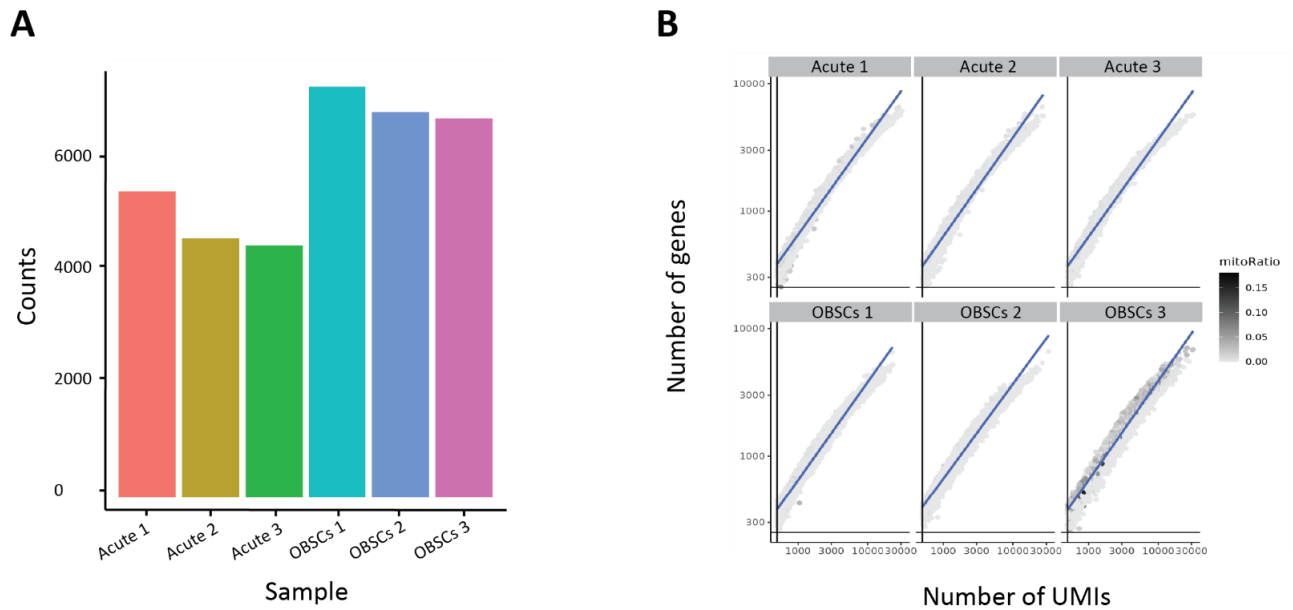


Figure 3.3) Quality control and normalization in organotypic brain slice cultures and acute brain slices. **A)** Bar plot showing the filtered counts of droplets (which correlate to the number of individual nuclei or cells) in each of the 6 samples after the quality control step. **B)** Scatter plots showing the linear correlation between the number of UMIs and the number of genes in the 6 samples.

3.4 Comparative analysis of cell clusters between organotypic brain slice cultures and acute brain slices

The UMAP of nuclei from the integrated samples (Figure 3.2F) demonstrates that the nuclei “cluster” according to differences in their gene expression profiles. Nuclei with similar gene expression profiles are in closer spatial proximity on the UMAP plot than nuclei with different gene expression profiles. To assign the nuclei to distinct clusters, I used the FindNeighbors() and FindClusters() functions in Seurat, which uses a K-nearest neighbor (KNN) graph-based method to group cells into clusters according to the largest sources of variation in the data (see Research Method section). At a clustering resolution of 0.4, 28 clusters were identified in the integrated dataset (Figure 3.4A). To investigate whether certain clusters may be unique to OBSCs or acute brain slices, or whether there were differences in the number of cells from the two conditions in any of the different clusters between OBSCs and acute brain slices, I compared the number of cells (nuclei counts) from each condition in each cluster (Figure 3.4B).

In Cluster 1 (Figure 3.4B), the mean nuclei count was significantly higher in the OBSCs group, with a mean of 610 when compared to the acute brain slices group, with a mean of 344.67 ($p = 0.02081$, Welch's t-test). Similarly, Cluster 10 with a mean nuclei count of 297.67 in OBSCs versus 43.33 in acute brain slices ($p = 0.01065$, Welch's t-test), Cluster 13 with a mean nuclei count of 145.67 in OBSCs versus 78.33 in acute brain slices ($p = 0.01414$, Welch's t-test), and Cluster 14 with a mean nuclei count of 195.33 in OBSCs versus 9.67 in acute brain slices ($p = 0.00580$) showed higher mean counts in OBSCs. In contrast, Cluster 5 had a significantly lower mean nuclei count of 5.73 in OBSCs compared to 253.67 in acute brain slices ($p = 0.00472$, Welch's t-test) as well as Cluster 16 with a mean nuclei count of 17 in OBSCs versus 164 in acute brain slices ($p = 0.00495$, Welch's t-test).

These mean nuclei counts provide an initial comparison between the two conditions but do not account for differences in sample sizes, which is a potential confounding factor given that the OBSC samples had more total cells than the Acute samples. To address this, I calculated the proportion of cells that belonged to each cluster within a sample and averaged this across all samples in the group. For example, 17% of the cells in the Acute 1 sample were from Cluster 0. I repeated this calculation for the remaining Acute 2 and Acute 3 samples and averaged the percentages. This method accounts for the differences in sample sizes within the two groups (OBSCs versus acute brain slices). When accounting for differences in sample sizes by comparing proportions, the percentage of samples made up by Cluster 10 was 4.74% in OBSCs and 0.95% in acute brain slices ($p = 0.02967$, Welch's t-test) and in Cluster 14 was 3.07% in OBSCs and 0.21% in acute brain slices ($p = 0.00292$, Welch's t-test). In addition to showing significantly higher count proportions in OBSCs compared to acute brain slices, these clusters also had significantly different mean counts between the two groups, as previously shown. Conversely, Cluster 4 had a significantly lower cell count percentage average in OBSCs (5.88%) compared to acute brain slices (7.41%) ($p = 0.01755$, Welch's t-test).

To compare clusters, which constitute a small fraction of the total number of cells in a sample, I normalised the proportions to ensure that the fraction of cells belonging to either OBSCs or acute brain slices in each cluster sum to 1 (100%), allowing for a more accurate comparison of how each group contributes to that cluster (Figure 3.4D). I considered proportions lower than 20% and higher than 80% to indicate substantial

differences between the slice preparations. Clusters 10, 14, 17, and 18 were substantially more composed of OBSC cells than Acute cells. In contrast, clusters 16 and 28 were predominantly constituted of cells from acute brain slices as opposed to OBSCs. Interestingly, most of these clusters are located to the right of larger superclusters (Figure 3.4A), which contain multiple clusters (e.g., 3, 10, 14), suggesting that the superclusters have a polarity affected by brain slice preparation. Clusters 10 and 14 can be seen clearly on the stacked proportions graph (Figure 3.4D) as above the 80% line with higher proportions of OBSCs compared to acute brain slices. Although I did not perform any pseudo-time analysis on the development of the clusters, there is a possible indication of a likely pseudo-time trajectory in these superclusters.

Collectively, this analysis reveals that other than Clusters 16 and 28, cells from OBSCs are well represented in the same clusters as those seen in Acute slices. However, four clusters (10, 14, 18, and 27) appear relatively unique to OBSCs. Furthermore, it is clear that in clusters where both brain slice preparations are well represented, the proportions differ. This suggests that the two brain slice preparations likely differ in terms of the presence of specific cell types and within cell-type abundance.

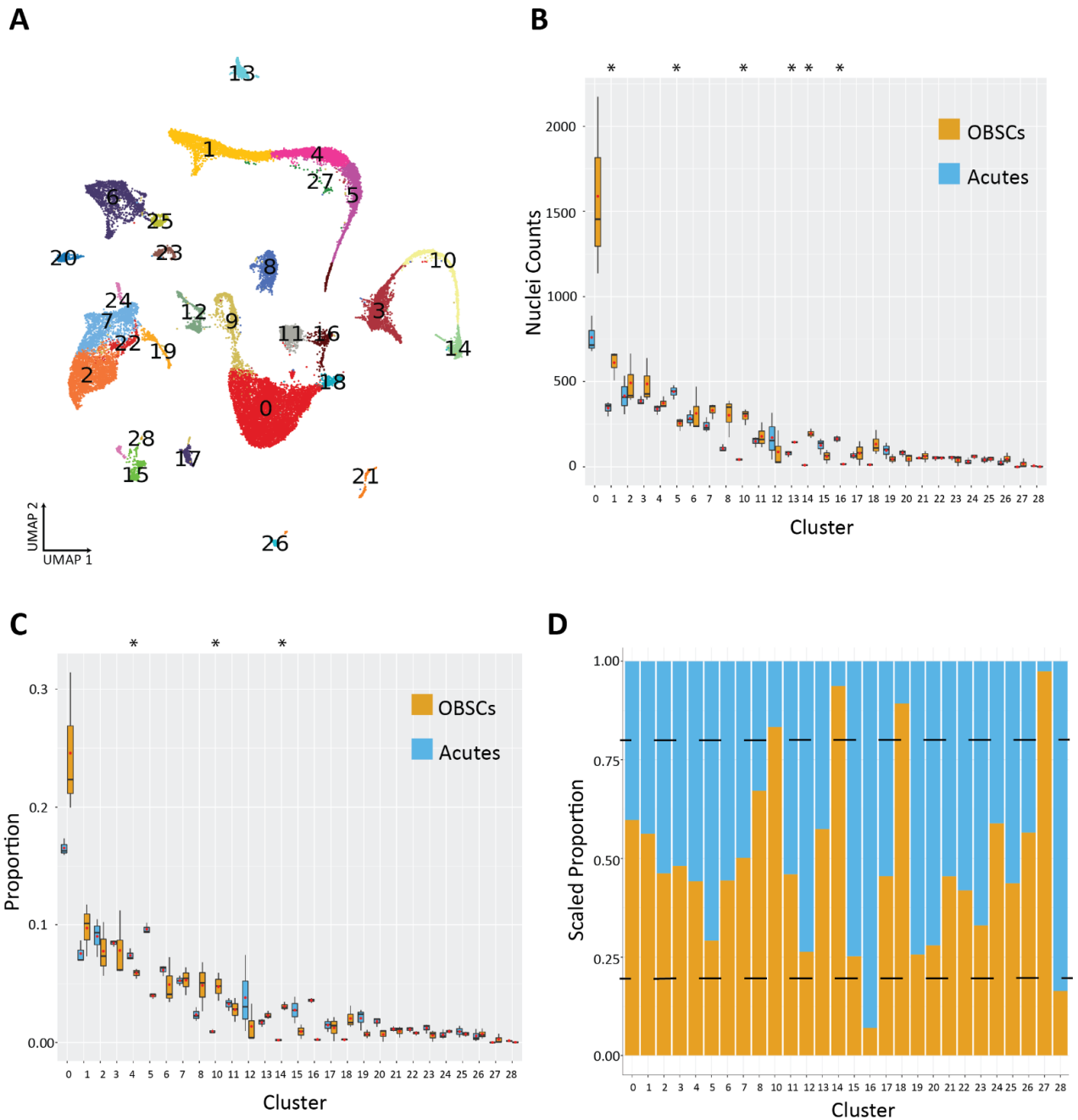


Figure 3.4) Comparative analysis of cell clusters between organotypic brain slice cultures and acute brain slices. **A)** UMAP plot with integrated samples where nuclei are clustered, coloured, and numbered using Seurat's K-nearest neighbor (KNN) graph-based method with a resolution of 0.4. **B)** Boxplot showing the differences in cell counts between the two groups across all the different clusters. (* $p < 0.05$; Welch's t-test). **C)** Boxplot showing the differences in the average proportion of cells that belong to a cluster between the two groups across the different clusters (* $p < 0.05$; Welch's t-test). **D)** Bar plot showing differences in scaled proportions between the two groups across all the different clusters. Dotted lines indicate 0.2 and 0.8 thresholds respectively.

3.5 Differential cell-type composition in organotypic brain slice cultures versus acute brain slices

To explore which cell types were present in the two brain slice preparations, I annotated the clusters using the steps described in the Research Method section. I identified 12 major cell types and labelled these as a primary ‘coarse’ level of annotation (Figure 3.5.1A). I then applied the Allen label transfer to further classify different excitatory neuronal populations (such as CA1, CA2/CA3, CA3, and Subiculum/Entorhinal cells) and inhibitory neuronal populations (such as Sncg, Vip, Lamp5, Sst, and Pvalb) found in the Allen Mouse Brain annotations onto my clusters. This process resulted in a further ‘nuanced’ level of annotations (Figure 3.5.1B, C). The label transfer also validated my coarse level of annotations, as it showed consistent classifications across the different clusters. Further validation was conducted using gene marker identification. I plotted known markers for the 12 cell types present in my data to verify their presence in the expected cell-type clusters (Figure 3.5.1D). Importantly, as a validation of the ambient RNA removal, RNA markers for specific cell types were not seen as being substantially expressed in other cell types, confirming the cell-type specificity of my dataset.

Next, I examined whether there were differences in the annotated cell-type clusters between OBSCs and acute brain slices. To quantify the differences in cell-type clusters between the two groups, I compared the number of nuclei from each group within a cluster. I also normalised these counts to the total number of nuclei in that sample to determine what proportion of nuclei comprise a particular cell-type in OBSCs versus acute brain slices.

The results showed significant differences in mean counts for several cell types. For instance, the number of nuclei (Figure 3.5.2A) annotated as oligodendrocytes (Figure 3.5.1B) was significantly higher in OBSCs compared to acute brain slices with a mean of 195.33 in OBSCs versus 9.67 in acute brain slices ($p = 0.00580$, Welch’s t-test). The oligodendrocyte cluster is composed of the predominantly OBSC unique cluster 14 identified in the previous section. In contrast, for dentate progenitors cells, OBSCs had a significantly lower mean nuclei count of 17 than the 164 in acute brain slices ($p=0.00495$, Welch’s t-test).

