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Morphological and Activity-Dependent Effects of Astrocyte Activation in the Orbitofrontal Cortex

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Morphological and Activity-Dependent Effects of Astrocyte Activation in the Orbitofrontal Cortex

by

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A THESIS

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Abstract

Astrocytes, the most abundant glial cell in the central nervous system (CNS), have significant roles in supplying energetic substrates to neurons, regulating blood brain barrier (BBB) permeability, homeostasis of ions and pH to neurons in the tripartite synapse. Much remains unknown about astrocytes due to the limitations of current tools to visualize and manipulate astrocyte activity. To understand astrocyte physiology and pathophysiology, we need methods to visualize and specifically assess the activity of astrocytes. A recent astrocyte promoter, gfaABC1D, may provide better astrocyte specificity and transduction efficiency in the cortex. S100β is a calcium-binding glycoprotein only expressed in the soma of astrocytes also can be used as a marker for astrocytes. To activate cortical astrocytes, we targeted excitatory Designer Receptors Exclusively Activated by Designer Drugs (hM3Dq DREADDs) to astrocytes of the orbitofrontal cortex (OFC), a region involved in decision making and injected the DREADD-specific, brain penetrant ligand, DCZ. I hypothesize that using a combination approach of S100β, and gfaABC1D-tagged DREADD virus will successfully label and activate astrocytes in the lateral OFC (LOFC), whilst not indirectly influencing neuronal activity (measured by cFos). This thesis used a novel IMARIS 3D visualization method to visualize astrocytes and the colocalization of the DREADD-reporter in astrocytes. The result suggests that there are more astrocytes in the LOFC compared to the medial (MOFC), independent of DCZ administration. There is intraregional heterogeneity between the LOFC and the MOFC. The gfaABC1D-DREADD virus successfully transfected astrocytes in LOFC. There was no increase in cFos intensity in NeuN cells in the LOFC or the MOFC, suggesting that activation of Gq-DREADDs in astrocytes was not sufficient to affect neuronal activation. Future research should address the calcium activity response to DCZ on excitatory DREADDS in astrocytes *in-vitro*. This thesis offers an acute method to assess astrocytes

in the cortex to further be used in a chronic model to induce inflammation, to better understand cortical reward system inflammation seen in addiction and obesity.

Key words: *DREADDs, Acute Activation, Astrocytes, DCZ, Reactive Astrocytes*

Preface

Land acknowledgement: This work was conceived and performed at The University of Calgary, located on the traditional territories of the people of the Treaty 7 region in Southern Alberta, which includes the Blackfoot Confederacy (including the Siksika, Piikuni, Kainai First Nations), the Tsuut'ina, and the Stoney Nakoda (including the Chiniki, Bearspaw, and Wesley First Nations). The City of Calgary is also home to Metis Nation of Alberta, Region 3.

Research: Research done in chapter two was conducted in the Borgland lab at the University of Calgary and in accordance with the approved animal protocols (AC17-0004 and AC21-0034). I designed and conducted all experiments under the supervision of Dr. Stephanie Borgland. I analyzed the data and wrote the manuscript with the guidance of Dr. Stephanie Borgland. This work was supported by the Alberta Graduate Excellence Scholarship I was awarded.

Credit: All graphical figures were made with BioRender.com.

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I am among those who think that science has great beauty.

— Marie Curie

Dedication

This thesis is dedicated to women in S.T.E.M -past, present, and future. Our world would not be where it is today without our efforts. Keep exploring.

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List of Abbreviations

AMPAR: Aminomethylphosphonic Acid Receptor

BAC: Bacterial Artificial Chromosome

BBB: Blood Brain Barrier

bHLH: Basic Helix–Loop–Helix

BMPs: Bone Morphogenetic Protein

CNO: Clozapine-N-Oxide

CNS: Central Nervous System

CNTF: Ciliary Neurotrophic Factor

C21: Compound 21

DCZ: Deschloroclozapine

DREADD: Designer Receptors Exclusively Activated by Designer Drugs

E: Embryonic

FGF: Fibroblast Growth Factor

GFAP: Glial Fibrillary Acidic Protein

GLAST: Glutamate-Aspartate Transporter

GLU: Glutamate Transporter-1

GPCR: G-Protein-Coupled Receptor

IL6: Interleukin-6

i.p: intraperitoneal

IPC: Intermediate Progenitor Cell

ISF: Interstitial Cerebral Fluid

LAR: Leukocyte Antigen-Related Receptor

MCT: Monocarboxylate Transporter

NEC: Neuroepithelial Cell

NMDAR: N-methyl-D-aspartate receptor

P: Postembryonic

PDGF: Platelet-Derived Growth Factor

PET: Positron emission tomography

PFA: Paraformaldehyde

RGC: Radial Glial Cell

RFP: RED Fluorescent Protein

sEPSC: Synaptic Excitatory Post Synaptic Potential

sEPSC: Synaptic Inhibitory Post Synaptic Potential

SHH: Sonic-Hedgehog

SPARCS: Secreted Protein Acidic Rich in Cysteine

s.q: subcutaneous

SR101: Sulforhodamine 101

SVZ: Subventricular Zone

TNFα: Tumour Necrosis Factor alpha

VEH: Vehicle

VZ: Ventricular Zone

CHAPTER ONE: INTRODUCTION

Astrocytes are the most abundant cell in the central nervous system (CNS), but paradoxically, so much remains a mystery on how these star-shaped cells function and are modulated in disorders (Argente-Arizón et al., 2015). This introductory chapter offers a literature review of the development of astrocytes and other cells in the CNS, the role of astrocytes in the CNS and their regulation of neurons, and how to visualize and target astrocytes. There will be discussion on tools used in glial research to better understand the roles of physiological astrocytes and how their function is perturbed in pathophysiology. This introduction emphasizes what is well researched in the astrocyte field and what remains unknown due to limitations with the current methods to visualize astrocytes.

1.0 Development of Different Cell Types in the CNS

1.1 Location of Proliferation, Migration, and Division of Cells

For decades, researchers have relied on the impressive fate-mapping technique to study embryonic development and the lineage of a particular cell's fate (Clarke & Tickle, 1999). Fate-mapping uses the Cre-recombinase system to label cells in the embryo to trace their trajectory to their final locations (Legué & Joyner, 2010). Throughout their travels, these cells undergo the processes of proliferation, migration, and division. All cell division and proliferation occur in the primary germinal ventricle's zones comprised of the ventricular zone, VZ (deepest layer), and the subventricular zone (SVZ) in the developing brain (Rowitch & Kriegstein, 2010). The formation of neurons (neurogenesis) occurs in the VZ. Neurogenesis begins at embryonic (E) day 12, peaks

at E14, and ends at E17 (Parnavelas, 1999). The SVZ is developed by E14 and is the region where glial lineages are formed (gliogenesis) (Parnavelas, 1999). Astrocytes, neurons, and other cells originate from stem cells, called neuroepithelial cells (NECs), and later differentiate into specialized cells. NECs line the cerebral ventricles and spinal canal and will proliferate to increase cell number (Rowitch & Kriegstein, 2010). NECs have two fates: asymmetrical self-renewal into either neurons, or primary progenitor cells termed radial glial cells (RGCs) (Figure 1A). The process of neurogenesis occurs before astrocyte formation (astrogenesis).

1.2 Neurogenesis

In the rodent cortex, neurons and RGCs originate from the VZ (Sauvageot & Stiles, 2002; Figure 1A). All cells in the central nervous system arise from RGCs in the ventricular zone during development (Rowitch & Kriegstein, 2010). RGCs are the primary proliferative cells of the embryonic telencephalon's SVZ (Rowitch & Kriegstein, 2010). RGCs ultimately produce intermediate progenitor cells (IPCs) that will become neurons or different types of glia (Haubensak, 2004; Miyata, 2004; Noctor, 2004; Noctor, 2008; Figure 1B). Once a neuron is formed through the process of neurogenesis, this new neuron will need to leave the VZ. Neurons use the long radial glial processes to migrate to the SVZ and then ultimately reside in the white matter (Cayre, 2009; Purves, 2001; Figure 1C). Neurogenesis peaks at E14, with the primary germinal zone formed by E19 (Parnavelas, 1999).

1.3 Astrogenesis

Researchers using Cre-*lox* fate-mapping found that RGCs differentiate into macroglia or astrocytes (Noctor et al., 2008; Figure 1D). RGCs proliferate and become intermediate progenitor

cells (IPC) to eventually differentiate into an astrocyte or repress back into a neuron (Noctor et al., 2008). In summary, NECs proliferate into RGCs, and then differentiate into neurons or ultimately into different types of glia or ependymal cells. Peak astrocyte formation happens between postembryonic (P) days, P0 – P2 (Parnavelas, 1999). Astrocyte development is still an emerging field that has much controversy regarding the complexities of astrogenesis (Noctor, 2008; Rowitch & Kriegstein, 2010). How astrocytes migrate is still not fully known. Researchers have relied on *in-vitro* experiments to generally accept that astrocytes first move tangentially in the white matter, and then in a radial direction in the grey matter (Cayre et al., 2009). Since development, astrocytes begin to specialize revealing heterogeneity phenotypes (Chaboub & Deneen, 2012). The concept of heterogeneity will be revisited throughout this thesis as it is foundational, yet not well-understood.

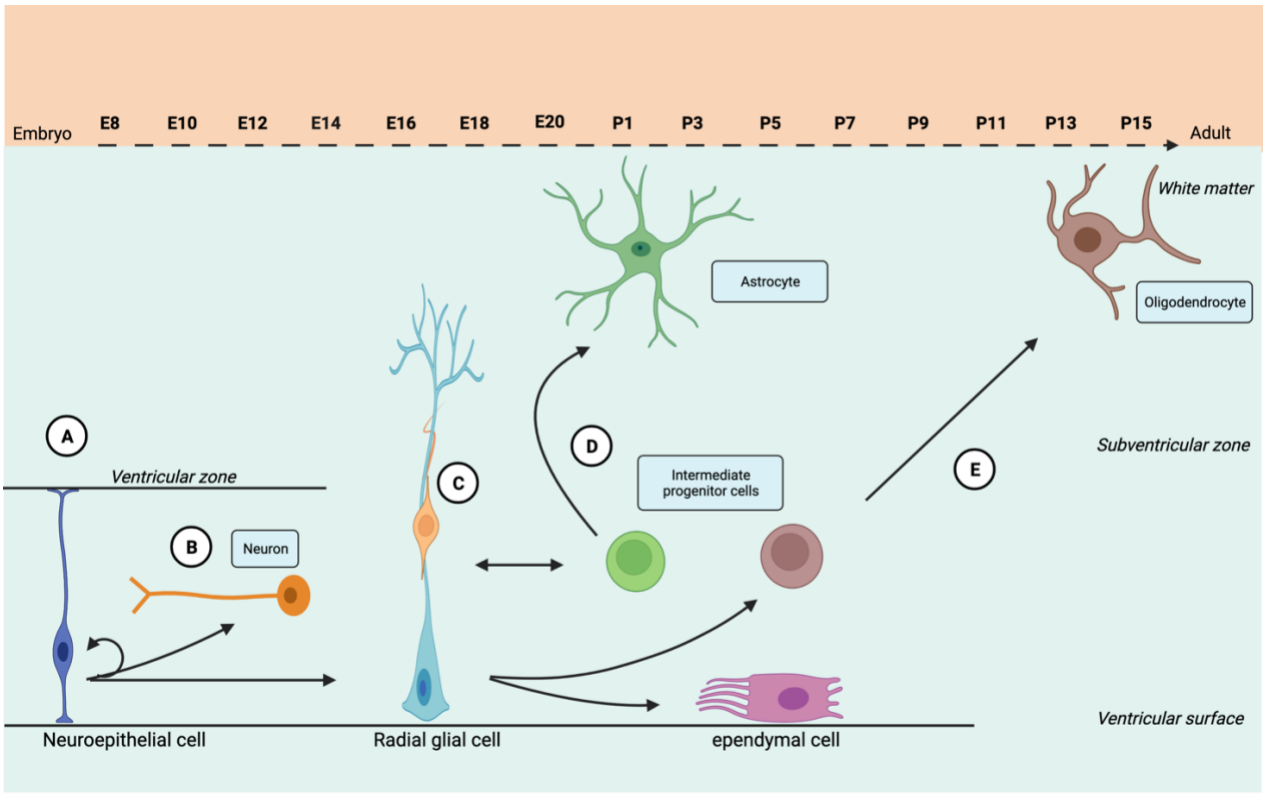

Figure 1: Simplified diagram of the developmental trajectory of different cells in the CNS.

Figure 1: The time course of cell fate beginning from an embryo to an adult as indicated by the timeline in the orange border. Embryonic (E) days are lineated followed by the post-embryonic (P) days.

A) NECs line the cerebral ventricles in the VZ. A NEC (dark blue) has the choice of undergoing self-renewal (indicated by the circular arrow) or will undergo neurogenesis and differentiate into a neuron. NECs can also proliferate into RGCs (light blue).

B) Neurogenesis. A neuron is formed around E12. A newly formed neuron will adventure out of the VZ to reside in the white matter.

C) For the neuron (orange) to leave the VZ it utilizes the long processes of a RGC. The neuron tethers to the RGC and migrates or climbs up to the subventricular zone. The RGC will differentiate into an IPC. The RGC can also differentiate into an ependymal cell (purple).

D) Different types of IPCs. The green IPC will differentiate into an astrocyte around P2, or it has the option of reverting back into a neuron (indicated by the double-headed arrow).

E) The brown IPC differentiates into an oligodendrocyte which will reside in the white matter.

2.0 Cellular Functions to Promote Either Neurogenesis or Astrogenesis

2.1 Morphogens

As mentioned, radial glia produce IPCs and oligodendrocyte precursor cells, which in turn produce neurons and oligodendrocytes, respectively. Radial glia can also become a different cell type, like an astrocyte (Rowitch & Kriegstein, 2010). The incredible *in-vivo* differentiation process of NECs, RGCs, and IPCs are impacted by the organizing signals of sonic-hedgehog (SHH), fibroblast growth factors (FGFs), Wnts, and bone morphogenetic proteins (BMPs) (Rowitch & Kriegstein, 2010). These signalling proteins, or morphogens, rely on competing concentration gradients over long distances to determine where they reside in the developing brain (Rogers & Schier, 2011).

SHH is the crucial organizational protein in the ventral midline of the neural tube (Placzek, 1995). It positions the notochord and the floor plate, which will eventually transform into the developing nervous system (Placzek, 1995). SHH signaling is repulsed by the concentration gradients of dorsal BMPs and Wnt-mediated signalling (Briscoe & Novtich, 2008; Rowitch & Kriegstein, 2010; Ulloa & Marti, 2010). SHH is necessary for NG2 (oligodendrocyte marker) expression, which was abolished when cells were treated with cyclopamine, an SHH antagonist (Tekki-Kessaris et al., 2001). SHH has a critical role in the later development of astrocytes and oligodendrocytes.

Consistent with prior research, later in development BMPs promote astrogenesis in cortical cultures (Mehler et al., 2000). In contrast, BMPs will promote neurogenesis in early development (Mabie et al., 1999). Since an astrocytic fate is later favoured, transduction pathways and molecular mechanisms promoting a neuronal phenotype, such as basic/helix-loop-helix (bHLH) factors, are

silenced (Nakashima et al., 2001). Morphogens work together, competing to express their desired cell fate in a critical development window.

2.2 Notch Signaling

Another mechanism to promote astrogenesis, is the NOTCH signaling pathway (Fox & Kornblum, 2005). Activation of NOTCH ligands on neurons activates NOTCH signaling in radial glia to differentiate into astrocytes. NOTCH signaling will inhibit bHLH neurogenic factors; whilst activating the JAK/STAT pathway that upregulates astrocyte-specific genes (Kamakura et al., 2004). NOTCH signaling is an excellent example of the on-off switch of neuronal factor suppression and the promotion of astrocyte factors. Notch signalling has an intimate relationship with pro-glial transcription factors that are required for astrocyte promotion (Rowitch & Kriegstein, 2010). Loss-of-function experiments, like knocking out or mutating *Sox9* (a pro-glial transcription factor), had defective astrocyte and oligodendrocyte production (Karcavich & Doe, 2005). Fascinatingly, *Sox9* deficiencies increase neurogenesis, suggesting that *Sox9* is necessary in the promotion of glial fate.

2.3 Growth Factors

External cues, like growth factors, influence the fate-determination of stem cells. Growth factors such as platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF), and interleukin 6 (IL6) promote neurogenesis (Sauvageot & Stiles, 2002). Isolated neuroepithelial cells in the neurogenesis phase treated with PDGF will differentiate cells into neurons (Williams et al., 1997). Researchers have found that removing pro-neuronal bHLH transcription factors will result in gliogenesis instead of neurogenesis (Sauvageot & Stiles, 2002).

In-vitro experiments using pro-gliogenesis growth factors, aids in the understanding of their *in-vivo* mechanisms. Cells that are typically going to differentiate into astrocytes *in-vivo* are treated with CTNF *in-vitro*, they will still express the astrocytic marker glial fibrillary astrocytic protein (GFAP) in a 24-hour window period (Bonni et al., 1997). The response of these extracellular cues is modulated by time. Conversely, if the same experiment is done with cells isolated during peak neurogenesis are exposed to CNTF, astrocytic differentiation will be delayed for a few days (Bonni et al., 1997). CNTF activates the STAT pathway, which results in astrocyte formation (Kiu & Nicholson, 2012; Sauvageot & Stiles, 2002). The STAT pathway plays a significant role in immune function as proinflammatory cytokines like IL-6 are involved (Sauvageot & Stiles, 2002). Neurons are also able to secrete IL-6 to promote gliogenesis (Barnabé-Heider, 2005; Bonni, 1997). Therefore, growth factors activate other molecules, like cytokines, to cause downstream signalling cascade effects.

