Glutamatergic regulation of adult goldfish radial glial cells via group III metabotropic glutamate receptors

by

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Abstract

Aromatase is an enzyme that converts androgens to estrogens. In teleosts, brain aromatase, also known as aromatase B (*cy19a1b*), is only expressed in radial glial cells (RGCs). This is in contrast to aromatase A, which is expressed in gonads. Estrogens such as estradiol (E2) modulate neurogenesis in the adult teleost brain. Recent studies show that E2 also differentially regulates aromatase B expression in goldfish RGCs. As a result, teleost RGCs are suggested to be involved in regulating neurogenesis. In addition, aromatase B expression in goldfish RGC is under the control of dopamine suggesting that neurons and neurotransmitters can regulate RGC function. Interestingly, goldfish RGC transcriptome data shows the expression of one group of metabotropic glutamate receptors (mGluRs), group III mGluRs, which suggests that glutamate may affect RGC function. In this thesis, I present my findings regarding potential glutamatergic regulation of RGCs. Firstly, I investigated the distribution of glutamatergic synaptic vesicles and RGCs in the female goldfish forebrain. Double-staining immunohistochemistry shows that vesicular glutamate transporter (vGLUT) 1/2-labelled glutamatergic synaptic vesicles are in close anatomical proximity to aromatase B-labelled RGCs, which suggests potential regulation of RGCs by glutamate. Glutamatergic regulation of *cyp19a1b*, cyclin D1 (*ccnd1*), cyclin A2 (*ccna2*), mGluR6b (*grm6b*), mGluR7 (*grm7*), and mGluR8b (*grm8b*) expression in cultured adult female goldfish RGCs was also examined. Results from pharmacological manipulations and qPCR data analysis show that selective activation of group III mGluRs decreased *cyp19a1b*, *ccnd1*, and *ccna2* mRNA via inhibition of cAMP/PKA signalling. Furthermore, *grm7* mRNA is positively regulated by cAMP-dependent signalling. The glutamate analog L-glutamic acid decreased *cyp19a1b* mRNA and increased *ccnd1* and *grm6b* mRNA in a dose-dependent manner. This suggests that *ccnd1* and *grm6b* expression may be regulated by glutamate receptors other than group III mGluRs, for

example, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are expressed in cultured goldfish RGCs. It was found that E2 upregulated *cyp19a1b*, *ccnd1* and *grm7* mRNA. However, selective activation of group III mGluRs decreases the stimulatory effect of E2 on *ccnd1* expression. My findings show that glutamate finely regulates RGC neurogenic and steroidogenic genes, which may implicate glutamate in the regulation of RGC differentiation, RGC proliferation, and neurogenesis in surrounding cells.

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List of Abbreviations

5-HT	serotonin
AMN082	N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
ATT	aspartate aminotransferase
AroB	aromatase B
BrdU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CDK	cyclin dependant kinase
CNS	central nervous system
CPPG	(*RS*)-α-cyclopropyl-4-phosphonophenylglycine
CRE	cAMP response element
CREB	cAMP response element binding protein
$CREM_{\tau}$	cAMP responsive element modulator transcriptional activator
CS	citrate synthase
CSF	cerebrospinal fluid
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
D1R	dopamine 1 receptor
DAPI	4',6-diamidino-2-phenylindole
E2	estradiol
ERE	estrogen response element
GDH	glutamate dehydrogenase
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase

IDH	isocitrate dehydrogenase
KID	kinase-inducible domain
L-AP4	L-2-amino-4-phosphonobutyric acid
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
ME	malic enzyme
mGluR	metabotropic glutamate receptor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
P2RX7	P2X purinoreceptor 7
p-CREB	phosphorylated CREB
PAG	phosphate activated glutaminase
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKA_C	catalytic protein kinase A subunit
PKA_R	regulatory protein kinase A subunit
qPCR	quantitative real-time PCR
RGC	radial glial cell
SEM	standard error of the mean
Sp1	Specificity protein 1
vGLUT	vesicular glutamate transporter
Vv	area ventralis telencephali pars ventralis

Chapter 1: General Introduction

1.1. Thesis Outline and Rationale

The goal of this thesis is to investigate the role of glutamate in regulating goldfish radial glial cells (RGCs) *in vitro*. In teleosts, RGCs are involved in organization of the central nervous system, act as stem-like neural progenitor cells, and are the only cells in the brain that express aromatase B (*cyp19a1b*), an estrogen synthesis enzyme. Estrogens such as estradiol (E2) play a significant role in regulating proliferation and differentiation in the adult teleost brain and positively regulate aromatase B expression in teleost RGCs. Therefore, RGCs are implicated in the regulation of neurogenesis. Recent studies in cultured goldfish RGCs have shown that dopamine can positively regulate *cyp19a1b* expression via cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/cAMP response element binding protein (CREB) signalling suggesting that input from neuronal cells can regulate cultured goldfish RGC steroidogenic and neurogenic function. In addition, dopamine differentially regulates estradiol-mediated changes in *cyp19a1b* expression. I have surveyed the transcriptomic data of goldfish RGCs provided by Da Fonte et al. (2017) and found that G protein-coupled and ionotropic glutamate receptors are expressed, which suggests that glutamate is a potential regulator of RGCs. Specifically, one family of G protein-coupled glutamate receptors are expressed, the group III metabotropic glutamate receptors (mGluRs). Canonically, group III mGluRs suppress cAMP/PKA signalling and inhibit downstream effectors by inhibiting adenylate cyclase activity. In mouse neural progenitor cells, selective activation of group III mGluRs decreased the expression of cyclin D1, an important regulator of cell proliferation and differentiation, via inhibition of cAMP-dependent signalling (Nakamichi et al., 2008). Furthermore, proliferation and differentiation of mouse neural progenitor cells were suppressed suggesting that glutamate may regulate neurogenic functions via group III

mGluRs. Cyclins such as cyclin D1 (*ccnd1*) and cyclin A2 (*ccna2*) are expressed in the goldfish RGC transcriptome (Da Fonte et al., 2017). Additionally, the transcription of both cyclins is regulated by cAMP-dependent CREB suggesting that group III mGluRs may modulate cyclin D1 and cyclin A2 expression (Mayr and Montminy, 2001). No study to this point has looked at how glutamate controls vertebrate RGCs via group III mGluRs. Given the involvement of group III mGluRs in negatively regulating mammalian neural progenitor function via PKA signalling, newly-acquired transcriptomic data, and current knowledge of goldfish RGC signal transduction mechanisms, my null hypothesis is that glutamate does not regulate RGCs via group III mGluRs.

To set the stage for testing of the proposed hypothesis, I provide an overview of RGC functions and discuss what is known about neuronal-glial interactions. Furthermore, I review literature pertaining to glutamate as a neurotransmitter. Topics covered include the relationship between neurons and glia with regards to glutamate synthesis and removal, glutamate receptor pharmacology, and the regulation of RGC-like functions such as proliferation and differentiation by group III mGluRs. In the second chapter of the thesis, I provide data from anatomical, pharmacological, and gene expression analysis studies indicating that group III mGluRs are functional in female goldfish RGCs, regulating *cyp19a1b*, *ccnd1*, and *ccna2* mRNA.

1.2. Radial Glial Cells

Radial glial cells are a type of neuroglial cell found in the developing central nervous system (CNS) of all vertebrates. The RGC precursors arise from neuroepithelial cells located along the ventricular and subventricular zones of cerebral cortex (Rakic, 2003). These radial neuroepithelial cells act as neural stem cells thus allowing the expansion of neurons and glia across the developing

CNS (Rakic, 1971). Following the completion of the early stages of neurogenesis, radial neuroepithelial cells transition to RGCs (Barry et al., 2014). Morphologically, RGCs are characterized by long processes protruding from a periventricular cell body and apical-basal polarity. Within the ventricular zone of mammals and teleosts, RGCs proliferate and their processes extend through the subventricular zone and the cortical plate towards the pial surface (Stevenson and Yoon, 1982; Barry et al., 2014). RGCs facilitate CNS development via their extended processes, which act as scaffolds for newborn neurons to migrate along (Marin and Rubenstein, 2003; Zupanc et al., 2012). Further into development, asymmetric cell division allows for one daughter cell to remain a self-renewing RGC while the other daughter cell can act as a neural progenitor cell and differentiate into neurons, astrocytes, and oligodendrocytes. Towards the end of embryonic CNS development, RGCs preferentially undergo asymmetric cell division (Voight, 1989; Tamamaki et al., 2001; Radkovits et al., 2009).

In postnatal mammals, RGC populations are restricted to the anterior subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus of the hippocampus, which are the two neurogenic regions of the adult mammalian brain (Kornack and Rakic, 1999; Pencea et al., 2001). Limited neurogenesis and neuroregeneration in adult mammals are suggested to be a result of the transient nature of RGCs and the lack of neurogenic sites (Brunne et al, 2010). In contrast to mammals, teleosts undergo extensive adult neurogenesis and neuroregeneration as a result of numerous neurogenic zones identified in the brain (Grandel et al., 2006; Kaslin et al., 2008). Reportedly, the regions with the most neurogenic potential in teleosts are along the ventricles within the telencephalon and diencephalon (Chapouton et al., 2007; Zupanc and Sirbulescu, 2011). Moreover, RGCs persist through adulthood in teleosts, which is suggested to contribute to their high neurogenic capacity (Zupanc and Clint, 2003; Strobl-Mazzulla et al., 2010).

In the forebrain of adult pejerrey and zebrafish, RGCs along the periventricular zone have significant bromodeoxyuridine (BrdU) immunoreactivity, which indicates proliferative activity (Pellegrini et al., 2007; Strobl-Mazzulla et al., 2010). Overall, these findings suggest that teleost RGCs maintain their proliferative capacity past embryogenesis and into adulthood. Interestingly, adult goldfish RGCs are able to grow and proliferate *in vitro* (Xing et al., 2015).

Besides self-renewal, newborn RGCs are reported to migrate away from the proliferative zone along radial processes where some differentiate into neurons (Zupanc and Horshke, 1995, Adolf et al., 2006; Zupanc et al., 2005; Grandel et al., 2006; Pellegrini et al., 2007). RGCs within the ventricular zone of adult zebrafish express C-X-C chemokine receptor type 4 and C-X-C motif chemokine 12, a chemokine-receptor tandem critical for neuronal migration and neurogenesis that are typically found in progenitor cells (Bajetto et al., 1999; David et al., 2002; Diotel et al., 2010a). Currently, the breadth of neurons that arise from migrating RGCs in adult teleosts are unclear. However, some studies suggest that newborn glutamic acid decarboxylase-positive GABAergic, tyrosine hydroxylase-positive dopaminergic, and serotonin (5-HT)-positive serotonergic neurons present in the telencephalon and the paraventricular region of the hypothalamus may arise from RGCs that migrated away from proliferative zones such as the anterior subpallium (Adolf et al., 2006; Pérez et al., 2013). Therefore, in addition to being able self-renew, RGCs appear to retain their progenitor cell function in adult teleosts too.

1.3. Steroidogenesis in Radial Glial Cells

In vertebrates, certain cell types within the brain are capable of synthesizing steroids from local or peripheral precursors. The abundant expression of the estrogen receptors throughout the

brain of all vertebrates suggests that estrogens play a significant role in regulating neural functions and development (Rao and Kölsch, 2003; Diotel et al., 2011; Ciu et al., 2013). Estrogens such as estrone (E1) and E2 are exclusively synthesized from androgens by aromatase (Callard et al., 1981; Santen et al., 2009). In mammals such as rodents and humans, the aromatase gene *cyp19* is expressed in the gonads, adipose tissue, and brain (Sanghera et al., 1991; Bulen and Simpson, 1994; Bulen et al., 1994; Simpson et al., 1994). As a result of a gene duplication event, teleosts have two forms of *cyp19* - *cyp19a1a*, which encodes for aromatase A and is expressed mainly in the gonads, and *cyp19a1b*, which encodes for aromatase B and is expressed mainly in the brain (Tchoudakova and Callard, 1998; Chiang et al., 2001). Aromatase expression in the brain of adult mammals is localized to neurons and reactive astrocytes (Sanghera et al., 1991; Balthazart and Ball, 1998). Interestingly, *cyp19a1b* expression in teleosts is exclusively found in RGCs (Forlano et al., 2001; Diotel et al., 2016). As a result, RGCs are the only source of locally-synthesized estrogens in the teleost brain.

The functional relevance of RGC-specific *cyp19a1b* expression is still unclear. As outlined earlier, adult zebrafish RGCs can undergo extensive proliferation and act as neuronal precursors, which suggests that aromatase B and estrogens potentially regulate RGC proliferation and neurogenesis (Pellegrini et al., 2007). Female rodents in estrus show a significant increase in cell proliferation within the subgranular zone of the hippocampus compared to female rodents in diestrus (Tanapat et al., 1999; Rummel et al., 2010). Furthermore, hippocampal neurogenesis is reduced in ovariectomized female rodents suggesting that peripheral E2 that can freely cross the blood-brain barrier is more significantly involved in regulating adult mammalian neurogenesis than neuronally-synthesized E2 (Tanapat et al., 1999; Bowers et al., 2010). Therefore, the functional link between brain aromatase and neurogenesis in mammals remains inconclusive. In

adult mammals, E2 also has neuroprotective properties and is thought to reduce neuronal damage by impeding apoptosis (Garcia-Segura, 2008). In rats, inhibition of phosphatidylinositol-3-kinase (PI3K)/ and Akt signalling with LY294002 suppresses the neuroprotective effect of E2 on dopaminergic and retinal neurons (Quesada et al., 2008; De Nicola et al., 2012). PI3K/Akt signalling activates downstream signalling proteins that act on apoptotic genes and proteins via phosphorylation to protect neurons from damage (Zhu et al., 2002; Hetman and Gozdz, 2004; Marino et al., 2006; Parcellier et al., 2008). Aromatase expression in the mammalian brain is primarily found in reactive astrocytes after injury supporting the idea that aromatase has a functional link to its region of expression (Garcia-Segura et al., 1999).

Some effects of estrogens on adult teleost RGC proliferation and neurogenesis have been documented but this certainly requires more research. In adult zebrafish, pharmacological inhibition of aromatase B and nuclear estrogen receptors increased the immunoreactivity of proliferative cell nuclear antigen, a marker for cell proliferation, in the junction between the olfactory bulbs and the telencephalon, the telencephalon, and the mediobasal hypothalamus, which are considered highly proliferative zones in teleosts. In contrast, E2 reduced proliferative activity in the same brain regions (Diotel et al., 2013). E2-induced decreases in proliferative activity in neurogenic zones such as the telencephalon and hypothalamus were also observed in another study performed on adult female zebrafish (Makantasi and Dermon, 2014). Both of these studies were in contrast to observations in mammalian models where estrogen promoted proliferation in neurogenic zones. Interestingly, the proximal promoter of *cyp19a1b* has an estrogen response element (ERE) suggesting that E2 can regulate its expression (Menuet et al., 2005). In female goldfish RGC cultures, low concentrations of E2 increase *cyp19a1b* expression supporting that RGCs have an auto-regulatory loop mechanism to control steroidogenesis and potentially

proliferation (Xing et al., 2016). Studies examining the preoptic region and hypothalamus of teleosts show that aromatase-expressing RGCs are distributed closely to estrogen receptors suggesting that locally-synthesized estradiol can affect adjacent cells thus further establishing the link between the steroidogenic ability of RGCs and neurogenesis (Menuet et al., 2002; Menuet et al., 2003; Pellegrini et al., 2005).

1.4. Neuronal Regulation of Radial Glial Cell Function by Dopamine and Secretoneurin

Considering the involvement of RGCs in neurogenesis and neurosteroidogenesis, regulation is necessary to maintain homeostasis of estrogens and cell populations thus ensuring optimal brain function. In the CNS, presynaptic neurons, postsynaptic neurons, and glia form a tripartite system in which they can each affect the other's function through the synaptic space by releasing neurotransmitters, neuropeptides, and hormones (Araque et al., 1999; Farhy-Tselnicker; 2018).

In mammalian models, astrocytes form a tripartite synapse with neurons. However, teleosts lack astrocytes. In teleosts, RGCs are thought to occupy the same niche as astrocytes in mammals considering they express similar genes exclusive to astrocytes (Verkhratsky and Nedergaard, 2016; Da Fonte et al., 2017). Recent studies suggest that RGCs are regulated by neurotransmitters and neuropeptides. In female goldfish RGC culture, selective activation of dopamine 1 receptor (D1R) increases *cyp19a1b* mRNA level via PKA signalling (Xing et al., 2016). In addition, *in vivo* immunofluorescence studies showed that catecholaminergic neurons were in close anatomical proximity to RGCs along the ventricular surface of the goldfish telencephalon (Xing et al., 2015). Serotonergic neurons were also shown to be in close proximity with RGCs in the paraventricular organ of zebrafish and *in vivo* inhibition of 5-HT synthesis reduced RGC proliferation (Pérez et

al., 2013). These findings suggest that RGC function such as steroidogenesis, neurogenesis, and metabolism are regulated by neurotransmitters from proximal neurons.

Neurons displaying immunoreactivity for the neuropeptide secretoneurin-A had a close anatomical relationship with radial glial in the periventricular preoptic nucleus of goldfish and zebrafish, Furthermore, intracerebroventricular injection of secretoneurin-A decreased *cyp19a1b* mRNA levels in the telencephalon and hypothalamus suggesting that RGC neurosteroidogenic function can be regulated by neuropeptides (Da Fonte et al., 2018). The vertebrate brain contains a number of neurotransmitters beyond dopamine and 5-HT that may affect RGC function. Transcriptomic data shows that goldfish RGCs express glutamate receptors, which suggests that they may be controlled by the neurotransmitter glutamate (Da Fonte et al., 2017).

1.5. Glutamate as a Neurotransmitter in the Brain

Glutamate is the most abundant amino acid in the brain and is the major excitatory neurotransmitter in the vertebrate central nervous system (Zhou and Danbolt, 2014). In addition to driving excitatory signal transmission, glutamate plays a critical role in regulating plasticity and long-term potentiation of synapses and neural development (Bliss and Collinridge, 1993; Nakanishi, 1994; Nakanishi et al., 1998, Nedergaard et al., 2002; Nicoll and Roche, 2013). Most neurotransmitters are only synthesized and found in neurons and certain endocrine cells. In contrast, glutamate is generated in all cell types within a biological system (Hackett and Ueda, 2015).

As an amino acid, glutamate can also function as a secondary fuel reserve in glial cells and neurons. Oxidative deamination of glutamate to oxaloacetate by glutamate dehydrogenase (GDH)

generates 12 ATP per molecule of glutamate (Balazs and Haslam, 1965; Hawkins, 2009, McKenna et al., 2016; Fendt and Verstreken, 2017). In rodents, it has been reported that the brain under low glucose conditions will increase glutamate output and metabolism for use as fuel (Miller et al., 1975; Rink et al., 2011; Torres et al., 2013). The use of glutamate as an additional source of energy is particularly important in the brain considering that neurons have intensive energy demands (Mergenthaler et al., 2013; Falkowska et al., 2015). Overall, glutamate is not only the most common neurotransmitter in the brain, its multifunctionality makes it one of the most important.