When accounting for differences in sample sizes by comparing proportions (Figure 3.5.2B), I observed a similar pattern as above when looking at mean nuclei counts. The

percentage of samples made up by the Oligodendrocyte cluster was 3.07% in OBSCs and 0.21% in acute brain slices ($p = 0.00292$, Welch's t-test). I observed the opposite for the dentate progenitors, where the percent of samples made up by this cell-type was 0.27% in OBSCs and 3.58% in acute brain slices ($p = 0.00015$, Welch's t-test). In addition, the dentate gyrus cell cluster was now significantly different, with 19.84% of nuclei in OBSCs versus 24.58% in acute brain slices being made up of this cell-type ($p = 0.04863$, Welch's t-test).

To more easily compare how each cell-type cluster is represented either in OBSCs or acute brain slices, even for less well-represented cell types, I normalised the proportions to ensure that the fraction of cells belonging to OBSCs and acute brain slices for each cell-type sum to 1 (100%) as shown in Figure 3.5.2C. Again, I considered proportions lower than 20% and higher than 80% to indicate substantial differences in cell-type composition between the slice preparations. This confirmed that oligodendrocytes (primarily present only in OBSCs) and dentate progenitors (mostly in acute brain slices) were the most substantially different. Nonetheless, it was also readily apparent that in the OBSC samples, the major glial cell types (astrocytes, microglia, OPCs, and oligodendrocytes) were overrepresented when compared to acute brain slices. Conversely, the neuronal cell types, dentate gyrus cells, excitatory neurons, and particularly inhibitory neurons, were underrepresented in OBSCs compared to acute brain slices (Figure 3.5.2C).

In summary, my data shows that oligodendrocytes are relatively unique to OBSCs, while dentate progenitor cells are unique to acute brain slices. Furthermore, glial cell types are overrepresented, and neuronal cell types are underrepresented in OBSCs compared to acute brain slices.

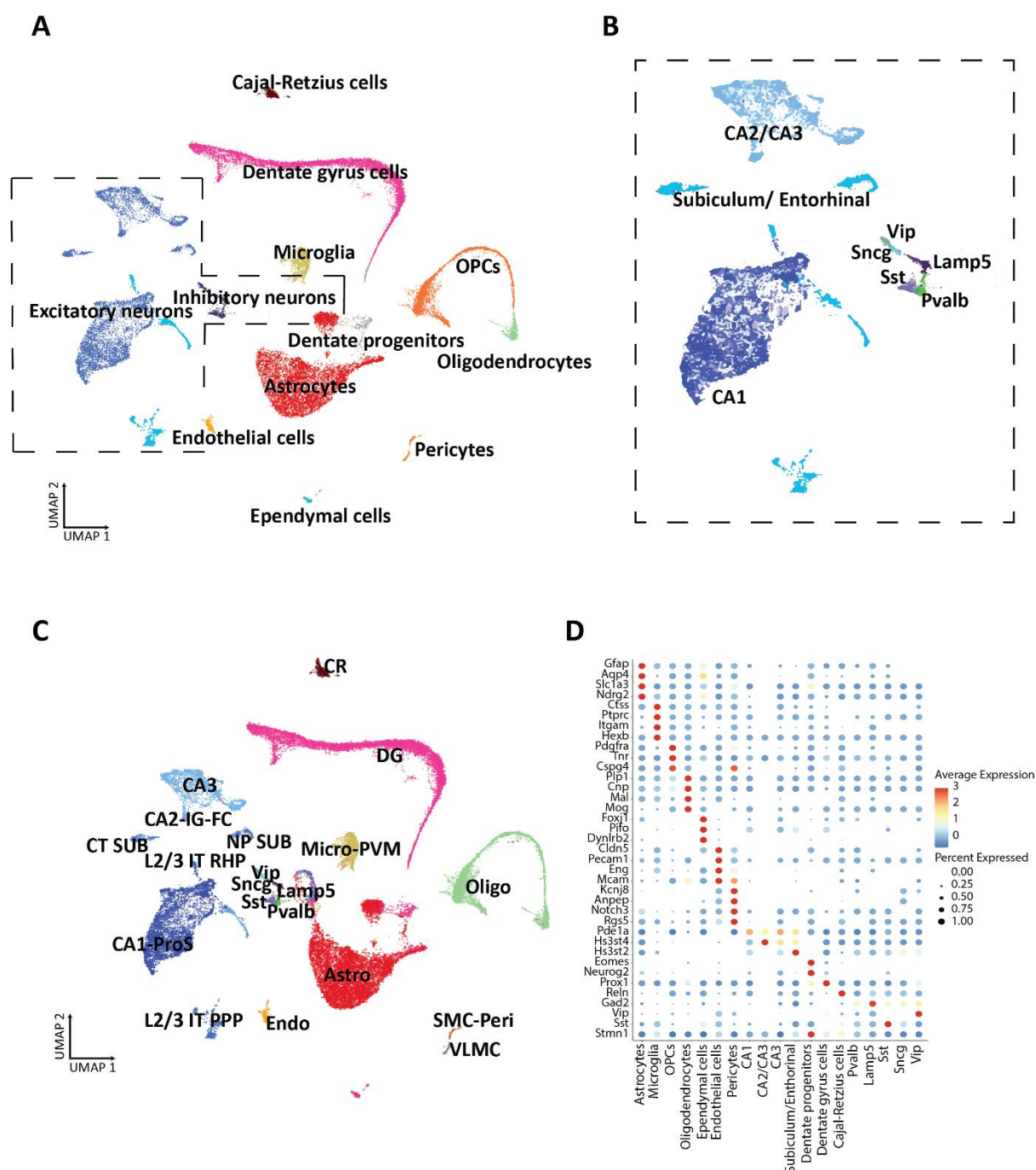


Figure 3.5.1) Cell-type composition in the integrated samples. **A)** UMAP plot of 12 user-defined coarse clusters using automated and manual methods; hashed inset denotes nuanced neuronal sub-populations annotated using the Allen Mouse Brain annotations in ‘**B**’. **C)** UMAP plot of sub-clusters annotated using the label transfer method with the Allen Mouse Brain hippocampal database as a reference. **D)** Bubble plot highlighting the percentage and average expression of cell-type-specific markers for the different cell-type cluster annotations, including the nuanced clustering of excitatory and inhibitory neuronal cells.

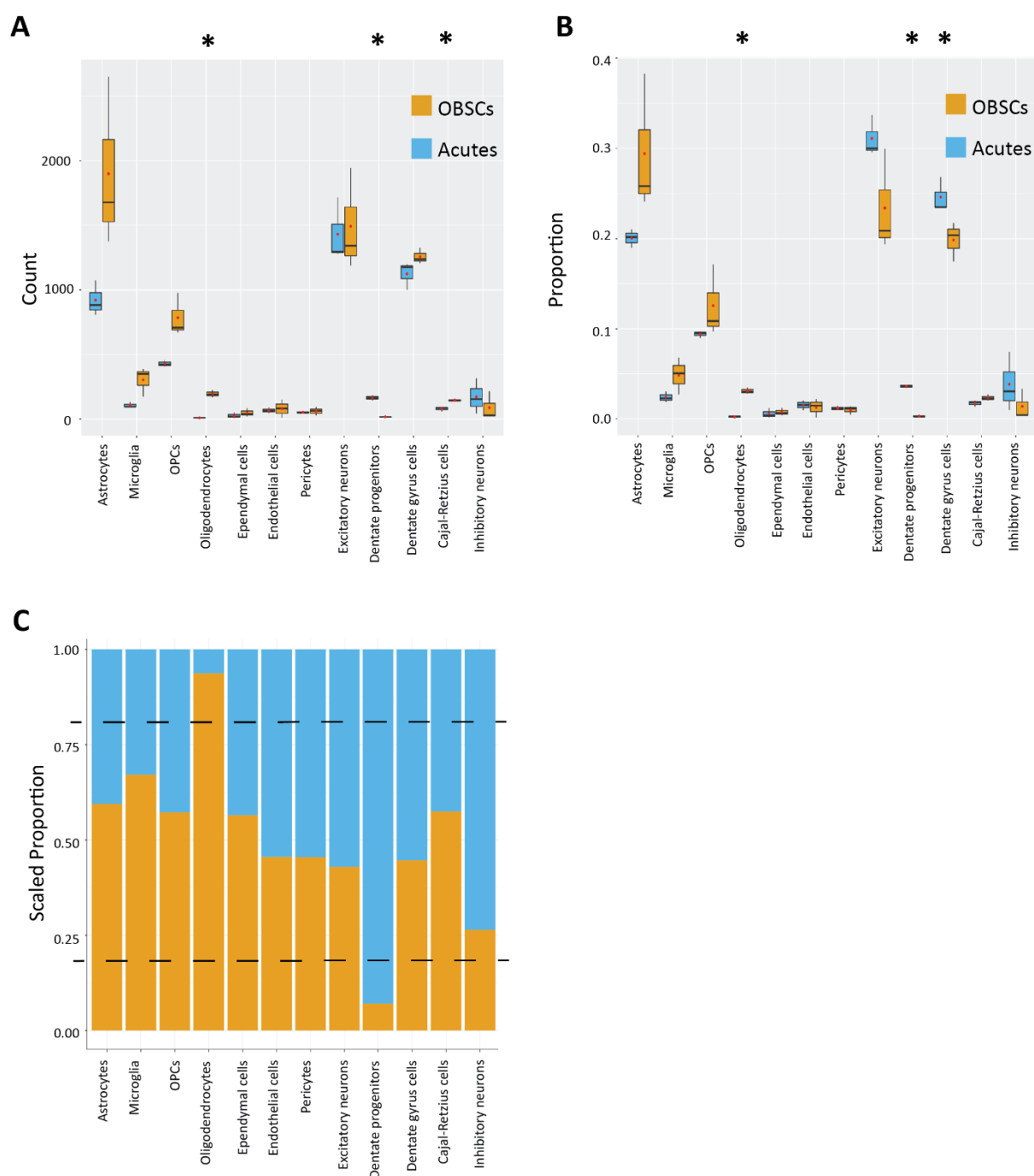


Figure 3.5.2) Differential cell-type composition in organotypic brain slice cultures versus acute brain slices. **A)** Boxplot showing the differences in nuclei counts between the two brain slice preparations across all the different cell-type annotations (* p < 0.05; unpaired t-test). **B)** Boxplot showing the differences in proportions between the two preparations across all the different cell-type annotations (* p < 0.05; unpaired t-test). **C)** Bar plot showing differences in normalized proportions between the two preparations across all cell-type annotations (these proportions are scaled and stacked). The dotted lines indicate 0.2 and 0.8 thresholds respectively.

3.6 Differential gene expression changes observed across multiple cell types between organotypic brain slice cultures compared to acute brain slices

To study cell-type-specific gene expression differences between OBSCs and acute brain slices, I ran a differential expression analysis using DEseq2 of OBSC and Acute samples across different cell types. There were 4 242 unique DEGs, both upregulated and downregulated, between the brain slice preparations (see Figure 3.6). I found that 1 554 DEGs were shared between two or more cell types (Figure 3.6B). The cell types with the most DEGs were excitatory neurons (3751), astrocytes (2780), dentate gyrus cells (1402), OPCs (1344), oligodendrocytes (722), endothelial cells (303), and microglia (278). The other cell types had 121 or fewer DEGs. A comparison to Figure 3.5.1A shows a strong correlation between the number of nuclei in a cell-type and the DEGs detected in that cell-type. This is likely because even though DEseq2 uses a pseudo-bulking approach with a single gene expression value per cell-type per sample, these values are likely more accurate for those cell types with many nuclei. This means that for cell types with more nuclei, it is likely easier to detect DEGs. There were some exceptions. For example, despite there being more nuclei in the microglial cluster in both brain slice preparations (a total of 1232 nuclei) compared to the oligodendrocytes clusters (a total of 615 nuclei), I still detected more than double the number of DEGs (722 versus 278) in oligodendrocytes versus microglia suggesting that oligodendrocytes undergo more gene expression changes following culturing.

Interestingly, I found that astrocytes, dentate gyrus, and endothelial cells were the only three cell clusters that had substantial differences between the number of upregulated and downregulated DEGs. Of the 2780 DEGs in astrocytes, 1759 were upregulated versus 1021 that were downregulated. This pattern is also observed in dentate gyrus cells; out of the 1402 DEGs, 843 were upregulated, while 559 were downregulated. In contrast, of the 303 DEGs in endothelial cells, 86 were downregulated, while 217 were upregulated.

To demonstrate cell-type-specific gene expression differences, I explored a subset of DEGs from various cell types which were uniquely differentially expressed (Figure 3.6C). These genes were chosen from a list of top DEGs that were unique to that cell-type; for instance, in Figure 3.6C, the *Igfbp5* gene is upregulated in OBSCs when compared to

acute brain slices only in astrocytes – this high expression is indicated by the red hue. In contrast, the same gene is not as expressed in all the other cell types (blue hues).

Enrichment analysis was done on all the top upregulated and downregulated DEGs to find the associated GO Biological Process 2021 functional pathways terms across all the clusters, comparing OBSCs to acute brain slices. Figure 3.6D shows upregulation in pathways related to cell signalling, neuronal development, and vascular interactions – which would support the growth of neurons in OBSCs. Pathways that were significantly upregulated included transmembrane receptor protein tyrosine kinase signalling pathway, axonogenesis, protein localization to membrane, protein phosphorylation, and cellular protein modification process (Figure 3.6D). On the other hand, downregulated DEGs were enriched in pathways relating to protein synthesis, and axon growth, suggesting that OBSCs exhibit some changes in axon guidance and outgrowth compared to acute brain slices (Figure 3.6E).

Taken together, these results highlight cell-type-specific gene expression changes, with excitatory neurons and astrocytes showing the most DEGs. They demonstrate both upregulation of pathways related to cell signalling and neuronal development in OBSCs when compared to acute brain slices and downregulation of certain axon guidance and protein synthesis pathways, consistent with the recovery process of slice cultures over time.