BMPs can further be classified as growth factors. Cytokines are a subtype of growth factor produced by hematopoietic and immune cell types, including microglia. Time critically determines the effect of growth factors on stem cells (Nakashima et al., 2001). For example, *in-vitro* and *in-vivo*, BMPs enhance neurogenesis or gliogenesis depending on the age of the cortical cells. Early prenatal cell culture exposed to BMP2/4 will differentiate into neurons (Nakashima et al., 2001). However, post-embryonic cells will still develop into astrocytes when treated with BMP2 (Gross, 1996; Nakashima, 2001). Depending on the critical timeframe of cell-fate determination, growth factors and morphogens will compete for either neural or glial expression.

3.0 General Astrocyte Function

3.1 Formation, Maturation, and Pruning of the Tripartite Synapse

Astonishingly, a single astrocyte can interact with multiple neurons and contact up to 100,000 synapses (Bushong, 2002; Halassa, 2007). Astrocytes are intimately near synapses to control synaptic transmission (Argente-Arizón, 2015; Chung, 2015). The structural and functional relationship between an astrocyte's perisynaptic processes with the neuronal pre- and post-synapse are collectively called the "tripartite synapse" (Araque et al., 1999). Astrocytes are integral in the in the beginning formation of synapses, a process called synaptogenesis (Clarke & Barres, 2013). For the context of this thesis, the aid of astrocytes in the three stages of excitatory synapse regulation will be explained.

The first stage of tripartite synapse formation, called synaptogenesis begins with immature synapse formation between axons and dendrites (Clarke & Barres, 2013). In the developing brain, astrocytes use the secretion of large extracellular matrix proteins, called thrombospondins (TSPs), to promote synapse formation between axons and dendrites (Christopherson et al., 2005). The postsynaptic neuronal cleft will secrete TSPs to bind to cell adhesion proteins called neuroligins and integrins (DeFreitas, 1995; Xu, 2010; Figure 2). Post-development astrocytes can modulate synaptic formation given the pressures of the surrounding environment. Astrocytes have a critical role in ensuring the correct number of synapses. Astrocytes can modulate the number of synapses by using two proteins called hevins and secreted protein acidic rich in cysteine (SPARCs) (Kucukdereli et al., 2011; Figure 2). If astrocytes are needed to upregulate the number of synapses and synapse size, hevins are released to bind to postsynaptic neurexins and neuroligins (Figure 2). However, if there needs to be a decrease in synapse number, then anti-adhesive SPARCs are released, counteracting the actions of hevins (Kucukdereli et al., 2011). Dual secreted protein release is an excellent example of astrocyte modulation and synaptic plasticity. SPARCs will then

interact with integrin receptors to mature inactive synapses into active synapses (Allen et al., 2012).

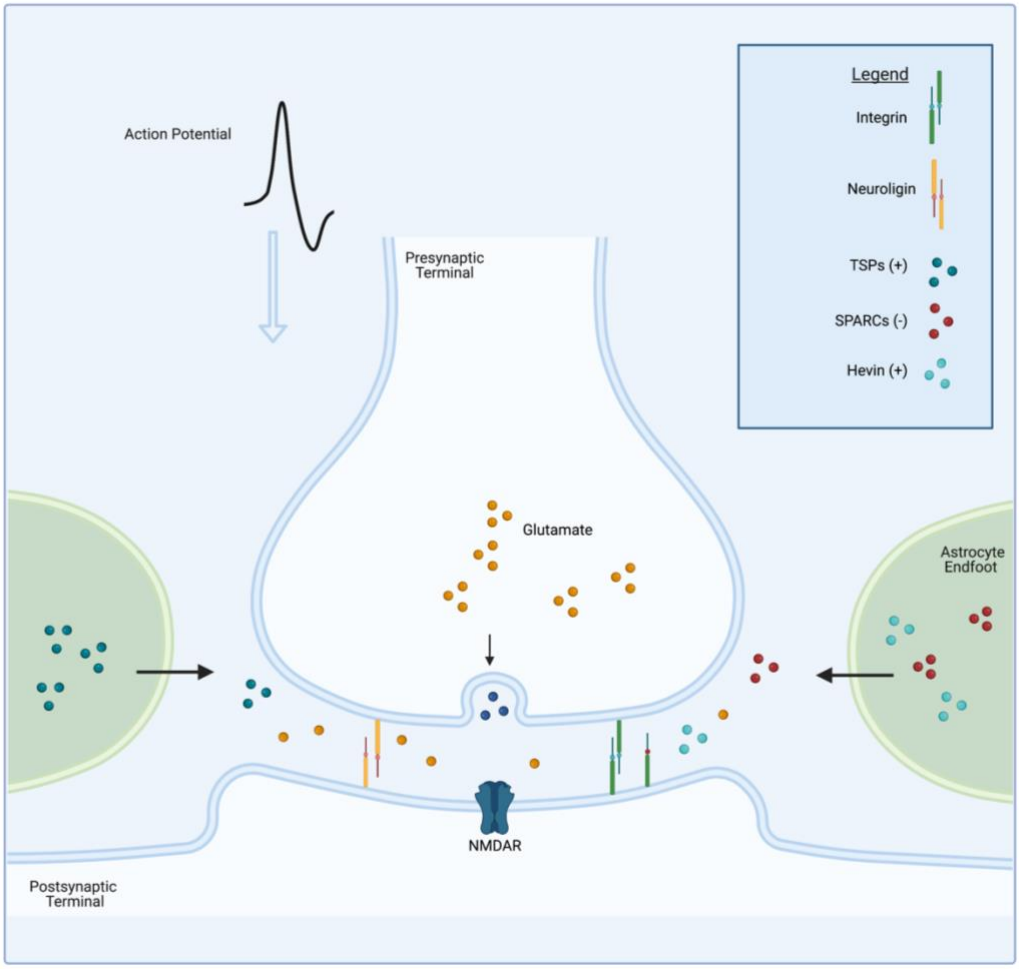

Figure 2: Astrocytes secrete several molecules to regulate synapse formation.

A presynaptic action potential will fail to evoke a detectable postsynaptic signal in immature synapses. To respond to activity, astrocytes mature the synapses (insert N-methyl-D-aspartate receptors (NMDARs) in the post synaptic cleft) to respond to a presynaptic action potential (Clarke & Barres, 2013). A postsynaptic potential is received by active NMDARs (Clarke & Barres, 2013). Inactive NMDARs have a magnesium voltage-dependent block. The activity of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPAR activation) caused by depolarization is sufficient to remove the block to allow for cation influx (Clarke & Barres, 2013; Figure 3). Astrocytes will then secrete proteoglycans, called glypicans, to bind to the leukocyte antigen-related receptor (LAR) (Figure 3). Triggering LAR increases synaptic activity and AMPAR activation, thus, insertion NMDARs into the postsynaptic plasma membrane (Allen, 2012; Dunah, 2005; Figure 3). Therefore, glutamate release from the presynaptic cleft through several mechanisms allow the influx of intracellular calcium in the post synaptic cleft (Clarke & Barres, 2013). Astrocytes have an integral role in synaptic signaling via neurotransmitter release and NMDAR insertion for calcium influx.

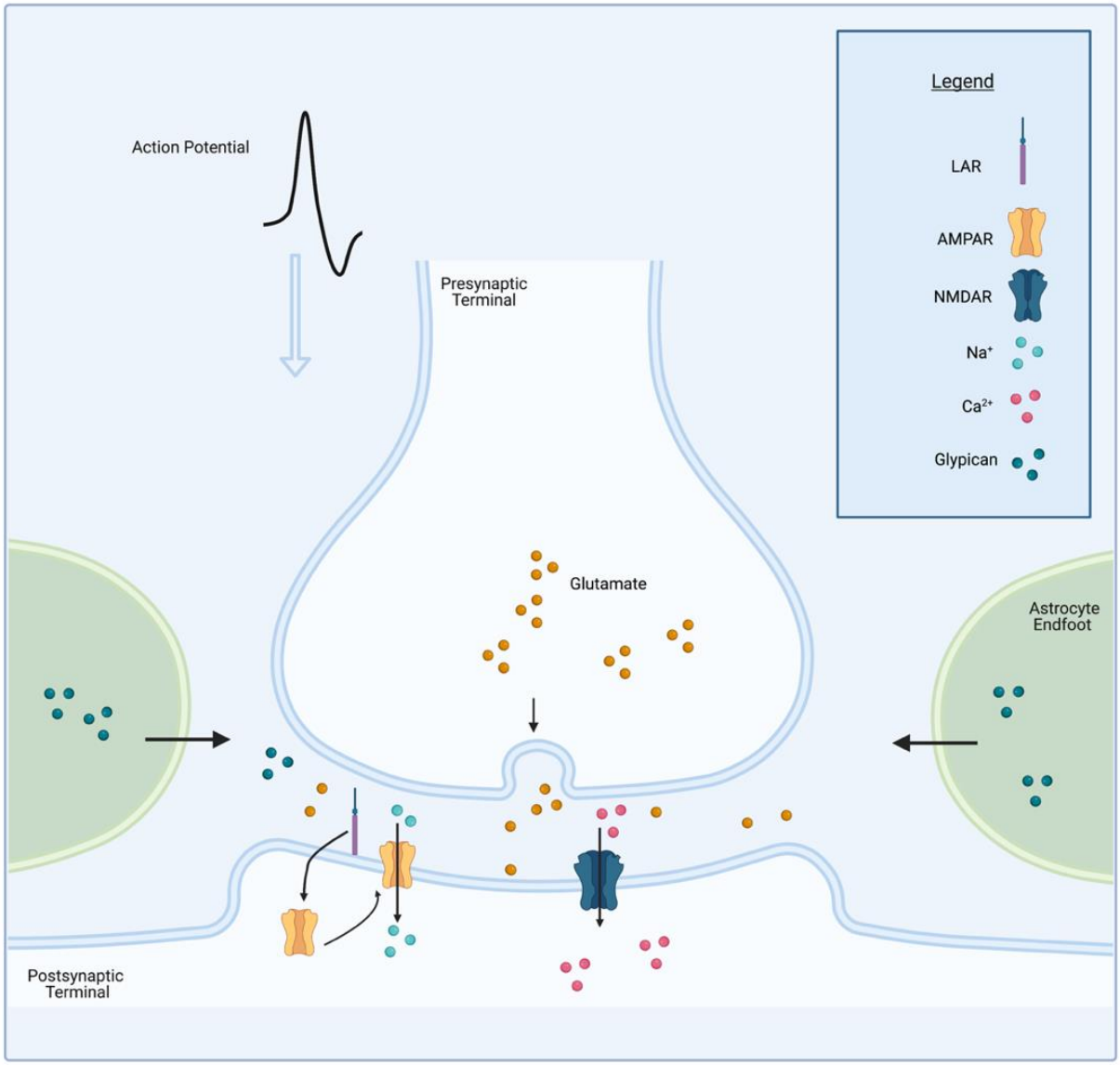

Figure 3: Astrocytes activate silent synapses to increase intracellular calcium.

Finally, during childhood and adolescent brain development, excess synapses are pruned to fine-tune connections in the neuronal circuit (Clarke & Barres, 2013). In pathological situations such as in response to trauma, there is evidence of both astrocytes and microglia engaging in phagocytosis of unwanted synapses (Clarke & Barres, 2013). This phagocytic process is likely relying on astrocytes emitting elimination signals of complement cascade proteins onto synapses

to be removed by microglia (Stevens et al., 2007). Neurodevelopmental disorders, like autism, schizophrenia, and fragile-x syndrome have surmounting evidence of a correlation between misfunctioning astrocytes and defective synapses (Auerbach, 2011; Bennet, 2009). Astrocytes are integral in maintaining normal synaptic formation and functioning and have a pivotal role in pathophysiology.

3.2 Regulating the Blood-Brain-Barrier

Astrocytes have significant roles in regulating the Blood-Brain-Barrier (BBB) (Argente-Arizón, 2015; Cabezas, 2014; Lyon & Allen, 2022; Satarker, 2022*).* The allows nutrients and important substrates into the brain, whilst expelling waste products, restricting ionic and fluid movement, and producing interstitial cerebral fluid (ISF) (Abbott et al., 2004). The BBB is a buffer to protect the brain from disturbances in the ionic gradient caused by food intake or aerobic exercise (Cserr & Bundgaard, 1984).

Endothelial cells, pericytes, and astrocyte end-feet make up the microvasculature of the BBB to maintain homeostasis (Ballabh et al., 2004; Figure 4). The endothelial cells reside between blood and the ventricular CSF, and the arachnoid epithelium is between the blood and subarachnoid CSF (Abbott et al., 2004). The endothelial cells have glucose transporter type 1 (GLUT-1), amino acid carriers, and transporters for nucleosides and other nutritive substances (Begley & Brightman, 2003). Complex nutritive molecules like glucose and amino acids cross the BBB with the help of different specialized transporters. Larger molecules like insulin or leptin use the process of receptor-mediated endocytosis to cross the BBB (Pardridge, 1985; Zhang & Pardridge, 2001). Endothelial cells line blood vessels and are found throughout the entire body (Grieb et al, 1985). Endothelial cells limit the intake of hydrophilic molecules across the BBB,

while allowing small molecules like oxygen and carbon dioxide to diffuse freely across the membrane in a concentration gradient-dependent manner (Grieb, 1985; Hawkins, 2005; Wolburg, 2002).

Astrocytes tile the CNS and interact with neurons, glial cells, and blood vessels (Yu et al., 2020). Astrocyte branches obey a border, which do not overlap with other astrocyte branches, creating a tiling effect of astrocytes (Eilam et al., 2016). Interestingly, astrocytes play a substantial role in regulating the connection between the BBB and neurons. Astrocytes position themselves to detect neural activity and transmit signals to nearby blood vessels (McCaslin et al., 2011). Astrocytes have several roles in maintaining the function of the BBB, such as compressing tight junctions (physical barrier) and expressing transporters such as GLUT-1, glutamate reuptake transporters, and metabolic barriers that are specialized enzyme systems (Argente-Arizón, 2015; Cabezas, 2014; Lyon & Allen, 2022; McCaslin, 2011; Satarker, 2022; Figure 4). There is a synergistic relationship between astrocytes and other cell types, like endothelial cells and pericytes, in maintaining the BBB (Abbott et al., 2006). Astrocyte endfeet wrap around the microvessel wall of blood vessels (Figure 4). The endfeet are involved in ion regulation by having high levels of orthogonal arrays of particles (OAPs), which contain the water channel, aquaporin 4, and the potassium channel, Kir4.1, for ionic flow (Abbot et al., 2006). Astrocytes secrete glial-derived neurotrophic factor, FGF, and angiopoietin 1, which modify the BBB phenotype depending on the environment (Lee et al., 2003).

While the BBB is a dynamic system; maladaptive modulation of the BBB is possible and is present in neuropathies. Several neuropathic phenotypes of the BBB are seen in neurological disorders causing inflammation. Brain edema will open the BBB tight junctions, while other

conditions, like starvation or hypoxia, increase the upregulation of GLUT-1 (Boado, 2002; Huber, 2001; Pardrige, 1998).

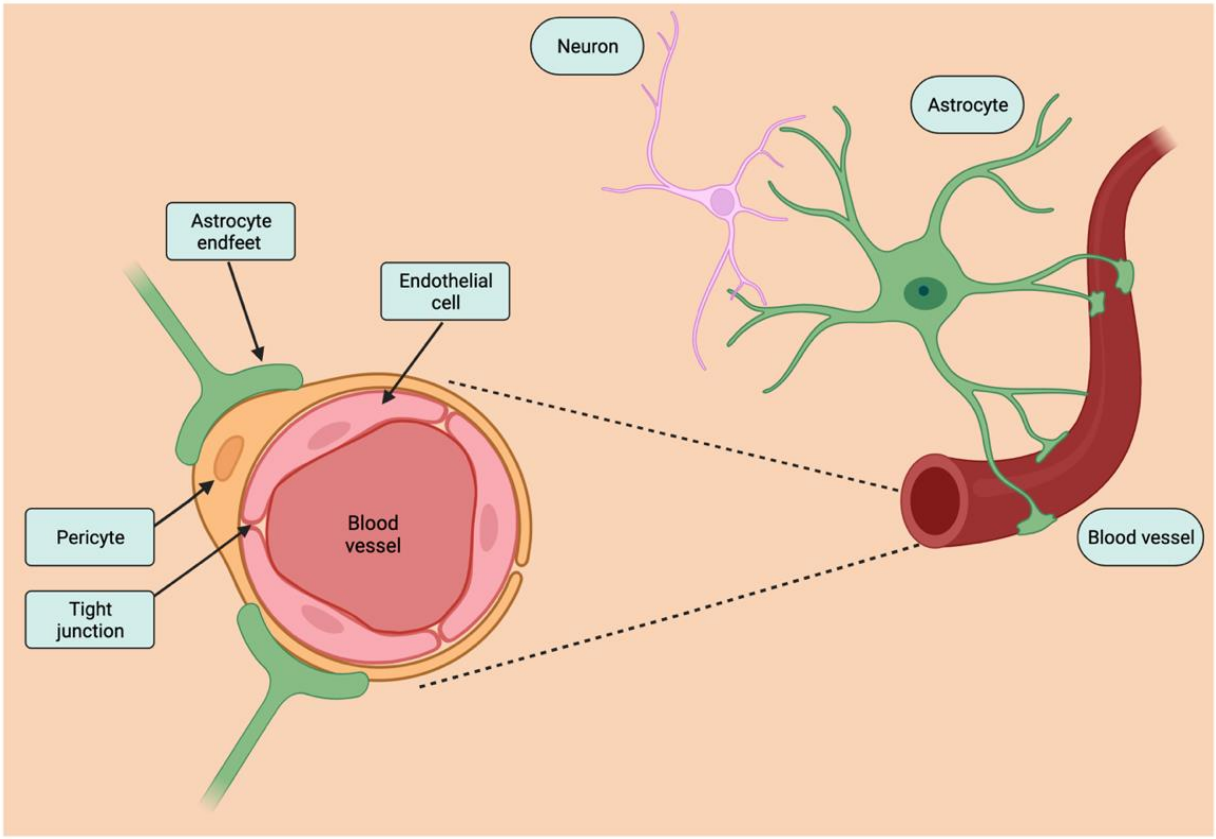

Figure 4: Diagram of neurovascular astrocyte coupling.