Although glutamate is synthesized in all cell types, peripheral glutamate is unable to reach the brain through the blood-brain barrier and can only access the cerebral extracellular fluid via limited regions of fenestrated capillaries found in circumventricular organs (Price et al., 1981; Viña et al., 1997; Hawkins and Viña, 2016). Restricted entry of peripheral glutamate is likely to prevent excitotoxicity in the brain. Glutamate can bind N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors leading to overexcitation of neuronal cells and an excessive influx of Ca^{2+}. Consequently, abnormally elevated levels of intracellular Ca^{2+} lead to the activation of nucleases, proteases, mitochondrial apoptotic pathways, and nitric oxide synthase, which eventually cause irreparable cell damage and neuronal death (Manev et al., 1989; Rothstein et al., 1996; Castillo and Babson, 1998; Jia et al., 2015). As a result, a localized mechanism to both synthesize and clear glutamate is necessary to facilitate normal excitatory synaptic transmission, provide metabolic fuel, and protect neurons from excitotoxicity by enforcing homeostasis.

The maintenance of glutamate homeostasis in the brain is crucially dependent on the exchange of metabolites between glutamatergic neurons and neighboring astroglial cells at the synapse (**Figure 1.1 and 1.2**). This exchange of metabolites ultimately generates the glutamate

necessary to drive proper brain function while also ensuring that excess glutamate is cleared and recycled. In glutamatergic neurons**,** glutamate can be synthesized *de novo* from citric acid cycle intermediates. Firstly, glucose is taken up into the cytosol and converted to pyruvate through glycolysis. Pyruvate is then transported into the mitochondrial matrix where it is oxidized to acetyl-CoA by the pyruvate dehydrogenase complex (PDH). Acetyl-CoA is condensed into citrate by citrate synthase (CS) thus initiating the citric acid cycle. Citrate is converted to isocitrate by aconitase, which is then oxidized and decarboxylated by isocitrate dehydrogenase (IDH) into α-ketoglutarate. From this point, α-ketoglutarate can act as an intermediate to synthesize glutamate via aminotransferases such as aspartate aminotransferase (AAT). Dicarboxylate transporters move α-ketoglutarate from the mitochondria to the cytosol via malate exchange, where cytosolic AAT catalyzes aspartate and α-ketoglutarate into oxaloacetate and glutamate. Interestingly, AAT is also bound to synaptic vesicles, creating a specialized microdomain for synthesis and uptake of glutamate. Cytosolic malate dehydrogenase (MDH) can convert oxaloacetate to malate to continue the transport of α-ketoglutarate into the cytosol through malate exchangers (**Figure 1.1**; Palaoilogos et al., 1988; McKenna et al., 2012; Mangia et al., 2012; Schousboe et al., 2013).

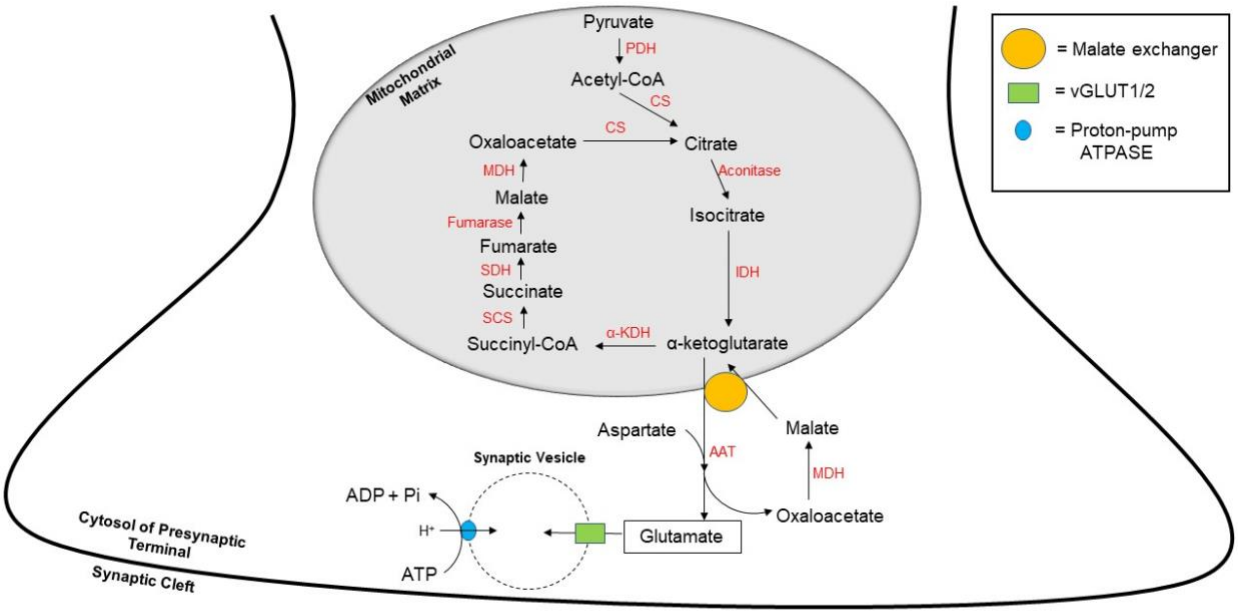

Figure 1.1. Glutamate synthesis in the presynaptic terminal of glutamatergic neurons. Abbreviations: α-KDH, α-ketoglutarate dehydrogenase; AAT, aspartate aminotransferase; CS, citrate synthase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinic dehydrogenase; vGLUT1/2, vesicular glutamate transporter 1/2.

Once in the cytosol, glutamate is packaged into synaptic vesicles via vGLUTs, specifically vGLUT1 and vGLUT2 (Morimoto et al., 2003; Herzog et al., 2006). A vesicle-bound proton-pump adenosine triphosphatase (ATPase) creates an electrochemical gradient, allowing for negatively-charged glutamate to pass through vGLUTs (Naito and Ueda, 1985; Omote et al., 2011) (**Figure 1.1**). The adenosine triphosphate (ATP) required to generate the proton gradient is locally

synthesized by a vesicle-bound glycolytic enzyme complex known as glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglycerate kinase (Ikemoto et al., 2003). At resting conditions, synaptic vesicles are enveloped by synapsin 1, a protein anchored to the cytoskeleton through F-actin. As a result, synaptic vesicles are immobilized and unable to release their contents into the synaptic cleft. Depolarization of the glutamatergic nerve terminal causes an influx of Ca^{2+}, which bind and activate calmodulins. In turn, Ca^{2+}/calmodulin complexes bind calmodulin-dependent protein kinase II (CaMKII), which phosphorylates synapsin 1, lowering its affinity for synaptic vesicles. Synaptic vesicles become untethered and are free to move and release their contents into the synaptic cleft through exocytosis (Llinás et al., 1985; Greengard et al., 1993; Hackett et al., 1990; Zhou and Danbolt, 2014).

Once glutamate is released into the synaptic cleft, it can bind metabotropic and ionotropic glutamate receptors on the surface of the postsynaptic membrane. Since glutamate cannot be degraded by any enzyme in the extracellular space it must be removed to prevent excitotoxicity. Presynaptic glutamatergic neurons and astroglial cells can re-uptake glutamate through three secondary active excitatory amino acid transporters (EAATs) (Bjørn-Yoshimoto and Underhill, 2016). In mammals, EAAT-1 and EAAT-2 are primarily expressed in astroglial cells whereas EAAT-3 is exclusively expressed by neurons (Gegelashvili and Shousboe, 1998; Danbolt, 2001). Although EAAT-3 returns glutamate directly back to glutamatergic neurons for compartmentalization, it is lowly expressed at the pre-synaptic terminal. In comparison with EAAT-1 and EAAT-2, EAAT-3 also has significantly lower density, affinity, and expression level (Shousboe, 1981; Holmseth et al., 2012). Furthermore, it has been reported that EAAT-2 is responsible for 90% of glutamate clearance in the brain (Lehre and Danbolt, 1998). Overall, high density and affinity glutamate transporters embedded in astroglial membranes suggest that

astroglia play a critical role in glutamate removal compensating for the lack of presynaptic glutamate transporters.

Astroglial uptake of extracellular glutamate in mammals has benefits beyond protection against excitotoxicity. This is particularly important considering that near the synapse, EAATs are numerous on the astroglial membrane whereas they are sparingly found on presynaptic membranes, making it difficult for glutamatergic neurons to recuperate glutamate (Chaudhry et al., 1995). Although astroglial cells have significantly greater capacity for glutamate clearance, which may appear to be a detriment to neighbouring neurons, they have mechanisms to recycle and replenish neuronal glutamate supplies (**Figure 1.2**). Through the glutamate-glutamine cycle, glutamate can be amidated to glutamine by glutamine synthetase (GS), an enzyme exclusively found in astroglia (Shousboe et al., 2013). Glutamine is expelled into the extracellular space through System N, a Na^+-glutamate symporter and H^+ antiporter, and System L, an alanine-leucine antiporter. On the presynaptic membrane, System A, a sodium-dependent, electrogenic transporter, takes up glutamine where it can be hydrolyzed by phosphate activated glutaminase (PAG) to generate glutamate and ammonia (Bröer and Brookes, 2001; Bak et al., 2006). Glutamate can then be released into the synaptic cleft or enter the citric acid cycle through aminotransferases.

Much like glutamatergic neurons, mammalian astroglial cells can synthesize glutamate from pyruvate through the citric acid cycle (**Figure 1.2**). In contrast to neurons, astroglia express pyruvate carboxylase (PC), an enzyme that can convert another pyruvate molecule into oxaloacetate, which will eventually yield glutamate after being processed through the citric acid cycle. Once again, glutamate is converted to glutamine and shuttled to a glutamatergic neuron where it can be reconstituted as glutamate (Shousboe et al., 2013). In astroglial cells, citric acid cycle intermediates must be replenished to compensate for the expenditure needed to generate

glutamate. Total oxidation of glutamate maintains stoichiometry but also acts as mechanism to remove glutamate thus avoiding excessive release. In this process, glutamate dehydrogenase converts glutamate to α-ketoglutarate and ammonia. Once in the citric acid cycle, α-ketoglutarate is eventually converted to malate, which can be decarboxylated to pyruvate by malic enzyme (ME). In turn, pyruvate can either enter the citric acid cycle allowing for intermediates to be replenished or it can be converted to lactate and released via lactate dehydrogenase (McKenna et al., 2012).

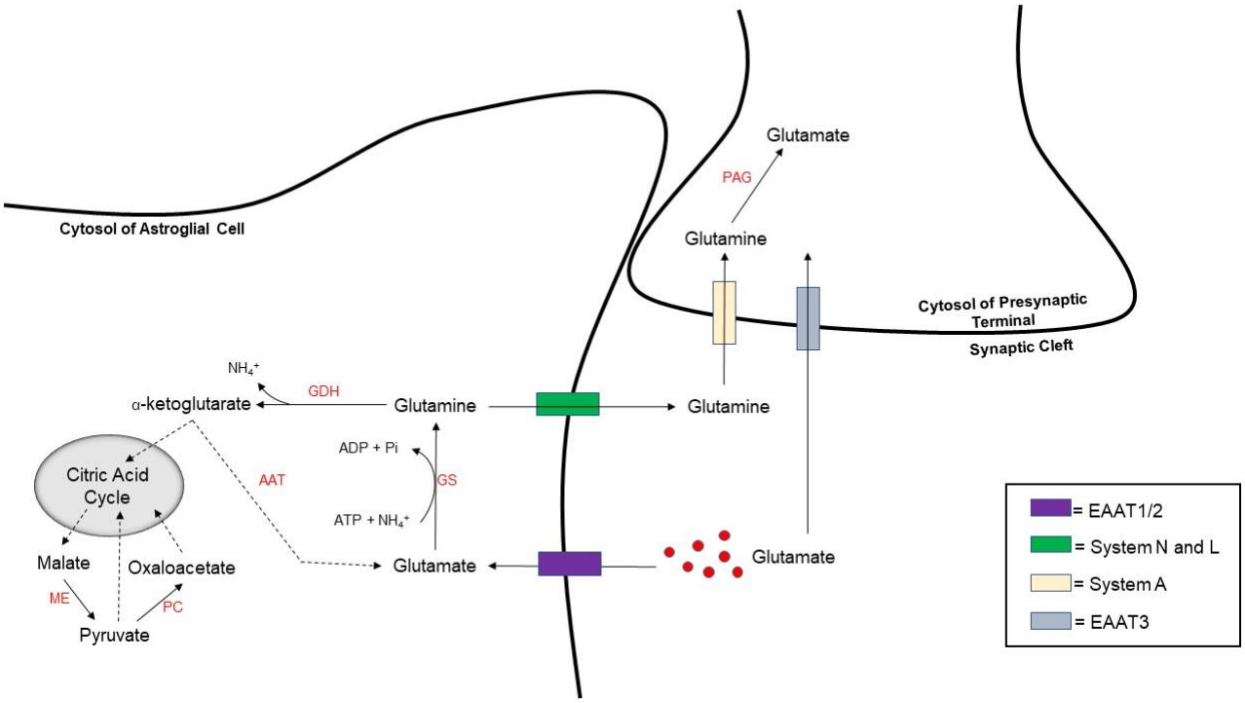

Figure 1.2. Glutamate-glutamine cycling between astroglial cells and presynaptic glutamatergic neurons. Abbreviations: AAT, aspartate aminotransferase; EAAT, excitatory amino acid transporter; GDH, glutamate dehydrogenase; GS, glutamine synthetase; ME, malic enzyme; PAG, phosphate activated glutaminase; PC, pyruvate carboxylase.

Glutamate can also be cleared from the extracellular space through the endothelial cells that form the blood-brain barrier. The EAATs on the abluminal membrane of endothelial cells can actively import glutamate into endothelial cells but cannot export it to the extracellular fluid of the brain. On the luminal surface, a bidirectional facilitative amino acid transporter shuttles glutamate in and out of the endothelial cells into the blood (Rothstein et al., 1994; Hawkins and Vina, 2016).

The symbiotic exchange of metabolites between glutamatergic neurons and astroglial cells to generate and remove glutamate highlights the codependent and compensative interactions that occur in a tripartite system. These glutamate handling mechanisms are thought to be highly conserved among all vertebrates. In elasmobranchs and teleosts, RGCs are the sole astroglial-like type cell found in the brain. Interestingly, zebrafish RGCs express several genes exclusive to astroglia such as glial fibrillary acidic protein (GFAP) and GS. In addition, they appear to perform the same homeostatic function as astrocytes, their higher vertebrate counterpart (Gumbmann and Tappel, 1962; Verkhratsky and Nedergaard, 2016; Da Fonte et al., 2017).

1.6. Glutamate Receptors and Post-Receptor Signalling

Glutamate exerts action on the CNS by activating an array of ionotropic and metabotropic receptors (Nakanishi, 1992; Kew and Kemp, 2005). Members of the ionotropic glutamate receptor family include NMDA, AMPA, and kainite receptors, which are tetrameric ligand-gated cation channels that mediate fast excitatory synaptic transmission and long-term potentiation by facilitating depolarization of the postsynaptic membrane (Malenka and Nicoll, 1999; Traynelis et al., 2010). The NMDA receptors allow the influx of Ca^{2+} and Na^{+}, depending on the electrochemical gradient, when co-activated by glutamate and glycine (Chatterton et al., 2002;

Vyklicky et al., 2014). Similarly, AMPA and kainate receptors primarily direct Na^+ and some Ca^{2+} into the postsynaptic neuron when activated by glutamate (Traynelis et al., 2010).

Members of the mGluR family are G protein-coupled receptors divided into 3 subtypes: group I, II, and III. Group I mGluRs, such as mGluR1 and mGluR5, mediate slow synaptic transmission via secondary intracellular messengers (Hollmann and Heinemann, 1994; Nakanishi et al., 1998). Stimulation of group I mGluRs activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5,-biphosphate (PIP_2). Diacylglycerol (DAG), one of the products of PIP_2 hydrolysis, activates protein kinase C (PKC), which is recruited to the membrane by intracellular calcium. Next, PKC exerts changes in gene expression by phosphorylating second messenger proteins and transcriptional activators (Willard and Koochekpour, 2013). Inositol triphosphate (IP_3) the other product of PIP_2 hydrolysis, opens IP_3-sensitive Ca^{2+} channels on the endoplasmic reticulum leading to increased intracellular Ca^{2+} levels. In turn, Ca^{2+} forms a complex with calmodulin that can subsequently bind and activate CaMKs (Tabata and Kano, 2004). Ultimately, CaMKs also phosphorylate transcriptional activators and ionotropic receptors that regulate synaptic remodelling and long-term potentiation of synapses (Anwyl, 1999; Hudmon and Schulman, 2002; Niswender and Conn, 2010).

Group II mGluRs, which include mGluR2 and mGluR3, and Group III mGluRs, which include mGluR4, mGluR6, mGluR7, and mGluR8, are coupled to $G_{i/o}$ proteins that inhibit stimulatory G-protein signalling (Mercier and Lodge, 2014; Muguruza et al., 2016). Group III mGluRs are of particular interest. I examined published transcriptomic data obtained for goldfish RGCs *in vitro* and noted that group III mGluRs are the only mGluRs expressed (Da Fonte et al., 2017). Previous studies in mammals indicate that group III mGluRs are functionally relevant to neuroprotection in other neuroglia such as astrocytes and microglia (Taylor et al., 2003; Zur Nieden and Deitmer,

2006; Zhou et al., 2006; Pinteaux et al., 2009; Loane et al., 2012). In primary cultured rat microglia, all group III mGluRs are expressed, whereas only mGluR4 is expressed in primary cultured rat cortical astrocytes (Besong et al., 2002; Taylor et al., 2003). Interestingly, mGluR4 is only expressed in reactive human astrocytes (Geurts et al., 2005). Furthermore, several group III mGluR subtypes and their paralogs are expressed in the RGC-rich forebrain and hypothalamus of teleosts. *In situ* hybridization revealed the expression of all group III mGluRs in the hypothalamic regions of the zebrafish brain. In the preoptic area, mGluR4, mGluR7, and mGluR8a are expressed while mGluR4, 6b, 8a, and 8b are expressed in the olfactory bulb. Strong mGluR7 expression was also found in the dorsal telencephalic area (Haug et al., 2012).