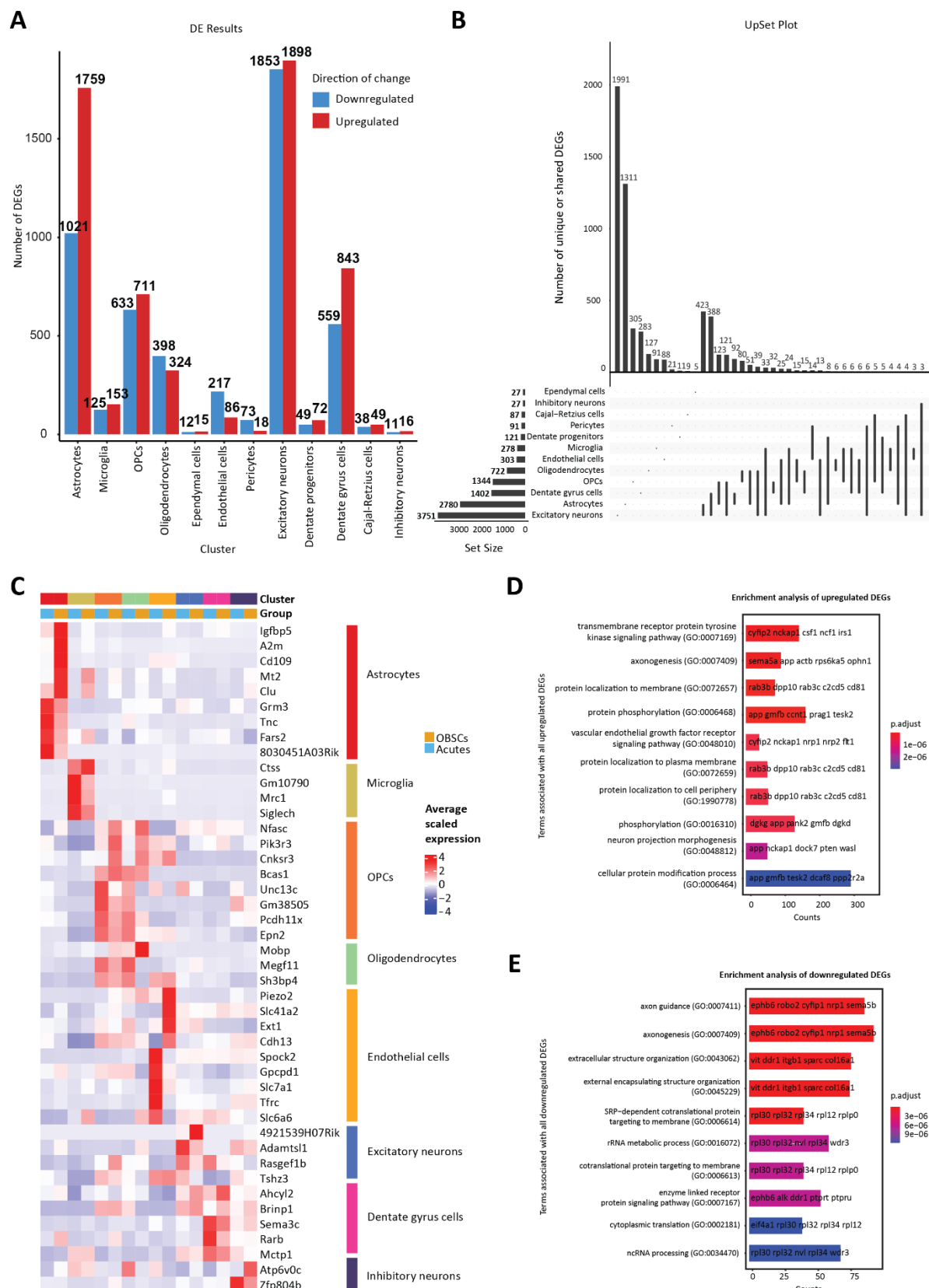


Figure 3.6) Hippocampal organotypic brain slice cultures had differentially expressed genes when compared to acute brain slices. **A)** Plot showing the number of DEGs that were upregulated and downregulated in the coarse annotation clusters of OBSCs when compared to acute brain

slices. **B)** Upset plot showing intersections of DEGs across the coarse annotation clusters. The dots represent DEGs that are unique to that cell-type annotation. The dots connected by a straight line represent DEGs found between two or more cell-type annotations. **C)** Heatmap showing the average expression of a sample of DEGs between OBSCs and acute brain slices across the different coarse annotation clusters. **D)** Gene Ontology Enrichment Analysis using the GO Biological Processes 2021 terms to elucidate the top 20 functional pathway terms associated with all the DEGs that were upregulated or **E)** Downregulated across the coarse annotation clusters, ranked by p-value and the names of the genes associated with that pathway written inside the bars. The width of the bars corresponds with the number of DEGs associated with the identified functional pathway or term. The bars include the names of the top DEGs associated with the given pathway or term. While the colour of the bars corresponds with the significance of the adjusted p-value ($p < 0.05$).

To explore cell-type-specific gene expression changes between OBSCs and acute brain slices, I focussed on each of the major cell types with over 150 DEGs, including astrocytes, microglia, oligodendrocyte precursor cells (OPCs), endothelial cells, excitatory neurons, and dentate gyrus cells. Oligodendrocytes were excluded from further analysis because this cell-type was almost exclusive to OBSCs. I performed a PCA for each cell-type to determine whether batch effects or brain slice preparation were the significant sources of variation in my data (Figures 3.6.1A to 3.6.6A). To do so, I used the regularized log transform (rlog) function after normalising the raw counts of the top 500 most variable genes before performing the PCA. In general, samples from the acute brain slices clustered more closely on the PCA plots than the OBSCs, likely because two Acute samples were from the same batch. This suggests that batch effects could be a source of some variance. Due to the particularities of sample collection only one of the OBSCs samples was collected on the same experiment run (i.e., same batch) as the two Acute samples. Nonetheless, I found that for all cell types, a clear separation between OBSCs and acute brain slices could be seen in the PCA plots, suggesting that brain slice preparation (and not batch effect) was the primary cause of variance in my samples. For each cell-type, using DEseq2, I calculated the top DEGs and generated volcano plots where differential gene expression changes could be observed in terms of adjusted p-value and log fold change for the top 20 DEGs. In addition to visualising the log fold changes of the significant DEGs, I also visualised their average normalised counts in a

dot plot to highlight the differences in counts between OBSCs and acute brain slices. Lastly, I performed an enrichment analysis on the list of all significant DEGs that were either upregulated or downregulated for each cell-type to identify GO biological terms associated with the sets of genes. Although all the pathways were significantly enriched ($p < 0.05$), I looked at the number of DEGs from a specific cell-type represented in a pathway when considering which pathways were functionally significant.

3.6.1 Astrocytic protein synthesis is promoted while axon guidance is downregulated in organotypic brain slice cultures when compared to acute brain slices

Astrocytes were the cell-type with the second highest number of DEGs (2780), with 47.15% (1311) being uniquely differentially regulated in this cell-type and not in any other. Of the top 20 DEGs in astrocytes, *Pik3r1* (Wei et al., 2021; Zhang et al., 2010), *Pvt1*, *Fn1*, and *Lncpint* were upregulated in OBSCs when compared to acute brain slices. These genes are related to reactive astrogliotic response in OBSCs, likely in response to the trauma of slicing and associated adaptive responses in culture. For example, *Pik3r1* (PI3K regulatory subunit) is involved in the PI3K/AKT signalling pathway, which is known to regulate astrocyte reactivity. When astrocytes become reactive, this pathway is often activated and contributes to inflammatory responses and cell proliferation (Wang et al., 2023). Similarly, *Fn1* (Fibronectin 1) is also indicative of reactive astrocytes. During reactive astrogliosis, astrocytes increase their production of extracellular matrix proteins, including fibronectin. This is part of the process of forming a glial scar and responding to injury (Chu et al., 2023; Li et al., 2020). Again, *Pvt1* is a long non-coding RNA that has been shown to be involved in inflammatory responses and cell proliferation, whilst *Lncpint* is involved in stress responses, which are likely part of the reactive astrocyte phenotype (Chuankun Li et al., 2024). Conversely *Tnc* (Evers et al., 2002), *Csmd2*, *Sncaip*, *Ero1l*, and *Grip1* were the top 5 downregulated genes in OBSCs when compared to acute brain slices, and involved in synaptic plasticity, synapse regulation (Gottschling et al., 2019; Poehler et al., 2014) and neuronal maturation (Gutierrez et al., 2019) (Figure 3.6.2B and C).

Gene Ontology (GO) enrichment analysis revealed that upregulated DEGs in OBSCs were significantly enriched in biological processes related to protein localization to plasma

membranes and vascular regulatory pathways, reflecting a potential reactive adaptation of astrocytes to the organotypic environment (Figure 3.6.1D). In contrast, the downregulated DEGs showed significant enrichment in pathways critical for nervous system development and synaptic integrity, suggesting a possible change in these functions in the OBSC model compared to Acute preparations (Figure 3.6.1E).

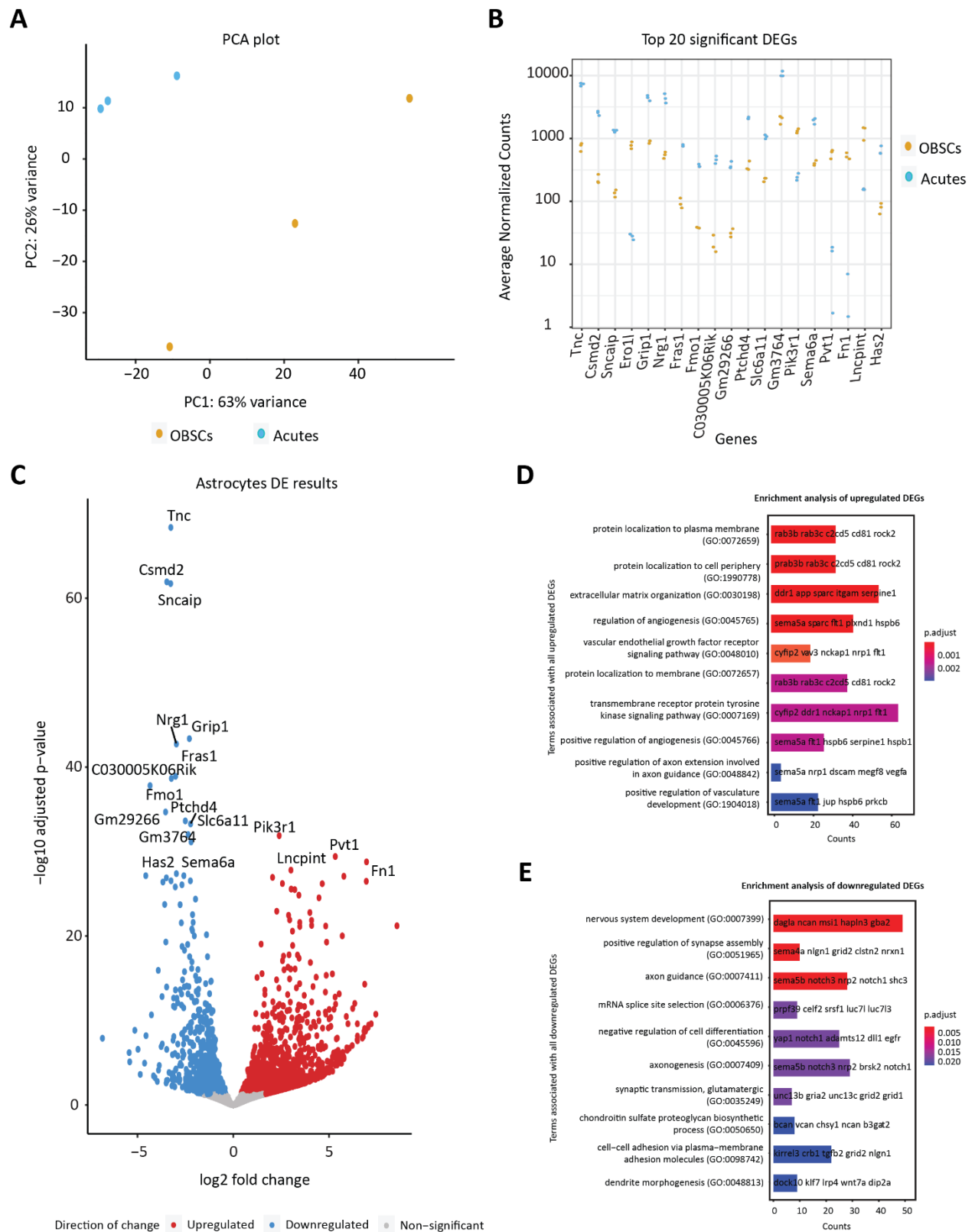


Figure 3.6.1) Hippocampal organotypic brain slice cultures had differentially expressed genes when compared to acute brain slices in astrocytes. **A)** PCA plot of the regularised log-transformed gene expression counts, showing the spatial separation between the OBSCs and acute brain slices groups in a reduced dimensional space. **B)** Dot plot showing the top 20 significant DEGs

and their log-transformed gene expression counts, with each dot representing a single sample from either the OBSCs or acute brain slices group. **C)** Volcano plot showing the log fold change and their associated p-values of the top 20 significant DEGs, with downregulated genes on the left and upregulated genes on the right. **D)** Gene Ontology Enrichment Analysis using the GO Biological Processes 2021 terms to elucidate the top 20 functional pathway terms associated with all the DEGs that were upregulated or **E)** Downregulated across the coarse annotation clusters, ranked by p-value and the names of the genes associated with that pathway written inside the bars. The width of the bars corresponds with the number of DEGs associated with the identified functional pathway or term. The bars include the names of the top DEGs associated with the given pathway or term. While the colour of the bars corresponds with the significance of the adjusted p-value ($p < 0.05$).

3.6.2 Microglia exhibit cell-type-specific differential expression differences in organotypic brain slice cultures compared to acute brain slices

I detected 278 DEGs in microglia, which was comparatively few compared to other cell types, particularly given that microglia were reasonably well represented in my samples. Nonetheless, the PCA analysis showed an apparent effect of brain slice preparation on variation in microglial gene expression (Figure 3.6.2A). The DEseq2 analysis showed that *Apoe*, *Mt1*, *Kncma1*, *Fat3* and *Arid5b* were the top 5 DEGs upregulated in the OBSCs group compared to acute brain slices. Enrichment analysis of the complete list of top upregulated DEGs showed that there was an upregulation in the pathways involved in neutrophil degranulation and neutrophil activation involved in immune response, suggesting that microglia may be part of some sort of inflammatory response when in culture (Figure 3.6.2D). These pathways were significant ($p < 0.003$) and included a count of 15 top DEGs from the microglia cluster, thus making them more reliable than the other pathways in this list that only had a count of 8 or fewer top DEGs.