3.3 Energetic Supply and Homeostasis of Neurons

The brain has high energy requirements; a quarter of oxygen and glucose consumed by the human body is utilized for cerebral functions (Bélanger et al., 2011). The brain needs metabolic substances for restoring ionic gradients dissipated by energy costly signalling processes, like action potentials, and uptake or recycling of neurotransmitters (Alle, 2009; Attwell & Laughlin, 2001). Synaptic potentials have greater energy requirements than action potentials. (Attwell & Laughlin,

2001). Excitatory glutamate synapses in the grey matter represent up to 80% of cortical synapses (Attwell & Laughlin, 2001; Hyder, 2006). Positron emission tomography (PET) reveals that blood flow also plays a significant role in delivering these substances to the most active brain regions (Figley & Stroman, 2011). Since neurons are not directly connected to the BBB; astrocytes dynamically regulate energetic supply to neurons depending on the environment. Astrocytes employ multiple membrane transporters, enzymes, and H^+ buffers to maintain physiological pH (Deitmer et al., 2019). Astrocytes maintain the homeostasis of neurotransmitters, like glutamate, or energy intensive substances (glucose or lactate) in the tripartite synapse.

3.31 Astrocyte-Neuron Lactate Shuttle

In 1994, the astrocyte-neuron lactate shuttle hypothesis was first demonstrated by researchers, Pellerin and Magistretti. In the rodent cortex, they found that astrocytic lactate production is tightly bound to neuronal activation through glutamate release and glutamate uptake in astrocytes. This glutamate release has direct effects on the signaling cascades of energy intensive substances, like glucose or lactate in astrocytes (Newington, 2013; Pellerin & Magistretti, 1994). The astrocyte-neuron lactate shuttle is one of the primary methods astrocytes use to regulate the synaptic environment.

Glutamate is one of the most prevalent neurotransmitters released by excitatory neurons in the synaptic cleft. However, too much extracellular glutamate has neurotoxic effects such as neuronal death (Mahmoud et al., 2019). Astrocytes use Na^+ independent or dependent transporters to regulate extracellular glutamate. Several types of sodium-independent transporters are essential in the removal of glutamate from the synaptic cleft. There are five isoforms of the excitatory amino acid transporters (EAATs) which are responsible primarily for glutamate uptake from the synaptic

cleft (Anderson, 2000; Rose 2018). Human EAAT-1 and EAAT-2 are known in rodents as glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), respectively (Pines, 1992; Storck, 1992). GLASTs and GLT-1s are found primarily in astrocytes, distributed in clusters on the peri-synaptic processes contacting glutamatergic neurons (Mahmoud, 2019; Nakagawa, 2008; Takasaki, 2008). GLASTs and GLT-1s are responsible for 80 - 90% of the total uptake of extracellular glutamate in the CNS (Eulenburg & Gomeza, 2010; Lehre & Danbolt, 1998). Glutamate is rapidly upregulated in astrocytes by GLAST and GLT-1 and is synthesized to intracellular glutamine by glutamine synthetase to ensure low levels of glutamate (Mahmoud et al., 2019). The uptake of glutamate into astrocytes stimulates both increased glucose uptake from surrounding capillaries via GLT-1 and increased aerobic glycolysis (Deitmer, 2019; Newington; 2013). The newly synthesized glutamine is then released into the extracellular space to be upregulated by neurons or resynthesized into glutamate or inhibitory GABA depending on energy requirements and the type of neuron (Mahmoud et al., 2019).

In response to an excitotoxic environment, astrocytes can also upregulate the amount of glutamate through sodium dependent transporters such as the Na^+/glutamate cotransporter (Deitmer et al., 2019). Alternatively, glutamate can undergo oxidative metabolism after conversion into α-ketoglutarate to be used in ATP synthesis (Longuemare, 1999; Rose, 1996, Rose, 1998; Silver, 1997). The sodium-dependent transporter, Na^+/K^+ ATPase uses enzymatic degradation of ATP to enhance glycolysis and produce pyruvate as a source of energy to the cell (Allaman, 2011; Chatton, 2016). In astrocytes, the pyruvate is converted to lactate and exported out of the cell by monocarboxylate transporters (MCTs) into neurons for oxidative energy (Newington, 2013; Schurr & Payne, 2007). Extracellular lactate is taken up in neurons and converted to pyruvate by lactate dehydrogenase (Newington et al., 2013). Briefly, in neurons pyruvate is converted to acetyl

CoA through pyruvate dehydrogenase complex to initiate the tricarboxylic acid cycle to produce nicotinamide adenine dinucleotide (NADH) to fuel oxidative phosphorylation (Newington et al., 2013). The interchange of glutamate and pyruvate between neurons and astrocytes regulates the synaptic environment.

3.32 Gliotransmission

The work by Pellerin and Magistretti is foundational for better understanding the relationship between astrocytes and neurons in the tripartite synapse (1994). However, *in-vitro* experiments like theirs and used in neuroscience today concern the validity of studying the naïve physiology of cells and the synapse. More specifically, this can be narrowed down to the debate on gliotransmission; the process of neuronal activity leading to Ca^{2+} dependent release of neurotransmitters from astrocytes (Fiacco & McCarthy, 2018). Researchers will often employ approaches like mechanical stimulation, astrocytic depolarization using whole-cell recordings, uncaging Ca^{2+} or BAPTA, all of which could perturb astrocyte physiology (Fiacco & McCarthy, 2018), which could potentially confound measurements of gliotransmitter release. The discovery of gliogenesis was first found *in-vitro* cultured astrocytes relied on Ca^{2+} dependent exocytosis of neurotransmitters (Parapura 1994; Parapura 1995). While there is still much debated about this phenomenon, astrocytes should still be appreciated for their critical role in neuronal synapse regulation.

4.0 Tools to Visualize Astrocytes

Rudolf Virchow established that "neuroglia comprised of cellular elements were part of the connective tissue of the brain" (1858). Ten years later, Camillo Golgi visualized and biographically

sketched astrocytes in the CNS. Golgi described these cells as “glue” and illustrated all the unique shapes and branching of astrocytes (1871). These revolutionary findings are nearly two centuries old, and they still hold great importance and appreciation today. The conclusion that astrocytes have unique shapes is paramount in glial research. Astrocytes are not uniform in appearance, nor function. Astrocytes are heterogeneous, their function differs depending on their type and location (Matias, 2019; Miller, 2018; Zhang & Barres, 2010). Astrocyte morphology and function differ between brain regions, and there are different subclasses of astrocytes within brain regions (Oberheim et al., 2012). The following section will discuss how to label different astrocytes, as there is not one marker that will label all types.

4.1 Methods to Study Astrocytes on Different Visualization Scales

The vast mechanisms of astrocyte functions are understood by our advancement in tools to probe and manipulate. To gain comprehension of how astrocytes communicate with themselves, and other cell types, there needs to be an effective way to label these cells visually. The crux of this thesis is to label cortical astrocytes, a well-known problem that has been in the field for years (Escartin, 2021; Oberheim, 2012; Yu, 2020). The astrocyte marker and method suited for a particular experiment depends on a few critical components. The first criteria to consider is the scale of visualization.

Astrocyte visualization from tissue (~ 1 mm) would be on the largest end of the scale. Methods used to visualize astrocytes in tissue include the use of transgenic mouse reporter-lines, dye-filling, viral-vector and mediated reporter expression, and plasmid vector-mediated reporter expression (Yu et al., 2020). Visualizing astrocytes on a broader scale allows for a greater field of view, but less detail of singular astrocytes. If the purpose is to better understand the morphology

of astrocytes, researchers can visualize astrocytes on the cellular scale (~ 50 μm). Visualization methods for the cellular level are dye filling, transgenic mouse reporter-lines, viral-vector and mediated reporter expression, plasmid vector-mediated reporter expression, and immunohistochemistry (Yu et al., 2020). At the subcellular level (~ 0.1–1 μm), immunohistochemistry is not successful in labelling the branchlets and leaflets of astrocytes (Yu et al., 2020). Lastly, super-resolution microscopy or electron microscopy is needed to examine astrocyte interactions with synaptic structure (~ 10–100 nm) (Yu et al., 2020). For the scope of this thesis, the cellular scale will be discussed.

Methods used to visualize astrocytes present challenges. Genetic techniques, like transgenic mouse reporter lines, are used in tandem with other cre-dependent mouse lines to manipulate astrocytes and their functions. There is no singular reporter mouse-line that can be used to target all astrocytes. Instead, it is imperative to know the types of astrocytes to be transfected to choose a specific promoter. This depends on where the astrocytes reside in the brain (Escartin, 2021; Oberheim, 2012; Yu, 2020). Commonly used reporter lines like GFAP, Aldehyde Dehydrogenase 1 Family Member L1 (ALDH1L1), GLU aspartate Transporter (GLAST), and GLT-1, all present limitations. There are issues of specificity, for example, GLT-1 is detectable in some neurons (Yu et al., 2020). Cre-transgenic mouse lines have also increased in popularity. Cre-transgenic mouse lines have promoters used to target astrocyte-marker genes. Cre-transgenic mouse lines have their own restrictions based on the astrocyte expression of the gene (Yu et al., 2020).

Of interest to this thesis, two Cre-transgenic mouse lines have been widely used to label astrocytes in the cortex, GFAP-Cre and ALDH1L1-Cre ERT2 BAC. The GFAP-Cre mouse has been used in combination with AAV1-CAG.FLEX-GCaMP6s to stimulate and visualize calcium

release in cortical astrocytes (Poskanzer & Yuste., 2015). These researchers and others rely on the Cre-*lox*P system to manipulate gene functions and the activity of astrocytes (Bailey & Shipley, 1993; Poskanzer & Yuste., 2015). However, GFAP and other astrocyte promoters are also expressed by non-astrocytes during development (Casper & McCarthy, 2006; Guo, 2018; Hirrlinger, 2006). Similarly, the ALDH1L1-cre line also relies on the efficacy of the Cre-*lox*P system transducible by tamoxifen (Srinivasan et al., 2016). Moreover, ALDH1L1, has been considered a pan-astrocytic marker (Beyer, 2021; Cahoy, 2008; Molofsky, 2013; Yoon, 2017).

The bacterial artificial constructs (BAC) which the ALDH1L1-Cre ERT2 BAC mouse line relies on have some limitations. BAC transgenes are created by nonspecific integration into the target genome, therefore multiple copies can be inserted into an unidentified locus (Beil et al., 2012). Cre-mouse lines are subjected to the issues with developmental expression of cre recombinase, leading to leaky expression of the transgene. BAC mouse lines were created to have more accurate Cre expression. However, distal *cis* – or *trans*-regulatory elements might be present and not included in the BAC, resulting in Cre expression in undesired cell types (Becher et al., 2018). Another common misconception with Cre-mouse lines is concluding that Cre-expression is proportionate to Cre-mediated recombination. Cre-reporter mice rely on a *loxP* flanked stop cassette in front of a reporter fluorescent protein inserted into a specific locus (Becher et al., 2018). Each locus has its own sensitivity to Cre-recombination. Thus, the locus could allow high efficiency or resistance to cell or tissue specific targeting using one Cre line, resulting in high or weak expression of the reporter (Becher et al., 2018).

4.2 Immunohistochemical and Viral Vectors to Visualize Astrocytes

4.21 GFAP

In development, astrocytes express canonical markers prior to differentiation into specialized cells (Akdemir et al., 2020). One common marker, glial fibrillary acidic protein (GFAP) is an intermediate filament composing cytoskeleton protein expressed in astrocytes (Serrano-Pozo et al., 2013). GFAP provides motility and stability and is expressed later in development (Akdemir et al., 2020). GFAP has been recognized as marker of astrocyte maturation, and to identify signaling pathways like BMPs and NOTCH (Barnabé-Heider, 2005; Bonni 1997). For the last 30 years, GFAP has been the most widely used astrocyte marker to identify astrocytes from other non-neuronal and neuronal cell types (Serrano-Pozo et al., 2013).

Labelling efficiency of a marker for astrocytes are dependent on the type of astrocyte and the intracellular localization of the marker. While the presence of GFAP is the primary labelling method for astrocytes, it has become increasingly clear that this method can have some limitations. GFAP does not correctly label all heterogenous astrocytes and even under labels astrocytes in specific brain regions, like the cortex (Oberheim et al., 2012). Currently, it has been estimated that GFAP only labels at most 15% of rodent astrocyte volume, and is mostly comprised of spongiform morphologies (Bushong, 2002; Oberheim, 2012). Furthermore, GFAP in mice, has been found to have minimal immunoreactivity for protoplasmic cortical astrocytes in normal physiological conditions (Serrano-Pozo et al., 2013).

In terms of astrocyte morphology, GFAP labels radial shaped astrocytes that have small somas, but under labels long processes with multiple branches (Zhang et al., 2019). GFAP has limited success in labelling protoplasmic human astrocytes, and poor labelling of fibrous astrocytes in later development (Zhang et al., 2019). Specificity of GFAP labelling is also dependent on the

time of development as it also is expressed by progenitor cells (Cahoy et al., 2008). Brain region for using GFAP is also limited; previous researchers found that there were 40% GFAP-negative astrocytes within the hippocampus, a region of the brain considered to typically have high GFAP expression (Zhang et al., 2019). In general, GFAP depending on location of brain region, can label the filaments of astrocytes, but fails to label the microfilaments (Escartin et al., 2021).

GFAP has been regarded for decades as the gold standard marker, but there is a need for a combination-validation method for locating and analyzing different types and parts of astrocytes. However, GFAP labels have some notable advantages. GFAP may be useful for other brain regions where it has had reproducible success, like the white matter (Zhang et al., 2021). For instance, GFAP is more accurate in labelling astrocytes in the corpus callosum and cerebral peduncle (Oberheim et al., 2012). Transgenic mouse lines with a GFAP promoter inducible by tamoxifen have also been used by researchers and have found the fusion protein to be mainly confined to astrocytes (Chow et al., 2008). GFAP has been used regularly in the field to study astrocytes for models of disorders or stress, as GFAP can be upregulated in these conditions (Escartin et al., 2019). GFAP immunolabelling of astrocytes in these pathological conditions will further be explored in the discussion.

4.22 GfaABC1D

GFAP can be used as a promoter to label astrocytes or as a molecular marker through immunohistochemistry. While neither method works for the visualization of all cortical astrocytes, a new abbreviated reporter derived from GFAP, called gfaABC1D, may be promising as a cortical astrocyte label (Escartin et al., 2021). Specifically, the gfaABC1D (681 - bp) is a shortened GFAP (2, 210 - bp) promoter and less reported drawbacks than GFAP (Griffin, 2019; Lee, 2008). The

gfaABC1D promoter offers greater astrocyte transduction efficiency, along with a decrease in non-specific targeting of neurons (Lee et al., 2008). The gfaABC1D promoter allows for greater flexibility with AAV constructs (Daya & Burns, 2008). One study found that the gfaABC1D promotor only transduced up to 25% of s100β expressing cortical astrocytes in a model for Huntington's disease (Vagner et al., 2016). Across species, the gfaABC1D promotor is successful in targeting astrocytes in macaques (Heffernan et al., 2022) and mice (Lee et al., 2008), but has lower efficiency in rats (Taschenberger et al., 2017). Excitingly, researchers have used an AAV5-gfaABC1D-cyto-GCaMp6f virus to drive calcium activity and monitor behaviour (Qin et al., 2020). However, the consensus on gfaABC1D being an effective promoter to target astrocytes still needs more exploration.

4.23 S100β

There needs to be a new protocol for astrocyte staining that will yield more selective labeling than GFAP; S100β as an astrocyte marker may be promising. S100β is a small calcium binding glycoprotein (Barateiro, 2016; Verkhratsky & Nedergaard, 2018). Interestingly, S100β in astrocytes may contribute to shaping calcium signals in freely-moving animals (Verkhratsky & Nedergaard, 2018). S100β is also integral in regulating cell proliferation, differentiation, and apoptosis (Verkhratsky & Nedergaard, 2018). Depending on the concentration of S100β, it will have either have neurotoxic or neuroprotective effects, thus activating astrocytes and microglia (Verkhratsky & Nedergaard, 2018). In pathological conditions, S100β is upregulated in serum and cerebral spinal fluids (Allore, 1988; Donato, 2001; Heizmann, 2002; Mrak, 2001; Van Eldik & Wainwright, 2003; Verkhratsky & Nedergaard, 2018).

At the present, s100β is an astrocyte marker in the developing and adult CNS (Du et al., 2021). In development s100β is expressed after GFAP expressing cells lose their neural stem cell potential, suggesting s100β is repressed by the adult SVZ microenvironment (Raponi et al., 2007). Moreover, *in-vitro* and *in-vivo*, epidermal growth factors repress 100β expression in GFAP-expressing cells. Specifically, s100β is expressed in astrocytes and is found in a small population of oligodendrocytes (Du et al., 2021). Within protoplasmic astrocytes, S100β is found diffusely in the cytoplasm and associated the assembly or microtubules, type III filaments and enzymatic activities (Brozzi et al., 2009). On its own S100β is not considered a selective universal marker for astrocytes as it can be expressed in oligodendrocytes and ependymal cells in the CNS (Du et al., 2021). S100β labels the cell bodies of astrocytes, but fails to label the microfilaments (Escartin et al., 2021). S100β can be used in tandem with another astrocyte marker to label astrocytes. Co-staining for other cell types should be done to ensure specificity to astrocytes (Du et al., 2021).