The activation of group III mGluRs induces the exchange of guanosine diphosphate for guanosine-5'-triphosphate at the $G_{i/o}$ protein causing its dissociation from the heterotrimeric G protein complex. In turn, $G_{i/o}$ protein inhibits adenylate cyclase, which prevents cAMP production. Four cAMP molecules are necessary to free the catalytic subunits of PKA, which can then regulate the function of downstream transcription factors, receptors, and ion channels (Taylor et al., 2005; Kim et al., 2006; Taylor et al., 2008). In essence, stimulation of group III mGluRs attenuates PKA activation hence the activity of any PKA target. One of the most well-characterized targets of PKA is CREB, a transcription factor that targets the highly conserved cAMP response element (CRE; 5'-TGACGTCA-3') found within the promoter regions of genes (Montminy et al., 1986). An extensive list of genes involved in a range of functions such as metabolism, transcription, transport, and cell cycling contain consensus CREB binding sequences (Mayr and Montminy, 2001). Active PKA catalytic subunits translocate into the nucleus and phosphorylate CREB at serine-133 in a 60-residue region referred to as the kinase-inducible domain (KID). CREB-binding protein (CBP) is recruited to phosphorylated CREB through a N-terminus region known as the KID interaction

domain (Chrivia et al., 1993). CBP binds RNA helicase A, which associates with RNA polymerase II (Nakajima et al., 1997a, Nakajima et al., 1997b). In addition, transcription factor IID interacts with the Q2 domain of CREB downstream of CRE and binds to an upstream TATA box via TATA-binding protein, laying the foundation for the transcription machinery to operate (Shaywitz and Greenberg, 1999) (**Figure 1.3**).

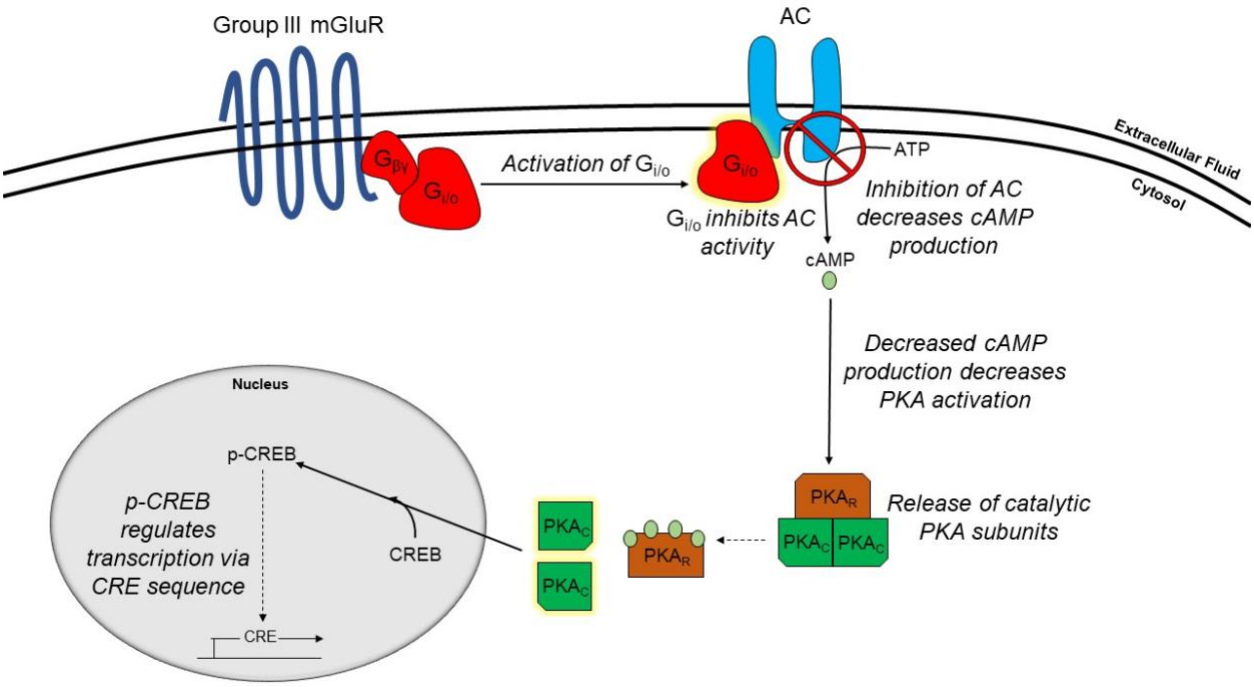

Figure 1.3. Canonical inhibition of PKA signalling via activation of group III mGluRs. Stimulation of group III mGluRs causes the dissociation of the $G_{i/o}$ protein. $G_{i/o}$ protein inhibits adenylate cyclase (AC) activity, inhibits cAMP production. Decreased cAMP production leads to decreased de-activation of regulatory PKA subunits (PKA_R) and therefore decreased catalytic PKA subunit (PKA_C) activation. In turn, phosphorylation of transcription factors, like CREB for example, is reduced and the gene transcription is downregulated.

Although CREB can be phosphorylated by kinases such as CaMKs and mitogen-activated protein kinases (MAPK), $G_{i/o}$-mediated inhibition of cAMP production via group III mGluRs overwhelmingly affects PKA, not other serine/threonine kinases (Pin and Duvoisin, 1995; Steven and Seliger, 2016). However, selective group III mGluR activation can produce effects that are not a result of decreased cAMP production. In cultured rat cerebellar granule cells, the selective activation of group III mGluRs using L-2-amino-4-phosphonobutanoate (L-AP4) improves neuronal survival and prevents apoptosis under nutrient-deficient conditions. Selective activation of group III mGluRs also increases phosphorylated extracellular signal-regulated kinase 1/2 and Akt levels, which are effectors in the MAPK and PI3K signalling cascade respectively (Graham and Burgoyne, 1994; Borodezt and D'Mello, 1998). The neuroprotective effect of the group III mGluR agonist L-AP4 on rat cerebellar granule cells was pharmacologically negated by PD98059 and UO126, which inhibit MAPK signalling, or by the drug LY294002, which inhibits PI3K signalling, suggesting that non-cAMP dependent kinases can regulate a group III mGluR-specific function (Iacovelli et al., 2002; Wortzel and Seger., 2011).

Another non-cAMP dependent regulatory mechanism involves $G_{\beta\gamma}$, a subunit linked to the $G_{i/o}$ heterotrimeric complex, which also dissociates when group III mGluRs are activated (Gilman, 1987). The $G_{\beta\gamma}$ subunit can directly inhibit voltage-gated dependent Ca^{2+} channels and activate inwardly rectifying K^{+} channels (Reuveny et al., 1994; Ikeda, 1996; De Waard et al., 1997; Dascal, 2001). In rat pyramidal neurons, selective activation of group III mGluRs with L-AP4 reduces Ca^{2+} current through voltage-gated dependent N-, R-, and Q-type Ca^{2+} channels (Stefani et al., 1998). In addition, L-AP4-induced inhibition of N-type Ca^{2+} channels is suppressed by (*RS*)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), a group III mGluR antagonist, and pertussis toxin, a G protein inhibitor, suggesting that group III mGluRs regulate voltage-gated dependent

Ca^{2+} channels via G protein coupling (Burns, 1988; Trombley and Westbrook., 1992; Guo and Ikeda, 2005; Bertaso et al., 2006). Voltage-gated dependent Ca^{2+} channels also affect CaMK-mediated release of neurotransmitters, especially glutamate, via group III mGluRs (Herrero et al., 1996; Martin et al., 2007; Niswender et al., 2008).

Having discovered that one group of mGluRs, group III mGluRs, is expressed in the goldfish RGC transcriptome, this suggests that glutamate may functionally regulate RGCs, which warrants testing of my null hypothesis. Canonically, activation of group III mGluRs regulates transcription via $G_{i/o}$-mediated inhibition of PKA signalling and cation channel activity (Anwyl, 1999). Their involvement in regulating key RGC functions such as steroidogenesis and neurogenesis remain unknown. However, *cyp19a1b* contains a CRE element at its promotor region and its expression is upregulated by dopamine-induced cAMP/PKA/CREB signalling suggesting that group III mGluRs may regulate steroidogenic function in goldfish RGCs (Kato et al, 1997; Sofi et al., 2003; Xing et al., 2016).

1.7. Regulation of Neural Progenitor Cell Function via Group III mGluRs

Currently, the understanding of mGluR regulation of radial glial cells is limited, especially regarding the potential role of group III mGluRs. López et al. (1998) showed that the mRNA and protein level of mGluR4 in two types of specialized radial glia, Bergmann and Müller glial cells, was modulated by glutamate and trans-1-amino-1,3-cyclopentanedicarboxylic acid, a group I/II mGluR agonist, *in vitro*. However, this study suggests that group I and II mGluRs regulate mGluR4 in RGCs and does not indicate whether mGluR4 affects RGC function.

In teleosts, RGCs function as endocrine cells and neural progenitor cells (Xing et al., 2014). Although there are no reports of group III mGluR-mediated regulation of radial glial cell function, there are several studies outlining group III mGluRs and their role in mammalian neural progenitor cells. In undifferentiated mouse neocortical neural progenitor cells that express mGluR4 and mGluR8, L-AP4 decreased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and BrdU incorporation, which are experimental markers for proliferation (Nakamichi et al., 2008). Application of group I and group II-specific mGluR agonists had no significant effect on proliferation. In addition, L-AP4 decreased the size of neurospheres suggesting that selective activation of group III mGluRs decreases proliferation of neural progenitor cells. Forskolin, an adenylate cyclase activator, increased MTT reduction and BrdU incorporation. Both of these effects were reversed when forskolin was applied in combination with L-AP4. Furthermore, H89, a PKA inhibitor, also significantly blocked the effect of forskolin suggesting that neural progenitor cell proliferation is regulated by PKA signalling (Nakamichi et al., 2008). The mouse neocortical neural progenitor cells express cyclin D1, a key cell cycle regulator that transitions the cell from G_1 phase to the proliferative S phase (Sherr, 1994, Nakamichi et al., 2008). Interestingly, prolonged L-AP4 exposure decreases cyclin D1 mRNA levels while forskolin increases cyclin D1 mRNA levels. The group III mGluR antagonist CPPG reversed the effect of L-AP4 suggesting that group III mGluRs may regulate proliferation by attenuating cyclin D1 expression (Nakamichi et al., 2008).

Pluripotent mouse P19 cells with similar mGluR expression patterns as undifferentiated mouse neocortical neural progenitor cells and a reporter plasmid of the cyclin D1 promoter region containing four CRE sites showed decreases in both cell cluster size and cyclinD1 promoter activity when treated with L-AP4. Once again, the inhibitory effect of L-AP4 on cyclin D1

promoter activity was reversed by CPPG. Forskolin increased promotor activity but had its effect blocked by L-AP4 and H89 suggesting that activation of group III mGluRs potentially attenuates proliferation in mouse neural progenitor cells via downregulation of cyclin D1 expression, which is likely because of decreased PKA signalling (Nakamichi et al., 2008).

Similar findings were observed in other neural progenitor cell types. In ReN human neural progenitor cells, L-AP4 decreased MTT reduction and BrdU incorporation in a CPPG-reversible manner (Vernon et al., 2011). Treatment with N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), a mGluR7-selective agonist, significantly reduced MTT reduction and BrdU incorporation while mGluR4 and mGluR8 agonists had no effect. In rat granular neural precursor cells, PHCCC, a mGluR4-specific agonist, significantly decreased BrdU incorporation. Moreover, the forskolin-induced increase in cAMP levels was reversed by PHCCC. In comparison with wild-type mice, granular neural precursor cultures from mGluR4 knockout mice treated with PHCCC did not show decreased BrdU incorporation (Canudas et al., 2004). Findings from Vernon et al. (2011) and Canudas et al. (2004) further support group III mGluR activation as a mechanism to regulate proliferation in mammalian neural progenitor cells. Furthermore, the effect of group III mGluRs on neural progenitor cell proliferation is potentially receptor subtype specific.

Besides regulating proliferation, group III mGluRs appear to affect fate determination in neural progenitor cells. In differentiating mouse neural progenitor cells, L-AP4 increased the immunoreactivity of GFAP, an astroglial marker in mammals, while also decreasing the immunoreactivity of microtubule-associated protein 2 (MAP2), a neuronal marker. In contrast, forskolin significantly decreased GFAP immunoreactivity and increased MAP2 immunoreactivity suggesting that activation of group III mGluRs preferentially induces astrocytic fate in neural progenitor cells (Nakamichi et al., 2008). Differentiated human neural progenitor cells cultured in

L-AP4 and AMN082 had decreased mRNA and protein levels of β-III tubulin, a neuronal marker. In contrast, selective activation of mGluR4 with VU0155041 increased β-III mRNA. Application of VU0155041 had no effect on the expression of the astroglial marker S100β whereas AMN082 increased S100β mRNA and protein level suggesting that activation of mGluR7 promotes astroglial fate while activation of mGluR4 may promote neuronal fate. These findings indicate that much like proliferation, fate determination in neural progenitor cells can be regulated by selective activation of specific group III mGluRs (Vernon et al., 2011).

Group III mGluRs in mammalian models appear to negatively regulate neural progenitor cell proliferation via PKA signalling. In addition, they appear to differentially affect fate determination. Currently, few studies on group III mGluR regulation of RGC proliferative and differentiative function have been conducted. However, past studies in mammalian neural progenitor cells elucidate some possible group III mGluR-mediated regulatory mechanisms to investigate such as PKA-mediated regulation of cyclins. Moreover, the expression of group III mGluRs in the goldfish RGC transcriptome suggests that glutamate may regulate RGC function. Considering the importance of PKA signalling in regulating goldfish RGC steroidogenic function as documented by Xing et al. (2015, 2016), I predict that glutamate may counteract PKA signalling via inhibitory group III mGluR-mediated signalling thus inhibiting aromatase B expression, which suppresses steroidogenesis and affects teleost neurogenesis, and inhibiting proliferation by downregulating cyclin D1 and A2 expression (**Figure 1.4**).

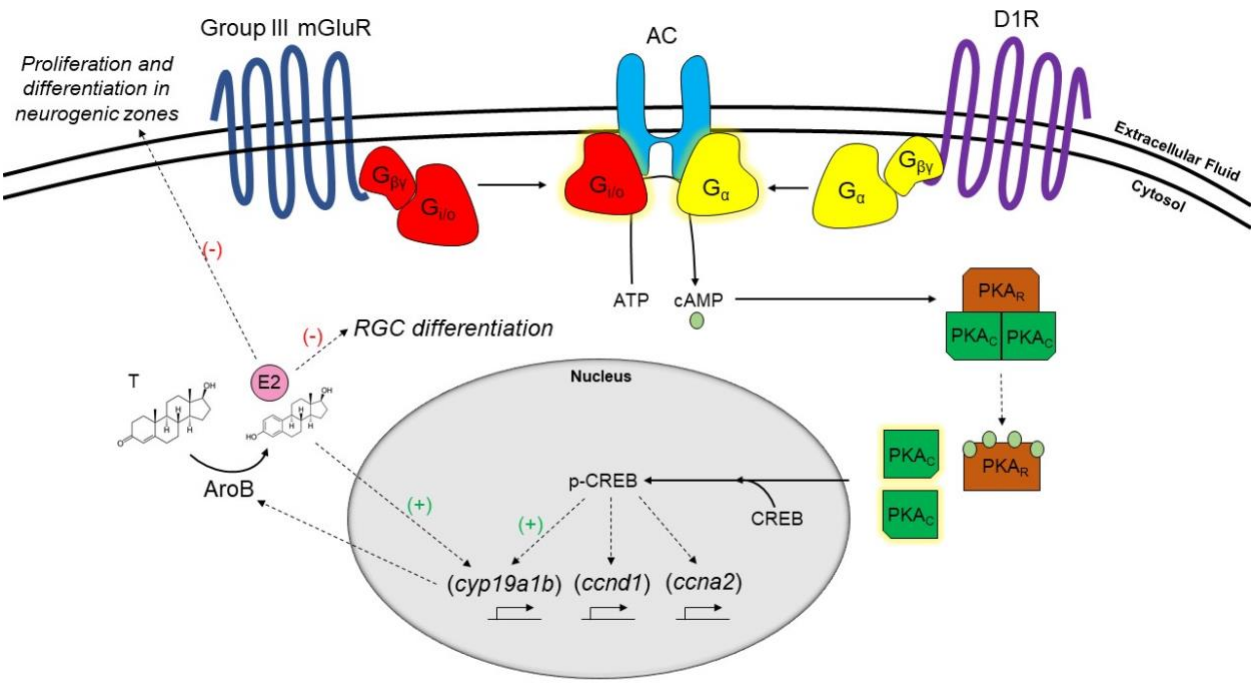

Figure 1.4. Predicted group III mGluR-mediated regulatory mechanism of D1R-induced cAMP-dependent signalling in goldfish RGCs. Activation of group III mGluRs leads to inhibition of adenylate cyclase (AC) activity, which in turn decreases cAMP/PKA signalling. Decreased cAMP/PKA signalling leads to less CREB phosphorylation, which potentially reduces *cyp19a1b*, *ccnd1*, and *ccna2* expression. Decreased *cyp19a1b* expression may result in reduced E2 synthesis and therefore decreased neurogenesis and increased RGC differentiation. Attenuation of *ccnd1* and *ccna2* expression may lead to decreased RGC proliferation but increased differentiation.

Chapter 2: Glutamatergic regulation of steroidogenic and proliferative gene expression in goldfish radial glial cells via group III metabotropic glutamate receptors

2.1. Introduction

Aromatase is an enzyme encoded by *cyp19* that converts the androgens androstenedione and testosterone respectively to E1 and E2. In mammals, aromatase is expressed in the gonads, adipose tissue, CNS neurons, and astrocytes (Simpson et al., 1994; Lephart, 1996). Teleosts exhibit a somewhat similar localization of aromatase expression as mammals; however, teleosts express two different aromatase genes because of a gene duplication event. Aromatase A, which is encoded by *cyp19a1a*, is expressed mainly in the gonad whereas aromatase B, which is encoded by *cyp19a1b*, is expressed mainly in the brain (Chiang et al., 2001; Diotel et al., 2010a). In contrast to the mammalian brain where aromatase expression can be found in both neuronal and glial cell bodies, aromatase B in the teleost brain is exclusively and strongly expressed in RGCs making them the sole source of brain-derived estrogens (Forlano et al., 2001; Xing et al., 2014; Diotel et al., 2018). In all vertebrates, RGCs contribute significantly to the development of the CNS (Zupanc et al., 2012; Barry et al., 2014). During the early stages of CNS development, elongated processes of the RGCs extend from the ventricular zone to the pial surface acting as a scaffold for newborn neurons to migrate along (Rakic, 1972). Furthermore, mammalian RGCs act as neural progenitor cells capable of differentiating into neurons, astroglia, and oligodendrocytes (Schmechel and Rakic, 1979; Radakovits et al., 2009). In mammals, the postnatal population of RGCs almost

entirely disappears and is suggested to be one of the reasons adult mammals have a limited capacity for neurogenesis and neuroregeneration (Malatesta et al., 2000; Rakic, 2003; Brunne et al., 2010). In marked contrast, the extensive adult neurogenesis and neuroregeneration exhibited by teleosts is suggested to be a result of persistent and abundant pools of RGCs located along proliferative zones of the brain (Strobl-Mazzulla et al., 2010; Kroehne et al., 2011; Diotel et al., 2013).