On the other hand, I found that *Sdk1*, *Mrc1*, *Top2a*, *Gm10790*, and *Bank1* were among the top 20 significant DEGs downregulated in OBSCs compared to acute brain slices. These top downregulated were enriched in mitotic sister chromatid segregation and mitotic spindle organization pathways (Figure 3.6.2E), suggesting that in addition to leaning

toward a more reactive and inflammatory phenotype while in culture, microglia cell growth is also altered in OBSCs compared to acute brain slices.

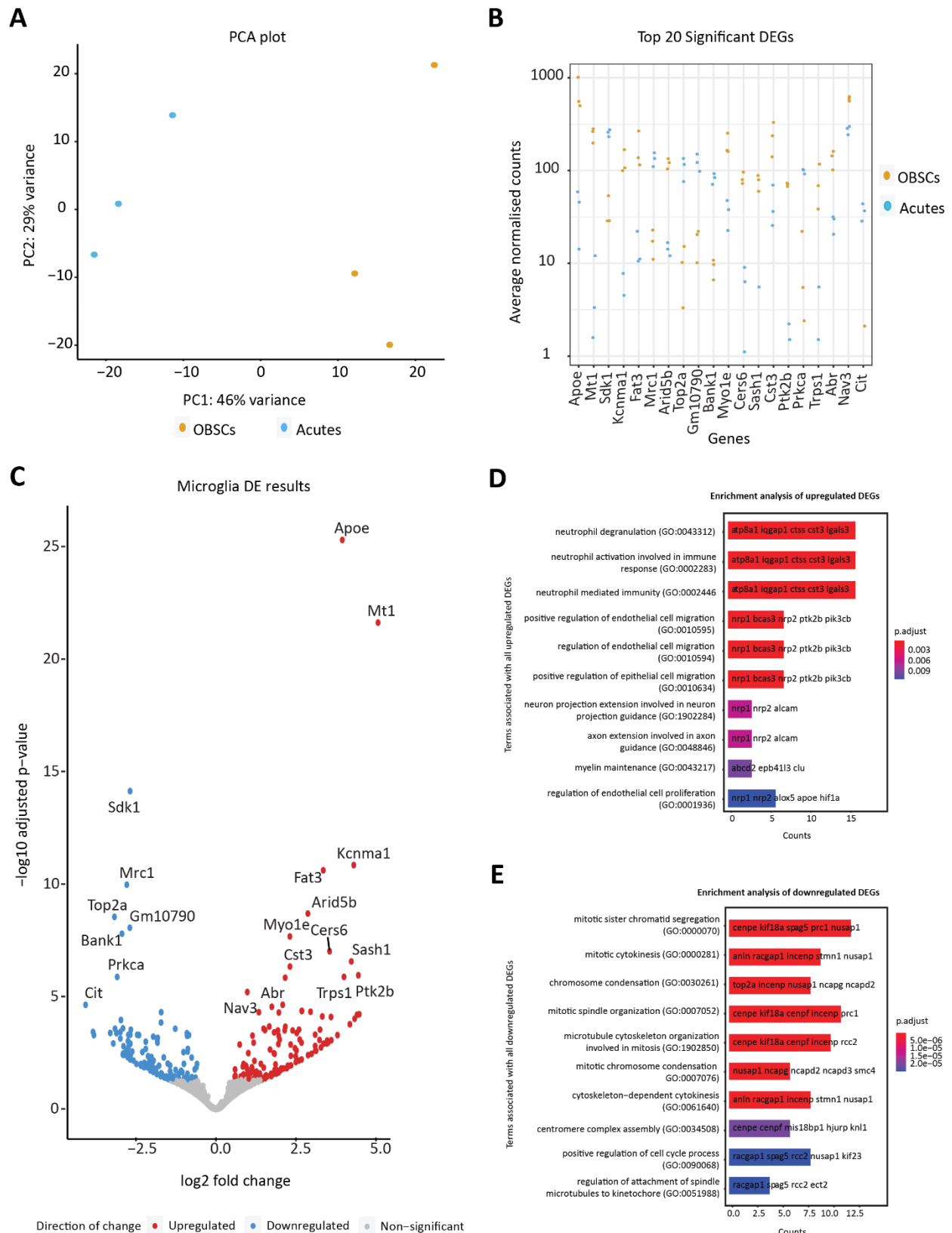


Figure 3.6.2) Microglia in hippocampal organotypic brain slice cultures had differentially expressed genes when compared to acute brain slices. **A)** PCA plot of the regularised log-transformed gene expression counts, showing the spatial separation between the OBSCs and acute brain slices groups in a reduced dimensional space. **B)** Dot plot showing the top 20 significant DEGs and their log-transformed gene expression counts, with each dot representing a single sample from either the OBSCs or acute brain slices group. **C)** Volcano plot showing the log fold change and their associated p-values of the top 20 significant DEGs, with downregulated genes on the left and upregulated genes on the right. **D)** Gene Ontology Enrichment Analysis using the GO Biological Processes 2021 terms to elucidate the top 20 functional pathway terms associated with all the DEGs that were upregulated or **E)** Downregulated across the coarse annotation clusters, ranked by p-value and the names of the genes associated with that pathway written inside the bars. The width of the bars corresponds with the number of DEGs associated with the identified functional pathway or term. The bars include the names of the top DEGs associated with the given pathway or term. While the colour of the bars corresponds with the significance of the adjusted p-value ($p < 0.05$).

3.6.3 OPCs had differentially expressed genes in organotypic brain slice cultures versus acute brain slice preparations

There is a clear indication of variation in the gene expression of OPCs due to brain slice preparation (i.e., OBSCs and acute brain slices), explaining 70% of the variation in the data (Figure 3.6.3A). The acute brain slices samples cluster closely together, showing slight variation within the group, while the OBSCs group has one outlier sample on the far bottom right quadrant. This OBSC 1 sample lies away from the other OBSC samples likely because it was collected in a separate batch from the other samples in the group, but just like the other OBSC samples, its variation is still explained mainly in PC1.

Consistent with being the 4th largest cell-type in my dataset, OPCs also had the 4th most extensive list of DEGs (1344). From these, the top 20 significant DEGs showed that *Slc24a2*, *Frmd4a*, *Lingo2*, *Mt1*, and *Cnksr3* were upregulated in OBSCs compared to acute brain slices. Performing an enrichment analysis on these upregulated genes and all the other top upregulated DEGs revealed that these genes were enriched in pathways involved in axonogenesis, axon guidance, nervous system development, and supramolecular fiber organization (Figure 3.6.3D). These may indicate that OPCs may be

involved in the maintenance of axons as the tissue matures in culture. Interestingly, *Frmd4a* is involved in oligodendrocytes maturation, showing that OPCs may be undergoing maturation in culture.

On the other hand, I found that *Thsd7b*, *Schip1*, *Gm3764*, *Lrrtm4*, and *Gpr158* were the top 5 downregulated DEGs in OPCs (Figure 3.6.3B). This list of top downregulated DEGs was enriched in various pathways, including chemical synaptic transmission, anterograde trans-synaptic signalling, calcium ion transport, and inorganic cation transmembrane (Figure 3.6.3E). These suggest an attenuation in the regulation of synaptic transmission in OBSCs compared to acute brain slices.

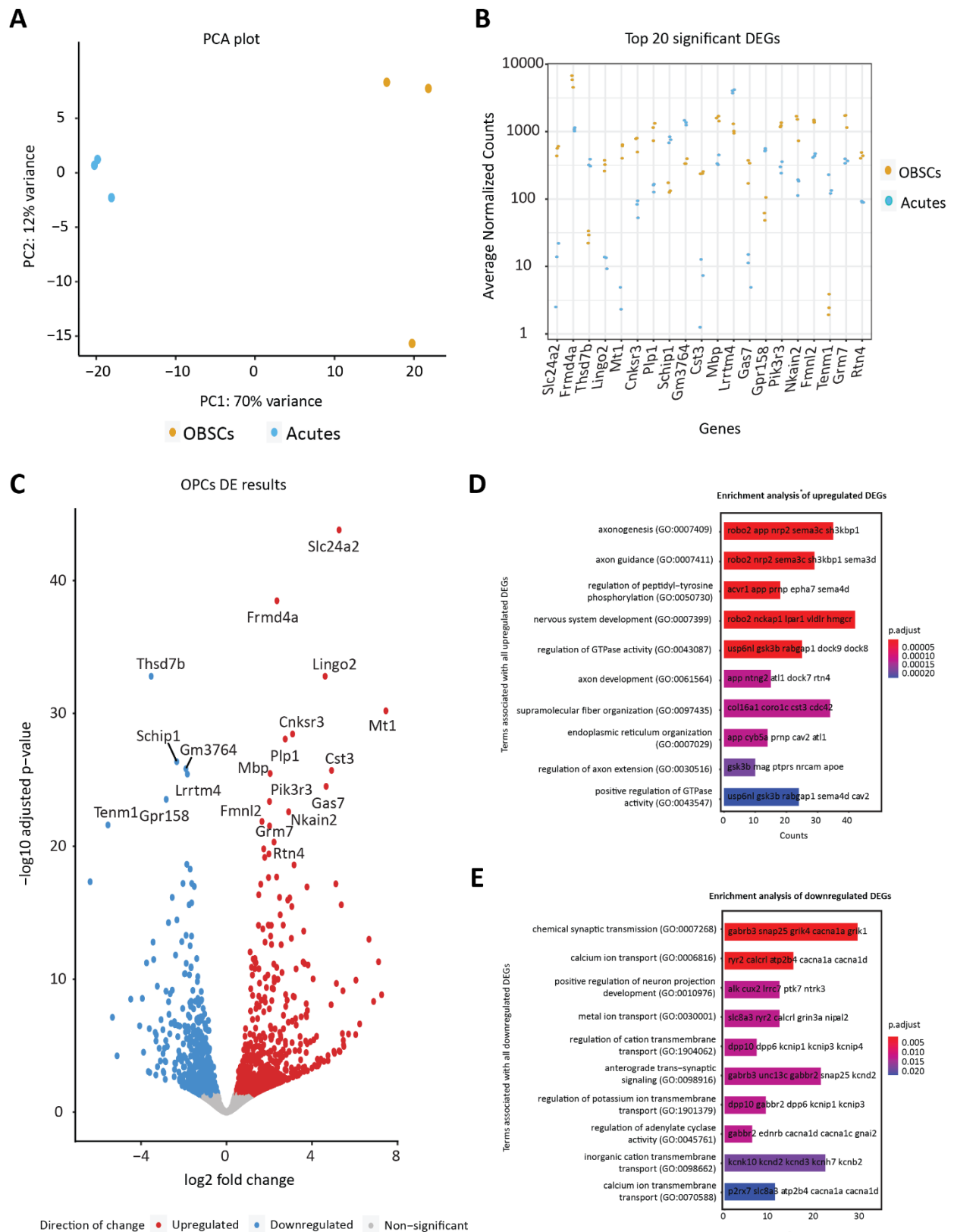


Figure 3.6.3) Differentially expressed genes in OPCs between the brain slice preparations. **A)** PCA plot of the regularised log-transformed gene expression counts, showing the spatial separation between the organotypic brain slice culture and acute brain slice groups in a reduced dimensional space. **B)** Dot plot showing the top 20 significant DEGs and their log-transformed

gene expression counts, with each dot representing a single sample from either the OBSCs or acute brain slices group. **C)** Volcano plot showing the log fold change and their associated p-values of the top 20 significant DEGs, with downregulated genes on the left and upregulated genes on the right. **D)** Gene Ontology Enrichment Analysis using the GO Biological Processes 2021 terms to elucidate the top 20 functional pathway terms associated with all the DEGs that were upregulated or **E)** Downregulated across the coarse annotation clusters, ranked by p-value and the names of the genes associated with that pathway written inside the bars. The width of the bars corresponds with the number of DEGs associated with the identified functional pathway or term. The bars include the names of the top DEGs associated with the given pathway or term. While the colour of the bars corresponds with the significance of the adjusted p-value ($p < 0.05$).

3.6.4 Endothelial cells show differences in active maintenance of vascular integrity in organotypic brain slice cultures and acute brain slices

Differential gene expression analysis of endothelial cells revealed significant changes between OBSCs and acute brain slices (Figure 3.6.4C). Despite being one of the minor groups of cells in my dataset (440 cells), endothelial cells had 303 DEGs, which is very close to the 278 DEGs in microglia, which have more than double the cell count (1232 cells) (Figure 3.6A). This indicates this cell-type undergoes comparatively large gene expression changes in OBSCs. Among the top 5 upregulated genes in OBSCs, *Cdh13* and *Kcnma1* showed the highest significance. Other significantly upregulated genes included *Piezo2*, *Hdac9*, and *Man1a*. These genes are associated with various cellular functions, including cell adhesion (Forero et al., 2017). On the other hand, *Slc7a1* was the most significantly downregulated gene in OBSCs compared to acute brain slices, followed by *Spock2*, *Prkg1*, *Gpcpd1*, and *Tfrc*. Many of these genes are involved in transport processes and cellular metabolism (Cikes et al., 2024; Moos & Morgan, 2000).

Enrichment analysis of upregulated (Figure 3.6.4D) DEGs revealed several overrepresented biological processes in OBSC endothelial cells. However, the gene counts for these enriched pathways were very low, with a maximum of only 4 genes per category. Hence, when compared to acute brain slices, it is difficult to confirm whether these pathways were truly enriched or represent any significant functional change in endothelial cells in OBSCs. These upregulated pathways include cellular response to

drug, regulation of positive chemotaxis, cellular response to epinephrine stimulus, and sensory perception to mechanical stimulus and sound.

On the other hand, the enrichment analysis of downregulated DEGs (Figure 3.6.4E) showed that many of the pathways were involved in protein synthesis and processing. The most significantly downregulated pathways included SRP-dependent cotranslational protein targeting to membrane, cytoplasmic translation, nuclear-transcribed mRNA catabolic process, and cellular macromolecule biosynthetic process. These results suggest that endothelial cells in OBSCs undergo substantial transcriptional changes, including downregulated protein synthesis and upregulated cell adhesion processes, compared to cells in acute brain slices.