5.0 Acute Astrogliosis

A major area of glial research studies how astrocytes can become activated and have a perturb response to the environment. There is debate on the terminology and classification of activated astrocytes, also called astrogliosis (Escartin et al., 2021). The term astrogliosis was first coined in the late 19 century when neuroanatomists recognized astrocytes had structural changes in response to CNS damage or injury (Sofroniew, 2015). In the glial field, there is debate on the names and classification of activated astrocytes (astrogliosis) (Escartin et al., 2021). Astrogliosis is graded in severity from mild (acute) to severe or chronic (Sofroniew et al, 2015). Pertaining to this thesis, acute activation will be the focus. Moreover, the term "activated astrocytes" is generally accepted for mild, not chronic astrogliosis (Sofroniew et al., 2015).

Astrocyte activation or astrogliosis still lacks a stringent definition and has been classified in several ways in recent years. Acute activation has been described as a change in astrocyte morphology and proliferation of scar tissue paired with the secretion of inflammatory cytokines, which activate the NFkB pathway and lead to increased cytokine release (Argente-Arizón, 2015; Choi, 2014). Other researchers have classified acute astrogliosis as mild to moderate in condition with changes in astrocyte gene expression, and varying hypertrophy of cell body and processes (Sofroniew, 2009; Sofroniew & Vinters, 2010). At most, there is a minor loss of individual astrocyte proliferation (Sofroniew, 2009; Sofroniew & Vinters, 2010; Sofroniew, 2015). The non-overlap nature of healthy astrocytes is also mostly conserved in cases of acute astrogliosis (Sofroniew, 2015; Wanner, 2013; Wilhelmsson, 2006).

Immunohistochemical markers can be used to visualize astrogliosis. Depending on severity, GFAP has been used previously as a marker of astrogliosis, as there is generally, increased proliferation of the astrocyte filaments (Sofroniew, 2015). In a mild model of astrogliosis, the GFAP label would be obsolete. For an acute model of astrogliosis there is a need for a combination of markers to account for the brain region and type of astrocyte. This thesis focuses on how astrocytes modulate their activity and surrounding neurons based on the environment. Here, we have developed a model to directly label and target astrocytes of the lateral orbitofrontal cortex (LOFC).

6.0 Tools to Target Astrocytes: Chemogenetics

6.1 Overview of GPCR Signaling

G protein-coupled receptors (GPCRs) are a group of membrane-bound proteins with a highly conserved seven-transmembrane domain motif (Allen & Roth, 2011; Meltzer & Roth,

2013). Currently, there are four main classes of GPCRs that vary in signaling mechanics from each other. GPCRs are activated by a wide range of ligands such as neurotransmitters, photons, lipids, hormones, and peptides, among other small molecules (Allen & Roth, 2011). Each type of GPCR responds to a variety of signalling ligands and allows the cells to adapt to the complex, ever-changing environments. When an agonist binds to its respective GPCRs it will rearrange itself to activate the neighbouring heterotrimeric G-proteins (Gq, Gs, Gi and G12/13) to produce a specific response (Allen & Roth, 2011).

Chemogenetic methods take advantage of G-protein receptor signaling through the design of Designer Receptor Exclusively Activated by Designer Drugs (DREADDs) (Armbruster, 2007; Roth, 2016). The first successful chemogenetic strategy mutated a human muscarinic receptor (hM3Dq) to be insensitive to its original ligand (acetylcholine), and instead respond to the designer drug clozapine-N-oxide (CNO) (Armbruster et al., 2007). DREADDs lack detectable activity *in-vitro* and thus provide a receptor-effector complex to modify astrocyte activity (Armbruster, 2007; Roth, 2016). There are three types of DREADDs based on Gα protein receptor coupling; Gq-coupled hM3Dq, or Gs-coupled rM3Ds, which typically activate a cellular response, and Gi-coupled hM4Di which typically inhibits a cellular response (Alexander, 2009; Armbruster, 2007; Farrell, 2013; Zhu & Roth, 2014). Artificial manipulation of GPCR signalling allows the opportunity to understand cellular and behavioural effects. Thus, DREADDS are commonly used in neuroscience to probe the function and signalling of different cells (Armbruster, 2007; Roth, 2016).

This thesis employs the hM3Dq DREADD. When activated, the hM3Dq receptor will initiate the Gq-pathway in neuronal and non-neuronal cell types (Alexander, 2009; Armbruster, 2007). For the term hM3Dq, hM3 denotes the mutated human muscarinic receptor DREADD

agonists target (Wess et al., 2013). In neurons and astrocytes, the Gq-coupled hM3Dq DREADD activates the phospholipase C (PLC) cascade to increase intracellular calcium and promote neuronal burst firing (Armbruster; 2007; Volterra, 2014). Gq-coupled hM3Dq interacts with PLC, which catalyzes the degradation of phosphatidylinositol 4, 5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). The latter binds to IP3 receptors and releases calcium from the endoplasmic reticulum (ER) (Volterra et al., 2014). Moreover, the activation of Gq-coupled hM3Dq has been shown to cause membrane depolarization and increase the neuronal firing rate (Shen et al., 2021). Researchers should take into consideration if their desired effects are acute or chronic. If quick cellular changes that are easy to turn off are desirable, researchers should rely on optogenetics which uses light-activated ion channels (Britt & Bonci, 2013; Kim, 2017; Xie; 2013). If slower, or long-term effects for chronic studies are desirable, chemogenetics may be more suitable (Pati et al., 2019).

6.2 Chemogenetic Modulation of Astrocytes

The localization of excitatory DREADDs to astrocytes to modulate their calcium activity and phenotype have gained attraction. Astrocytes display complex intracellular calcium signals in response to an increase of extracellular glutamate (Cornell-Bell et al., 1990). Kang and colleagues activated astrocytes expressing excitatory DREADD to increase the amplitude of spontaneous excitatory postsynaptic currents (sEPSC) and decrease the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) (2015). This increase in astrocyte intracellular calcium can occur from direct stimulation of astrocytes or by the increased firing of the presynaptic glutamatergic afferent. Calcium increases in response to glutamatergic activity and results in the release of gliotransmitters to regulate neurons and the BBB (Bazargani & Attwell, 2016; Volterra, 2014).

GPCR activation has been primarily studied in neurons, but its functional consequences in astrocytes are less known (Durkee et al., 2019). To better understand their glial signaling, astrocytes have been activated by AAVs expressing DREADDs in the striatum and hippocampus (Chai et al., 2017). Comparatively, astrocyte activation in the striatum and hippocampus significantly differed in calcium signaling among other variables like electrophysiological properties. Using AAVs *in vitro*, Chai and researchers probed intracellular calcium release and assessed calcium signals in the astrocyte cell body, major branches and microdomains of the processes (2017). In astrocytes expressing hM3Dq receptors, 1 μM CNO evoked robust and equivalent increases in intracellular calcium in the hippocampal and striatal astrocyte cell body and processes (Chai et al., 2017). Astrocytes expressing hM3D activated by 1 μM CNO evoked robust and similar increases in intracellular calcium in hippocampal and striatal astrocyte cell bodies and processes. Calcium-induced activation of astrocytes has been shown in the hippocampus and can be further explored in terms of behavioural modulation.

Adamsky and colleagues expressed the Gq-coupled receptor hM3Dq in astrocytes of the CA1 region of the hippocampus to find out whether astrocyte activation is sufficient to produce synaptic potentiation and enhance memory (2018). In the hippocampus, DREADD activation of astrocytes led to an increase in synaptic transmission through the upregulation of D-serine and FOS expression in neurons recruited during fear conditioning (Adamsky et al., 2018). Furthermore, astrocyte activation is necessary for synaptic plasticity and sufficient to induce NMDA-dependent de novo long-term potentiation in the hippocampus after astrocyte activation ceases (Adamsky et al., 2018).

The activation of hM3Dq receptors and subsequent GPCR signaling led to cellular activation in neurons and astrocytes. Specifically, Gi-GPCR activation inhibits neuronal activity

but produces cellular activation in astrocytes (Durkee et al., 2019). Activation of hM4Di receptors in astrocytes increases calcium and gliotransmitter (glutamate) release, thus increasing neuronal excitability (Durkee et al., 2019). Therefore, activation of the hM3Dq or hM4Di receptors causes an increase in intracellular calcium, similarly to endogenous GPCR signalling (Chai, 2017; Durkee, 2019). However, in the hippocampus, activating hM4Di receptors compared to hM3Dq receptors in astrocytes produced smaller amounts of intracellular calcium (Chai et al., 2017). Chai and researchers further examined astrocyte activation effects on c-Fos signaling in striatal and hippocampal astrocytes expressing hM4Di receptors. *In vivo*, 1 mg/kg increased c-Fos more in the striatum than in the hippocampus (Chai et al., 2017). Expression of intermediate genes, like cFos, may reflect the differences in Gi-coupled signaling in different brain regions.

Astrocyte activation has been employed to probe the cellular mechanisms and signalling pathways of disorders, such as chronic pain or inflammation. As previously discussed, astrocytes are physically close to neurons, and through secreted molecules, astrocytes can regulate synaptic function, synaptogenesis, and modulation of synaptic plasticity (Chung, 2015; Clarke, 2013). These astrocytic synaptic signalling pathways are involved in peripheral nerve injury and contribute to this remodelling of the cortical (S1) pain circuit (Kim et al., 2011). Activation of hM4Di receptors has also been shown to reduce inflammation (Kim et al., 2021). Using AAVs, Kim and colleagues virally expressed hM4Di in hippocampal astrocytes in lipopolysaccharide (LPS)-induced neuroinflammation mouse models. Corrective remodelling of these S1 circuit synapses may effectively reverse chronic pain. Similarly other researchers, in mice employed DREADDS to create a model of chronic pain behaviour induced by nerve injury (Takeda et al., 2022). Takeda and colleagues reversed allodynia-like behaviours caused by partial sciatic nerve ligation using chemogenetics to activate astrocytes in the somatosensory cortex (S1) (2022).

Therefore, corrective remodelling of these S1 circuits may be effective in reversing chronic pain behaviour. As discussed, there has been several studies using activation of astrocytes to better understand their role in calcium signaling, gliotransmission, and thus perturbing behaviour.

7.0 Strengths and Limitations of Different DREADD Agonists

This section discusses CNO, compound 21 (C21), and deschloroclozapine (DCZ); three different DREADD agonists that have been used to activate excitatory and inhibitory DREADDs. The strengths and significance of each agonist will be debated whilst highlighting their unique limitations. Lastly, rationale for choosing the DREADD agonist utilized in this thesis will be given.

7.1 Clozapine-N-Oxide

CNO was first used to activate hM3Dq receptors in neurons to mobilize intracellular calcium (Armbruster, 2007; Roth, 1994). CNO is the most widely used DREADD agonist and has been for decades (Roth, 1994). Previous researchers were successful with CNO and considered it pharmacologically and behaviourally inert at low doses (0.1 – 3.0 mg/kg) (Alexander, 2009; Farrell, 2013; Krashes, 2011; Zhu, 2014). Previous research found that CNO had excellent BBB permeability after 45 minutes following intraperitoneal (i.p.) injection (Hellman et al., 2016). The long-lasting effects of CNO are desirable for chronic experimental design.

While CNO is considered an inert ligand, it has issues with metabolizing into clozapine resulting in off-target effects at different organs and brain regions (Jann et al., 1994). I.p. doses of CNO (0.5mg/kg) delivered in guinea pigs were detected in the plasma, liver, frontal cortex, and caudate (Jann et al., 1994). CNO being considered an inert ligand is founded on the assumption that CNO, alone, does not activate any signalling cascades or unwanted side effects *in-vivo.* CNO can bind to non-DREADD (histamine H1, muscarinic M1, and dopamine D1) receptors at

concentrations typical for DREADD activation (Gomez et al., 2017). CNO (varying concentrations) can revert to its original metabolite, clozapine, in humans, monkeys, guinea pigs, and rats (Araque, 2014; Bosson, 2015; Kuchibhota, 2009; Martorana, 2012; Tian, 2005). Jendryka and colleagues assessed plasma concentrations and found systemic administration of CNO (3.5 mg/kg) metabolized into clozapine in mice (2019). Other researchers have found that higher doses of CNO, 10 mg/kg or greater, administered systemically, produced several off-target effects in rats and mice resulting in behavioural effects not produced by DREADD activation (Bærentzen, 2019; Gomez, 2017; Goutaudier, 2019; MacLaren, 2016). Moreover, Gomez and team found that CNO does not readily cross the BBB (1997).

CNO has undesirable effects in other organs from other routes of administration. Oral CNO (5mg/L, delivered in drinking water for seven days) produced unwanted effects on gut microbiota composition after repeated administration (Guo et al., 2021). The gut-brain axis, a bidirectional relationship between the stomach and the brain, allows the gut to modify the brain's signalling directly. It is crucial when choosing a DREADD agonist to research its effects in the CNS and the periphery. Guo and colleagues found that CNO does not affect gut microbiota biodiversity (2021). However, CNO effects produce unusual organization of the gut microbiota (*Muribaclum intestinale)* in adult mice, which may alter the CNS and produce pathologies (Guo et al., 2021). Taken together, the use of CNO as a DREADD agonist is foundational in chemogenetic research; however, a DREADD agonist that is inert and BBB permeable is desired.

7.2 Compound-21

The limitations described with CNO pushed researchers to develop other non-CNO chemical actuators (Chen et al., 2015). Compound 21 (C21), 11-1-piperazinyl- 5H-dibenzo [*b,e*]

[1,4] diazepine, were found to have minimal off-target activity and high selectivity for the excitatory DREADD, hM3Dq (Thompson et al., 2018). C21 is very unlikely to metabolize to clozapine or another metabolite (Roth, 2017). Compared to CNO, C21 does not revert to clozapine and has a >10-fold affinity for the hM3D and hM4D receptors whilst lacking activity at wildtype receptors (Thompson et al., 2018). Taken together, C21 offers notable advantages over CNO.

Researchers found in control animals, a dose of 1 mg/kg (i.p.) of C21 strongly increased neuronal activity creating undesirable effects and questioning the selectivity of C21 (Goutaudier et al., 2020). Moreover, C21 is a selective agonist for the angiotensin AT_2 receptors which prevent inflammation, specifically, tumour necrosis factor alpha (TNFα)-induced, and high-fat diet induced, inflammation (Sampson et al., 2016). Other researchers found that C21 supresses inflammation *in-vivo* and *in-vitro* (Zhao et al., 2020). C21 also attenuates to demyelination in mice in the cuprizone multiple sclerosis model, which induces pathological demyelination and inflammation (Zhao et al., 2020). An anti-inflammatory mechanism may be of interest to other researchers in different fields of neuroscience. However, it is a confounding limitation in studies interested in inflammatory mechanisms associated with different pathologies. Pertaining to this thesis and future aims, a DREADD agonists cannot influence or alter cytokine inflammatory pathways.

7.3 Deschloroclozapine

CNO and C21 are well researched, but they require large systemic doses to activate DREADDs which may have off-target actions (Nagai, 2020; Thompson, 2018). A DREADD agonist with more potency at low systemic doses is needed to reduce the possibility of off-target effects. A new DREADD agonist, Deschloroclozapine, DCZ (11-(4-methyl-1-piperazinyl)-5H-

dibenzo(*b*,*e*)(1,4)diazepine), was found to have a lower affinity for serotonergic and dopaminergic receptors than clozapine, another DREADD agonist (Nagai, 2020; Phillips, 1994). Compared to CNO and C21, DCZ has a 100-fold affinity and greater agonist potency for excitatory and inhibitory DREADDs (Nagai et al., 2020). Moreover, Nagai and team took advantage of PET imaging in macaques (2020). They found that DCZ is BBB permeable and selective for doses of DREADD occupancy, 20-fold, and 60-fold lower than CNO and C21, respectively (Nagai et al., 2020). To determine if DCZ binds to DREADD receptors effectively, Nagai and colleagues performed [11C] DCZ PET imaging with a hM4Di transgenic mouse line expressing under the control of Thy, a neuron-specific promoter (2020). Compared to controls, researchers found that DCZ increased the binding signal in striatum and cortex compared to wild-type mice (Nagai et al., 2020).

The researchers also utilized two-photon calcium imaging in the central amygdala of mice. They found that DCZ can selectively and rapidly enhance neuronal activity (40% dF/F) of the hM3Dq DREADD *in-vivo* (Nentwig et al., 2022). Low doses of DCZ (0.001 – 0.1 mg/kg) were sufficient to induce neuronal activity and increase cFos expression in $hM3Dq^{+}$ cells without unwanted side effects (Nentwig et al., 2022). Other researchers found in mice, DCZ is comparable to CNO in decreasing locomotion when activating inhibitory DREADD in neurons in the ventrolateral-ventromedial hypothalamic nucleus (Krause et al., 2021).

Research in non-human primates found that DCZ in the absence of DREADDs could alter memory performance at 0.3 mg/kg in two of the four rhesus monkeys tested (Upright & Baxter, 2020), suggesting that DREADD actuators may be more prone to off-target effects acting at endogenous monoaminergic receptors in non-human primates. More research needs to be done on the cellular signalling potentiated by DCZ. Translating DCZ properties between animals is

complex, and there needs to be more validity experiments of appropriate DCZ doses in mice. Previously, researchers found that in monkeys, oral administration of DCZ effects lasted for longer than four hours, but in mice, oral bioavailability needs to be determined (Oyama et al., 2022). Other researchers have successfully used DCZ orally, which offers a feasible alternative for chemogenetic activation in marmosets (Mimura et al., 2021). DCZ is a novel DREADD agonist that is impressively, more potent than C21 or CNO; however, more validity experiments are encouraged.

8.0 Tools for Studying Astrocytic Calcium Responses

8.1 Organic and Genetically Encoded Calcium Indicators

In 1990, Cornell-Bell and colleges, demonstrated dynamic calcium fluctuations in hippocampal astrocyte cultures. The researchers employed organic calcium indicator dyes to illustrate that astrocytes exhibited "spontaneous" complicated patterns of calcium elevations that developed into waves propagating over long distances (Cornell-Bell et al. 1990; Charles et al. 1991; Dani et al. 1992). *In-vitro* and *in-vivo* experiments, the investigators found the activation of neurotransmitter receptors increased calcium signaling within astrocytes (Cornell-Bell et al. 1990; Charles et al. 1991; Dani et al. 1992). Since then, there are several successful methods to employ to increase astrocytic intracellular calcium in the CNS.