Teleost neurogenesis is reported to be closely linked to the ability of RGCs to synthesize E2. In adult zebrafish, E2 inhibits cell proliferation in highly neurogenic regions of the forebrain such as the olfactory bulb, telencephalon, and mediobasal hypothalamus (Diotel et al., 2013; Makantasi and Dermon, 2014). Within the periventricular layer of the forebrain, aromatase B-positive RGCs are reported to be actively proliferating (Pellegrini et al., 2007). In addition, aromatase B expression in the teleost brain disappears as RGCs differentiate into neurons, which could lead to decreased E2 synthesis, indicating that E2 may also maintain their progenitor state (Pellegrini et al., 2007; Diotel et al., 2010a, Xing et al., 2014; Coumailleau et al., 2015). These findings suggest that RGCs may affect the function of neighbouring cells in the brain through the synthesis and release of E2 while also affecting their own proliferative and progenitor capabilities.

Since RGCs appear to modulate neuronal commitment and proliferation via E2, regulation of *cyp19a1b* is critical for ensuring balanced cell production and therefore normal brain function. The presence of an ERE on the proximal promoter of *cyp19a1b* shows that E2 can increase *cyp19a1b* expression and potentially aromatase B synthesis thus creating a positive auto-regulatory loop that maintains *cyp19a1b* expression and RGC fate (Tchoudakova et al., 2001; Menuet et al., 2005; Diotel et al., 2010a). Neighbouring neurons also appear to influence RGC function through neurotransmitters. In cultured goldfish RGCs, activation of D1Rs increases *cyp19a1b* expression via cAMP/PKA/ phosphorylated CREB (p-CREB) signalling showing that dopamine regulates the

expression of steroidogenic genes implicated in neurogenesis and RGC differentiation. Furthermore, low concentrations of E2 enhance the stimulatory effect of dopamine on *cyp19a1b* expression. In contrast, high concentrations of E2 inhibit dopamine-mediated upregulation of *cyp19a1b* expression (Xing et al., 2016). The neuronally-released peptide secretoneurin-A has also been shown to differentially regulate neurogenic proteins and transcriptional networks and decrease *cyp19a1b* expression in female goldfish RGCs (Da Fonte et al., 2017, 2018).

Although the mechanisms by which RGCs regulate neurogenesis are steadily being elucidated, the involvement of other neurotransmitters and neuropeptides remains unknown. Analysis of goldfish RGC transcriptome data obtained from Da Fonte et al. (2017) revealed the expression of mGluRs suggesting that the neurotransmitter glutamate may regulate RGC function. Interestingly, goldfish RGCs appear to only express one group of mGluRs known as the group III mGluRs, which canonically inhibit cAMP/PKA/p-CREB signalling by preventing the synthesis of cAMP. The *cyp19a1b* promoter contains a CRE and it has been shown that *cyp19a1b* expression in goldfish RGCs can be upregulated by dopamine-mediated increases in CREB phosphorylation (Xing et al., 2016). Activation of group III mGluRs could counteract this mechanism through $G_{i/o}$-mediated inhibition of the cAMP-generating enzyme, adenylate cyclase, potentially leading to a decrease in *cyp19a1b* expression and, therefore, encouraging proliferation of neurons and RGC differentiation (Conn and Pin, 1997; Anwyl, 1999; Spooren et al., 2003).

In the context of neurogenesis, selective activation of group III mGluRs in fetal mouse progenitor cells decreases the expression of cyclin D1, a key regulator of cell proliferation that also contains a CRE sequence on its promoter, via inhibition of cAMP/PKA/p-CREB signalling. Furthermore, neural progenitor cell proliferation and neuronal differentiation were suppressed (Nakamichi et al., 2008). Significant decreases in mammalian neural progenitor cell types

following selective activation of group III mGluRs were also reported in other studies supporting the idea that group III mGluR activation prevents neural progenitor cell proliferation (Canudas et al., 2004; Vernon et al., 2011). Cyclin D1 and cyclin A2 are expressed in goldfish RGCs (Da Fonte et al., 2017) and are critically important to regulating cell proliferation (Blanchard, 2000; Stacey, 2003; Bendris et al., 2015; Loukil et al., 2015). Past studies have suggested that RGCs can auto-regulate proliferation through E2 but have not provided concrete evidence (Diotel et al., 2013). Analyzing cyclin expression may indicate whether well-characterized regulators of RGC function, such as E2, and potential regulators, such as glutamate, modulate RGC proliferation mechanisms.

Based on the framework of our current understanding of RGC regulatory mechanisms, previous findings in mammalian neural progenitor cells, and transcriptomic data from goldfish RGCs, I have formulated a null hypothesis in which glutamate does not regulate goldfish RGC function via group III mGluRs. Using an *in vitro* culture system developed by Xing et al. (2015), primary goldfish RGCs were submitted to pharmacological manipulations designed to investigate the involvement of glutamatergic signalling pathways in regulating the expression of neurogenic genes, such as cyclin D1 (*ccnd1*) and cyclin A2 (*ccna2*), and the steroidogenic gene *cyp19a1b*. In addition, the anatomical relationship between glutamate sources and RGCs was studied to further determine the potential of glutamate as a regulator of RGC function. Finally, since teleost RGCs are known to produce estrogens, the effect of E2 on *cyp19a1b*, *ccnd1*, and *ccna2* expression were quantified and the involvement of group III mGluRs in regulating E2-mediated responses were examined.

2.2. Materials and Methods

2.2.1. Experimental animals

All animal procedures performed were approved by the University of Ottawa Protocol Review Committee and are in accordance with the Canadian Council on Animal Care guidelines on the use of animals. Adult female goldfish (*Carassius auratus*) purchased from Aquality Fish Wholesale (Mississauga, Canada) were acclimated for a minimum of 3 weeks prior to experimentation and maintained in 18°C water under a natural simulated photoperiod for Ottawa, Canada (45.4215° N, 75.6972° W). Goldfish were fed a diet of Finfish Bronze 3.0 mm pellets (Ziegler Bros.).

2.2.2. Radial glial cell culture

Cell culture methods employed were previously developed and validated by Xing et al. (2015). Prior to cervical transection, sexually mature, non-gravid female goldfish were anesthetized with tricaine methanesulfonate. Female goldfish telencephalon and hypothalamus were dissected and extracted. Subsequently, these tissues were washed twice with Hanks Balanced Salt Solution (HBSS; 400 mg KCL, 600 mg KH_2PO_4, 350 mg $NaHCO_3$, 8 g NaCl, 48 mg Na_2HPO_4, 1 g D-glucose, in 1 L of double distilled water) containing 0.5% antibiotic-antimycotic solution (Gibco). HBSS was removed and tissues were chopped into small explants then dissociated with 2 mL of 0.25% trypsin for 1 hour. Trypsin reaction was quenched with Leibovitz's L-15 Medium (Gibco) with 15% fetal bovine serum (Gibco) and antibiotic-antimycotic solution, and cells were subsequently cultured in L-15 media with serum. This cell culture medium was replaced after 5 days and plates were gently rinsed twice with HBSS to remove potential tissue

explants. After the first changeout, cells were given new cell culture media every 5-7 days and were passaged once 80%-90% confluence was reached. The RGCs were sub-cultured following 0.125% trypsin treatments for three passages.

2.2.3. Drug exposures

Cells were exposed to the selective group III mGluR agonist L-AP4 (10 µM; Abcam), adenylate cyclase activator forskolin (10 µM, Sigma-Aldrich) selective group III mGluR antagonist CPPG (10 µM; Tocris), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-bromo-cAMP, 10 µM; Abcam), E2 (1 µM, Sigma-Aldrich), and L-glutamic acid (1 µM, 10 µM, 100 µM, and 1 mM; Tocris) to investigate their effects on *cyp19a1b*, *ccnd1*, *ccna2*, *grm6b*, *grm7*, and *grm8b* mRNA. Water was used as a vehicle to dissolve L-AP4, forskolin, CPPG, and L-glutamic acid whereas 0.1% dimethyl sulfide (DMSO) in water was used as a vehicle to dissolve CPPG and forskolin. DMSO had no effects on *cyp19a1b*, *ccnd1*, *ccna2*, *grm6b*, *grm7*, and *grm8b* mRNA levels in RGCs *in vitro* (See appendix - **Figure A1**). For analysis of the involvement of selective group III mGluR activation and E2, cells were treated for 48 hrs with L-AP4, forskolin, CPPG, L-AP4 + forskolin, L-AP4 + CPPG, E2 , and L-glutamic acid. A 4 hr CPPG pre-treatment was applied to cells prior to 48 hr L-AP4 + CPPG exposure. For analysis of the involvement of PKA signalling with 8-bromo-cAMP and L-AP4 + 8-bromo-cAMP treatments, cells were incubated for 24 hrs without 8-bromo-cAMP then given 8-bromo-cAMP for the remaining 24 hrs. In addition, media containing L-AP4 was removed and replaced following 24 hrs. For the full duration of all treatments, cells were kept in serum-free L-15 media in a 25°C, low CO_2 incubator.

2.2.4. RNA extraction and cDNA synthesis

RNA was extracted using the RNeasy Microkit (Qiagen) as specified in the manufacturer's protocol. After extraction, RNA concentration and quality were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was determined by running isolated RNA on a 1% agarose gel with 0.5% SYBR Safe DNA gel stain (Thermo Fisher Scientific). 3 μg of total cDNA was synthesized from extracted untreated RGC sample RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Reverse transcriptase (RT)-PCR reaction parameters for cDNA synthesis were: 25°C for 10 mins, 50°C for 30 mins, and 85°C for 5 mins.

2.2.5. Primer validation

Primers for aromatase B (*cyp19a1b*), cyclin D1 (*ccnd1*), cyclin A2 (*ccna2*), mGluR6b (*grm6b*), mGluR7 (*grm7*), mGluR8b (*grm8b*), and 18S ribosomal subunit (*18S*) were designed with Primer3 (Untergasser et al., 2012) and synthesized by Integrated DNA Technologies (**Table 2.1**). Primer products were generated by mixing untreated RGC pooled cDNA with GoTaq Green Master Mix (Promega) and performing PCR amplification. Thermal cycling parameters for amplification were a 95°C denaturation step for 5 mins followed by 40 cycles of a 95°C denaturation step for 1 min, an annealing step for 1 min (predicted annealing temperatures provided by primer manufacturer), and a 72°C extension step for 1 min. A final 72°C extension step was performed for 10 mins after completion of the 40 cycles. Primer sets containing the GoTaq Green Master Mix stain were tested for specificity by running PCR products on a 5% agarose gel. Products were excised from the excised gel, extracted with NucleoSpin Gel and PCR

Clean-up kit (Machery-Nagel), and sequenced by StemCore Laboratories at the Ottawa Hospital Research Institute to establish primer specificity. Primer product specificity was also used to validate transcriptomic data available in Da Fonte et al. (2017).

Table 2.1. Primers (5'→3') validated and used for qPCR of primary goldfish RGCs.

Gene	Primer Sequence (Forward)	Primer Sequence (Reverse)	Annealing Temperature (°C)
cyp19a1b	AGCGACAGGCCATCAATAAC	ATCACCATCTCCAGCACACA	62
ccnd1	GTTCATAGCCAGCGGAAAGA	CAATGCTCAAAGCAGAGGAA	61
ccna2	TGCTGCTCCAACAATCAATC	GCTCAGCTCACCAAGAAACA	57
grm6b	ATTCTGCTCTGGGTGGAAGA	GACCAACCACCTACGACTCAA	59
grm7	CGCCGGTATGATTTCTTCTC	GCAGATTCCACCTGCTTCTC	57
grm8b	GGCCTTCATTCCCATCTTCT	AGCTGGCTGAGCTTGTTTGT	57
18S	AAACGGCTACCACATCCAAG	CACCAGATTTGCCCTCCA	60

2.2.6. Quantitative real-time PCR

Quantitative real-time PCR based on Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) intercalation with cDNA and fluorescence detection was used to analyze relative gene expression. Amplification of cDNA and detection of SYBR green fluorescence was performed by the CFX96 Real-Time PCR Detection System (Bio-Rad). Thermal cycling parameters for amplification were a 95°C Taq activation step for 3 mins proceeded by 40 cycles of a 95°C denaturation step for 10 s and a primer annealing step (optimized through thermal gradient assays) for 30 s. After completion of the 40 cycles, a 65°C-95°C melt curve step with a

temperature increment of 0.5°C over 5 seconds was performed to confirm the amplification of a single PCR product.

2.2.7. Immunohistochemistry

Whole brains were dissected from female goldfish and placed in 4% paraformaldehyde in 1x PBS solution overnight at 4°C. After overnight fixation, brains were washed four times for 5 minutes in 1x PBS at room temperature. Brains were then placed in a 30% sucrose in 1x PBS solution overnight at 4°C. Afterwards, brains were removed from the sucrose solution and placed in 2:1 Optimal Cutting Temperature (OCT) compound (Thermo Fisher Scientific)/30% sucrose in 1x PBS solution for 30 mins at room temperature. Next, brains were placed in cryomolds and covered in 2:1 OCT compound/30% sucrose in 1x PBS solution. Cryomolds were then submerged in -80°C isopentane for 30 s. Resulting cryoblock was then placed in a cryo-sectioning chuck with OCT compound and submerged in -80°C isopentane for 30 s. Frozen cryoblocks were cut as 16µm transverse sections onto Superfrost Plus slides (Thermo Fisher Scientific) and allowed to dry for a minimum of 30 mins. Once slides were dry, they were placed in blocking buffer (1% bovine serum albumin, 0.3% TritonX100 in a 100mL 1x PBS solution) at room temperature. Each individual slide was then incubated in 200 µL of 1:800 primary monoclonal mouse anti-GFAP antibody (EMD Millipore; Cat. # MAB360) and 200 µL of 1:2000 primary polyclonal rabbit anti-vGLUT1/2 antibody (Synaptic Systems; Cat. # 135 503) overnight at 4°C. After incubation, slides were washed twice with PBS for 10 mins and then incubated with 200 µL of 1:500 anti-mouse goat Alexa fluor 647 (Invitrogen) and 200 µL of 1:500 anti-rabbit chicken Alexa fluor 488 (Invitrogen) for 1 hr at room temperature. Slides were once again washed twice in PBS for 10 minutes. Coverslips were mounted on slides with Vectashield containing 4,6-diamino-2-phenylindole

(DAPI) (Vector Laboratories) for 30 minutes at room temperature and left to further dry at 4°C overnight. The anti-vGLUT1/2 antibody was generated against a conserved amino acid sequence (AA 324-339) in rat vGLUT1 that shares 99% sequence identity with goldfish vGLUT1 and vGLUT2. Images were captured using a Nikon A1RsiMP confocal microscope and Nikon's Imaging Software-Elements.

2.2.8. Statistics

Quantitative real-time PCR data were analyzed with CFX Manager Software (Bio-Rad). A standard curve generated from pooled cDNA was ran with each assay. Efficiency of each assay was 95% - 110% and the R^2 of each standard curve was ≥ 0.99. Relative mRNA level was determined using the relative standard curve method based on C_q values and normalized against mRNA level of the endogenous control gene *18S* as a ratio. Percent fold-change was calculated as the mean normalized ratio over the mean normalized ratio of the control. Statistical analyses of qPCR data were performed using SigmaPlot 12. Data was tested for normality with the Shapiro-Wilk's test and homogeneity of variance with the Levene's test prior to any statistical analysis. Comparison between groups was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Post-hoc tests were only performed if results from one-way ANOVA were statistically significant. P-value less than 0.05 was considered statistically significant. Data are presented as a mean + standard error of the mean (SEM), wherein each sample was assayed in duplicate.

2.3. Results

2.3.1. Validation of group III mGluR and neurogenic genes in cultured goldfish RGCs

To validate the expression of group III mGluRs and neurogenic genes identified in the female goldfish RGC transcriptome, primer pairs were designed based on assembled unigene basepair sequences available in Da Fonte et al. (2017). Of the group III mGluRs identified in the RNA sequencing data, the expression of mGluR6b (*grm6b*), mGluR7 (*grm7*), and mGluR8 (*grm8b*) was validated by sequencing of gel-extracted PCR products. The nucleotide sequence identity of our genes of interest were assessed using the BLASTn sequence alignment tool (Pearson, 2013) (**Figure 2.1**). In the case of *grm6b* expressed in female goldfish RGCs, it shares 97% sequence identity with common carp *grm6*-like, and *Sinocyclocheilus anshiensis* and *Sinocyclocheilus grahami grm6*-like. The goldfish *grm7* shares 87% sequence identity with zebrafish *grm7*. Regarding *grm8b*, it shares 99% sequence identity with *Sinocyclocheilus rhinocerous grm8*-like, 97% sequence identity with common carp *grm8*-like, and 93% sequence identity with zebrafish *grm8b*. Sequence alignments show that *grm6b*, *grm7*, and *grm8b* sequences generated from PCR products share 100%, 97%, and 97% similarity with their assembled unigene counterpart.

The cell cycle regulator gene *ccnd1* from goldfish RGCs shares 97% sequence identity with common carp *ccnd1* and 95% sequence identity with zebrafish *ccnd1*. In addition, *ccna2* shares 98% sequence identity with common carp *ccna2*-like and 94% sequence identity with *ccna2* identified in full-grown oocytes derived from goldfish. Sequence alignment shows that *ccnd1* and *ccna2* sequences generated from PCR products share 100% and 94% similarity with their assembled unigene counterpart.

Sequenced Gene (identified in female goldfish RGCs)	Gene (with sequence similarity)	Sequence Identity (%)	Accession Number
grm6b TGGATACGGTCAGTGTGGTGGT CTGAATGAACATCTTTTCTGTGG ATTGTGATGTGCCAAAAAATA	*Cyprinus carpio grm6*-like	97	XM_019090789.1
	Sinocyclocheilus anshiensis grm6-like	97	XM_016489957.1
	Sinocyclocheilus grahami grm6-like	97	XM_016249740.1
grm7 CCTCGACTCCTTTCTCGCCATA GCTTCCCTCCGAGGCCACTGTA GACCGTCTTCCAACCCATCAGT TTCACTATGTCCAC CATGACC	*Dario rerio grm7*	87	NM_001302247.1
grm8b ATGCCCAAAGTCTACATCATTAT CCTGCACCCGGAACAGAACGTG CCCAAACGCAAGCGCAGCTTCA AGGCCATCGTCAC CGCTGCCACCATGACAAACAAG GTCAGCCAGC	*Sinocyclocheilus rhinocerous grm8*-like	99	XM_016569877.1
	Cyprinus carpio grm8-like	97	XM_019114052.1
	Dario rerio grm8b	93	NM_001287539.1
ccnd1 CATGTGGCGACGATTTTCCTCA TTTTAGGTACAATTTCTTTCTGA ACACACTTGAAATAATTTGGTGA TGGAAGGTAGTTTTCCTCTGCTT TGAGCAT	*Cyprinus carpio ccnd1*	97	XM_019106913.1
	Dario rerio ccnd1	95	NM_131025.4
ccna2 TGTGAGCAACAAAGTGGAAGCT TATCAATGTTTCTTGGTGAGCTG AGC	*Cyprinus carpio ccna2*-like	98	XM_019114924.1
	Carassius auratus ccna2	94	AF273493.1

Figure 2.1. Group III mGluR and cyclin genes identified in the cultured goldfish RGC transcriptome share high sequence identity with cyprinid variants. Newly characterized basepair

sequences of gel-extracted PCR products were analysed with BLASTn to determine their sequence similarity in comparison with published basepair sequences from other species.