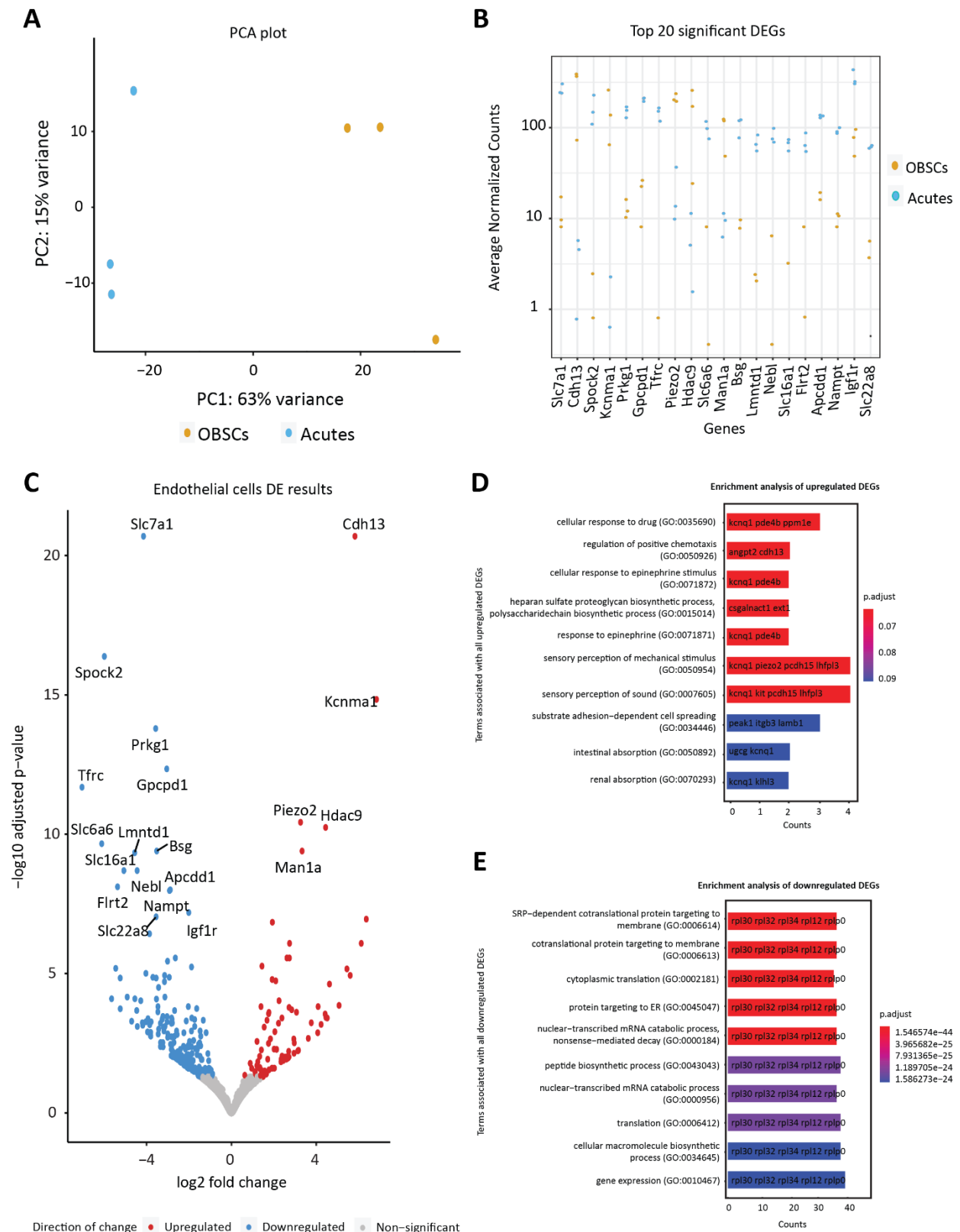


Figure 3.6.4) Endothelial cells had differentially expressed genes between hippocampal organotypic brain slice cultures and acute brain slices. **A)** PCA plot of the regularised log-transformed gene expression counts, showing the spatial separation between the OBSCs and acute brain slices groups in a reduced dimensional space. **B)** Dot plot showing the top 20

significant DEGs and their log-transformed gene expression counts, with each dot representing a single sample from either the OBSCs or acute brain slices group. **C)** Volcano plot showing the log fold change and their associated p-values of the top 20 significant DEGs, with downregulated genes on the left and upregulated genes on the right. **D)** Gene Ontology Enrichment Analysis using the GO Biological Processes 2021 terms to elucidate the top 20 functional pathway terms associated with all the DEGs that were upregulated or **E)** Downregulated across the coarse annotation clusters, ranked by p-value and the names of the genes associated with that pathway written inside the bars. The width of the bars corresponds with the number of DEGs associated with the identified functional pathway or term. The bars include the names of the top DEGs associated with the given pathway or term. While the colour of the bars corresponds with the significance of the adjusted p-value ($p < 0.05$).

3.6.5 Excitatory neurons had the largest number of uniquely differentially expressed genes between organotypic brain slice cultures and acute brain slices

Excitatory neurons had the highest number of DEGs (3751), which was consistent with their making up the largest proportion of cells in my dataset (Figure 3.6A). Notably, 1991 (53.08%) of these DEGs were unique to excitatory neurons and not observed in the other cell types. Genes upregulated in OBSCs compared to acute brain slices include *Specc1*, *Tanc1*, *4921539H07Rik*, and *Adcy9*. While *Scn9a*, *Styk1*, *A530065N20Rik*, *Gm16083* and *Srgap1*, were downregulated in OBSCs (Figure 3.6.5B and C).

Enrichment analysis of all the upregulated DEGs (Figure 3.6.5D) highlights several enriched pathways in the OBSC excitatory neurons compared to acute brain slices. The most significant pathways include Fc-gamma receptor signalling involved in phagocytosis, phosphatidylinositol biosynthetic process, and Fc receptor mediated stimulatory signalling pathway. Other enriched processes involve ubiquitin-dependent protein catabolism, transmembrane receptor protein tyrosine kinase signalling, and regulation of intracellular signal transduction.

For all the downregulated DEGs (Figure 3.6.5E), the enrichment analysis reveals pathways related to axon guidance and axonogenesis. Additionally, cytoskeleton organization and supramolecular fiber organization were also downregulated. I also found several downregulated biosynthetic processes (related to secondary alcohol,

cholesterol, and sterol) involving less than 12 DEGs from my data as opposed to over 20 DEGs in the other significant pathways.

These findings indicate that when comparing excitatory neurons from OBSCs to those from acute brain slices, there are significant changes in gene expression patterns.

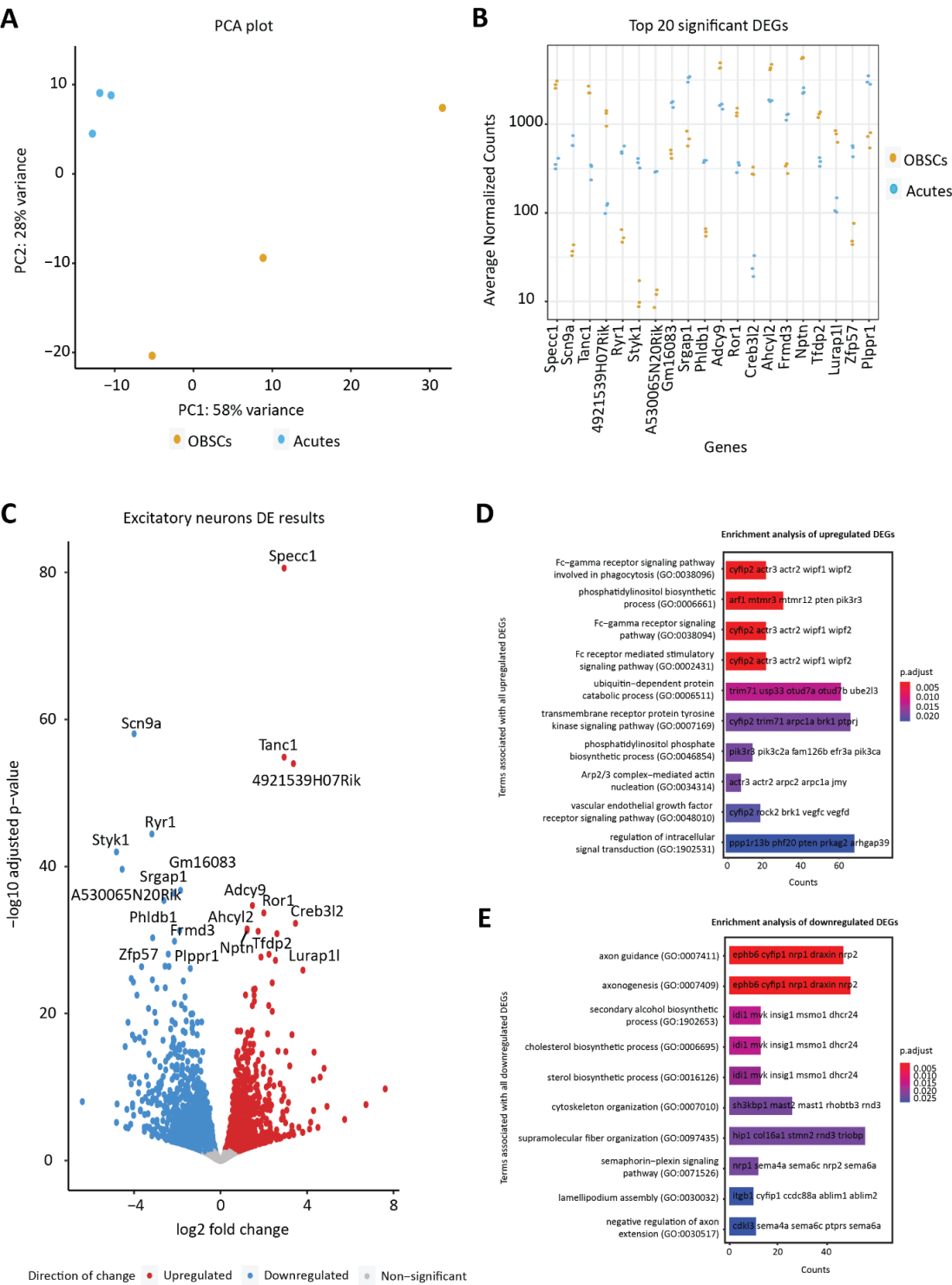


Figure 3.6.5) Hippocampal organotypic brain slice cultures had differentially expressed genes when compared to acute brain slices in excitatory neurons. **A)** PCA plot of the regularised log-transformed gene expression counts, showing the spatial separation between the OBSCs and acute brain slices groups in a reduced dimensional space. **B)** Dot plot showing the top 20 significant DEGs and their log-transformed gene expression counts, with each dot representing a single sample from either the OBSCs or acute brain slices group. **C)** Volcano plot showing the log fold change and their associated p-values of the top 20 significant DEGs, with downregulated genes on the left and upregulated genes on the right. **D)** Gene Ontology Enrichment Analysis using the GO Biological Processes 2021 terms to elucidate the top 20 functional pathway terms associated with all the DEGs that were upregulated or **E)** Downregulated across the coarse annotation clusters, ranked by p-value and the names of the genes associated with that pathway written inside the bars. The width of the bars corresponds with the number of DEGs associated with the identified functional pathway or term. The bars include the names of the top DEGs associated with the given pathway or term. While the colour of the bars corresponds with the significance of the adjusted p-value ($p < 0.05$).

3.6.6 Dentate gyrus cells showed differentially expressed genes between organotypic brain slice cultures and acute brain slices

Finally, I also explored the differential expression of genes in dentate gyrus cells in OBSCs and acute brain slices. I found 1402 DEGs, with only 283 (20.19%) unique to this cell-type (Figure 3.6A). Among the top 20 DEGs, these 5 genes were upregulated: *Nptn*, *Cdr1os*, *Ldlrad4*, *Maml2*, and *Dock10*. Enrichment analysis on all the top upregulated pathways revealed that these genes were enriched in various pathways, including chemical synaptic transmission, anterograde trans-synaptic signalling, transmembrane receptor protein tyrosine kinase signalling pathway, phosphorylation and neuron projection morphogenesis (Figure 3.6.6E).

On the other hand, I found that *Gabrg3*, *Myo1b*, *Tenm3*, *Myo5b*, and *Rasgef1b* were amongst the top 20 significant DEGs that were downregulated (Figure 3.6.6B and C). An enrichment analysis on all the top downregulated DEGs revealed that several pathways relating to extracellular matrix organization and axon guidance were enriched. For instance, the supramolecular fiber organisation, collagen fibril organization, extracellular structure and matrix organization, and axon guidance pathways. Interestingly, there was

a downregulated pathway, regulation of cardiac muscle cell contraction, which had less than 10 DEGs count from my dataset, so the functional significance of this pathway remains unclear.

These findings suggest that dentate gyrus cells in OBSCs undergo significant transcriptional changes compared to acute brain slices, which may be due to the time spent in culture. The upregulation of DEGs involved in synaptic function and neuronal development processes, coupled with the downregulation of extracellular matrix and axon guidance DEGs, may indicate the growth of neurons but with less guided precision over time in culture.