Measuring bulk cytosolic calcium signals with organic indicator dyes, such as Fluo-4 and Fura, can be successful (Khakh & McCarthy, 2015). Specifically, Fura-2 which is a predominant indicator is a calcium chelating complex conjugated to a fluorescent promoter and shifts the excitation spectra in the presence of calcium (Gorzo & Gordon, 2022). Organic indicator dyes have the potential to buffer calcium because they all rely on calcium binding (Khakh & McCarthy,

2015). Calcium indicator dyes has been paramount in exploring calcium dynamics and the physiology of astrocytes. These dyes are available in a wide range of affinities, binding kinetics, and spectral properties for astrocyte calcium dynamics (Neher, 2000; Nett, 2002). Bulk and patch loading of calcium indicators requires co-loading with a secondary fluorescent probe to identify astrocytes (Gorzo & Grant, 2022). Sulforhodamine 101(SR101) is a secondary fluorescent probe typically chosen to increase validity due to its preferential uptake by astrocytes and its feasibility with loading (Gorzo & Grant, 2022; Nimmerjahn, 2004). SR101 has a deep-red emission that minimally overlaps spectrally with GFP or green fluorescein-based calcium indicators (Gorzo & Grant, 2022). However, this method would be difficult to employ for this thesis, as bulk loading is problematic in adult tissue and lacks labeling of finer astrocyte branches (Khakh & McCarthy, 2015). While organic calcium indicator dyes have limitations for this thesis and *in-vivo* designs, calcium dyes are successful *in-vitro* with patch pipette-mediated loading of indicator calcium dyes (Nett et al., 2002). Depending on the experiment, indicator dyes are successful.

Single-wavelength genetically encoded calcium indicators (GECIs) are based on circularly permuted green fluorescent protein and derived from fluorescence resonance energy transfer indicators to measure calcium signals in astrocytes (Atkin, 2009; Hires, 2008; Russel, 2011; Tian et al. 2009). GECIs are now regularly used *in-vitro* and *in-vivo* experiments. Researchers have developed subcellular (cytosol, plasma-membrane, endoplasmic reticulum, mitochondria, etc) targeted GECIs, which are useful tools to measure calcium signals in adult tissue (Gorzo &Grant, Shigetomi, 2013). The biggest challenge with GECIs, is the need for critical methods to deliver the genes to astrocytes. Impressively, GECIs have been used to assess different types of calcium signals. For example, researchers have found a transmembrane calcium flux pathway mediated by TRPA1 channels in astrocytes (Shigetomi et al., 2011). The utilization of GECIs allowed

researchers to conclude that TRPA1 channels mediate calcium microdomains independent of calcium release from endoplasmic reticulum stores. GECIs are impressive in measuring calcium signals.

8.2 Two-Photon Imaging

Another impressive method in measuring transient calcium signals is two-photon microscopy to further explore signaling dysfunction in disorders. An example of two-photon measurement of astrocyte calcium signaling was used to study whole brain irradiation (WBI) therapy is a treatment for brain metastases and microscopic malignancies that contribute to cognitive dysfunction (Institoris et al., 2020). Previously, Institoris and researchers, used two-photon microscopy to test the hypothesis of whether WBI-induced impairment associates with persistent impairment of astrocyte calcium signalling and/or gap junction coupling (2020). Mice underwent a WBI protocol to induce cognitive impairment that persistent for up to 15 weeks after. To test the integrity of astrocyte-to astrocyte gap junction coupling astrocytes were loaded Alexa-488-hydrazide via patch-based dye infusion and was assessed with two-photon microscopy in acute slices of the sensory-motor cortex. Two-photon imaging revelated that astrocyte gap junctions were not affected by WBI. However, calcium dynamics were attenuated by theta bursts revealing transient responses in the astrocyte arbour and soma in WBI (Institoris et al., 2020). Therefore, WBI causes a decrement in synaptic-evoked astrocyte calcium signals up to 15 months post-irradiation (Institoris et al., 2020).

Two-photon fluorescence-imaging has been used in an *in-vivo* awake model to examine the complexities of calcium signaling between astrocytes or within astrocyte compartments near arterioles and capillaries that have vasomotor responses to vibrissae stimulation (Sharma et al.,

2020). Researchers found that whisker stimulation inconsistently produced astrocytic calcium responses (Sharma et al., 2020). Two-photon imaging was useful in revealing that calcium responses were heterogenous among subcellular structures within and between astrocytes. Sharma and team also found that whisker stimulation induced discrete calcium "hot spots" that regionally spread in astrocyte endfeet (2020). Therefore, astrocyte endfeet neurovascular coupling aid to the heterogeneity of calcium dynamics.

9.0 The Reward Circuit and Behavioural Implications

9.1 The orbitofrontal Cortex and Decision making

Evolutionarily speaking, making decisions are crucial for any species' survival. Compared to primitive species, humans engage in a much more elaborate decision-making process that requires the collaboration between several different brain regions. Here, several brain regions such as the OFC, the ventral striatum, the limbic system, and the striatum underpin the complex circuitry in reward decision-making (Guo et al., 2013). While a decision can seem impulsive or easy to make, our brains weigh the benefits and costs of possible outcomes.

The OFC is in the prefrontal cortex, an area of the brain responsible for decision-making processes, higher cognition, and emotional salience (Grossberg et al., 2018). The OFC encodes stimuli and assigns value to the stimuli, positive or negative, and updates actions based on this information (Moorman et al., 2018). Moreover, when addressing the value of predicted outcomes, this can be modulated in terms of reward probability, latency, and magnitude (Burton, 2015; Moorman, 2018; Roesch, 2006; van Duuren, 2008; van Duuren, 2009). Therefore, impairments in OFC function can influence decision making leading to impulsivity or perseveration. In an obesity model, astrocytes within the lOFC were hypertrophic and had reduced glutamate reuptake via

GLT-1, leading to enhanced extrasynaptic glutamate disrupting excitatory and inhibitory synaptic transmission (Lau et al., 2021). However, it is unknown how changes in lOFC astrocyte function contribute to decision-making behaviour. This purpose of this thesis is to design a model of selective astrocyte activation within the lOFC that can be used in future studies to address the acute or chronic activation of lOFC astrocytes to cortical synaptic transmission, neuroinflammation, and behaviour.

Thesis Aims and Hypotheses

Visualizing OFC astrocytes is challenging due to immunohistochemical limitations, but necessary to understand their function in physiological or toxic environments. The main objective of this thesis is to create a model of acute activation of astrocytes within the LOFC using a combination immunohistochemical approach with IMARIS 3D-rendering. It's imperative to have a sensitive immunohistochemical protocol that will selectively label astrocytes. The overarching hypothesis is LOFC astrocytes can selectively express and be activated by an excitatory DREADD. We offer a new 3D visualizing protocol that allows us to assess astrocytes labeled with S100β and their colocalization with the DREADD reporter, mCherry, to determine the effectiveness of DREADD transfection. This thesis also posits a method to assess if astrocyte activation affects neuronal activity.

Aim 1: I will determine whether the hM3Dq-DREADD can be selectively expressed in astrocytes in the LOFC.

Hypothesis 1: hM3Dq-DREADDs can be targeted to LOFC astrocytes using the gfaABC1D promotor expressing the mCherry reporter.

Aim 2: I will determine if acute astrocyte activation is sufficient to indirectly influence neighbouring neuronal activity, indicative of increased cFos expression.

Hypothesis 2: Acute activation of astrocytes will not be sufficient to influence neuronal activity indirectly.

CHAPTER TWO: METHODS

Subjects

Male (n = 6) and female (n = 2) DAT IRES-cre mice (post-natal day 60-90) were initially bred locally at the University of Calgary Clara Christie Center for Mouse Genomics. Mice were group-housed (3-5 per cage) with ad libitum access to water and food. Mice were housed in ventilated cages in a temperature (21 ± 2 ºC) and humidity-controlled (30-40 %) room on a 12h reverse light/dark cycle (lights off at 10:00 AM MST). All experimental procedures adhered to ethical guidelines established by the Canadian Council for Animal Care and animal use protocols approved by the University of Calgary Animal Care and Use Committee (Protocol: AC21-0004).

Viral Infusion Surgeries

All mice received a bilateral infusion of the excitatory DREADD (hM3Dq) (AAV2/8-gfaABC1D-hM3Dq-mCherry; Neurophotonics, Centre de Recherche CERVO, Quebec City, QC, Canada) virus in the LOFC. Mice were anesthetized with isoflurane gas (4% for induction; 2% for maintenance) and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). All measurements were made relative to bregma for viral infusions. Viral injections were performed using a microinjector (Nano-inject II; Drummond Scientific Company, Broomall, PA, USA). Each mouse received two bilateral infusions (110.4 nl per hemisphere; 55.2 nl/s per injection) in the LOFC at the coordinates of anterior-posterior (AP) -2.58; mediolateral (ML) ± 1.3; dorsoventral (DV) -1.945, -1.925 (one injection per DV value). After the first infusion, the microinjector was left in place for an additional 3 minutes, The injector was then lowered to -1.925 for the final infusion. After 3 minutes, the injector was raised back to -0.245 DV for another 3 minutes. All mice received postoperative analgesia (Meloxicam 5 mg/kg, subcutaneous (s.c.)) and

saline (0.5 ml, s.c.) were returned to their home cages and allowed to recover for five weeks before further experimental procedures (Figure 5).

Acute DREADD Activation Protocol

DCZ Preparation

DCZ (Hello Bio - HB9126) was dissolved in DDH20 to create a stock solution of 2.6 mg/2ml. The stock solution was diluted with saline to a final concentration of 1 ug/kg. The solution was separated into 1.5ml aliquots and stored at -20 degrees Celsius.

Acute Administration

Intraperitoneal injections (i.p.) of either DCZ (n = 4) or saline vehicle (VEH, n = 4) were given at a final volume based on body weight with a concentration of 1 μg/kg. Mice were habituated to i.p. injections for two consecutive days before testing. On the third day, mice received an i.p. dose of DCZ or VEH and were euthanized 60 minutes later. Mice were split equally based on body weight into the VEH (n = 4) and DCZ (n = 4) groups. (Figure 5).

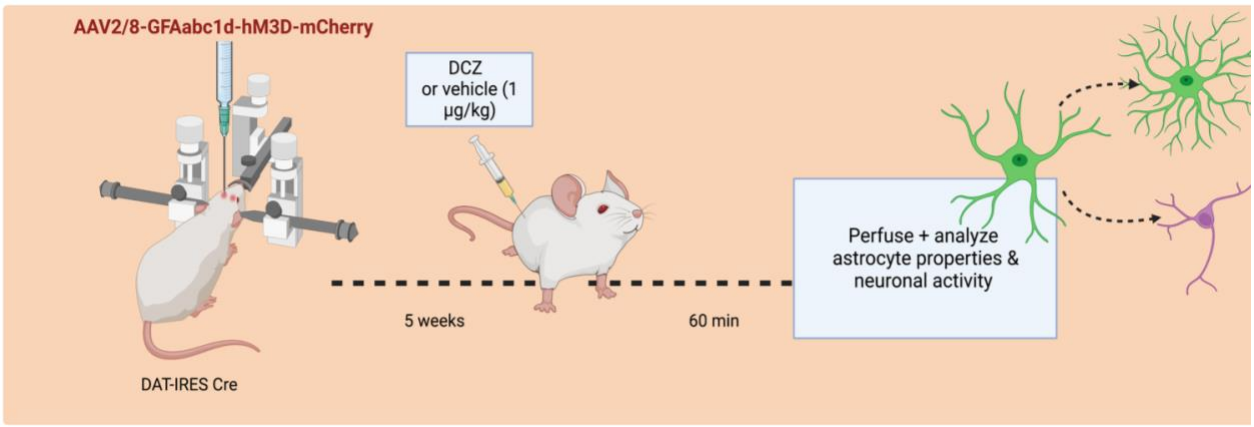

Figure 5: Schematic of acute astrocyte activation protocol.

Histology

To check viral transfection mice were deeply anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde (PFA). Brains were dissected and post-fixed in 4% PFA at 4°C overnight, then switched to 30% sucrose. Coronal frozen sections were cut at 30 μm using a cryostat. Slices were then warmed to room temperature. The following immunohistochemical protocols were followed for each specific aim:

Aim 1 Staining for AAV2/8-gfaABC1D-hM3Dq-mCherry and s100β

Slides were first washed with 2 x 10 min washes of PBST and 3 x 10 min washes of PBS. 10% goat serum was applied to slides to block non-specific binding for 1 hour. A cocktail of primary anti-chicken RFP to enhance the mCherry reporter (1:2000 in BSA) (Rockland, code # 600-901-379) and mouse anti-S100β (Sigma, S2532) (1:1000 in Sharkey Dilutant (comprised of 200 ml PBS; 200 μl Titron-X; 200 mg BSA; 2000 μl; sodium EDTA 80 mg)) to label astrocytes was applied to the sections and were incubated for 24 hours at -4 degrees Celsius. The following day slides were washed with PSBT and PBS. Then a secondary antibody cocktail of Alexa Fluor 594 goat anti-chicken (Invitrogen) (1:400) and Alexa Fluor 488 goat anti-mouse (Invitrogen) (1:400) was applied to the slides and left to incubate in the dark for 2 hours. Slides received final washes of PBST and PBS and then mounted with fluoromount and coverslips.

Each animal had two-channel immunohistochemistry for expression of AAV2/8-gfaABC1D-hM3Dq-mCherry (enhanced with RFP) and s100β. For each mouse, two-channel staining was done on two slides with five OFC slices each. From each slide, confocal images were taken from two OFC sections per animal. The LOFC was the injection site and then we used a

within-slice control design to take images from the medial OFC, where there was no injection. Per section, there was total of 4 images taken, 2 LOFC images (bilateral) and 2 medial OFC (MOFC) control images (bilateral), for a total of 8 images per mouse. There were 4 mice in the DCZ treatment group and 4 mice in the control VEH group, for a total of 32 images per group: LOFC VEH, LOFC DCZ, MOFC VEH and MOFC DCZ.

Aim 2 Staining for AAV2/8-gfaABC1D-hM3Dq-mCherry, NeuN, and cFos

Slides were first washed with 2 x 10 min washes of PBST and 3 x 10 min washes of PBS. A 10% goat serum was applied to slides for 1hour to block non-specific binding. A primary antibody cocktail of anti-chicken RFP (Rockland, code #600-901-379) (1:2000) to enhance mCherry, anti-mouse NeuN (Cell Signalling Technology, E4M5P) (1:1000) to label neurons, and rabbit anti-c-Fos (Cell Signalling Technology, 9F6) (1:300) to detect neuronal activation, in Sharkey Dilutant was applied to the sections to incubate for 24 hours at -4 degrees Celsius. The following day slides were washed with PSBT and PBS. Then a secondary antibody of Alexa Fluor 594 goat anti-chicken 1:400, Alexa Fluor 488 goat anti-mouse 1:400, Alexa Fluor far red 647 goat 1:400 was applied for 2 hours. Sections adhered to slides received final washes of PBST and PBS and then mounted with fluoromount and coverslips.

For each mouse, three-channel staining was done on two slides with five OFC slices each. From each slide, confocal images were taken from two OFC sections per animal. Per section, a total of 4 images, 2 LOFC images (bilateral) and 2 MOFC control images (bilateral), for a total of 8 images per mouse. There were 4 mice per group enabling a total of 32 images per group: LOFC VEH, LOFC DCZ, MOFC VEH and MOFC DCZ.

Confocal Imaging

Image Properties

To identify the expression of the viral reporter, whole LOFC slice images were collected on an Olympus Virtual Slide Microscope VS120-L100-W with a 10x objective (Olympus Canada Inc., Ontario, Canada). Confocal images were then collected with a Leica confocal microscope TCS SP8 with a 25x objective (Leica Microsystems Inc., Ontario, Canada). The settings for all imaging were as follows: each image was taken in the format of 2976 by 2976 pixels at a speed of 600 Hz. 3D images of slices were taken in the XYZ plane. The phase X constant, which compensates for any image distortion while taking z-stacks was set to 35.32. Z-stacks framerate was set to 0.198 frames/second. The total image size was 1024 pixels by 1024 pixels. The optical sectioning, or the process by which suitable clear images of focal planes within a tissue is produced, was set to 1.705 μm.

Lasers Properties

Alexa Fluor 594 is excited by the 594 laser which has an excitation peak at 590 nm and an emission peak at 618 nm. The gain coefficient, or the measure of the amplification applied to the detection system, was set to 640.9 on the PMT 2 laser and the laser power was 1.83%. Alexa Fluor 594 is excited by the 488 laser with an excitation peak of 499 nm and an emission peak of 520 nm. The gain was set to 21.5 and the HyD laser power was set to 4.60 %. Alexa fluor 647 is excited by either the 594 nm or 647 nm laser with an emission peak at 671 nm. The gain for the far-red laser was set to 23.7 for the HyD filter, and the laser power was set to 2.7%. Since the 594 laser was used to excite the Alexa Flour 594, there is an issue of the 594-laser bleeding from the red channel into the far-red channel. To circumvent this issue, two sperate time sequences were used to image

which reduced the amount of crosstalk between the 594 and 647 lasers. For the 3-channel imaging, the red channel and green channel were imaged first and then the far-red followed in the z-stack acquisition.

3D IMARIS Protocol

This section describes the IMARIS protocol for visualizing and quantifying transfection of the AAV2/8-gfaABC1D-hM3Dq-mCherry virus in astrocytes, and if activation of DREADD increases cFos intensity. IMARIS 9.9 3D-imaging allows researchers to robustly examine results on a greater dimension (Althammer, 2022; Testen 2020). Using IMARIS, confocal data was assessed qualitatively and quantitatively. All IMARIS images were kept blind to treatment until sorted for statistical analyses.