2.3.2. RGCs in the goldfish forebrain are in close proximity to numerous glutamatergic synaptic vesicles

Xing et al. (2016) previously identified that aromatase-B-positive, GFAP-labelled RGCs were localized along the ventricular surface of the female goldfish telencephalon. Furthermore, Da Fonte et al. (2018) previously showed that GFAP-labelled RGCs were found in the periventricular preoptic area of the female goldfish hypothalamus. Double staining immunohistochemistry with the RGC marker GFAP (Zupanc and Sîrbulescu, 2011; Xing et al, 2016), and vGLUT1/2, a glutamatergic synaptic vesicle marker, was used to determine the relative localization of RGCs and potential synaptic glutamate sources in the female goldfish forebrain. Double immunofluorescence detection shows that RGC fibers located along the ventricular surface of the ventral telencephalic area (**Figure 2.2B and Figure 2.2G**) are in close proximity to highly abundant glutamatergic synaptic vesicles (**Figure 2.2C**), which are also distributed throughout the area ventralis telencephali pars ventralis (Vv) (**Figure 2.2D**). Control reactions showing no immunoreactivity (data not shown) included omission of primary vGLUT1/2 antibody and 24 hr pre-incubation of this antibody (0.5 μg/μL at 1:2000 dilution) with a vGLUT1/2 blocking peptide (SQPAYFEEVFGFEISK; Bio Basic) at a ratio of 20:1. The X-axis and Y-axis views of merged confocal slice images further indicate that glutamatergic synaptic vesicles are anatomically close to RGCs along the ventricle but are not co-localized (**Figure 2.2E and 2.2F**). Double immunofluorescence detection also shows that RGCs are located along the third ventricle of the hypothalamus and that their fibers extend into the periventricular preoptic area (**Figure 2.3B**).

Additionally, RGC fibers are surrounded by discrete, punctate glutamatergic synaptic vesicles, which are also located around the periventricular preoptic area (**Figure 2.3C and 2.3D**).

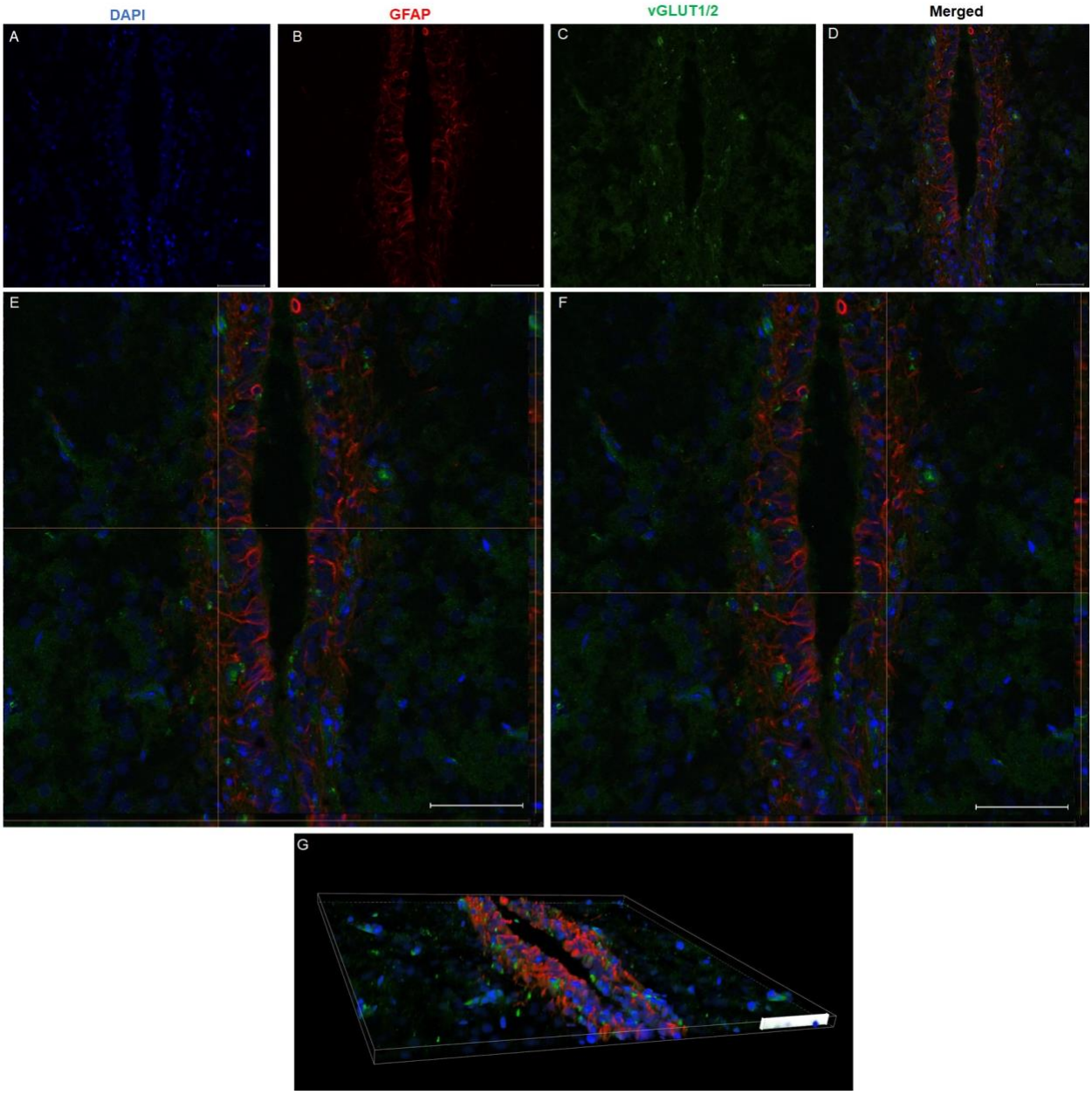

Figure 2.2. Double fluorescent detection of GFAP (red) and vGLUT1/2 (green) around the ventricular surface in the ventral telencephalic area of the female goldfish brain. Fluorescent confocal microscopy of transverse brain sections shows that GFAP-labelled RGCs surround the

ventricular surface of the ventral telencephalon (red; **B, G**). Merged images (**D**) show that vGLUT1/2-labelled glutamatergic synaptic vesicles (green; **C**) are localized around RGCs and abundant along the ventricular surface and the area ventralis telencephali pars ventralis (Vv). The crosshair in the optical slice scan view of (**D**) shows that RGC fibers and glutamatergic synaptic vesicles can be anatomically close but not directly in contact or co-localized along both sides of the ventricular surface (**E, F**). Blue fluorescence denotes nuclear staining with DAPI (**A**). Scale bar = 50 µm.

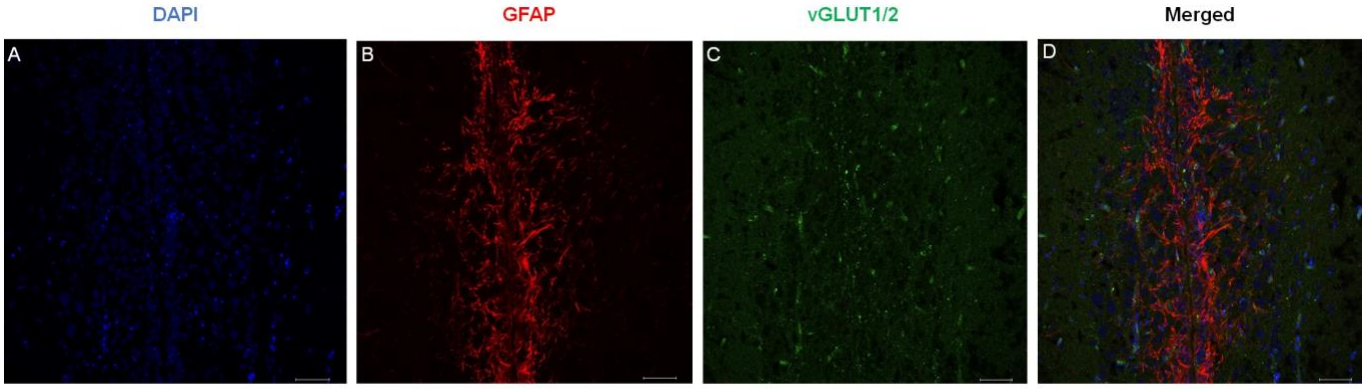

Figure 2.3. Double fluorescent detection of GFAP (red) and vGLUT1/2 (green) in the periventricular preoptic area of the hypothalamus of the female goldfish brain. Fluorescent confocal microscopy of transverse brain sections shows that GFAP-labelled RGCs line the third ventricle and extend into the periventricular preoptic area (red; **B**). Merged images (**D)** show that vGLUT1/2-labelled glutamatergic synaptic vesicles (green; **C**) are distributed around RGC fibers and the periventricular preoptic area. Blue fluorescence denotes nuclear staining with DAPI (**A**). Scale bar = 50 µm.

2.3.3. Group III mGluR activation downregulates *cyp19a1b*, *ccnd1*, and *ccna2* mRNA in cultured RGCs via inhibition of cAMP/PKA signalling

Our qPCR data shows that pharmacological manipulation of receptor signaling pathways modulated *cyp19a1b*, *ccnd1*, and *ccna2* mRNAs (one-way ANOVA; $p < 0.001$). Firstly, RGCs were treated *in vitro* with the selective group III mGluR agonist L-AP4 to investigate glutamatergic regulation of *cyp19a1b*, *ccnd1*, and *ccna2* mRNAs. After 48 hr exposure with 10 µM L-AP4, Tukey's post-hoc tests between treatments showed that activation of group III mGluRs significantly decreased *cyp19a1b* (**Figure 2.4A**), *ccnd1* (**Figure 2.4B**), and *ccna2* (**Figure 2.4C**) mRNA by 60% relative to the control group ($p_{cyp19a1b} = 0.003$, $p_{ccnd1} < 0.001$, $p_{ccna2} < 0.001$). Treatment with the group III mGluR antagonist CPPG was used to determine whether the inhibitory effect of L-AP4 was receptor-mediated. Tukey's post-hoc tests also show that the increase in *cyp19a1b* and *ccnd1* mRNA level following 48 hr exposure with 10 µM CPPG was not significantly different in comparison with the control group ($p_{cyp19a1b} = 0.156$, $p_{ccnd1} = 0.743$), forskolin group ($p_{cyp19a1b} = 0.061$, $p_{ccnd1} = 0.054$), and L-AP4 + forskolin group ($p_{cyp19a1b} = 0.070$, $p_{ccnd1} = 0.117$). Moreover, CPPG had no affect on *ccna2* mRNA relative to the control group ($p = 0.247$). Pretreatment of RGCs with CPPG prior to application of L-AP4 maintained mRNA levels at baseline ($p_{cyp19a1b} = 0.303$, $p_{ccnd1} = 0.381$, $p_{ccna2} = 0.198$) thus blocking the inhibitory effect of L-AP4. Furthermore, *cyp19a1b*, *ccnd1*, and *ccna2* mRNA level of L-AP4 + CPPG-treated RGCs were different from the L-AP4 treated group ($p_{cyp19a1b}$, p_{ccnd1}, $p_{ccna2} < 0.001$).

Since activation of group III mGluRs canonically inhibits adenylate cyclase activity thus decreasing cAMP production (Niswender and Conn, 2010), the involvement of cAMP-dependent signalling was investigated with the adenylate cyclase activator forskolin. Tukey's post-hoc tests showed that 48 hr treatment with 10 µM forskolin significantly increased *cyp19a1b*, *ccnd1*, and

ccna2 mRNA in comparison to the control ($p_{cyp19a1b} < 0.001$, $p_{ccnd1} = 0.003$, $p_{ccna2} < 0.001$) and L-AP4-treated cells ($p_{cyp19a1b}$, p_{ccnd1}, $p_{ccna2} < 0.001$). Application of forskolin in combination with 10 µM L-AP4 also yielded an increase in *cyp19a1b* mRNA by 3.7-fold, *ccnd1* mRNA by 2-fold, and *ccna2* mRNA by 2.8-fold relative to the control ($p_{cyp19a1b} < 0.001$, $p_{ccnd1} = 0.008$, $p_{ccna2} < 0.001$) and significantly negated the inhibitory effect of L-AP4 ($p_{cyp19a1b}$, p_{ccnd1}, $p_{ccna2} < 0.001$).

The activation and release of PKA catalytic subunits, which regulate transcription factors, is cAMP-dependent (Beavo et al., 1974; Meinkoth et al., 1993). Therefore, the involvement of cAMP/PKA signalling downstream of group III mGluRs was investigated with the PKA activator 8-bromo-cAMP. Similar to the continuous 48 hr L-AP4 exposures, Tukey's post-hoc tests showed that treatment of RGCs *in vitro* with 10 µM L-AP4 for 48hrs with a changeout and re-administration of 10 µM L-AP4 after 24 hrs significantly decreased *cyp19a1b* (**Figure 2.4D**), *ccnd1* (**Figure 2.4E**), and *ccna2* (**Figure 2.4F**) mRNA relative to the control group ($p_{cyp19a1b}$, p_{ccnd1}, $p_{ccna2} < 0.001$) albeit to a stronger degree. Much like forskolin-treated RGCs, the qPCR data indicates that treatment with 8-bromo-cAMP for 24 hrs following a 24 hr serum-free incubation period significantly increased *cyp19a1b* and *ccnd1* mRNA by 6-fold and *ccna2* mRNA by 2-fold relative to the control group ($p_{cyp19a1b} = 0.005$, $p_{ccnd1} < 0.001$, $p_{ccna2} = 0.023$). RGCs treated with 8-bromo-cAMP also had significantly increased *cyp19a1b*, *ccnd1*, and *ccna2* mRNA in comparison with the L-AP4-treated cells ($p_{cyp19a1b}$, p_{ccnd1}, $p_{ccna2} < 0.001$). Application of 8-bromo-cAMP after 24 hrs completely reversed the inhibitory effect of L-AP4 ($p_{cyp19a1b}$, p_{ccnd1}, $p_{ccna2} < 0.001$) and increased *cyp19a1b* and *ccnd1* mRNA relative to untreated RGCs ($p_{cyp19a1b} = 0.001$, $p_{ccnd1} < 0.001$). Although the combination treatment did increase *ccna2* mRNA relative to L-AP4 treated cells ($p < 0.001$), Tukey's post-hoc tests showed that it was not significantly different from the control group ($p = 0.072$) and the 8-bromo-cAMP-treated group (0.872). The mRNA level of *cyp19a1b*

and *ccnd1* following the combination treatment were also not significantly different from the 8-bromo-cAMP group ($p_{cyp19a1b}$ = 0.810, p_{ccnd1} = 1.000).

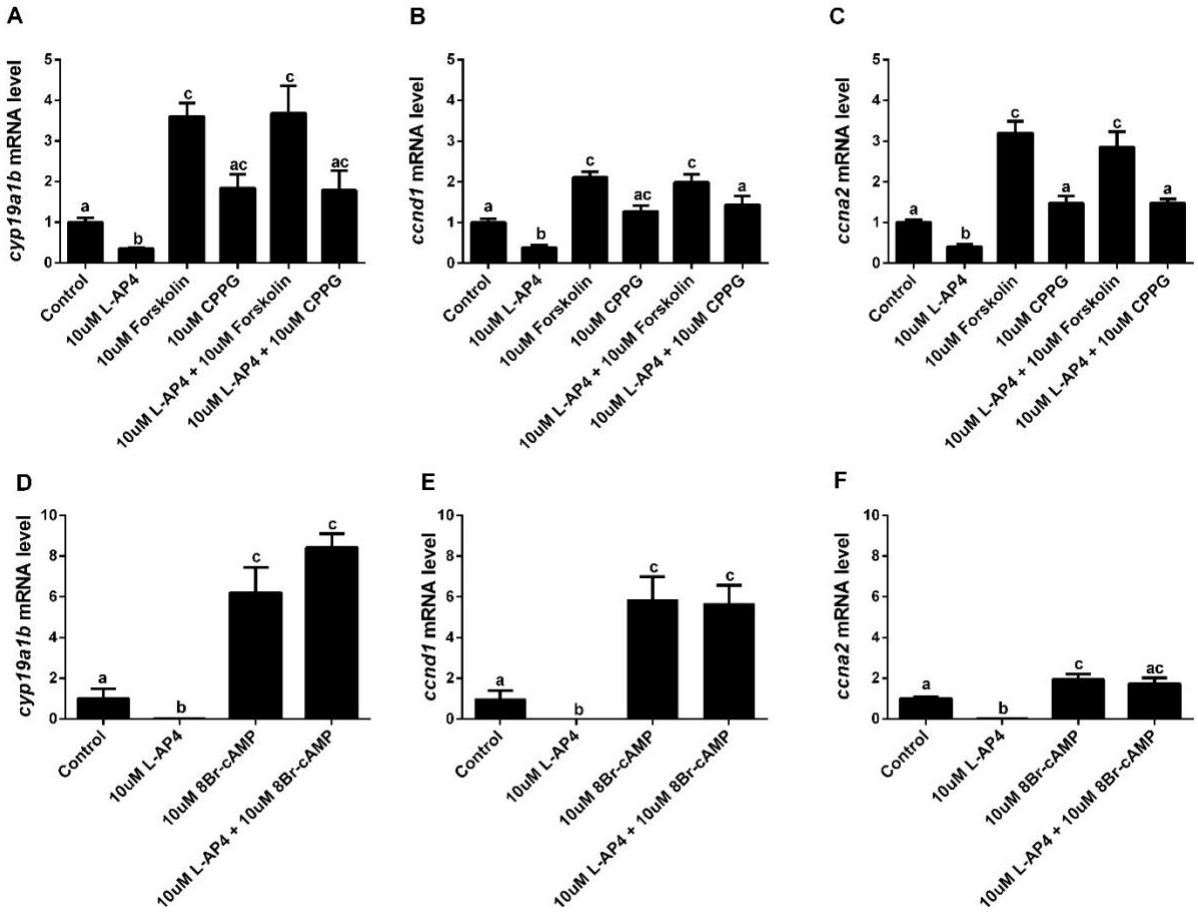

Figure 2.4. Glutamatergic regulation of *cyp19a1b*, *ccnd1*, and *ccna2* mRNA in cultured goldfish RGCs via group III mGluR/cAMP/PKA signalling. Quantitative real-time PCR analysis showing changes in *cyp19a1b* (**A**), *ccnd1* (**B**), and *ccna2* (**C**) mRNA level in primary RGC culture following treatment with 10 µM L-AP4, 10 µM forskolin, 10 µM CPPG, and combinations of 10 µM L-AP4, 10 µM forskolin, and 10 µM CPPG. All treatments were administered as a continuous 48 hr dose with the exception of L-AP4 + CPPG treatments in which RGCs were pre-treated with CPPG for 4 hrs. Data were normalized to 18S and defined as a fold change relative to control. Bars

represent the mean + SEM (n = 4). Quantitative real-time analysis also showing changes in *cyp19a1b* (**D)**, *ccnd1* (**E**), and *ccna2* (**F**) mRNA level after treatment with 10 µM L-AP4, 10 µM 8-Br-cAMP, and 10 µM L-AP4 + 10 µM 8-Br-cAMP**.** L-AP4 was applied for 48 hrs total with a refresher dose after 24 hrs while 8-Br-cAMP was applied for 24 hrs after 24 hrs of incubation in L-AP4-treated serum-free media or serum-free media only. Data were normalized to 18S and defined as a fold change relative to control. Bars represent mean + SEM (n = 4, except for "Control" group where n = 3). Treatment groups noted with different letters are statistically significantly different ($p < 0.05$).