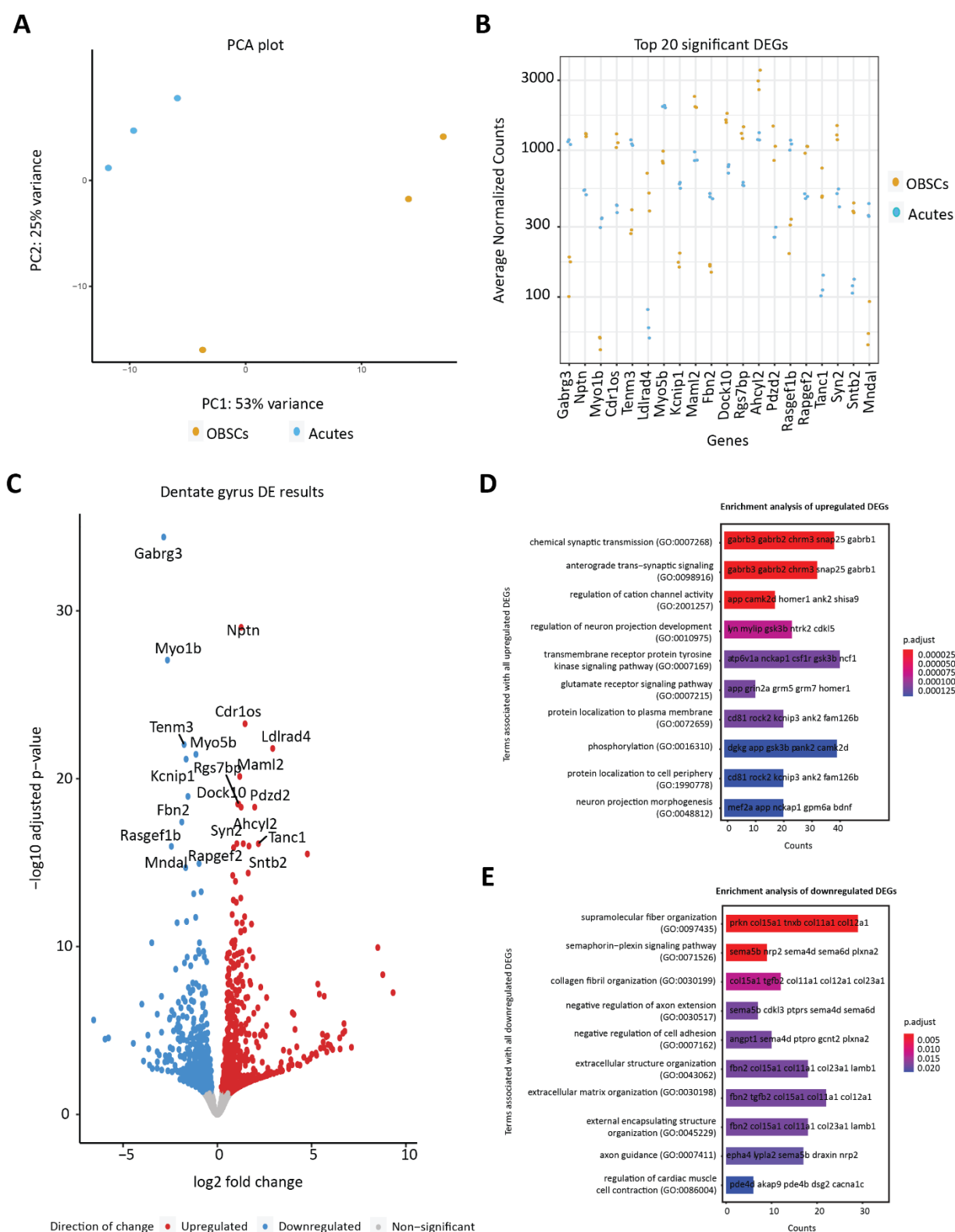


Figure 3.6.6) Hippocampal organotypic brain slice cultures had differentially expressed genes when compared to acute brain slices in dentate gyrus cells. **A)** PCA plot of the regularised log-transformed gene expression counts, showing the spatial separation between the OBSCs and acute brain slices groups in a reduced dimensional space. **B)** Dot plot showing the top 20 significant DEGs and their log-transformed gene expression counts, with each dot representing a

single sample from either the OBSCs or acute brain slices group. **C)** Volcano plot showing the log fold change and their associated p-values of the top 20 significant DEGs, with downregulated genes on the left and upregulated genes on the right. **D)** Gene Ontology Enrichment Analysis using the GO Biological Processes 2021 terms to elucidate the top 20 functional pathway terms associated with all the DEGs that were upregulated or **E)** Downregulated across the coarse annotation clusters, ranked by p-value and the names of the genes associated with that pathway written inside the bars. The width of the bars corresponds with the number of DEGs associated with the identified functional pathway or term. The bars include the names of the top DEGs associated with the given pathway or term. While the colour of the bars corresponds with the significance of the adjusted p-value ($p < 0.05$).

Together my data shows DEGs in all major cell types indicating that the culture process induces gene expression changes which are likely important for cell and circuit (synaptic) function.

Chapter 4: Discussion, limitations, and conclusion

4.1. Summary

OBSCs and acute brain slices are widely used *in vitro* models to study diseases of the central nervous system. However, the transcriptomic differences between these brain slice preparations have not been explored. To address this gap, the overall aim of my project was to characterize cell-type-specific gene expression differences between OBSCs and acute brain slices. I used snRNA-seq to compare OBSCs and acute brain slices from the P7 mouse hippocampus, revealing significant differences in cell composition and gene expression. OBSCs had more glial cells, fewer neurons, and unique oligodendrocyte and dentate progenitor distributions. Over 4200 genes were differentially expressed, with excitatory neurons and astrocytes showing the most changes. OBSCs showed upregulated cell signalling and neuronal development pathways but downregulated axon guidance. Cell-type analysis revealed adaptations, including altered inflammation in microglia and astrocytes and modified synaptic signalling in dentate gyrus cells. These findings highlight how brain slice cultures adapt to *ex vivo* conditions.

4.2. Similarities

While this study highlights differences in cellular composition and gene expression between OBSCs and acute slices, it is also important to recognize the preserved features that make OBSCs valuable models. Despite some transcriptional shifts and changes in cell proportions, core hippocampal cell types were consistently detected in both preparations. This preservation of neural architecture and cellular diversity supports the use of OBSCs for studying neuronal connectivity, synaptic plasticity, and cellular responses.

Specifically, I found that ependymal cells, pericytes, Cajal-Retzius cells, and inhibitory neurons had similar proportions in OBSCs and acute brain slices, with relatively few DEGs. This suggests that these cell types may be more transcriptomically stable between the two conditions compared to others. One possible explanation is that these cells may have already undergone key transcriptional maturation by postnatal day 7, and further

changes in culture are limited due to the absence of developmental cues that would normally be present *in vivo*.

4.3. Oligodendrocyte-specific enrichment and enhanced glial representation in OBSCs.

In this thesis, I used my snRNA-seq datasets to quantify and compare the relative abundance of the different cell types in the two preparations. To this end, one of the most striking findings was that the oligodendrocyte cell-type was almost exclusively found in OBSCs and not in acute brain slices. This observation suggests that brain slices are likely to be undergoing continued development over time in culture. The relative abundance of oligodendrocytes in OBSCs is likely due to OPCs maturing into oligodendrocytes during the week in culture as it is well known that the final maturation of OPCs into mature oligodendrocytes occurs during the first 6 postnatal weeks (Tripathi et al., 2017). A study in rats by Dawson et al. (2003) demonstrated that oligodendrocytes continue to mature in culture from NG2-expressing OPCs (Valério-Gomes et al., 2018). Interestingly in OBSCs, my results showed that oligodendrocytes were the least abundant glia, which is not consistent with other studies that have shown oligodendrocytes to be the most abundant glia in the intact brain (Pelvig et al., 2008; von Bartheld et al., 2016), particularly in the hippocampus (Valério-Gomes et al., 2018). It is important to note that the study by Valério-Gomes et al., (2018) used postnatal day 90 to 100 adult mice, while I used postnatal day 7 juvenile mice in my study with 7 days in culture (i.e. equivalent to postnatal day 14). Therefore, this suggests that oligodendrocyte populations still need time to fully develop in the intact brain before their large numbers in brain slice cultures can be observed (for full review see Sams, 2021). The differences may also be due to the fact that my preparation contained relatively little white matter which is the predominant location of oligodendrocytes in the mature brain. Due to the almost complete lack of oligodendrocytes in the acute slices, I did not perform a differential gene expression analysis on the oligodendrocyte population between the two brain slice preparations.

One of the challenges encountered in long-term OBSCs is the tendency for glial cells, particularly astrocytes, to proliferate excessively. My observation of increased glial cell population in the OBSCs aligns with findings from several other studies in the field (Delbridge et al., 2020a; Kernie et al., 2001; Laskowski et al., 2005; Raineteau et al., 2004;

Rio et al., 1991). For instance, a study by Chechneva et al. (2005) reported that the initial trauma during preparation and subsequent culturing stimulates astrocyte proliferation, leading to the formation of a glial barrier encapsulating the slice. The pronounced difference in glial cell populations between OBSCs and acute brain slices in my study likely reflects this adaptive response to the slicing trauma.

In line with this glial proliferation, the differential expression analysis revealed that astrocytes had the second-highest number of DEGs between OBSCs and acute brain slices. More on this is discussed below.

4.4. Selective changes in neuronal populations including dentate progenitor loss and Cajal-Retzius cell persistence

In tandem with the changes I observed in glial cell populations discussed above, I found an accompanying overall relative underrepresentation of the neuronal populations. Given that the absolute numbers of excitatory neurons and dentate gyrus cells were not different between populations and that the differences were found when comparing the proportion of total cells that were made up by the neuronal population, a major reason for the neuronal underrepresentation could likely be a simple overabundance of glia in OBSCs, although I cannot exclude a comparatively reduced survival of neurons as a contributing factor to their underrepresentation. For example, due to the fact that OBSCs develop in isolation from the brain, the vascular system is compromised, resulting in the absence of blood flow despite the presence of capillaries, endothelial cells, and pericytes (Kovács et al., 2011). This means that some nutrients that would have been derived from the blood cannot reach the slices. Secondly, afferent connections from distant brain areas are also lost due to axotomy, leading to the reorganization of axonal connections in the brain slice (Frotscher & Gähwiler, 1988). While exogenous growth factor supplements used in my growth media could mitigate some of these issues (Moser et al., 2003), the lack of a complete vascular system and severed connections could contribute to the underrepresentation of the neuronal populations in OBSCs compared to acute brain slices. That said my findings align with previous work suggesting that the deafferentation associated with OBSCs does not result in the selective loss of any particular neuronal cell types (Finsen et al., 1992; Gähwiler, 1984). Interestingly, in confirmation of previous findings I found that unlike other neuronal cell-types, cajal–

Retzius cells were overrepresented in OBSCs compared to acutes. This has been demonstrated before and is thought to be due to the fact that these cells survive in culture whereas *in vivo* most of these cells undergo progressive cell death toward the third postnatal week (Del Río et al., 1996, 1997).

Another difference I observed between the two preparations was that a cluster of cells representing dentate progenitor cells in the acute brain slices, which was mostly absent in the OBSCs. Again, this observation likely reflects the ongoing maturation of the hippocampal slices over the week in culture with the dentate progenitor cells observed in the acute slices likely continuing to differentiate into mature dentate granule cells over the culture period (Namba et al., 2005). My work supports this hypothesis as I found a high expression of proliferating markers (data not shown) in the dentate progenitor cells cluster. Namba et al. (2005) also showed that some of these dentate progenitor cells also differentiated into glial cell types such as astrocytes, which is consistent with their increase in the OBSCs.

4.5. Differential gene expression and enrichment analysis

Differential gene expression analysis revealed substantial differences between the preparations with over 4200 unique DEGs identified across the major cell types. Excitatory neurons and astrocytes had the highest number of DEGs, making up 61.73% of all the DEGs between OBSCs and acute brain slices across all the cell types. In addition, they each had the largest number of unique DEGs compared to the other cell types. It is worth noting that these were the two largest populations and hence I had the greatest statistical power for detecting DEGs in these two cell types. Nonetheless, the large number of detected DEGs does suggest that these populations are undergoing substantial adaptation to the culture conditions necessary for survival outside the brain.

On the other hand, the upregulation of axonogenesis-related genes points to increased axonal growth and neural network remodelling in culture. This is supported by evidence showing that astrocytes in spinal cord lesions form scars that support axon regeneration (Anderson et al., 2016). They performed RNA-seq and found that there was an upregulation of axon growth-promoting genes such as *Tnc* (Gotz et al., 1996) which was also differentially expressed in the astrocytes in my data. Given this evidence and

assuming that axon regeneration may occur in OBSCs, my data interestingly revealed high expression of *Cspg5* predominantly in astrocytes and OPCs, which has been implicated in blocking axon extension (McKeon et al., 1991). This suggests that while axons may regenerate, they might fail to grow sufficiently or form the necessary connections required for functional neural networks. Supporting this, pathways associated with axon guidance in my data are notably downregulated, as is extracellular structure organization, which further highlights a disorganized environment where axons lack the structural cues to guide their growth. This disarray suggests that astrocytes may play a dual role: facilitating regeneration to some extent but simultaneously contributing to an environment that hinders proper axonal extension and connectivity. Consequently, this highlights the complexity of axonal regeneration in OBSCs and the critical influence of astrocytes on neural network formation in such systems.

4.6. Cell-type specific effects

The effects described above were looking at the total number of DEGs between OBSCs and acute brain slices. Below, I focus on specific cell types.

4.6.1. Astrocytes

Consistent with the high number of DEGs, astrocytes also had the biggest population of cells in my dataset. As discussed earlier, astrocytes are overrepresented in OBSCs. One possible reason could be the upregulation of the *Pik3r1* gene, which may support astrocyte survival and growth in culture. This is because *Pik3r1* encodes the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), which has been implicated in various astrocyte functions, including cell survival, proliferation, and inflammatory responses (Wei et al., 2021; Zelenai et al., 2000; Zhang et al., 2010). Enrichment analysis of upregulated DEGs revealed a significant focus on protein localization to plasma membranes and vascular regulatory pathways. This shift may represent an adaptive response of astrocytes in the OBSC environment to maintain cellular structure and support BBB functions. This is interesting because there is no BBB in OBSCs, so the astrocytes could potentially be running some damage control mechanism to mitigate the effects of this. Alternatively, this could be an intrinsic property of astrocytes in supporting

the connection of neurons with the BBB – and so these astrocytes are habitually (and blindly) trying to form a connection with a non-existent BBB. Similar adaptations have been observed in 3D culture models, where astrocytes show enhanced expression of genes related to extracellular matrix organization and angiogenesis (Simão et al., 2018). Conversely, the downregulation of genes like *Tnc*, *Csmd2*, *Sncaip*, *Ero1l*, and *Grip1* was enriched in pathways involved in synaptic plasticity, synapse regulation, and neuronal maturation (Gutierrez et al., 2019; Poehler et al., 2014). *Tnc*, showing the most significant log 2-fold change, is an extracellular matrix glycoprotein that plays crucial roles in neural development, synaptic plasticity, and regeneration after injury (Gottschling et al., 2019; Laywell et al., 1992). Its downregulation in OBSCs may reflect a steady recovery from slicing-induced trauma in culture.

Another important factor to consider is that the overrepresentation of astrocytes in OBSCs may be due to the use of horse serum in the culture medium. Kim et al. (2013) showed that slices cultured with serum developed increasing inflammatory cell-like masses over time, which they speculated might potentiate slicing-induced trauma and lead to astrocyte proliferation (Kim et al., 2013). On the other hand, serum-free culture did not produce the inflammatory masses, and the astrocyte population decreased and stabilised after 15 days in culture. Hence, the use of serum in the OBSCs may have contributed to the overrepresentation of astrocytes, and a serum-free culture using Neurobasal medium would be useful to control for this effect (Kim et al., 2013). Nonetheless, even accounting for proliferation, it is possible that astrocytes would still be significantly overrepresented in OBSCs, albeit at a lower level.