IMARIS Processing of DREADD Transfection of Astrocytes

Z-stacks were opened in IMARIS in their native format. Z-stacks are automatically reconstructed into a multi-channel 3D model during input into IMARIS, requiring no further image pre-processing. To designate individual S100β-labeled astrocytes, a surface creation tool was used to identify astrocytes based on absolute intensity (threshold) or background intensity of a signal. The size and shape of the generated surface were a direct map of the intensity distribution of s100β immunolabeling as detected by IMARIS. The system quantified signal based on background intensity of the green channel (Table 1A). The background subtraction (diameter of the largest cell body of astrocyte) was set to separate the cell in the foreground from the background environment (Table 1B). The images were filtered by the number of voxels (10), as recommended by the

program (Table 1C). The object-object statistics used to assess the volumetric properties of the green and red channel.

The surface plot steps, described above were repeated for the RFP signal. To enable a clean border around a desired cell, smoothing surface detail was enabled with the surface grain size set to 0.1 μm. The cell width was measured at its widest point in slice mode and entered as the diameter of the largest sphere which fits into the object in the surface creation wizard. This was set to 0.7 μm. Filter by number of voxels was set to 10 (Table 1C). For the question of if virus present in astrocyte, the RFP intensity signal was masked channel as a binary code. The object-object statistics were used to assess the signal intensity properties of the far-red channel, and green channel.

The following statistical outputs for the green channel and the red channel were collected:

I. Number of astrocytes
II. S100β (green) channel data volume
III. RFP (red) channel data volume occupying total green channel volume
IV. Total pixel volume of all data

IMARIS Processing for Signal Intensity of cFos Within NeuN Cells

Surfaces distorted the shape of NeuN cells. To quantify the number of NeuN immunolabeled neurons, we used IMARIS' spots creation tool. The spot function works similarly to surfaces except it is used for more uniform shapes like cell bodies. IMARIS' integrated machine learning function successfully detected neurons (NeuN cells) in the green channel and labelled them with spots. IMARIS identified a spot versus a non-spot, or a NeuN cell versus a non-NeuN cell, respectively. The spot detection parameter worked by using the average size of a NeuN cell

(10 μm). Spots with a diameter smaller than this were not detected. Every image was manually visually checked and corrected if IMARIS' spot detection needed revision. Background subtraction was enabled to smooth the image. The quality filter was set to 1.43 μm. The Mean volume of cFos with NeuN (volume of far-red channel in green channel) statistical output was collected.

Aim(s)	Channel:	A. Surface/spot detail	B. Background subtraction	C. Filter type:	
				Voxels	Quality
1	S100β (astrocytes)	0.208 μm (surface)	10 μm	10	N/A
1,2	RFP (DREADD virus)	0.1 μm (surface)	0.7 μm	10	N/A
2	NeuN (neurons)	10 μm	Enabled	N/A	1.43

Table 1: IMARIS processing properties for s100β, RFP and NeuN

Data Analysis

All statistical analyses were completed using GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA). All values are expressed as median value and n, unless expressed as a percentage and n. The alpha risk for the rejection of the null hypothesis was set to 0.05. All data was tested for normality using the Shapiro-Wilk test for normal distribution. The null hypothesis of the Shapiro-Wilk test is if the p-value is less than 0.05, the data was not normally distributed. All data met criteria for normality, except one animals' value in the lateral and medial OFC. Therefore, a non-parametric Mann-Whitney's U t-test was used to assess differences between the two regions of the OFC, or if there was a difference between DCZ and VEH. Asterisks were used to express statistical significance in figures: **P < 0.01 and ***P< 0.001. Figures were generated using GraphPad Prism 9.0 and Adobe Illustrator CS4 (Adobe Inc., San Jose, CA, USA) software.

CHAPTER THREE: RESULTS

Aim 1 Qualitative Results: Visualization of DREADD and OFC Astrocytes

Immunohistochemical Results of AAV2/8-gfaABC1D-hM3Dq-mCherry Virus and s100β

S100β primarily labels cell bodies and some filaments of astrocytes (Figures 6A,7A). However as expected, microfilaments were not detected in our s100β immunolabeled images. RFP antibody amplified the expression of the reporter from AAV2/8-gfaABC1D-hM3Dq-mCherry transduction (Figures 6B,7B). The two-channel confocal imaging of S100β and RFP show morphological variances within slices (Figure 6C). Some astrocytes have characteristic cell bodies, which s100 effectively labels. Other astrocytes present smaller cell bodies that morph into their filaments (Figures 7A, 6C, 7C).

IMARIS 3D surface rendering was able to outline astrocytes effectively, and then identify the RFP-enhanced mCherry reporter for DREADD expression (Figures 6D, 7D). Merging confocal images of both channels reveal two characteristics: the mCherry reporter was either located within an astrocyte (shown by RFP cells being in the astrocytic surface domain (Figure 6D)) or was outside the astrocyte (typically in a cluster shape (Figure 6B)). Note that IMARIS 3D surface rendering was able to effectively label the cell bodies, however, it was less efficient with the filaments.

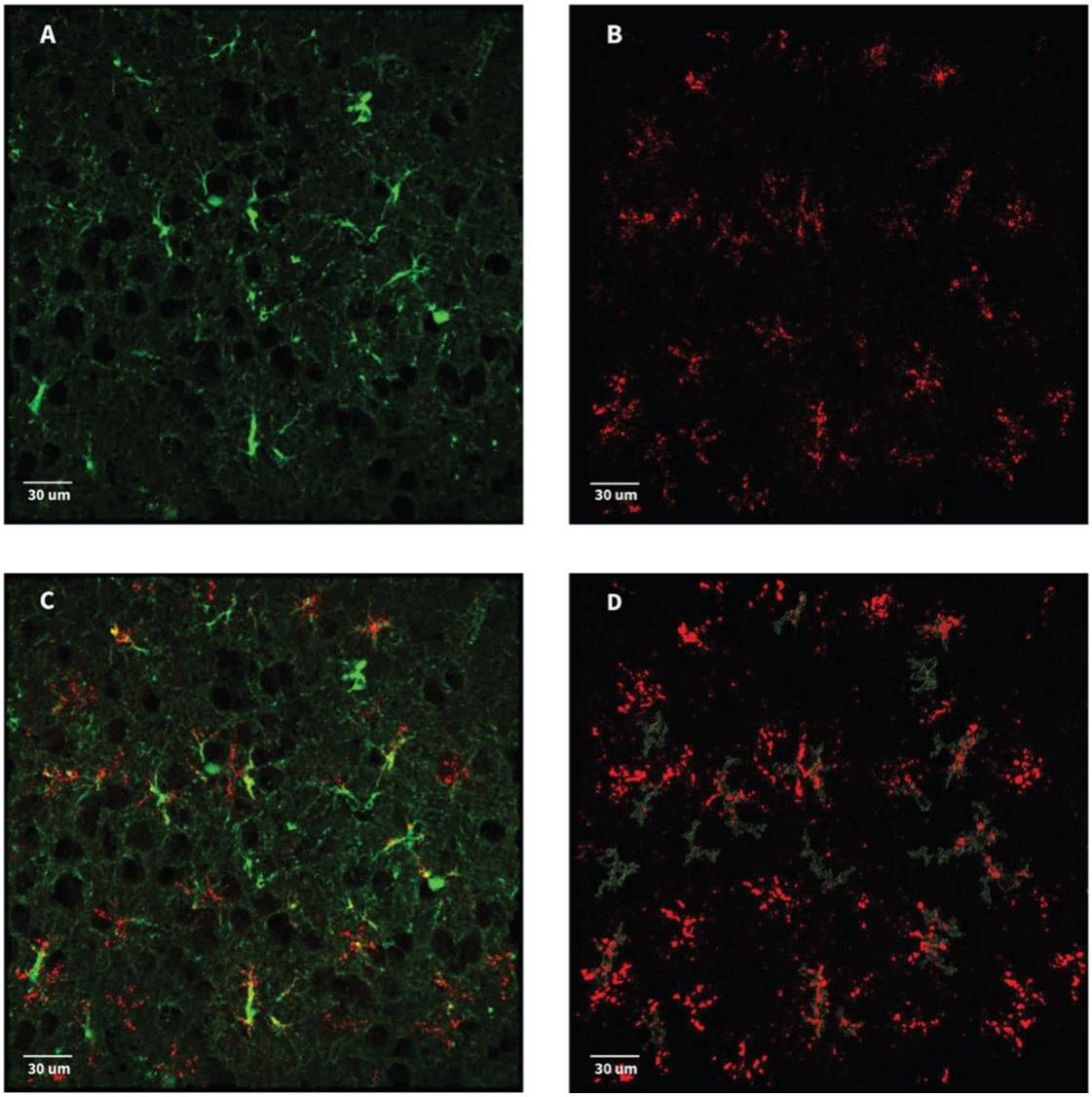

Figure 6: Confocal images of transfection of astrocytes immunolabeled with s100β and hM3Dq-with RFP-enhanced mCherry expression.

A) Confocal 25X imaging of s100β positive astrocytes (green). S100β largely labels the cell body and less so the microdomains of astrocytes.

B) Confocal 25X imaging of RFP (red) amplified immunohistochemical staining of the mCherry-tagged hM3Dq DREADD.

C) Merged confocal imaging of s100β (green) and RFP (red) in the LOFC.

D) 3D-Imaris imaging of overlap confocal image (Figure 6C), showing location of the hM3Dq-mCherry immunolabel in the astrocyte, and the hM3Dq-mCherry immunolabel occupying space outside of an astrocyte domain.

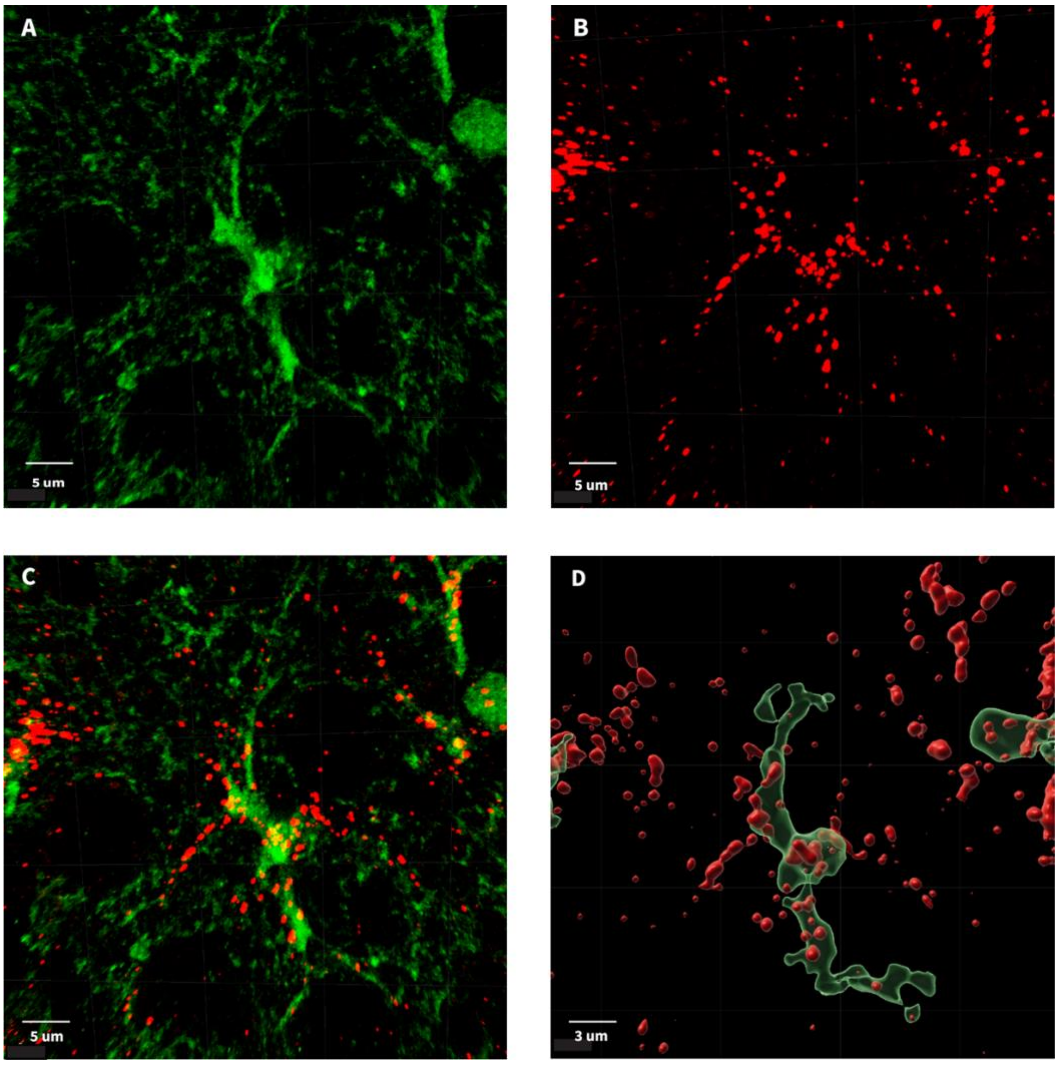

Figure 7: Close-up of DREADD location in astrocytes.

A) Enlarged Confocal 25X image of s100β (green) positive astrocytes. Example of S100β labeling the cell body and less so the microdomains of astrocytes.

B) Enlarged Confocal 25X imaging of RFP (red) amplified hM3Dq-mCherry.

C) Enlarged merged confocal 25X imaging of s100β (green) and RFP (red) in the LOFC. The virus labels the filaments more than s100β.

D) Enlarged 3D-Imaris imaging of merged s100β and RFP confocal image (Figure 7C). 3D astrocytes were masked and outlined in green.

Aim 1 Quantitative Data Results

Aim 1 Quantitative Results I: OFC Regional Heterogeneity of Astrocyte Number

Astrocyte number varied between regions within the OFC of VEH mice. Slices from the LOFC (median = 27.50, n = 32) had significantly more astrocytes immunolabeled with s100β than MOFC slices (20.0, n = 32; U = 276, p = 0.001; Figure 8). This suggests that either LOFC astrocytes express more s100β than MOFC astrocytes, or that there are a greater number of astrocytes in the LOFC compared to MOFC.

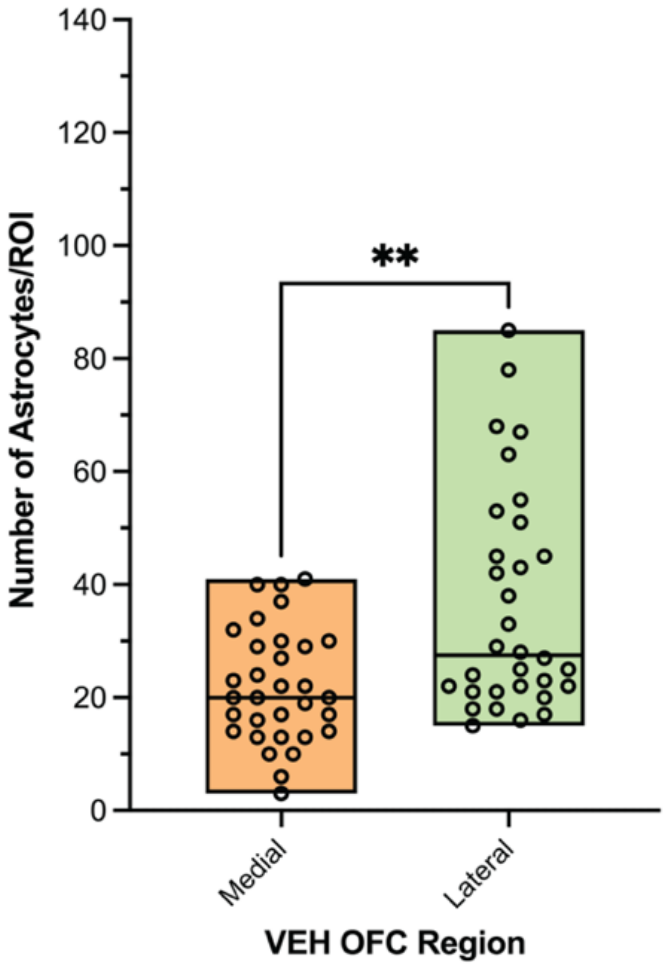

Figure 8: Comparison of VEH control groups between OFC regions.

Aim 1 Quantitative Results II: LOFC Astrocytes are Transfected with DREADD Virus

The percent of hM3Dq-mCherry immunolabel occupying the total volume of an astrocyte was analyzed. In the lateral region, the acute administration of the DREADD agonist, DCZ (0.087%, n = 32), did not affect the amount of astrocyte volume occupied when compared to the vehicle treatment group (0.082%, n = 32; Mann Whitney U = 489, $p = 0.764$; Figure 9A). As a control for viral injection spread, we assessed the amount of hM3Dq-mCherry immunolabel occupying the total astrocyte volume in the MOFC. As expected, there was less hM3Dq-mCherry expression in the MOFC than in the LOFC, which was the site of viral injection (Figure 9A, B). Furthermore, there was no significant difference in hM3Dq-mCherry occupying total astrocyte volume in the MOFC when treated with VEH (0.0001%, n = 31) or DCZ (0.0005%, n = 32) (Mann Whitney U = 455, $p = 0.4324$; Figure 9B). Taken together, these data suggest that hM3Dq-mCherry is expressed in S100β labeled cells in the LOFC, but not the MOFC.

To determine if DCZ activation of hM3Dq-mCherry influenced astrocyte size immunolabeled with s100β, we measured the percent of total pixel volume from the s100β's green channel. There was no significant difference in astrocyte size in the LOFC between vehicle (0.537%) or DCZ (0.725%) treated mice (Mann Whitney U = 469.5, $p = 0.573$; Figure 9C). Thus, an acute activation of astrocytes does not influence astrocyte size.