2.3.4. Adenylate cyclase activation increases *grm7* mRNA

Selective activation of group III mGluRs with L-AP4 was used to study glutamatergic regulation of group III *grm* mRNA as a potential autoregulatory mechanism in primary RGC cultures. Results from qPCR data shows that pharmacological manipulation of receptor signaling pathways significantly affected *grm6b* and *grm7* mRNAs (one-way ANOVA; $p_{grm6b} = 0.017$, $p_{grm7} < 0.001$). Tukey's post-hoc tests showed that L-AP4 has no effect on *grm6b* mRNA compared with the control group ($p = 0.954$) (**Figure 2.5A**). In addition, there was no difference between control *grm6b* mRNA and increased *grm6b* mRNA levels following forskolin ($p = 0.083$), L-AP4 + forskolin ($p = 0.074$), CPPG ($p = 0.771$). There was also no difference between *grm6b* mRNA after the L-AP4 + CPPG treatment and mRNA levels following forskolin ($p = 0.631$), L-AP4 + forskolin ($p = 0.999$), CPPG ($p = 0.358$). However, treatment with the L-AP4 + CPPG combination significantly increased *grm6b* mRNA relative to the control group ($p = 0.035$). According to Tukey's post-hoc tests, application of L-AP4 also had no effect on *grm7* mRNA ($p = 0.514$) in comparison with the control group and CPPG-treated cells ($p < 0.001$) (**Figure 2.5B**). Furthermore,

relative to the control group, *grm7* mRNA level increased after CPPG ($p = 0.012$), and L-AP4 + CPPG treatment ($p = 0.040$). Interestingly, *grm7* mRNA increased by 7 to 8-fold in comparison with the control following forskolin ($p < 0.001$) and L-AP4 + forskolin ($p < 0.001$) treatments. Tukey's post-hoc tests indicated that the increase in *grm7* mRNA after forskolin treatment is higher than increases in *grm7* mRNA following CPPG ($p < 0.001$) and L-AP4 + CPPG treatment ($p < 0.001$). Moreover, the increase in *grm7* following L-AP4 + forskolin treatment is also higher than increases in *grm7* mRNA following CPPG ($p < 0.001$) and L-AP4 + CPPG treatment ($p < 0.001$). No individual drug or drug combination in the glutamatergic signalling pharmacological assay had a significant effect on *grm8b* mRNA (one-way ANOVA; $p = 0.754$) (**Figure 2.5C**).

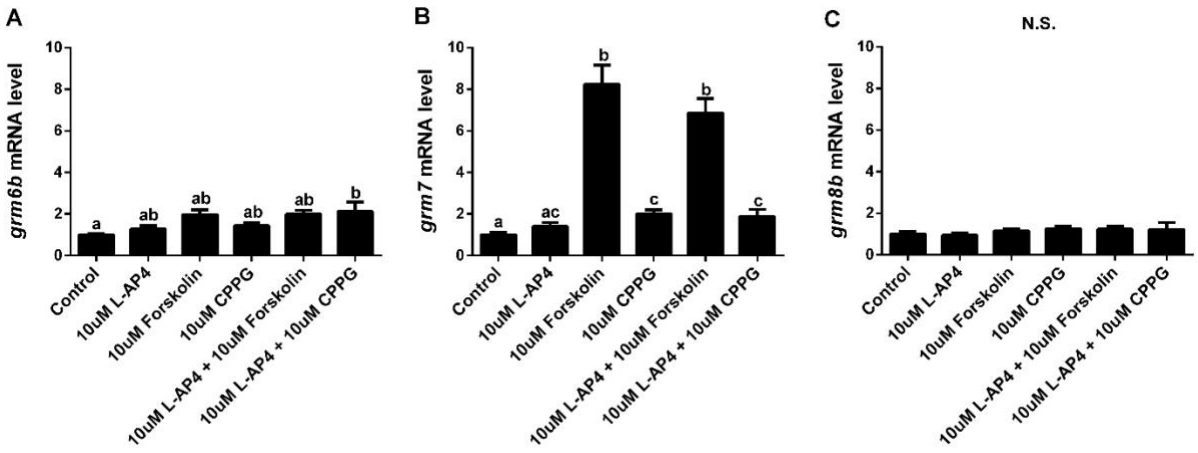

Figure 2.5. Involvement of glutamatergic signalling in regulation of group III *grm* mRNA in cultured goldfish RGCs. Quantitative real-time PCR analysis showing variations in *grm6b* (**A**), *grm7* (**B**), and *grm8b* (**C**) mRNA level in primary RGC culture following treatment with 10 µM L-AP4, 10 µM forskolin, 10 µM CPPG, and combinations of 10 µM L-AP4, 10 µM forskolin, and 10 µM CPPG. All treatments were administered as a continuous 48 hr dose with the exception of L-AP4 + CPPG treatments in which RGCs were pre-treated with CPPG for 4 hrs. Data were

normalized to 18S and defined as a fold change relative to control. Bars represent the mean + SEM (n = 4). Treatment groups noted with different letters are statistically significantly different ($p < 0.05$).

2.3.5. Glutamate differentially regulates *cyp19a1b*, *ccnd1*, and *grm6b* mRNA in a dose-dependent manner

Specific pharmacological activation of group III mGluR-related signalling mechanisms with L-AP4 has clear effects on RGC mRNAs. It was also important to determine the effects of the endogenous ligand glutamate, which could potentially activate multiple receptors subtypes simultaneously. To assess this, a 48 hr dose response exposure with L-glutamic acid, a protonated glutamate analog, was performed. Analysis of the qPCR data with one-way ANOVAs shows that the dose response treatment significantly affected *cyp19a1b*, *ccnd1*, and *grm6b* mRNA ($p_{cyp19a1b}$, $p_{ccnd1} < 0.001$, $p_{grm6b} = 0.016$). Tukey's post-hoc tests indicated a dose-dependent decrease in *cyp19a1b* mRNA, which decreased by 80% only after exposure to 1 mM L-glutamic acid ($p = 0.001$) (**Figure 2.6A**). Conversely, *ccnd1* mRNA significantly increased by 4-fold after treatment with 1 mM L-glutamic acid ($p = 0.001$) (**Figure 2.6B**). Similarly, *grm6b* mRNA significantly increased following treatment with 1 mM L-glutamic acid ($p = 0.029$) (**Figure 2.6D**). One-way ANOVAs showed that the L-glutamate treatment did not affect *ccna2* (**Figure 2.6C**), *grm7* (**Figure 2.6E**), and *grm8b* (**Figure 2.6F**) mRNA ($p_{ccna2} = 0.135$, $p_{grm7} = 0.096$, $p_{grm8b} = 0.294$).

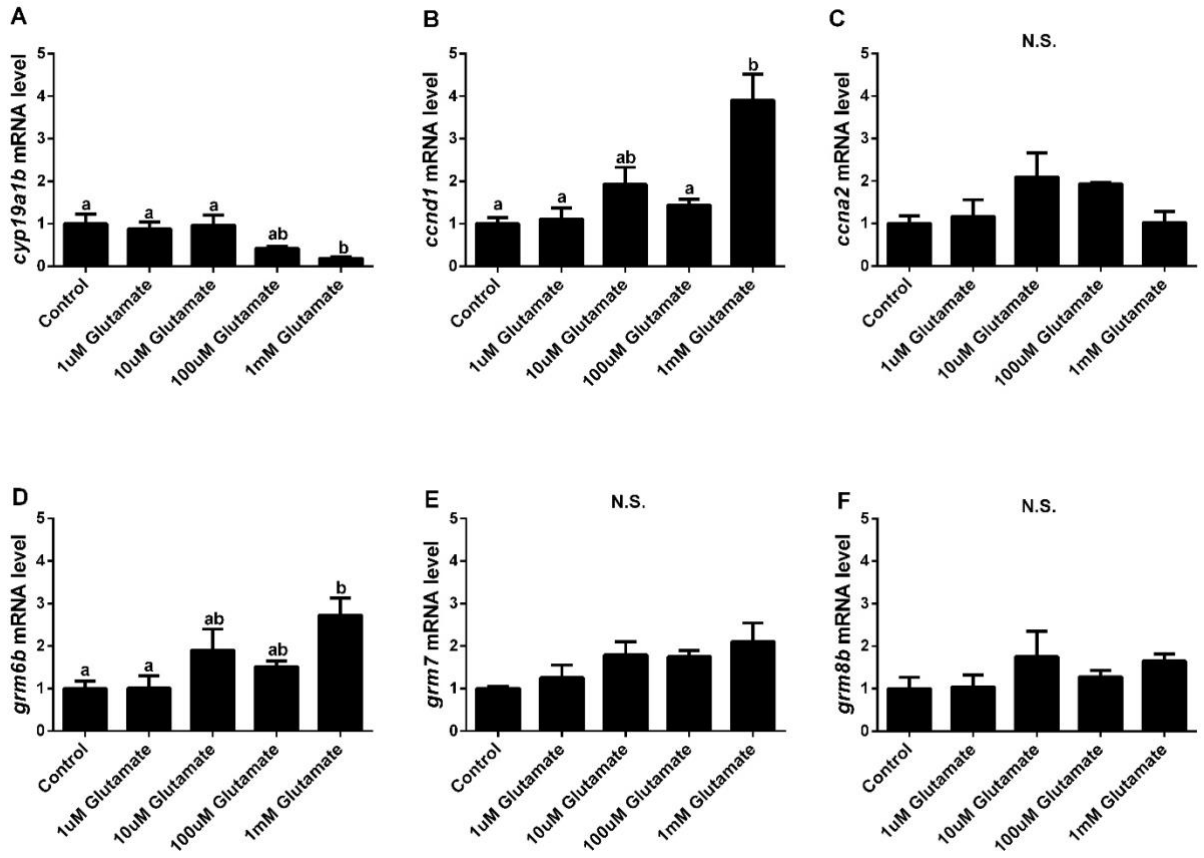

Figure 2.6. Dose-dependent glutamate response of *cyp19a1b*, *ccnd1*, *ccna2*, and group III *grm* mRNA in cultured goldfish RGCs. Quantitative real-time PCR analysis showing changes in *cyp19a1b* (**A**), *ccnd1* (**B**), *ccna2* (**C**), *grm6b* (**D**), *grm7* (**E**), and *grm8b* (**F**) mRNA level in primary RGC culture following 48 hr L-glutamic acid dose response treatment. Data were normalized to 18S and defined as a fold change relative to control. Bars represent the mean + SEM (n = 3, except for "1 mM Glutamate" group where n = 4). Treatment groups noted with different letters are statistically significantly different ($p < 0.05$).

2.3.6. Estradiol-induced upregulation of *ccnd1* mRNA but not *cyp19a1b* and *grm7* mRNA is blocked by selective activation of group III mGluRs

Xing et al. (2016) previously demonstrated that 24 hr 1 µM E2 exposure significantly increases *cyp19a1b* mRNA level in cultured goldfish RGCs. Here, RGCs were treated with 1 µM E2 for 48 hrs *in vitro* to determine whether E2 also regulates *ccnd1*, *ccna2*, and group III *grm* mRNA. In addition, cells were co-treated with 10 µM L-AP4 to determine whether selective activation of group III mGluRs could affect E2-induced *cyp19a1b* mRNA levels. One-way ANOVAs determined that estrogenic treatments had an effect on *cyp19a1b*, *ccnd1*, and *grm7* mRNA ($p_{cyp19a1b} < 0.001$, $p_{ccnd1} = 0.003$, $p_{grm7} = 0.030$). Similar to Xing et al. (2016), Tukey's post-hoc tests showed that 48 hr E2 treatment significantly increased *cyp19a1b* mRNA by 11-fold relative to the control ($p < 0.001$). However, L-AP4 had no effect on the E2-induced increase in *cyp19a1b* mRNA and expression levels were similar to the E2-only exposed cells ($p = 0.758$) (**Figure 2.7A**). Treatment with E2 also significantly increased *ccnd1* mRNA ($p = 0.013$) but less strongly compared to the induction of *cyp19a1b* mRNA. Radial glial cells treated with L-AP4 in tandem with E2 had *ccnd1* mRNA levels that were not significantly different from the control group ($p = 0.925$). Therefore, co-treatment with L-AP4 negated the stimulatory effect of E2 on *ccnd1* mRNA (**Figure 2.7B**). Additionally, application of E2 significantly increased *grm7* mRNA ($p = 0.033$). However, the *grm7* mRNA level of L-AP4 + E2-treated cells was not different from the E2-treated group ($p = 1.000$) (**Figure 2.7E**). Furthermore, qPCR data and one-way ANOVAs show that the E2 and E2 + L-AP4 treatment had no effect on *ccna2* (**Figure 2.7C**), *grm6b* (**Figure 2.7D**), and *grm8b* (**Figure 2.7F**) mRNA ($p_{ccna2} = 0.120$, $p_{grm6b} = 0.053$, $p_{grm8b} = 0.237$).

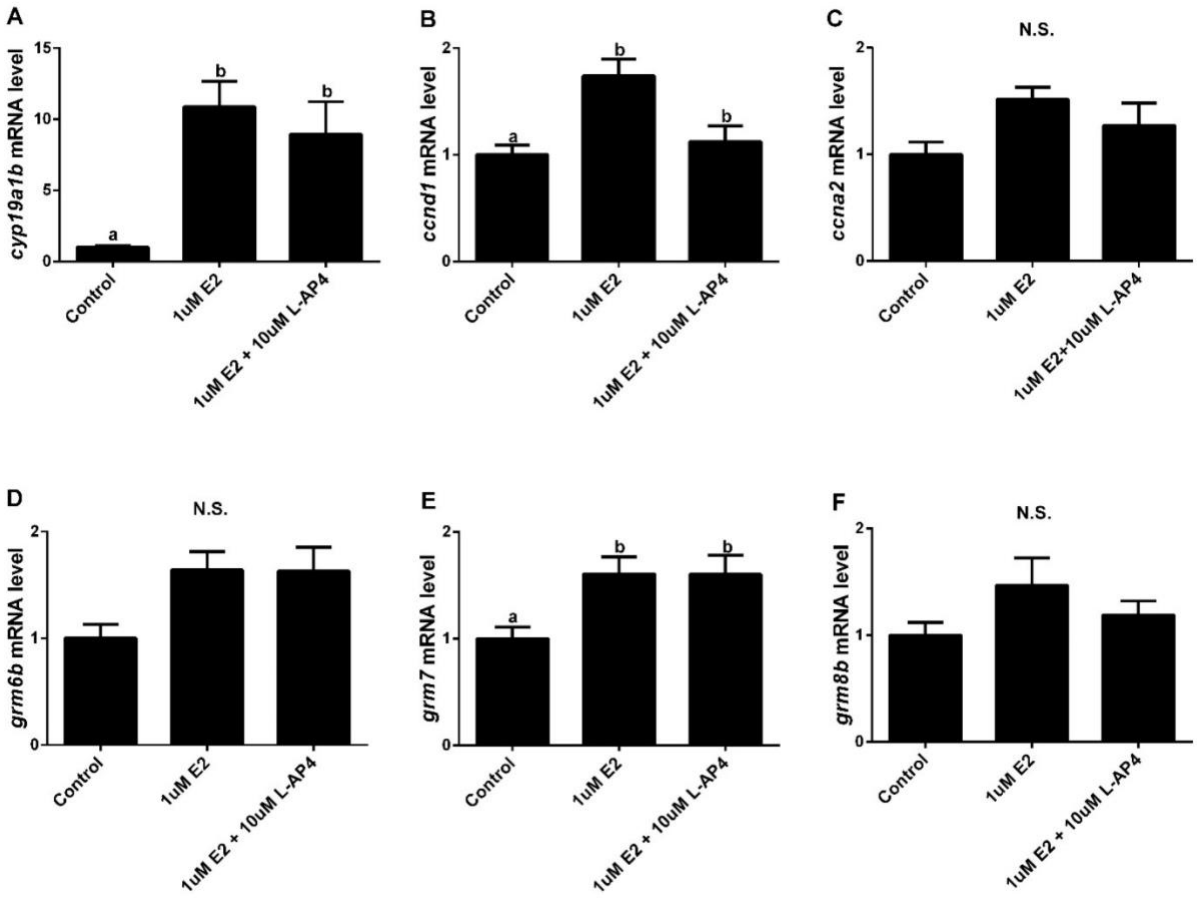

Figure 2.7. Effect of E2 and E2 + L-AP4 on *cyp19a1b*, *ccnd1*, *ccna2*, and group III *grm* mRNA in cultured goldfish RGCs. Quantitative real-time PCR analysis showing changes in *cyp19a1b* (**A**), *ccnd1* (**B**), *ccna2* (**C**), *grm6b* (**D**), *grm7* (**E**), and *grm8b* (**F**) mRNA level in primary RGC culture following 48 hr 1 μM E2 exposure and 48 hr 1 μM E2 + 10 μM L-AP4 exposure. Data were normalized to 18S and defined as a fold change relative to control. Bars represent the mean + SEM (n = 4). Treatment groups noted with different letters are statistically significantly different ($p < 0.05$).