4.6.2. Microglia

Microglia, like the other glial cells, were overrepresented in OBSCs but had relatively few DEGs compared to the other glial cells. Microglia exhibited 278 DEGs, a relatively modest number compared to other cell types, despite being well-represented in the dataset (5th most populous cell-type but 7th most number of DEGs). PCA analysis indicated that brain slice preparation strongly influenced microglial gene expression (Figure 3.6.2A). Among the top upregulated DEGs in OBSCs were *Apoe*, *Mt1*, *Kncma1*, *Fat3*, and *Arid5b*. The upregulation of *Apoe* is consistent with another study that used Fluorescence-Activated Cell Sorting (FACS)-sorted microglia of 8-week-old mice and found that this was

associated with worsened neurodegeneration outcomes in a model of facial nerve axotomy (Tay et al., 2018). This suggests a role of *ApoE* in neurodegeneration both in pathological states, as demonstrated by Tay et al. (2018), and also in my OBSCs. This highlights the importance of considering the limitations of studying microglia in culture, as they can become activated under these conditions and may not accurately reflect their normal physiological state in the brain.

Enrichment analysis revealed that the upregulated genes were associated with pathways related to neutrophil degranulation and activation in immune responses, suggesting that microglia may indeed be participating in some sort of inflammatory response in culture. These pathways, supported by 15 DEGs, were among the most reliable in the analysis compared to other pathways that were associated with gene counts between 3 to 10. At the time of writing this thesis, I found that only one study had compared the gene expression differences of microglia between OBSCs and acute brain slices. Delbridge et al. (2020) used FACS to isolate microglia from both slice preparations and used bulk RNA sequencing to analyze their transcriptomic profile. Interestingly, they identified 280 DEGs when comparing day 1 (acute) brain slices to week 1 (i.e., P7) OBSCs, which is similar to the 278 DEGs that I identified in my dataset. They explained that the slicing trauma leads to an acute inflammatory response in acute brain slices which is downregulated over time in culture. They were able to demonstrate elevated inflammatory response in acute brain slices by measuring elevated levels of pro-inflammatory cytokines, IL-6, and TNF which decreased at week 1 in culture. Conversely, the levels of anti-inflammatory cytokines, IL-10, were initially low in their acute brain slices but increased in the OBSCs, suggesting that there is active resolution of the inflammatory response. Complementary to this, they correlated the resolution of the inflammatory response with the downregulation of inflammatory genes such as *Nfkb1* and *Rela*. However, these genes were not differentially expressed in my dataset. Secondly, the enrichment analysis of my microglia DEGs revealed only 2 pathways relating to inflammation, namely “neutrophil-mediated immunity” and “neutrophil activation involved in immune response”. These were from the upregulated DEGs, indicating some upregulation in at least some inflammatory genes in my OBSCs after 7 days in culture. However, it is important to note that these pathways in my study, as mentioned before, were associated with only 15 gene counts, so their significance remains unconfirmed. Unlike Delbridge et al. (2020), my

study only had 1-time point for OBSCs and would benefit from adding more time points to see if the inflammation response decreases over time in culture. Lastly, they found that microglia homeostatic genes (Keren-Shaul et al., 2017) such as *Cst3* were upregulated in OBSCs, and this was also true for my dataset. This shows that microglia in culture are more mature than those in acute brain slices, highlighting their adaptation to culture conditions, and continued maturation in OBSCs. Moreover, my study presents these findings on microglia differential gene expression in the context of other cells, which is more representative of the intact brain, as opposed to the FACS sorting done in the study by Delbridge et al. (2020).

4.6.3. Oligodendrocyte precursor cells (OPCs)

OPCs were also found to undergo differential gene expression between the two preparations. GO enrichment analysis showed dysregulation of pathways related to axonogenesis, axon guidance, nervous system development, and fiber organization. This suggests an active role for OPCs in axonal maintenance and support and is consistent with several potential differences between the preparations. Firstly, it is likely that gene expression differences in OPCs between acute brain slices and the OBSCs likely reflect the fact that OPCs are continuing their maturation trajectories toward an oligodendrocyte fate in OBSCs (Gähwiler et al., 1997; Tripathi et al., 2017). Secondly, OPC responses to the axotomy of slicing as well as their potential involvement in aberrant fiber sprouting that is known to occur in OBSCs could underly the observed DEGs in these pathways (Berdichevsky et al., 2013b; Zimmer & Gähwiler, 1984).

OPCs not only differentiate into myelinating oligodendrocytes but also interact with excitatory neurons in the hippocampus (Bergles et al., 2000). This is due to the expression of AMPA-preferring glutamate receptors in the membrane of OPCs that facilitate the formation of glutamate-mediated synapses. These AMPA receptors are permeable to calcium ions, thereby establishing a connection between axonal activity and calcium concentration in OPCs (Bergles et al., 2000). In other words, the activation of excitatory neurons can lead to increased uptake of calcium into OPCs and ultimately regulate their function. My GO analysis showed that DEGs were enriched in chemical synaptic transmission and calcium ion transport pathways demonstrating that these processes

are likely altered between OBSCs and acute brain slices, however one should exercise caution given the small sample sizes involved in this study.

4.6.4. Endothelial cells

Aside from glial cells, there are also other cells, such as endothelial cells, that are crucial for the functioning of the brain; in this case, endothelial cells aid in regulating the vascular system within the brain. I observed that the pathways associated with downregulated DEGs had higher gene counts (<38) compared to those associated with upregulated DEGs (>2). This suggests that the most significant effects of endothelial cells in culture are related to the reduction or suppression of their activity. Thus, while the number of endothelial cells in acute brain slices versus OBSCs is broadly the same (i.e., this cell-type is not lost in culture) their function is substantially altered. This is somewhat unsurprising given the cessation of vascular perfusion. Indeed, GO analysis demonstrated that the genes involved with the large ribosomal protein (*Rpl30*, *Rpl32*, *Rpl34*, *Rpl12*, and *Rpl0*) were all downregulated indicating a change in translational machinery in this cell-type in response to being in culture. Such changes have been observed in endothelial cells before in response to stress or infection (Patel et al., 2021). Some genes were upregulated in OBSC endothelial cells including *Piezo2*, a gene encoding the Piezo ion channel proteins which regulate mechano-transduction (Coste et al., 2010). It is possible that *Piezo2* may be serving some function in maintaining the shapes of blood capillaries in OBSCs despite the lack of blood flow (Hutter-Schmid et al., 2015).

4.6.5. Excitatory neurons

Excitatory neurons were very well represented in my data set, with the highest number of DEGs (3751), with over half (53.08%) of these DEGs being unique to excitatory neurons.

It is possible that many of these DEGs may be related to developmental processes that are ongoing in culture. For example, *Specc1* plays a crucial role in maintaining cellular structure and adhesion through tight and adherens junctions during development (Lee & Choi, 2021). Indeed, a previous study by Ayupe et al. (2023) used snRNA-seq to analyze gene expression in the superior colliculus across developmental stages and identified

significant upregulation of *Specc1* from the 1st to 3rd postnatal weeks. Another example of excitatory neuron DEG, which was upregulated in my dataset is *Tanc1*. *Tanc1* is essential for maintaining dendritic spine density and synaptic connectivity in the hippocampal CA3 region, where it interacts with PSD-95 to regulate excitatory synapses and supports hippocampus-dependent spatial memory (Han et al., 2010). However, the relationship between the differential expression of *Tanc1* to development or the culturing process remains uncertain.

GO enrichment analysis revealed an upregulation of Fc-gamma receptor signalling pathways, which is surprising, as these pathways are typically associated with immune function (Stamou et al., 2018). Therefore, this perhaps reveals some sort of progressive response to the tissue injury following slicing.

It is widely known that in prolonged culturing periods, spontaneous seizure-like events (SLEs) can develop (Berdichevsky et al., 2012; J. Liu et al., 2017). It is thought that the slice preparation process itself induces deafferentation, triggering compensatory but aberrant axonal sprouting (Zimmer & Gähwiler, 1987). In line with this, my GO enrichment analysis revealed the downregulation of genes associated with axon guidance and axogenesis (e.g. *Ephb6* and *Draxin*). Both of these genes affect axon guidance via repulsive chemorepellent signalling (Sloniowski & Ethell, 2011; Tawarayama, 2018). Therefore, their downregulation would be associated with aberrant axogenesis in line with axon sprouting and aberrant excitatory connections which are well known to occur in OBSCs and contribute to seizure-like development (Berdichevsky et al., 2013).

4.6.6. Dentate gyrus cells

Finally, dentate gyrus cells in OBSCs were also found to undergo significant transcriptional changes compared to acute brain slices. For example, the top downregulated gene between OBSCs and acute brain slices was *Gabrg3*, $\gamma 3$ subunit of GABA-A receptors whose expression is thought to decrease during maturation of the dentate gyrus. In fact, multiple genes for GABA-A receptor subunits were differentially regulated (e.g. *Gabrb3*, *Gabrb2*, and *Gabrn1*). These differences are most likely linked to the ongoing maturational processes that are occurring in culture (Dieni et al., 2013; Farrant & Kaila, 2007) and probably underscore the top GO enrichment analysis of

upregulated DEGs term “chemical synaptic transmission”. That said, GABA-A receptor subunits can change expression in response to injury, such as in post-traumatic epilepsy, and presumably in response to the trauma of slicing, so this can’t be excluded as a possible cause of their differential expression (Becerra et al., 2021). Additionally, my data confirmed that axon guidance and fiber sprouting were very likely dysregulated in OBSCs as has previously been observed (Zimmer & Gähwiler, 1987). For example, *Tenm3* was downregulated in OBSC granule cells. A study by Berns et al. 2018) showed that *Tenm3* is essential for precise axon targeting in hippocampal circuits. Further the GO term, “negative regulation of axon extension” was downregulated, as was “axon guidance”, again supporting this idea.

4.7. Future directions

My study has many limitations that can be considered for future work. Firstly, I only had 3 samples for each group which limits the extent to which I can generalise my findings and has implications on the statistical power of my analyses. Secondly, whilst each sample contained 36 slices from multiple mice, the small sample size (3 per condition) means that variability was still an issue, and it was difficult to account for possible batch effects. So while snRNA-seq generates large amounts of data and has the potential to produce statistically significant results due to the high number of cells analyzed, I did use pseudobulking and the appropriate statistical tests to ensure the rigorousness of my findings. Nonetheless, they are based on a small number of samples collected at a single time point. This raises concerns about the repeatability of the results. Therefore, it would be useful to repeat this study with a bigger sample size. Nonetheless, I still found significant changes in cell-type composition and gene expression. Future work could add more samples at different time points. One of the most valuable additional comparisons would be to prepare snRNA-seq libraries from acute slices of P14 mice. These could be compared to my current data from acute slices of P7 mice and the OBSCs which are also P14 with 7 of those days in culture. In this way, I would be able to directly compare putative maturational changes which I think are continuing in the slices *in vitro* to those that occur *in vivo*. I would be able to determine which maturational changes are halted, and which continue at various speeds compared to the *in vivo* situation. I would also more easily be able to separate which changes are due to the culturing process itself

rather than those that are due to ongoing developmental processes. In addition, temporal and maturational dynamics could be effectively studied using pseudotime analysis such as monocle (Trapnell et al., 2014). Further, advanced multimodal sequencing techniques, as demonstrated by Cao et al. (2018) and Zhu et al. (2019), who successfully combined DNA and RNA measurements in single cells could be used. This multimodal approach could be further enhanced by incorporating RNA velocity analysis to predict the trajectories of cellular state changes during the culture period (La Manno et al., 2018).

Secondly, the substantial transcriptional changes identified in neurons and astrocytes need deeper functional investigation. In neurons this could be accomplished by adapting the patch-seq approach demonstrated by Cadwell et al. (2015) and Fuzik et al. (2015), who successfully combined whole-cell patch recordings with transcriptome sequencing. Gouwens et al. (2020) profiled 3700 GABAergic neurons in the mouse visual cortex, demonstrating how transcriptomic analysis can be integrated with detailed electrophysiological characterization. This could be employed in OBSCs and acute brain slices to understand how gene expression changes affect neuronal function.

Thirdly, the differential gene expression of genes related to axon guidance suggests significant circuit reorganization in OBSCs. Future studies could employ high-resolution spatial transcriptomics to map gene expression in the brain tissue (Ståhl et al., 2016). Furthermore, to explore the circuit reorganization, in situ sequencing can be used to map long-range neuronal projections. The MAPseq approach described by Kebschull et al. (2016) could be particularly valuable for tracing multiple projections from individual neurons in OBSCs, providing insight into how culture conditions affect circuit formation.

Finally, and more simply, there are running studies in our lab using in situ hybridization to validate the DEGs that I found. However, these could be expanded using emerging techniques like MERFISH which allows for highly multiplexed detection of RNA species while preserving spatial information (Xia et al., 2019).

These various research directions would not only build upon my current findings but also significantly advance our understanding of brain slice models and their utility in neuroscience research.

4.8. Conclusion

In conclusion, my snRNA-seq study revealed significant molecular and cellular differences in organotypic brain slice cultures compared to acute brain slices. The findings demonstrate substantial changes in cellular composition, with increased glial representation and selective changes in neuronal populations. The identification of over 4200 differentially expressed genes, particularly in excitatory neurons and astrocytes, highlights the extensive transcriptional remodelling that occurs during culture. These changes reflect both developmental processes and adaptation to ex vivo conditions, particularly in axon guidance and inflammatory pathways. These insights are crucial for understanding the capabilities and limitations of organotypic brain slice cultures as experimental models in the field of neuroscience.

4.9. Academic acknowledgments

Biorender was used to generate some figures included in this thesis.

Teresa Steyn provided guidance on the snRNA-seq pipeline code and allowed adaptations of her code.

Teresa Steyn, Josh Selfe, Emily Higgitt, Rachael Dangarembizi, and Joseph Raimondo assisted in the generation of snRNA-seq libraries.

Rodney Lucas taught proper mouse-handling techniques, helping to overcome an initial fear of mice.