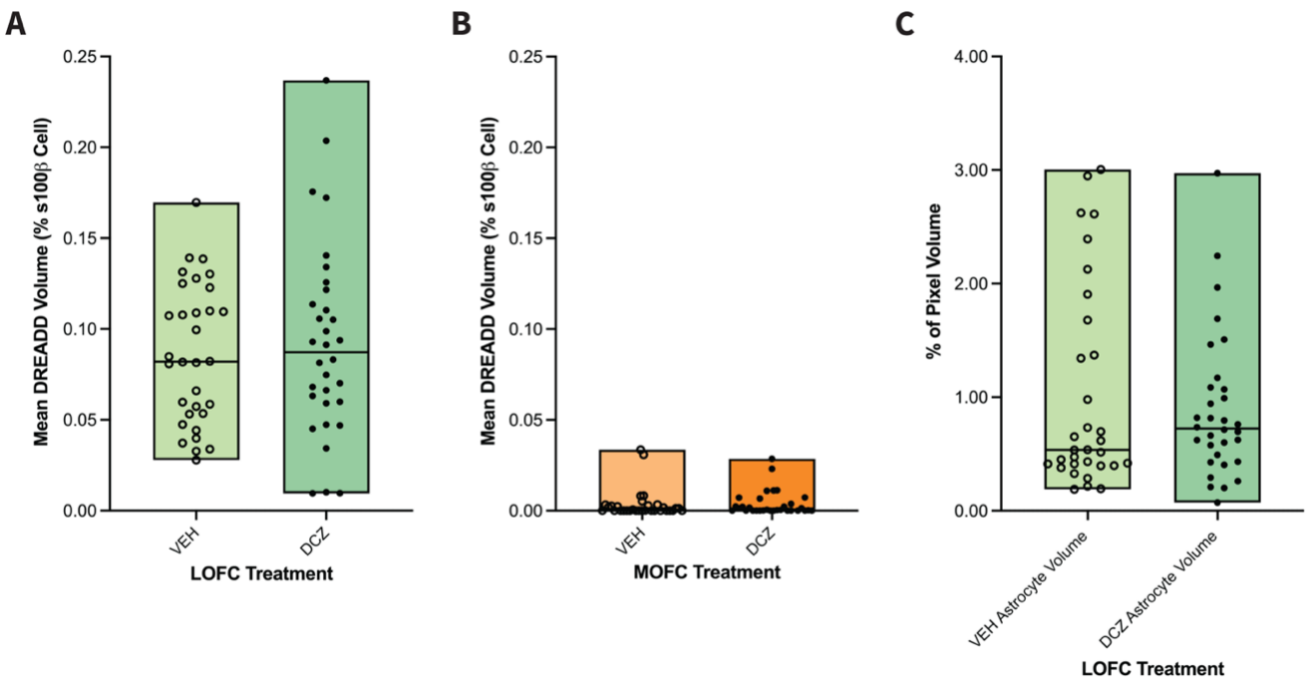

Figure 9: Mean hM3Dq-mCherry volume occupying s100β immunolabeled cells per OFC region.

A. Mean hM3Dq-mCherry volume occupying an astrocyte. In the LOFC, DCZ does not influence hM3Dq-mCherry immunolabelling in astrocytes.

B. Mean hM3Dq-mCherry volume occupying an S100β-labeled astrocyte. There is less hM3Dq-mCherry in the MOFC compared to LOFC, and no difference in mean hM3Dq-mCherry volume that is occupied by s100β between VEH and DCZ treatment groups.

C. Percent of total slice pixel volume that is occupied by astrocytes. DCZ did not affect the total astrocyte volume.

Aim 1 Quantitative Results III: DCZ Increased Number of Astrocytes in the MOFC.

We next determined if there was an effect of DCZ- activation of hM3Dq expression on astrocyte number in the region of injection (LOFC). The number of astrocytes were quantified in the DCZ and the VEH treatment (27.50, n = 32) groups. The hM3Dq activation in astrocytes by DCZ (38.50, n = 32) did not influence the number of astrocytes in the LOFC compared to the VEH treated group (27.50, n = 32) (Mann Whitney U = 390, $p = 0.102$; Figure 10A). Conversely, in the MOFC, the DCZ group, had a significant increase in the number of astrocytes (30, n = 32) compared to the VEH group (20, n = 32) (Mann Whitney U = 291, p = 0.003; Figure 10B).

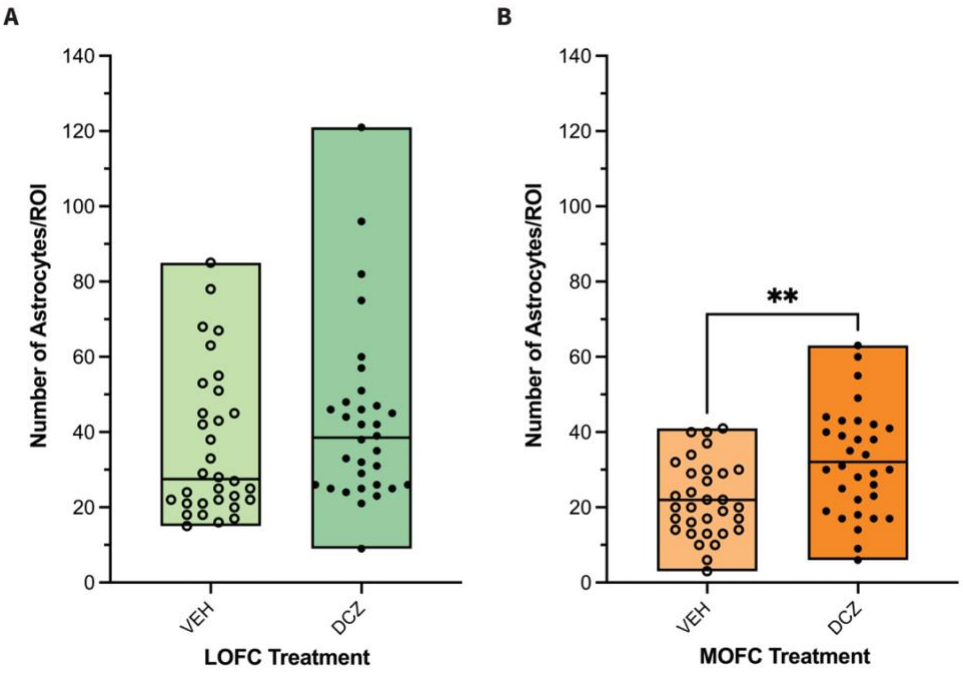

Figure 10: Number of astrocytes per OFC region.

A. Number of astrocytes in LOFC for the VEH group vs the DCZ treatment group. DCZ did not influence astrocyte number.
B. Number of astrocytes in the MOFC for the VEH group vs the DCZ treatment group. DCZ increased the number of astrocytes.

Aim 2 Qualitative Data Results

IMARIS 3D Rendering Successfully Identify NeuN Cells

NeuN immunolabeled neurons showed uniform shapes (Figure 11A). The majority of cFos cells resembled a granulated spherical shape with varied optical intensity (Figure 11B). IMARIS integrated machine learning spot detection tool was able to identify NeuN immunolabeled neurons and correctly label them with a spot (Figure 11C). This allowed for effective quantification of cFos and NeuN colocalization. The RFP-enhanced hM3Dq-mCherry immunolabel was used to confirm viral transduction and the presence of astrocytes (Figure 11D). This process was done for images from both the MOFC and LOFC.

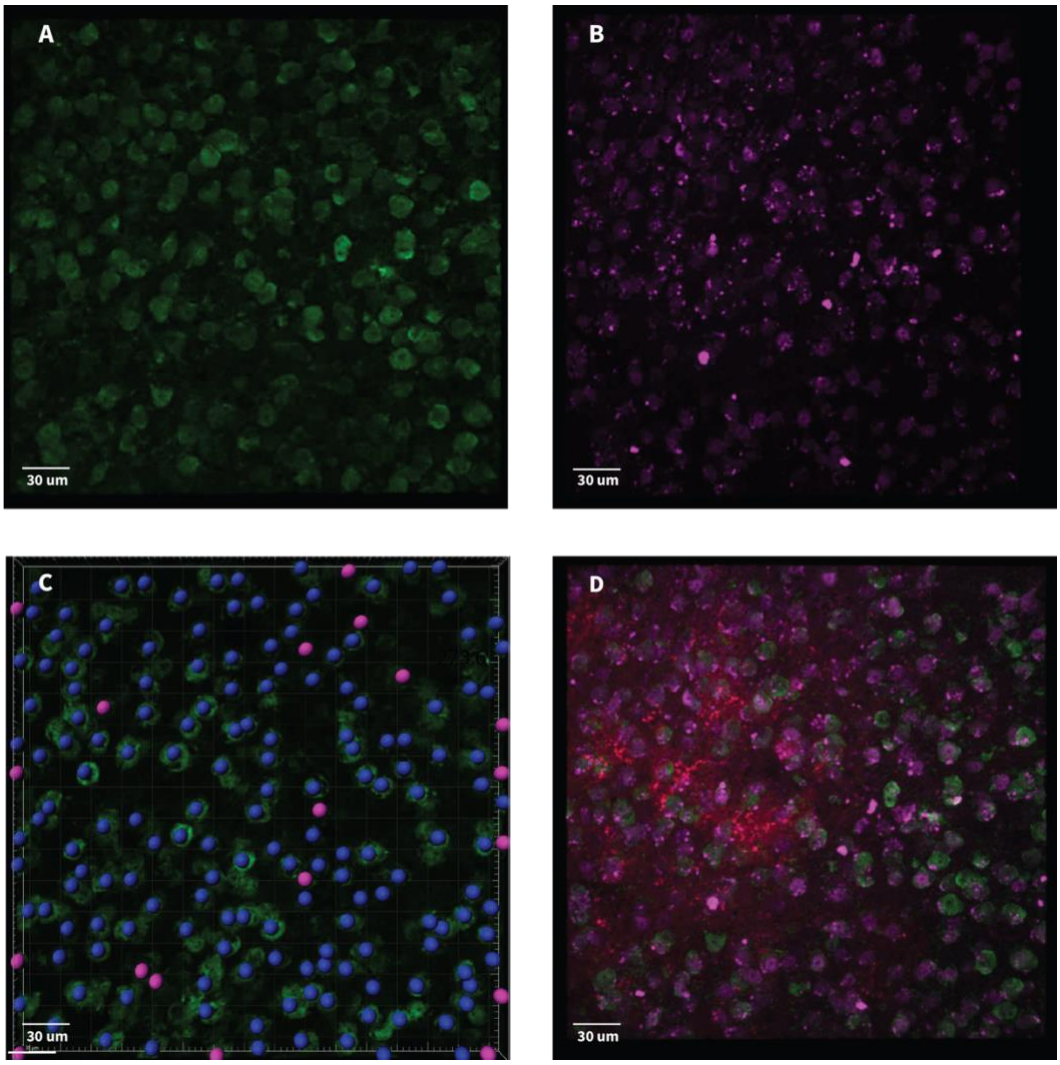

Figure 11: 3-channel confocal 25X images and IMARIS 3D-rendering for spot detection.

A) Confocal 25X imaging of NeuN (green) labelled neurons.

B) Confocal 25X imaging of cFos (magenta).

C) 3D-Imaris imaging and Machine learning process of categorization of a prototypical NeuN cells. Pink spheres are non-NeuN cells and blue spheres are NeuN cells.

D) Confocal 25x of triple channel image of NeuN (green), cFos (magenta) and RFP (red).

Aim 2 Quantitative Data Results

hM3Dq Activation in Astrocytes Does Not Affect Neuronal cFos Expression

The second aim was to determine if acute activation of astrocytes with DREADD will indirectly influence neuronal activity, indicated by an increase in cFos expression. We assessed cFos expression intensity in NeuN cells, measured in voxel intensity values in both the LOFC and MOFC. There was no regional difference in cFos intensity in NeuN cells in the LOFC (5.977, n = 32) compared the MOFC (4.790, n = 32) of vehicle treated mice (Mann Whitney U = 412, p = 0.1815; Figure 12A). In the LOFC, the DCZ treatment group (7.383, n = 32) and the VEH treatment group (5.977, n = 32) did not significantly differ (Mann Whitney U = 489, p = 0.7642; Figure 12B). Likewise, in the MOFC region the DCZ (6.656, n = 32) and the VEH treatment groups (4.790, n = 32) were not significantly different (Mann Whitney U = 397, p = 0.1240; Figure 12C). Taken together the acute activation of hM3Dq in astrocytes does not indirectly activate neurons in the LOFC or MOFC.

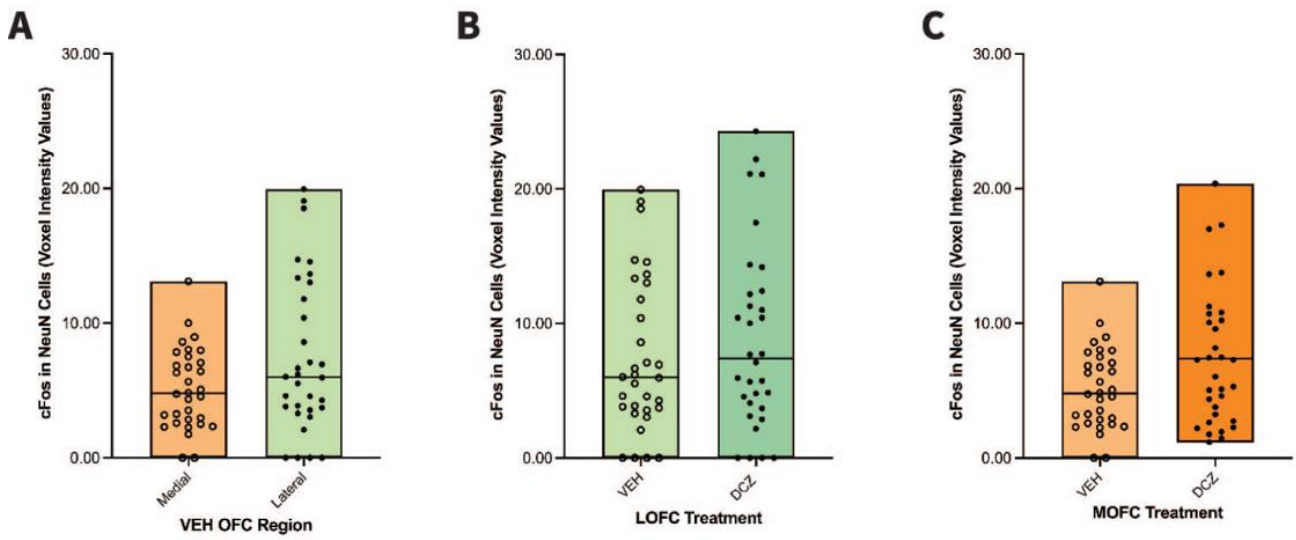

Figure 12: Overall signal intensity of cFos is not increased by DREADD activation.

A. cFos intensity in NeuN cells did not differ for the VEH controls in the medial and lateral regions.
B. cFos intensity in NeuN cells in the LOFC region did not differ from each other for the VEH and DCZ group.
C. cFos intensity in NeuN cells in the MOFC region did not differ from each other for the VEH and DCZ group.

CHAPTER FOUR: DISCUSSION

Overview

This study's goal was to create a model for acute activation of astrocytes for future use in neuroinflammation research. As discussed in chapter one, chemogenetics has been employed in neuroscience and has advanced knowledge of specific cellular contributions to behaviour. There were several novel aspects of this thesis advancing our understanding of using chemogenetics in astrocytes. We found that the gfaABC1D promotor was effective in targeting hM3Dq-mCherry to astrocytes, IMARIS software allowed 3D-visualization and quantification of astrocytes expressing hM3Dq-mCherry, and the potent DREADD agonist DCZ did not induce cFos activation of neurons.

Aim 1 Immunohistochemical Assessment: DREADD Transfection in OFC Astrocytes

The aim 1 hypothesis is that the AAV2/8-gfaABC1D-hM3Dq-mCherry virus would successfully transfect astrocytes. The initial challenge was to create a robust immunohistochemical protocol that ensured good penetration of astrocytes with s100β. Compared to other astrocyte markers, like GFAP, s100β localizes in astrocytic cytoplasm (Brozzi et al., 2009). Here, s100β effectively labels the cell body and the major filaments (Figure 6A, 7A). There is less cytoplasm in the microfilaments, which could be why the s100β antibody was less effective at labelling them. Interestingly, s100β resided outside the masked astrocyte's domains (Figure 6D, 7D). The domains of astrocytes were masked at the surface using IMARIS (Figure 6D, 7D). If there were cases in which IMARIS made a mask for the green fluorescence background signal that was not an astrocyte, it was omitted from the analyses.

The expression of hM3Dq-mCherry amplified by RFP immunofluorescence indicated that AAV2/8-EF1a-gfaABC1D-hM3Dq-mCherry could effectively transduce astrocytes in the LOFC and did not spread to the MOFC (Figure 6B, 6C, 7B and 7C). Previous researchers used a similar protocol with the combination of confocal imaging and IMARIS 3D-rendering to image expression of hM3Dq-Lck-GFP in astrocytes due to viral mediated gene transfer using the same promotor (Testen et al., 2020). Testen and colleagues took advantage of the lymphocyte-specific fluorescent green protein, tyrosine kinase (Lck) (Scofield, 2016; Shigetomi, 2010). Lck binds to an astrocyte's plasma membrane, allowing the labelling of peripheral processes. Lck had a surface area 10-fold greater than that of GFAP (Testen et al., 2020). This study was done in rats and needs to be tested if it is as effective in mice. Using an Lck membrane-bound virus may show better labeling of astrocytic processes that s100β, but so far, it has not been directly compared.

Aim 1 Immunohistochemical Caveats

My results indicate that I can express hM3Dq-mCherry in s100β labeled cells. However, I did not specifically address the selectivity of the viral transfection of AAV2/8-gfaABC1D-hM3Dq-mCherry in astrocytes. While I observed no overlap with NeuN, suggesting that it does not target neurons, I did not address whether this virus could transduce other glial cells, including microglia or oligodendrocytes. Follow-up immunohistochemical staining for microglia using Iba-1 and hM3Dq-mCherry colocalization could address this. I also noted that expression of hM3Dq-mCherry was primarily localized to astrocyte filaments, whereas s100β primarily labeled astrocyte cell bodies, thus giving the appearance of a lack of colocalization. This study reiterates the limitations of tools used in neuroscience to visualize astrocytes.