2.4. Discussion

2.4.1. Cultured goldfish RGCs express *grm6b, grm7,* and *grm8b*

This study is the first to elucidate the functionality of group III mGluRs and group III mGluR-mediated signal transduction mechanisms in teleost RGCs. My analysis of the *de novo* transcriptome of cultured goldfish RGCs provided by Da Fonte et al. (2017) indicated that the only group of mGluRs expressed are group III mGluRs. Validation of PCR clone products via sequencing confirms that *grm6b, grm7,* and *grm8b* are expressed in cultured female goldfish RGCs originating from the telencephalon and hypothalamus. The presence of group III mGluR mRNA suggests that RGCs potentially possess the machinery necessary to be regulated by glutamate.

Although there are no published studies in teleosts or mammals that assess group III mGluR mRNA or protein expression in RGCs, group III mGluRs are expressed in RGC-rich regions of the teleost forebrain. *In situ* hybridization of whole adult zebrafish brain revealed that *grm6b* is expressed in the olfactory bulb (Haug et al., 2012). Additionally, *grm7* is strongly expressed in the dorsal telencephalic area and moderately expressed in the preoptic region whereas the expression of *grm8b* was observed in the olfactory bulb and the dorsal division of the subpallium. Interestingly, RGCs in the zebrafish forebrain are most abundant in the olfactory bulb, the pallial and subpallial regions, and the preoptic region (Meneut et al., 2005; Pellegrini et al., 2007). Furthermore, RGCs are abundant in the hypothalamus but primarily along the lateral and posterior recesses of the mediobasal hypothalamus (Menuet et al., 2005). *In situ* hybridization of the whole adult zebrafish brain also showed that *grm7* was expressed in the dorsal zone of the periventricular hypothalamus, the lateral hypothalamic nucleus, torus lateralis, central nucleus of inferior lobe, and the diffuse nucleus of inferior lobe. However, *grm6b* or *grm8b* were not expressed in the

hypothalamus of adult zebrafish (Haug et al., 2012). The expression of group III mGluRs has also been observed in other types of neuroglia. In primary cultured rat microglia, mGluR6 and mGluR8 protein and mRNA were detected but mGluR7 was not expressed (Taylor et al., 2003). The expression of *grm6, grm7,* and *grm8* was observed in mixed neuronal/glial cultures derived from rats (Faden et al., 1997). In addition, mGluR8 was expressed by microglia and reactive astrocytes derived from human brain tissue lesioned by multiple sclerosis (Geurts et al., 2005). Successful PCR cloning of *grm6b*, *grm7*, and *grm8b* in primary cultured goldfish RGCs supports previous findings in mammals that neuroglia can express group III mGluRs *in vitro*.

2.4.2. Glutamatergic synaptic vesicles and RGCs are anatomically close in the goldfish forebrain

Our double staining immunohistochemical data shows that vGLUT1/2-labelled glutamatergic synaptic vesicles are anatomically close to GFAP-labelled RGCs in the ventral telencephalic area and the periventricular preoptic area of the goldfish brain. Canonically, cytoplasmic glutamate within glutamatergic presynaptic terminals accumulate into synaptic vesicles, via vGLUT1 and vGLUT2, which can then release glutamate into the synaptic cleft (Naito and Ueda, 1985; Ozkan and Ueda, 1998; Hackett and Ueda, 2015; Rees et al., 2017). The concentration of glutamate within the synaptic cleft of mammals is reported to be 1-7 mM (Clements et al., 1992; Clements, 1996; Rodriguez et al., 2013). The mechanism for synaptic vesicular glutamate transport is highly conserved between mammals and goldfish (Tabb and Ueda, 1991). The anatomical proximity between glutamatergic synaptic vesicles and RGCs suggests the potential for glutamatergic regulation of RGCs. Immunohistochemistry shows that glutamatergic CA3 neurons in the rat hippocampus directly synapse onto GluR cells, which are oligodendrocyte precursors that co-

express glial markers S-100β and GFAP (Bergles et al., 2000). In addition, immunostaining of rat hippocampal slices shows that glial amino acid transporter 5-labelled astroglial processes are in close contact with vGLUT1-labelled glutamatergic presynaptic terminals and envelope the synapse (Cubelos et al., 2005). Although the tripartite system around the synaptic cleft is tightly sealed by perisynaptic glial cells, there is evidence to suggest that synaptic glutamate can leak into the non-synaptic extracellular space and interact with extrasynaptic glutamate receptors (Asztely et al., 1997; Araque et al., 1999; Vizi and Mike, 2006; Reichenbach et al., 2010; Kessler, 2013). However, the concentration of glutamate in the non-synaptic extracellular space is 4000-12000 times lower than the reported concentration of glutamate in the cytoplasm of glutamatergic neurons (5-10 mM; Cubelos et al., 2005; Featherstone, 2010). Previous studies showing intimate glutamatergic neuronal-glial interactions support our finding that glutamatergic synaptic vesicles can be in close anatomical proximity to RGCs. In turn, their proximity potentially allows for extracellular synaptic and non-synaptic glutamate to regulate RGC function.

Our immunohistochemical data also shows that RGCs are located along the ventricular surface of the female goldfish brain, confirming the observations of Xing et al. (2015). Therefore, RGCs may receive glutamatergic signals from the cerebrospinal fluid (CSF) produced and contained within the ventricles. The presence of glutamate in human and rodent CSF has been confirmed via high-performance liquid chromatography (Ludewig, 1953; Spink et al., 1986; Eckstein et al., 2008). In rats, the concentration of glutamate in the CSF is reported to be approximately 10 µM (Vannucci et al., 1999). Data from our vGLUT1/2 immunostaining shows that some glutamatergic synaptic vesicles are located along the surface of the third ventricle. This implies that glutamatergic terminals are in close proximity to the ventricle so glutamate release into the CNS may be possible. In macaques, glutamatergic neuronal fibers within the periventricular zone of the hypothalamus

contact the third ventricle. Glutamate can then be released into the CSF and exert changes on cells around the ventricular surface via volume transmission (Okubo and Iino, 2011; Leak and Moore, 2012). The position of goldfish RGCs and synaptic vesicles along the ventricular surface support the possibility of glutamatergic regulation of RGCs, not only through the synaptic inputs but also through the CSF.

2.4.3. Group III mGluRs negatively regulate cultured goldfish RGC steroidogenic and neurogenic gene expression via inhibition of cAMP/PKA-dependent signalling

Pharmacological experiments demonstrated that selective activation of group III mGluRs with L-AP4 in cultured goldfish RGCs significantly decreased *cyp19a1b*, *ccnd1*, and *ccna2* mRNA levels. This inhibitory effect is reversed by increasing cAMP production with forskolin and PKA activation with 8-bromo-cAMP demonstrating that group III mGluRs negatively regulate *cyp19a1b*, *ccnd1*, and *ccna2* expression by inhibiting cAMP/PKA signalling. Furthermore, blocking group III mGluRs with CPPG prevents L-AP4-induced downregulation of *cyp19a1b*, *ccnd1*, and *ccna2* mRNA levels indicating that the observed inhibitory effect is specific to group III mGluRs. Our transcriptional data also revealed that forskolin increased *grm7* mRNA, which suggests that *grm7* is positively regulated in a cAMP-dependent manner. Upregulation of *grm7* mRNA by cAMP or cAMP-dependent effectors potentially acts as an autoregulatory system to increase mGluR7 expression thus increasing the inhibitory potential of glutamate via group III mGluRs and further attenuating cAMP/PKA signalling. Activation of AMPA and kainate receptors in chick Bergmann cells has been shown to increase mGluR4 mRNA and protein expression while decreasing mGluR4 mRNA and protein expression in Müller glial cells (López et al., 1998). However, cAMP-dependent regulation of *grm7* and group III mGluR transcription in

general remains unexplored and further analysis of elements within the promoter region of mGluR7 is necessary to support our speculative regulatory mechanism.

As a serine/threonine kinase, PKA can phosphorylate numerous downstream targets which include transcription factors that regulate gene expression (Daniel et al., 1998; Turnham and Scott, 2016). One of the best characterized downstream transcription factors phosphorylated by PKA is CREB, which induces gene expression by a CRE on gene promotors (Montminy et al., 1986; Mayr and Montminy, 2001). Interestingly, CRE-like consensus sites are present on the *cyp19a1b* promoter of zebrafish suggesting that teleost brain aromatase expression can be regulated via PKA signalling (Tong and Chung, 2003). In addition, CRE sequences have also been found on the *ccnd1* promoter region of zebrafish and mammalian cells and *ccna2* promoter region of mammalian cells (Herber et al., 1994; Desdouets 1995; D'Amico et al., 2000; Dworkin et al., 2007; Klein and Assoian, 2008; Guo et al., 2011). Therefore, a decrease in CREB phosphorylation, which in turn reduces target gene transcription, is a potential mechanism by which group III mGluR-mediated inhibition of cAMP/PKA signalling leads to decreased *cyp19a1b*, *ccnd1* and *ccna2* mRNA levels in cultured goldfish RGCs. Previous *in vitro* studies in goldfish RGCs have shown that *cyp19a1b* mRNA is positively regulated by DIR activation via cAMP/PKA/p-CREB signalling, which further supports the idea that *cyp19a1b* transcription is regulated by a cAMP/PKA-dependent pathway (Xing et al., 2015; Xing et al., 2016).

Our study is the first in any *in vitro* or *in vivo* model to demonstrate that *cyp19a1b* expression can be regulated via selective activation of group III mGluRs. In male quail hypothalamic preoptic area explants, selective activation of ionotropic glutamate receptors such as AMPA and kainate receptors has been shown to rapidly decrease brain aromatase activity (Balthazart et al., 2006). Furthermore, *in vivo* injection of kainate into the medial preoptic nucleus inhibits aromatase

activity in the male quail brain (de Bournonville et al., 2017). The inhibitory effects documented in quail studies are ionotropic glutamate receptor-specific whereas those in goldfish are group III mGluR-specific, supporting that glutamate is an important regulator of brain aromatase in vertebrates.

Much like our observations in goldfish RGCs, selective activation of group III mGluRs with L-AP4 in cultured mouse neocortical progenitor and murine pluripotent P19 cells decreased *ccnd1* mRNA levels (Nakamichi et al., 2008). Forskolin treatments reversed the inhibitory effect of L-AP4 suggesting that cAMP-mediated signalling is critical to regulating *ccnd1* expression (Nakamichi et al., 2008). In the pluripotent P19 cells, the promoter activity of CRE sequences within the full-length promoter region of cyclinD1 decreased following L-AP4 exposure. This inhibitory effect was reversed by forskolin suggesting that group III-mediated inhibition of cAMP signalling decreases *ccnd1* expression by potentially reducing CREB phosphorylation as evidenced by decreased CRE site promoter activity after L-AP4 treatment (Nakamichi et al., 2008). Although CREB can be phosphorylated by other serine/threonine kinases, inhibition of cAMP production via group III mGluRs predominantly affects PKA activity (Pin and Duvoisin, 1995; Steven and Seliger, 2016). Overall, the regulatory mechanism documented by Nakamichi et al. (2008) supports our finding that selective activation of group III mGluRs can decrease *ccnd1* mRNA level via inhibition of cAMP-dependent signalling in cultured goldfish RGCs and implicates CREB in regulating *ccnd1* expression *in vitro*.

In contrast to *ccnd1*, the involvement of group III mGluRs in regulating *ccna2* expression was unknown until our current study. It has been shown that inhibition of glutaminase-1, which metabolizes glutamine to glutamate and ammonia, decreases cyclin A (*ccna*) promoter activity, mRNA levels, and protein levels in cultured human endothelial cells (Durante et al., 2017; Peyton

et al., 2018). An increase in *ccna2* mRNA in mouse neural progenitor cells following treatment with a mGluR3 and mGluR5 agonist shows that metabotropic glutamatergic regulation of *ccna2* expression is possible (Di Giorgi-Gerevini et al., 2005). In human diploid fibroblasts, *ccna* mRNA levels were increased by 8-bromo-cAMP suggesting cAMP/PKA signalling can positively regulate *ccna* expression (Desdouets et al., 1995). Furthermore, application of the PKA inhibitor H89 decreased *ccna* mRNA level suggesting that inhibition of cAMP/PKA signalling can negatively regulate *ccna*. Moreover, pharmacological activation of cAMP/PKA signalling strongly induced the cyclin A promoter specifically through a CRE site indicating that cAMP-dependent signalling may regulate *ccna* expression via CREB. The results reported by Desdouets et al. (1995) support our finding that *ccna2* can be negatively regulated by cAMP/PKA signalling through group III mGluRs and suggest that CREB can be involved in regulating *ccna2* expression.

Besides CREB, members of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family are transcription factors that can be regulated in a cAMP-dependent manner by PKA phosphorylation (Daniel et al., 1998). Direct phosphorylation of serine residues at PKA recognition sites on NF-κB subunits such as p50, p65 and c-Rel by PKA has been shown to stimulate transcription, nuclear localization, and DNA binding affinity *in vitro* (Zhong et al., 1998; Hou et al., 2003; Yu et al., 2004; Christian et al., 2016). Therefore, NF-κB is responsive to changes in cAMP/PKA signalling. Interestingly, sequence alignment performed against mammalian cyclin D1 promoters shows that a highly conserved region of the zebrafish cyclin D1 promoter contains NF-κB consensus sequences (Klein and Assoian, 2008). It has been shown in a wide array of mammalian cells that NF-κB directly upregulates cyclin D1 transcription by binding to NF-κB consensus sequences on the promoter region and inducing cyclin D1 kinase holoenzyme complex activity (Guttridge et al., 1999; Hinz et al., 1999; Joyce et al., 1999; Joyce et al., 2001). In addition,

activation of glutamatergic ionotropic and group I metabotropic receptors appears to regulate NF-κB activity and NF-κB-mediated mammalian neuronal development, synaptic plasticity, and glial immune responses, which demonstrates that glutamate can regulate downstream neuronal and neuroglial functions via NF-κB (Guerrini et al., 1995; O'Neill and Kaltschmidt, 1997; O'Riordan et al., 2006).

Unlike cyclin D1, no consensus NF-κB sites have been identified on cyclin A2 (Klein and Assoian, 2008). However, cAMP responsive element modulator transcriptional activator (CREMτ) can bind to CRE sequences on the rodent and human cyclin A2 and cyclin D1 promoter region and regulate transcription in a cAMP/PKA-dependent manner (Gonzalez and Montminy, 1989; Laoide et al., 1993; Desdouets et al., 1995; Lamas et al., 1996; Blanchard, 2000). For future research, CREB still remains the most intriguing cAMP-dependent transcription factor to study in the context of glutamatergic regulation of goldfish RGC gene expression. Furthermore, studying the involvement of transcription factors such as NF-κB and CREMτ further elucidates the role of effectors downstream of cAMP/PKA signalling in regulating gene expression. Chromatin immunoprecipitation may reveal potential interactions between suggested transcription factors and *cyp19a1b*, *ccnd1*, and *ccna2* DNA sequences. In addition, proteomic analysis with mass spectrometry of CREB and other transcription factors following co-immunoprecipitation may reveal additional binding partners such as co-transactivators that engage in transcriptional regulation (Free et al., 2009; ten Have et al., 2011).

2.4.4. Glutamate negatively regulates *cyp19a1b* expression but positively regulates *ccnd1* and *grm6b* expression in cultured goldfish RGCs

Group III mGluRs are the only metabotropic glutamate receptors expressed in the goldfish RGC transcriptome. However, transcriptomic data provided by Da Fonte et al. (2017) also shows the expression of ionotropic glutamatergic AMPA receptors suggesting an additional mechanism for regulation of RGC function. Our pharmacological dose-dependent study shows that 1 mM of glutamate halves *cyp19a1b* mRNA to a similar degree as 10 µM L-AP4. This suggests that glutamate regulates *cyp19a1b* expression primarily through negatively-coupled group III mGluRs in cultured goldfish RGCs rather than through other glutamate receptors. In contrast, 1 mM of glutamate increased *grm6b* and *ccnd1* mRNA, which suggests that glutamate differentially regulates *grm6b* and *ccnd1* expression through receptors other than group III mGluRs. The effects observed following treatment with 1 mM of glutamate are likely physiologically relevant as synaptic glutamate concentrations have been reported to be around 1-4 mM and as high as 7 mM in mammals (Clements et al., 1992; Clements, 1996; Rodriguez et al., 2013). Pharmacological experiments using the AMPA receptor antagonist CNQX should be performed to determine the involvement of AMPA receptors in regulating glutamate-mediated changes in goldfish RGC gene expression.

In cultured rodent neural progenitor cells, Bergmann glia, and the radial processes of radial glia-like cells, Ca^{2+}-permeable AMPA receptors are reported to increase intracellular Ca^{2+} concentration and elicit an array of Ca^{2}-dependent responses (Mayer and Westbrook, 1987; Dingledine et al., 1999; Saab et al., 2011; Jansson et al., 2013; Renzel et al., 2013; Cervetto et al., 2015). In the cytosol, Ca^{2+} can bind with calmodulin and activate CaMKs and calcineurin (Soderling, 1999; Kahl and Means, 2003). In mammalian cells, calcineurin upregulates cyclin D1

transcription and translation and CaMKI increases cyclin D1 accumulation in the nucleus (Schneider et al., 2002; Kahl and Means, 2004). In addition, pharmacological inhibition of CaMKII enzymatic activity decreased cyclinD1 protein levels (Morris et al., 1998). In the future, pharmacological assays in combination with fura 2 acetoxymethyl ester fluorescence imaging and transcriptional analyses may elucidate Ca^2-dependent signal transduction mechanisms downstream of AMPA receptor activation in RGCs.

The involvement of AMPA receptors, which may affect transcription factor activity via second messenger signalling, could explain the increase in *grm7* mRNA levels we observed following CPPG treatment in goldfish RGCs *in vitro* (Sun et al., 1994). Since CPPG blocks glutamate access to group III mGluRs, it potentially produces a bias towards AMPA receptor activation. Although glial cells predominantly uptake glutamate, it is reported that glutamate, which is a small anion, can be released by opening of anion channels, suggesting that glial cells might be capable of glutamatergic autoregulation via gliotransmitter release (Bonansco et al., 2011; Wang et al., 2013). Volume-sensitive channels, calcium-activated anion channels, P2X purinoreceptor 7 (P2RX7), and connexin hemichannels are reported be permeable to glutamate (Takano et al, 2005). Of note, voltage-dependent anion channel 3 and P2RX7 were identified in the cultured goldfish RGC transcriptome, which suggests that some of the elements necessary for glutamatergic autoregulation are present (Da Fonte et al., 2017).