Supplementary Data

Supplementary Table 1: CellBender optimization parameters

Using low threshold 100	OBSC 1	OBSC 2	OBSC 3	Acute 1	Acute 2	Acute 3
total_raw_counts	33796348,00	31404298,00	44835224,00	34465367,00	27253630,00	28366839,00
total_output_counts	26117447,00	26221684,00	35638205,00	28176657,00	22289765,00	24017920,00
total_counts_removed	767890,00	5182614,00	9197019,00	6288710,00	4963865,00	4348919,00
fraction_counts_removed	0,23	0,17	0,21	0,18	0,18	0,15
total_raw_counts_in_cells	33796348,00	31404298,00	44835224,00	34465367,00	27253630,00	28366839,00
total_counts_removed_from_cells	7678901,00	5182614,00	9197019,00	6288710,00	4963865,00	4348919,00
fraction_counts_removed_from_cells	0,23	0,17	0,21	0,18	0,18	0,15
average_counts_removed_per_cell	760,21	479,52	677,45	952,84	932,71	858,62
target_fpr	0,01	0,01	0,01	0,01	0,01	0,01
expected_cells	3431,00	2987,00	3609,00	2499,00	2141,00	2047,00
found_cells	10101,00	10808,00	13576,00	6600,00	5322,00	5065,00
Cellranger output of number of cells present	9989,00	11729,00	14391,00	6569,00	5776,00	5206,00
output_average_counts_per_cell	2585,63	2426,14	2625,09	4269,19	4188,23	4741,94
Cellranger median UMI counts per cell	2414,00	1521,00	1913,00	3783,00	3456,00	3953,00
ratio_of_found_cells_to_expected_cells	2,94	3,62	3,76	2,64	2,49	2,47
found_empties	3400,00	1777,00	6487,00	2844,00	5735,00	4852,00
fraction_of_analyzed_droplets_that_are_noner	0,75	0,86	0,68	0,70	0,48	0,51
convergence_indicator	0,09	1,06	0,12	0,38	1,90	0,55
overall_change_in_train_elbo	2171,75	2085,81	1577,03	3340,85	2225,15	2753,46

Supplementary Table 2.1: Mean nuclei count calculations for each cluster

Clusters	Acute 1	Acute 2	Acute 3	Total Acutes	Mean nuclei proportions in Acutes	OBSC 1	OBSC 2	OBSC 3	Total OBSCs	Mean nuclei proportions in OBSCs
0	885	713	679	2277	759	2175	1454	1137	4766	1588,666667
1	358	379	297	1034	344,6666667	507	657	666	1830	610
2	533	408	309	1250	416,6666667	393	665	417	1475	491,6666667
3	415	374	368	1157	385,6666667	427	395	638	1460	486,6666667
4	360	350	305	1015	338,3333333	413	352	356	1121	373,6666667
5	479	445	396	1320	440	283	267	211	761	253,6666667
6	331	278	240	849	283	238	470	233	941	313,6666667
7	290	229	205	724	241,3333333	279	354	363	996	332
8	94	132	96	322	107,3333333	350	173	387	910	303,3333333
9	0	0	0	0	0	0	0	0	0	0
10	43	50	37	130	43,33333333	244	312	337	893	297,6666667
11	172	164	114	450	150	260	115	161	536	178,6666667
12	154	43	316	513	171	28	214	23	265	88,33333333
13	92	58	85	235	78,33333333	140	144	153	437	145,6666667
14	15	4	10	29	9,666666667	191	225	170	586	195,3333333
15	140	72	165	377	125,6666667	66	99	18	183	61
16	184	164	144	492	164	16	14	21	51	17
17	46	89	65	200	66,66666667	150	8	82	240	80
18	15	6	13	34	11,33333333	216	110	75	401	133,6666667
19	141	45	102	288	96	74	44	22	140	46,66666667
20	101	58	87	246	82	72	64	3	139	46,33333333
21	49	61	46	156	52	92	27	64	183	61
22	61	57	37	155	51,66666667	42	56	55	153	51
23	39	66	59	164	54,66666667	52	65	3	120	40
24	56	15	23	94	31,33333333	66	71	45	182	60,66666667
25	22	62	40	124	41,33333333	53	59	28	140	46,66666667
26	19	51	6	76	25,33333333	83	41	20	144	48
27	0	0	1	1	0,333333333	4	49	3	56	18,66666667
28	5	5	10	20	6,666666667	5	1	0	6	2
					0					0
Total	5099	4378	4255	13732	4577,333333	6919	6505	5691	19115	6371,666667

Supplementary Table 2.2: Scaled proportion calculations for each cluster

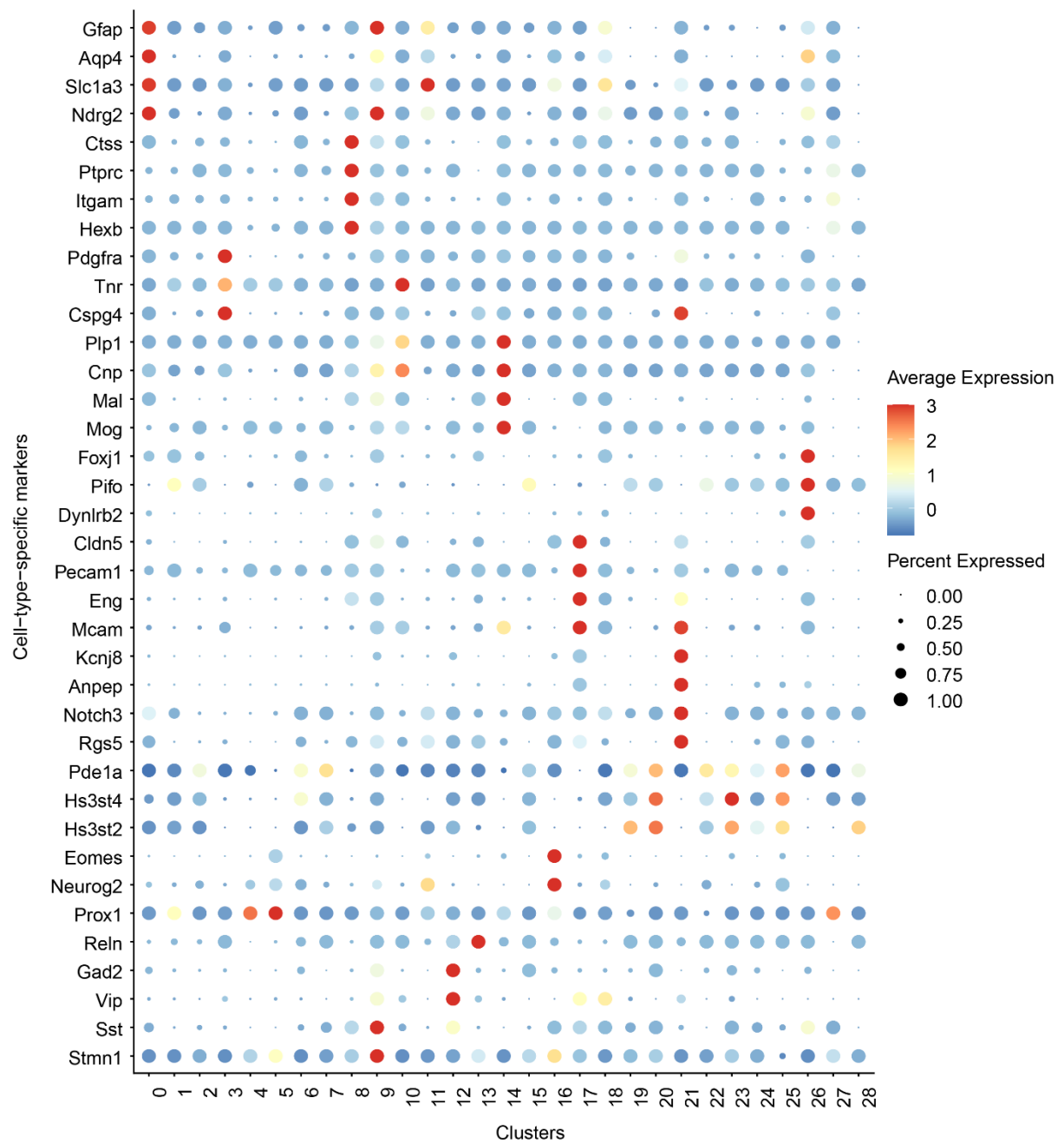
Clusters	Average proportion in Acutes	Number of cells in Acutes	Average proportion in OBSCs	Number of cells in OBSCs	factor = (1/average proportion in Acutes + average proportion in OBSCs)	Scaled proportion in Acutes (factor*average proportion in Acutes)	Scaled proportion in OBSCs (factor*average proportion in OBSCs)	Check (Scaled proportion in Acutes + Scaled proportion in OBSCs)
0	0,165333	2277	0,2458871	4766	2,431785459	0,40205533	0,59794467	1
1	0,075526	1034	0,09710087	1830	5,792826615	0,437511513	0,562488487	1
2	0,090115	1250	0,07743425	1475	5,968406294	0,537840915	0,462159085	1
3	0,084434	1157	0,07818116	1460	6,149486556	0,519226014	0,480773986	1
4	0,074076	1015	0,05878595	1121	7,526616474	0,557540727	0,442459273	1
5	0,096217	1320	0,03967443	761	7,358805603	0,70804356	0,29195644	1
6	0,061606	849	0,04919733	941	9,024993265	0,55599444	0,44400556	1
7	0,052453	724	0,05284278	996	9,497041534	0,498149902	0,501850098	1
8	0,023716	322	0,04839413	910	13,86771408	0,328884087	0,671115913	1
9	0	0	0	0				
10	0,009516	130	0,04748154	893	17,54447095	0,16696148	0,83303852	1
11	0,032661	450	0,02784889	536	16,52611961	0,539765879	0,460234121	1
12	0,038096	513	0,01366202	265	19,32050128	0,736042877	0,263957123	1
13	0,017089	235	0,02308517	437	24,89155156	0,425374298	0,574625702	1
14	0,002069	29	0,03068855	586	30,52775182	0,063147558	0,936852442	1
15	0,02756	377	0,00930697	183	27,12451896	0,74755299	0,25244701	1
16	0,035796	492	0,00271823	51	25,9644106	0,92942267	0,07057733	1
17	0,014875	200	0,01243932	240	36,61018401	0,544594057	0,455405943	1
18	0,002456	34	0,02043572	401	43,68425569	0,107280832	0,892719168	1
19	0,020634	288	0,00710832	140	36,04559944	0,743776251	0,256223749	1
20	0,017834	246	0,00692395	139	40,39084372	0,720335658	0,279664342	1
21	0,011451	156	0,0095644	183	47,58351833	0,544892209	0,455107791	1
22	0,011226	155	0,00811446	153	51,70469332	0,580444227	0,419555773	1
23	0,012197	164	0,00601167	120	54,91993457	0,669839685	0,330160315	1
24	0,006605	94	0,00945362	182	62,27292867	0,411295528	0,588704472	1
25	0,009292	124	0,00721669	140	60,57295217	0,562863946	0,437136054	1
26	0,005595	76	0,00727104	144	77,72302734	0,434872811	0,565127189	1
27	7,83E-05	1	0,00287931	56	338,1062226	0,026486974	0,973513026	1
28	0,001491	20	0,00029213	6	560,8304378	0,836167281	0,163832719	1

Supplementary Table 2.3: Mean nuclei count calculations for each cell-type

Cell types	Acute 1	Acute 2	Acute 3	Total Acutes	Mean nuclei counts in Acutes	OBSC 1	OBSC 2	OBSC 4	Total OBSCs	Mean nuclei counts in OBSCs
Astrocytes	1072	883	806	2761	920,3333333	2651	1679	1373	5703	1901
Microglie	94	132	96	322	107,3333333	350	173	387	910	303,3333333
OPCs	458	424	405	1287		429	671	707	2353	784,3333333
Oligodendrocytes	15	4	10	29	9,666666667	191	225	170	586	195,3333333
Ependymal cells	19	51	6	76	25,33333333	83	41	20	144	48
Endothelial cells	46	89	65	200	66,66666667	150	8	82	240	80
Pericytes	49	61	46	156		52	92	27	64	183
Excitatory neuron:	1719	1295	1277	4291	1430,333333	1340	1948	1187	4475	1491,666667
Dentate progenitor	184	164	144	492		164	16	14	21	51
Dentate gyrus cells	1197	1174	999	3370	1123,333333	1207	1325	1236	3768	1256
Cajal-Retzius cells	92	58	85	235	78,33333333	140	144	153	437	145,6666667
Inhibitory neuron	154	43	316	513		171	28	214	265	88,33333333
Total	5099	4378	4255	13732		6919	6505	5691	19115	

Supplementary Table 2.4: Scaled proportion calculations for each cell-type

Cell types	Proportion in Acutes	Number of cells in Acutes	Proportion in OBSCs	Number of cells in OBSCs	factor = (1/prop in acute+ prop in organo)	Scaled proportion in Acutes (factor*proportion in Acutes)	Scaled proportion in OBSCs (factor*proportion in OBSCs)	Check (Scaled proportion in Acutes + Scaled proportion in OBSCs)
Astrocytes	0,200450593	2761	0,294171709	5703	2,021744666	0,405259916	0,594740084	1
Microglia	0,023715811	322	0,048394127	910	13,86771408	0,328884087	0,671115913	1
OPCs	0,093950516	1287	0,1256627	2353	4,553460021	0,427799919	0,572200081	1
Oligodendrocytes	0,00206853	29	0,03068855	586	30,52775182	0,063147558	0,936852442	1
Ependymal cells	0,00559516	76	0,007271039	144	77,72302734	0,434872811	0,565127189	1
Endothelial cells	0,01487548	200	0,012439324	240	36,61018401	0,544594057	0,455405943	1
Pericytes	0,01145128	156	0,0095644	183	47,58351833	0,544892209	0,455107791	1
Excitatory neurons	0,311013201	4291	0,233902167	4475	1,835147362	0,570755055	0,429244945	1
Dentate progenitors	0,035796024	492	0,002718233	51	25,9644106	0,92942267	0,07057733	1
Dentate gyrus cells	0,245897833	3370	0,198440558	3768	2,250537028	0,553402177	0,446597823	1
Cajal-Retzius cells	0,017089103	235	0,02308517	437	24,89155156	0,425374298	0,574625702	1
Inhibitory neurons	0,038096469	513	0,013662023	265	19,32050128	0,736042877	0,263957123	1



Supplementary figure 1) Cluster markers. Bubble plot highlighting the percentage and average expression of cell-type-specific markers for 28 clusters.

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