Aim 1 Regional Difference of Astrocyte Number Discussion

The first analyses of aim 1 was to address if astrocyte number varied between OFC brain regions. I observed that there is a regional difference of astrocyte number, such that LOFC has a greater number of s100β labeled cells than MOFC. However, if s100β is poorly expressed in LOFC compared to MOFC astrocytes, it would appear as differences in astrocyte number, as low s100β expressing astrocytes may not be detectible with immunolabeling. Alternatively, this heterogeneity between the LOFC and the MOFC could be due to regional variation during and post astrogenesis, morphological variation, and molecular variation.

Developmental Influence on Astrocyte Region Specificity

In the introduction, morphogenic proteins influence on fate-mapping was discussed. Neurons express SHH, and astrocytes have SHH receptors (Cahoy, 2008; Farmer, 2016). Neuronal SHH signalling plays a vital role in astrocyte heterogeneity. SHH influences the expression of the astrocytic potassium channel, Kir4.1, and electrophysiological properties of the hippocampal and cortical astrocytes *in-vivo,* thus, altering astrocyte expression and identity (Farmer et al., 2016). Neuronal SHH influencing astrocytes is evident in the cortex, hippocampus, and the cerebellum (Farmer et al., 2016). Astrocytes have remarkable plastic properties that allow them to be influenced by morphogens. Morphogens can pressure for an astrocytic fate over a neuronal fate.

Confirmation experiments of morphogen's roles in astrocyte migration were done *in-vitro* with stem cells. Researchers found that when diffusible morphogens pressure stem cells, they will migrate to regionally distinct astrocyte populations (Bradely, 2019; Krencik, 2011). Clarke and colleagues found regionally distinct astrocytes on the dorso-ventral and rostro-caudal axes of the cortex (2021). The cortex's SHH receptors are on a proportion of astrocytes suggesting that

local synaptic activity may influence astrocytes at the transcriptomic level (Hill et al., 2019). Other morphogens play a role in astrocyte presentation. Astrocytes gain pathological phenotypes if fibroblast growth factor (FGF) signalling is inhibited; FGF is required to maintain healthy physiological astrocytic phenotypes (Farmer, 2016). The combination of signalling cues in tandem influences astrocyte diversity and patterning. The processes of gliogenesis migrate astrocytes allowing for interregional variation. However, intraregional variation in the cortex occurs after gliogenesis (Clavreul, 2019). The following section offers a greater comprehension of cortical astrocyte heterogeneity and how this could explain the discrepancy between the LOFC and MOFC astrocyte numbers (Figure 8).

Developmental Regional Variation of Cortical Astrocytes

Of interest to this thesis, researchers have found regional variation of astrocytes within the different cortical layers (Lanjakornsiripan et al., 2018). Unlike the spinal cord, where astrocytes are systemically organized, astrocytes in the cortex present in a scattered randomized manner (Clavreul et al., 2019). Specialized local environmental factors influence astrocytes in the cortex to have extensive intraregional morphological heterogeneity (Clavreul, 2019; Garcia, 2013). Neuronal environmental signaling via morphogens, such as SHH, influences astrocyte patterning. Other neuronal transcriptional factors play a role in astrocyte patterning. The knockout of the neuronal transcription factor, Satb2, affected astrocytic organization in the cortex (Bayraktar et al., 2020). The pressure of different transcription factors and morphogens secreted by neighbouring neurons influence astrocytes' location**.**

Molecular Heterogeneity and Gene Expression in the Cortex

There are limited studies on the molecular heterogeneity of astrocytes (Chaboub & Deneen, 2012). Functional diversity and morphological heterogeneity of astrocytes suggest underpinning molecular heterogeneity. However, it is challenging to study the complex molecular signalling within astrocytes and difficult to narrow down patterns of molecular heterogeneity and is an avenue of astrocyte research to explore.

One way to understand molecular heterogeneity is to examine gene expression in the cortical layers. Tissue microdissection and intersectional labelling strategies reveal unique regional variances between the layers of the cortex (I-VI) (Lanjakornsiripan et al., 2018). There is a contrast between the gene expression in the upper (II/III and IV) and deeper (V and VI) layers of the cortex (Bayraktar, 2020; Lanjakornsiripan, 2018). Genes like *sparc* and *merkt* are in the upper layers of the cortex, whilst *Chrdl1*, and *Il33* are in the deeper layers of the cortex (Bayraktar, 2020; Lanjakornsiripan, 2018). Regarding GFAP, cortical layer I has strong expression but reduced astrocyte density (Lanjakornsiripan et al., 2018). Therefore, the laminal and related gene expression should be considered when understanding intraregional heterogeneity. In relevance to the thesis results, the difference in the number of astrocytes between the MOFC and LOFC could be due to intraregional differences in the thickness of different layers and their respective gene expression (Figure 8).

Morphological Heterogeneity

Astrocytes in white matter are termed fibrous, and grey matter astrocytes are called protoplasmic astrocytes (Ben Haim & Rowitch, 2017). Researchers have classified up to nine different types of astrocytes, however, there is a general push to move away from the traditional

nomenclature of astrocytes (Ben Haim & Rowitch, 2017; Escartin, 2021). Even between regions, astrocytes can vary in density, but this is stringent upon rigorous immunohistochemical methods to ensure validity (Emsley, 2006; Keller, 2018). Due to the limitation of tools, the research on astrocyte morphology and location is heavily debated. Generally, s100β can label protoplasmic astrocytes, and GFAP can label fibrous astrocytes. Protoplasmic astrocytes are classified by highly branched bushes, whereas fibrous astrocytes have long fibre-like processes (Sofroniew & Vinters, 2010). Our finding suggests regional heterogeneity in the OFC which could be furthered explained by morphological different phenotypes of astrocytes (Figure 8). Morphological differences between astrocytes are due to different expressions in common marker proteins like GFAP and GLAST (Ben Haim, 2017; Khakh, 2015). Intraregional differences in proteins in association with brain region was present in the somatosensory cortex (Houades et al., 2008). The research on astrocyte morphology is an exciting avenue of research, and more investigation should be done on the morphology of cortical astrocytes.

Aim 1 DREADD Specificity to Astrocytes

We found that hM3Dq-mCherry and s100β labeled different compartments of the astrocyte making a straightforward co-localized measurement challenging. To get around this issue, we measured how much of the s100β immunolabeled volume that hM3Dq-mCherry expression occupied. Not surprisingly, the injection site in the LOFC had a significantly higher percentage of the hM3Dq-mCherry occupying the s100β volume than in the MOFC, where no viral mediated gene transduction occurred (Figure 9A). This suggests that the hM3Dq-mCherry expression was localized to the injection region and had low spread. This is important for future studies using this virus as a titre of 110.4 nl seems appropriate for region selective effects. Secondly, this confirms

that quantifying the volume of S100β labeled astrocytes occupied by hM3Dq-mCherry is an effective way to assess co-localization. Virtually no colocalization occurred in the region where there was no viral-mediated transduction.

Treatment with DCZ did not influence hM3Dq-mCherry co-localization with s100β labeled cells. Astrocytes in the LOFC take up similar amounts of pixel volume when treated with DCZ compared to controls (Figure 9C). Furthermore, activation of hM3Dq-mCherry did not increase the number of astrocytes or the pixel volume of astrocytes in the LOFC (Figures 9C, 10A). Given that increase astrocyte proliferation and hypertrophy are associated with astrogliosis, an acute activation of astrocytes was not expected to change astrocyte size or number in this proof-of-concept experiment. Surprisingly, administration of DCZ increased the number of astrocytes expressing s100β in the MOFC (Figure 10B). This effect was not due to hM3Dq activation in astrocytes, as this region was not transduced by the virus. This effect could potentially be an off-target effect of DCZ unmasking an effect at endogenous receptors specific to the MOFC. However, it is likely a spurious effect due to the high variability when automating masked astrocyte counting.

Aim 2 Activating hM3Dq-mCherry in Astrocytes Does Not Alter cFos Expression in Neurons

The hypothesis for aim 2 was that acutely activating hM3Dq-mCherry with DCZ would be sufficient in increasing neuronal cFos. Combined with confocal imaging, IMARIS 3D analysis was an effective way of identifying the colocalization of cFos with the neuronal marker, NeuN. We noted that there was no overlap between NeuN and the hM3Dq-mCherry immunolabels, suggesting that the DREADD is not being targeted to neurons. This was also evident through a lack of cFos activation in neurons with administration of DCZ.

We hypothesized that activation of hM3Dq-mCherry in astrocytes would indirectly influence neuronal activation as astrocytes can release glutamate via exocytotic mechanisms as well as through the cystine-glutamate transporter. However, this did not occur as there was no difference in cFos expression in the LOFC of DCZ and VEH treated mice, nor was there a difference in cFos expression between the LOFC and MOFC (Figure 12 A, B). This could be due to several reasons. First, it is possible that the dose of DCZ used in this study was ineffective. Only one study so far has used DCZ in mice, there needs to be more replication studies. Secondly, it is possible that the timescale we used (perfusing 60 min after DCZ or VEH injection) was not long enough to observe neuronal activity from acute astrocyte activation. Thirdly, it is likely that acute activation of astrocytes is not sufficient to induce neuronal activation such that an early intermediate gene would be expressed.

Other researchers have measured the effects of DCZ on cFos in neurons expressing excitatory DREADDs. Here, Nentwig and colleagues, in a rat model, examined the effectiveness of systemic of a similar dose DCZ for DREADD administration within slices and assessed the electrophysiology and behavioural outputs (2021). Instead of activating astrocytes they used the excitatory DREADD, AAV8-hSyn-hMDq-mCherry virus to excite neurons. They were able to increase neuronal activation of neurons and induce cFos activity, opposed to aim 2's design of activating neurons indirectly. Nentwig and team were also able to inhibit behaviour amygdala dependent activity with an inhibitory DREADD activated by DCZ, at a higher concentration of 0.1 mg/kg (i.p.) compared to our dose of 1 μg/kg (i.p.) (Nentwig et al., 2021).

Since we did not observe indirect neuronal activity from acute astrocyte activation, we were unable to verify if the hM3Dq expression in astrocytes was functional. Future experiments

should assess this measuring calcium activation in LOFC astrocytes after application of DCZ. This would be a more direct measure of the functionality of DREADD expression in the LOFC.

The advantages of IMARIS compared to other visualization methods like ImageJ are now discussed. Some studies use ImageJ to quantify cFos cells (Nentwig et al., 2022). Here, the advantages of IMARIS outweighs what ImageJ offers. Colocalization of reporters within cells occurs in 3D, and thus should be addressed as a 3D image. The output of IMARIS is much more robust than what is offered by the spot function of ImageJ. Instead of using cell count, which IMARIS automatically does, it quantifies volumetric parameters. This is especially desirable if triple labelling is used, and analysis of multiple channels is to be quantified. Thus, the IMARIS protocol is offered for future analyses.

Overview for Future Directions

This thesis's primary research question was whether we could develop an acute astrocyte activation model using chemogenetics. Once validated, this method could then be used to determine if chronic astrocyte activation can induce reactive astrogliosis or neuroinflammation. The first aim determined that the AAV2/8-gfaABC1D-hM3Dq-mCherry virus transduces astrocytes to express hM3Dq-mCherry. It remains unknown if the excitatory DREADD virus is transfecting other glial cell types in the LOFC. Confirmation specificity experiments need to be done before pursuing further research avenues.

Reactive Astrogliosis

The change in astrocytes' physiology and phenotype is on a continuous timescale ranging from acute to chronic activation. On the greater extreme, chronic activation leads to

pathophysiological astrocytes. Astrocytes become "reactive" in response to physiological conditions in the CNS, such as after acute injuries, like a stroke or trauma, or more progressive conditions, like tumours and epilepsy (Ben Haim, 2015; Escartin, 2021; Sofronview, 2015). Astrocytes taking on this reactive phenotype are evolutionarily conserved across species (Ben Haim et al., 2015). There is a need for these terms, like "reactive," to be clearly defined. In a recent review paper, researchers described reactive astrogliosis as the response to pathology, in which astrocytes will modify their transcriptional regulation and undergo biochemical, morphological, metabolic, and physiological remodelling, that depending on the circumstance will result in a gain or loss of function (Escartin et al., 2021).

Previously, reactive astrocytes were categorized by increased quantity and modification of morphology, such as branching and ramification. This increase in astrocyte quantification is accompanied by the release of growth factors, cytokines, neurotrophic factors, and other substances (Argente-Arizón et al., 2015). Moreover, numerous molecular changes occur from astrogliosis. One is the high expression of aquaporins, specifically AQP1 and AQP9, that would be considered abnormal if found in healthy tissues (Argente-Arizón et al., 2015). There has been a large amount of research done on reactive astrocytes. This field is increasing in popularity and producing impressive results, but the findings are not unanimous. There is much conflict on what qualifies an astrocyte to be reactive, and it has changed dramatically over the last few decades.

GFAP as a Marker of Reactive Astrocytes

The molecular markers used in the acute model of astrogliosis can be used in a model of reactive astrocytes; however, there are limitations. GFAP alone, used to be the gold standard

marker to label reactive astrocytes (Escartin et al., 2021). This thesis has highlighted the limitations of using GFAP as a solitary marker for astrocytes. The brain region of astrocyte expression plays a significant role in visualizing physiological and pathological astrocytes. There is a common misconception that reactive astrocytes upregulate the amount of GFAP expressed, but this is problematic for a few reasons. GFAP seems promising as marker for reactivity, as it is present in a diverse range of CNS disorders; has an early response to injury; and is sensitive even when there is overt neuronal death (Ben Haim, 2015; Griemsmann, 2015). While this gold standard maker would be beneficial in labeling in high expressing GFAP regions like the cerebellum, it would be limited at labelling in lower GFAP expressing regions like the cortex, thalamus, and striatum (Kriegstein et al., 2009).

The cortex even presents greater restrictions of GFAP labeling as researchers found as GFAP was not upregulated in a model of repeated trauma, (Escartin et al., 2019). Increased GFAP expression has also been found in physiological homeostatic responses such as exposure to enriched environments and physical activity, and it fluctuates with the natural circadian rhythm (Gerics, 2006; Rodríguez, 2013). Therefore, a common misconception is that increased GFAP cells are caused by proliferation and local recruitment of astrocytes instead of the caveats presented above.

Future Aims: Chronic Model of Cortical Reactive Astrogliosis

The field of reactive astrocytes now generally pushes for the use of a proliferation marker (Ki67) with GFAP and another ubiquitous astrocyte marker like ALDH1L1 (Serrano-Pozo, 2013; Escartin, 2021). I piloted this triple-marker method, and unpublished preliminary data revealed a few concerns that required a new approach to visualize astrocytes. While this tandem staining method may work in regions like the hippocampus, it was problematic in the cortex, particularly

in the LOFC, due to the lack of expression of GFAP in mice. Although GFAP appears to label LOFC astrocytes in rats (Lau et al., 2021). Therefore, s100β was used instead of GFAP. The Borgland lab also piloted using ALDH1L1 CRE-ERT2 transgenic mice but had issues with conditional expression of Cre-recombinase, resulting in weak expression of ALDH1L1 in astrocytes.

A chronic model of astrocyte activation that could produce a reactive astrocyte state or astrogliosis benefits understanding how inflammation can promote neuropathophysiological disorders. A chronic model of astrogliosis could probe phenotype changes and behavioural modifications. This thesis sets up a method for the chronic activation model of reactive astrocytes. The model allows visualization of hM3Dq-mCherry expression in astrocytes. While several measures of astrocyte reactivity were mentioned above, the proposed chronic model will focus on morphology and impacting neuronal activity. The IMARIS method for two-channel imaging of s100β and hM3Dq-mCherry, should be used to assess potential changes in astrocytes morphology, indicative of reactivity after chronic hM3Dq activation. A potential chronic regimen of DCZ for ≥14 days at a dose of 10 μg/kg s.q. could identify reactive astrocytes.

DCZ is a relatively new DREADD agonist, and there are few studies on the chronic validity of the drug. However, there has been recent success in macaques with oral administration (Oyama et al., 2022). Repeated doses of DCZ activated an inhibitory DREADD and impaired working memory (Oyama et al., 2022). Other researchers have tested the effects of chronic activation of α cell Gq signaling on glucose homeostasis by having mice receiving daily injections of DCZ (10 μg/kg i.p.) for seven days (Liu et al., 2021).

A chronic model to create reactive astrocytes offers novelty on several scales. To date, no studies have been published using DCZ with hM3Dq-mCherry to activate astrocytes and alter

physiological responses. The method of this thesis is an alternative to the traditional use of controversial CNO and problematic GFAP. The chronic model can assess neuronal activity with the same immunohistochemical method used in aim 2 of the acute model. One dose of DCZ is not sufficient to indirectly change neuronal activity. Chronic DREADD exposure can cause biochemical, morphological, metabolic, and physiological changes to astrocytes, altering neighbouring neuronal activity and thus increasing cFos (Liu et al., 2021). Previous researchers have found that chronic excitatory DREADD activation in astrocytes increased cFos expression in the ventromedial hypothalamus (Liu et al., 2021). Since there needs to be a way to assess whether DCZ is causing physiological changes in astrocytes, researchers should study acute astrocyte activation *in-vitro*. If functional validity is strengthened with calcium imaging, the chronic model offers several avenues to research how astrocytes manipulate neuronal activity and could be examined on a molecular level.

CONCLUSION

Overall, this thesis offers a novel chemogenetic approach for visualizing and manipulating astrocytes in the OFC. The hypothesis supports that the gfaABC1D-promoter virus can transfect astrocytes in the LOFC. This thesis is a foundational acute model with suggestions of future directions to strengthen its validity. Lastly, this thesis offers a chronic model to study reactive astrocytes to better understand how inflammation in disorders like addiction and obesity, affects decision making behaviour.

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