2.4.5. Group III mGluRs reduce E2-induced stimulation of *ccnd1* expression but not *cyp19a1b* and *grm6b* expression in cultured goldfish RGCs

Previous research has shown that E2 promotes *cyp19a1b* expression in teleost RGCs. Specifically, this positive autoregulatory loop is driven by EREs on the teleost *cyp19a1b* gene (Callard et al, 2001; Forlano and Bass; 2005; Menuet et al., 2005; Diotel et al., 2010a). In cultured goldfish RGCs, E2 upregulates *cyp19a1b* mRNA by recruiting estrogen receptors such as estrogen receptor α (ERα; *esr1*) and estrogen receptor β (ERβ; *esr2a*) and increasing estrogen receptor γ (ERγ; *esr2b*) expression thus maintaining the positive feedback loop between aromatase and its product E2 (Xing et al., 2016). Since RGCs are steroidogenic and dependent on autocrine/paracrine E2 regulation to maintain aromatase function, it is important to elucidate the role of neurotransmitters in modifying E2-driven expression of *cyp19a1b*. Selective activation of D1R with SKF38393 in tandem with E2 has been shown to up or downregulate *cyp19a1b* mRNA in cultured goldfish RGCs depending on SKF38393 concentration suggesting that neurotransmitters can finely regulate *cyp19a1b* expression in highly active RGCs (Xing et al., 2016). Similar to results reported by Xing et al. (2016), we show that 1 µM E2 increases *cyp19a1b* mRNA in goldfish RGCs *in vitro*. Furthermore, E2 increased *grm7* mRNA. In the case of the mGluR7 gene, it remains unknown whether ERE or CRE sequences are present in the promoter region. In rat hypothalamic neurons, hypothalamic astrocytes, and dorsal root ganglion neurons, it is hypothesized that E2 can activate group I and II mGluRs through caveolin-dependent interaction with ERs resulting in a conformational change that mimics glutamate binding thus inducing both G_s and $G_{i/o}$ signalling (Boulware et al., 2007; Dewing et al., 2007; Kuo et al., 2009; Chaban et al., 2011). Although potentially applicable in the context of CREB regulation, their studies focus on

the effect of E2 treatment on mGluRs at the receptor protein level rather than transcriptional regulation.

Our study also shows that E2 increased *ccnd1* mRNA in cultured goldfish RGCs. Selective activation of group III mGluRs had no effect on E2-mediated upregulation of *cyp19a1b* and *grm7* mRNA whereas the stimulatory effect of E2 on *ccnd1* mRNA was blocked by selective activation of group III mGluRs. Interestingly, the mammalian and zebrafish cyclin D1 gene are reported to not have an ERE, which suggests that the conventional ER-ERE interaction does not regulate E2-mediated changes in *ccnd1* mRNA (Lam et al., 2011). However, Sabbah et al. (1999) isolated a region in between -96 and -26 of the cyclin D1 promoter of human mammary carcinoma cells that contains a CRE that is strictly induced by ERα. In addition, pull-down experiments and measurement of cyclin D1 promoter activity illustrate a cAMP-independent mechanism in which ATF-2/c-Jun heterodimers bind the hormone-dependent CRE to control activation of the cyclin D1 promoter by ERα, which suggests that E2 can control cyclin D1 expression without EREs and supports our findings in relation to *ccnd1* (Sabbah et al., 1999). In ER-positive ZR-75 breast cancer cells, E2 also induced transcriptional activation of the cyclin D1 gene (Castro-Rivera et al., 2001). Chromatin immunoprecipitation elucidates a mechanism in which ERα forms a complex with specificity protein 1 transcription factor (Sp1) that binds a proximal CRE and Sp1-binding sites. Moreover, inhibition of PKA with SQ22536 inhibited the E2-mediated induction of cyclin D1 demonstrating that cAMP-dependent signalling can also regulate E2-induced cyclin D1 expression (Castro-Rivera et al., 2001).

The G protein-coupled estrogen receptor GPR30 can regulate cAMP-dependent signalling in some zebrafish and mammalian cell types (Prossnitz et al., 2007; Prossnitz et al., 2008; Prossnitz and Maggiolini, 2009). However, targeted PCR did not identify GPR30 in cultured goldfish RGCs

while RNA sequencing suggests very low levels of expression (Xing et al., 2016). This implies that GPR30 is unlikely to be a major player in regulating cyclin D1 expression. Our finding that L-AP4 blocks the stimulatory effect of E2 on *ccnd1* mRNA points to cAMP-dependent regulation of the cyclin D1 gene estrogen response mechanism considering that selective activation of group III mGluRs inhibits cAMP/PKA signalling.

2.4.6. Implications of a group III mGluR-mediated regulatory mechanism for goldfish RGC function

The elucidation of group III mGluRs as regulators of *cyp19a1b*, *ccnd1*, and *ccna2* in goldfish RGCs is important for our understanding of their function in the brain. This study further establishes that RGCs with documented stem cell-like potential and steroidogenic capacity can be regulated by neurotransmitters (Schmechel and Rakic, 1979; Zupanc and Clint, 2003; Zupanc et al., 2012; Xing et al., 2015, Xing et al., 2016). Firstly, our data show that activation of group III mGluRs via glutamate and a group III mGluR-specific agonist downregulates the expression of estrogen-synthesizing aromatase B, which is important for the regulation of critical brain functions such as behaviour, differentiation, neuroendocrine function, neuroregeneration, and proliferation (Garcia-Segura et al., 1999; Pellegrini et al., 2007; Cornil et al., 2013; Ubuka and Tsutsui, 2014). In mammals, E2 promotes developmental neurogenesis and reactive neurogenesis following brain injury (Martínez-Cerdeño et al., 2006; Brown et al., 2009; Mahmoud et al., 2016). In contrast to mammals, recent studies demonstrate that E2 inhibits neurogenesis in the zebrafish brain (Diotel et al., 2013; Makantasi and Dermon, 2014). Furthermore, aromatase B expression in zebrafish brain is lost as RGCs differentiate into neurons, which suggests that local E2 synthesis maintains RGC fate in teleosts (Pellegrini et al., 2007; Diotel et al., 2010a; Xing et al., 2014). Although

speculative, by decreasing *cyp19a1b* mRNA, activation of group III mGluRs may attenuate the E2-synthesizing mechanism thus potentially promoting the transition of RGCs to a neuronal fate. The resulting decrease in local E2 production perhaps increases proliferation in surrounding cells.

Secondly, our data also shows that selective activation of group III mGluRs downregulates cyclin D1 and cyclin A2 expression, which play crucial roles in progression of the cell cycle and are therefore involved in regulation of proliferation and differentiation. Cyclin D1 promotes progression through the G_1-S phase by complexing with cyclin-dependent kinase (CDK) 4 and deactivating retinoblastoma protein via phosphorylation (Matsushime et al., 1992; Diehl, 2002). The retinoblastoma protein is thought to silence genes involved in S-phase of the cell cycle by binding the transcription factor E2F and preventing E2F-mediated transcriptional activity (Baldin et al., 1993; Hu et al., 2001; Fu et al., 2004). Interestingly, the cyclin A2 gene is regulated by E2F (Soucek et al., 1997). Cyclin A2 links with CDK2 to initiate and complete the DNA-replicating S-phase and associates with CDK1 to progress cells through late S-phase until late G_2-phase to ensure entry into the mitotic, proliferative phase (Pagano et al., 1992; Jeffrey et al., 1995; Yam et al., 2002).

Both cyclins have been shown to induce progenitor cell proliferation in mammals and zebrafish and glial cell proliferation in mammals (Bessa et al., 2008; Das et al., 2012; Yamamoto et al., 2012; Chung et al., 2013; Nobs et al., 2013; Nobs et al., 2014; Tsunekawa et al., 2014; Lee et al., 2016; Lien et al., 2016). In cultured mouse neural progenitor cells, activation of G_s-coupled mGluR5 increases cyclin D and cyclin A2 mRNA level and promotes proliferation indicating that glutamate-related changes in cyclin expression can have functionally significant effect on proliferation (Di Giorgi-Gerevini et al., 2005). In rat neocortical progenitor cells, selective activation of group III mGluRs inhibits cyclin D1 expression via group III mGluR/cAMP

signalling, leading to decreases in self-replication and preferential differentiation towards neuronal fate (Nakamichi et al., 2008). In addition, selective activation of mGluR7 inhibits proliferation of human ventral mesencephalon neural progenitor cells (Vernon et al, 2011). Also, selective activation of mGluR4 decreases proliferation of cerebellar granule cell neuroprecursors and promotes differentiation towards a neuronal fate (Canudas et al., 2004). By decreasing *ccnd1* and *ccna2* mRNA, selective activation of group III mGluRs in RGCs potentially decreases proliferation and promotes neuronal differentiation. However, glutamate potentially increases *ccnd1* mRNA through glutamate receptors other than mGluRs as suggested by our findings. Therefore, by increasing *ccnd1*, glutamate could have the opposite effect and increase proliferation and maintain RGC fate by preventing differentiation.

The expression of *ccnd1* in cultured goldfish RGCs was also increased by E2. Although E2 is reported to be anti-neurogenic in teleost brains, studies suggest that E2 can promote proliferation of normal and cancerous mammary cells and endometrium through upregulation of cyclin D1 (Neuman et al., 1997; Giulianelli et al., 2012; Pellegrini et al., 2012; Lamb et al., 2013). By increasing *ccnd1* mRNA, E2 may promote proliferation of RGCs while also negatively regulating neurogenesis of the other surrounding cells. In turn, expansion of the RGC pool may increase neuroestrogen synthesis thus further inhibiting neurogenesis. Increasing *ccnd1* expression also potentially prevents exiting of the cell cycle before initiation of the G_1-phase thus suppressing differentiation (Hu et al., 2001; Hindley and Philpott, 2012). Therefore, E2 may maintain RGC fate via an E2-aromatase positive feedback loop and increased cyclin D1 expression. Furthermore, our study shows that the stimulatory effect of E2 on *ccnd1* mRNA was suppressed by selective activation of group III mGluRs, which suggests that glutamate may also attenuate proliferation in

highly active RGCs. By decreasing RGC proliferation, the ability of E2 to maintain RGC fate potentially weakens as a result of fewer neuroestrogen-synthesizing cells in the brain.

Overall, by decreasing *cyp19a1b*, *ccnd1*, and *ccna2* mRNA via group III mGluRs, glutamate may synergistically induce RGC differentiation and suppress RGC proliferation resulting in a decrease in the RGC progenitor pool. In addition, by decreasing *cyp19a1b* expression and theoretically reducing the RGC population due to increased differentiation and decreased proliferation, glutamatergic activation of group III mGluRs may also synergistically suppress total E2 synthesis leading to increased proliferation of surrounding cells and further promoting the transition of RGCs to neurons (**Figure 2.8**). Beyond driving adult neurogenesis in teleosts, glutamate could prove important in a neuroregenerative context following ischemic or traumatic brain injuries, in which abnormally high levels of glutamate leak into the extracellular fluid of the central nervous system due to mechanical disruption of the blood-brain barrier or changes in ionic homeostasis (Katayama et al., 1990; Bullock et al., 1998; Hazell, 2007; Hinzman et al, 2010; Kostandy, 2012; Guerriero et al., 2015). In response to increased extracellular glutamate levels, perhaps the excitotoxic death of neurons can be mitigated via activation of RGC group III mGluRs leading to the reactive replacement of neuronal populations (Mark et al., 2001; Dong et al., 2009).

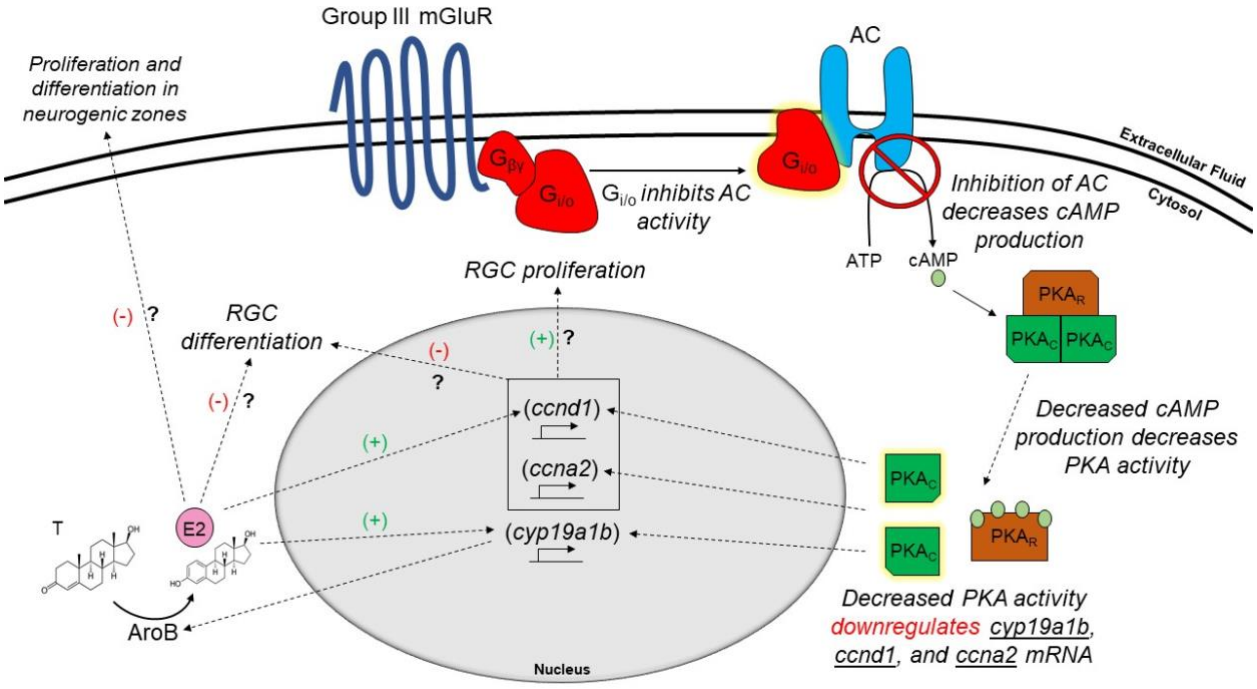

Figure 2.8. Speculated effects of group III mGluR activation and E2 on proliferation and differentiation of goldfish RGCs. Activation of group III mGluRs leads to downregulation of *cyp19a1b*, *ccnd1*, and *ccna2* mRNA via inhibition of cAMP/PKA-dependent signalling. Both *ccnd1* and *ccna2* are known to promote self-proliferation and prevent differentiation, therefore decreased expression potentially reduces RGC proliferation and drives differentiation to a neuronal state. Downregulation of *cyp19a1b* may decrease the synthesis of E2, which potentially leads to increased proliferation of neighboring cells and RGC differentiation.

2.5. Future Directions

Including potential experiments outlined in the "Discussion" section of this thesis, future experiments should be designed to analyze the functional effects and relative importance of group

III mGluRs and AMPA receptors for proliferation and differentiation in goldfish RGCs *in vitro*. Performing BrdU assays after pharmacological manipulation of glutamate receptors and post-receptor signalling may determine whether activation of group III mGluRs and/or AMPA receptors is sufficient to induce changes in proliferation. In addition, immunohistochemical analyses of neuronal markers such as Hu and acetylated-tubulin transcriptional analyses of early neuronal marker genes such as polysialylated-neural cell adhesion molecule (*PSA-NCAM*) and achaete-scute homolog 1 (*ascl1*) may reveal the effect of activation of group III mGluRs and/or AMPA receptors on differentiation (Gultekin et al., 2000; Schmidt et al., 2013; Pellegrini et al., 2016). Furthermore, analyzing changes in hypothalamic and telencephalic expression of *cyp19a1b*, *ccnd1*, and *ccna2* following *in vivo* intracerebroventricular injection of glutamate, L-AP4, and an AMPA receptor agonist into the third ventricle of the goldfish brain may lead to a better understanding of glutamate interactions *in vivo*. Proliferative and differentiation studies used for future *in vitro* experiments could also be applied to *in vivo* experiments. Considering that glutamate levels in the brain increase following brain injury, studying hypothalamic and telencephalic *grm6b*, *grm7*, and *grm8b* following *in vivo* stab-lesion assays in goldfish brains may elucidate reactive receptor-related regulatory mechanisms that minimize neuronal damage by taking advantage of elevated glutamate.

2.6. Conclusion

The results presented here offer the first evidence for glutamatergic control of adult goldfish RGCs through activation of group III mGluRs. Sequencing of cloned PCR products confirms the expression of *grm6b*, *grm7*, and *grm8b* in cultured goldfish RGCs. Additionally,

anatomical data shows that RGCs along the ventricular surface are localized closely to glutamatergic synaptic vesicles indicating the potential for glutamate interactions. Furthermore, pharmacological activation of group III mGluRs downregulates the expression of *cyp19a1b*, *ccnd1*, and *ccna2* by canonical inhibition of cAMP/PKA signalling, which potentially acts as a regulatory mechanism to reduce E2 synthesis and cell cycle progression. Considering the role of E2 in inhibiting neuronal proliferation in zebrafish, decreases in *cyp19a1b* expression may allow for increased neurogenic proliferation while decreases in *ccnd1*, and *ccna2* expression may lead to decreased RGC proliferation, increased neuronal fate commitment, and increased neurogenesis (Diehl, 2002; Diotel et al., 2013; Makantasi and Dermon, 2014). Treatment of RGCs with E2 confirms that E2 upregulates *cyp19a1b* as previously shown by Xing et al. (2015, 2016) and *ccnd1* expression. In addition, pharmacological activation of group III mGluRs suppressed E2-mediated increases in *ccnd1* but not *cyp19a1b*. All in all, the regulation of *cyp19a1b*, *ccnd1*, and *ccna2* by glutamate and E2 can potentially affect neurogenic and neurosteroidogenic function of goldfish RGCs. In turn, this could lead to changes in local E2 levels thus affecting neighboring E2-responsive cells (**Figure 2.9**).

Our results do not support the proposed null hypothesis and support the alternative hypothesis that glutamate regulates RGCs, notably via group III mGluR-mediated inhibition of genes implicated in neurogenesis and neurosteroidogenesis. However, further studies regarding the functional significance of expression modulation are necessary to determine whether changes in *cyp19a1b*, *ccnd1*, and *ccna2* mRNA tangibly alter proliferation and differentiation. Although this study lays the foundation for glutamatergic regulation of RGC function, further studies on group III mGluR signalling are also needed to identify the transcriptional regulators downstream of cAMP/PKA signalling that are affecting gene expression. Additional pharmacological studies

related to AMPA receptor signalling must be performed to determine the overall role of glutamate in regulating RGC function. In the brain, RGCs are surrounded by multitude of neurotransmitter-releasing neurons. Dopamine was previously identified as a regulator of RGC function through upregulation of *cyp19a1b* expression via cAMP/PKA signalling (Xing et al., 2015). As a result, the group III mGluR inhibitory signalling mechanism may counteract dopamine signalling. Therefore, pharmacological studies are needed to analyze cross-talk and integration between group III mGluR and D1R signal transduction pathways to further elucidate the mechanisms regulating neurogenic and steroidogenic genes in RGCs. The novel goldfish RGC culture system developed by Xing et al. (2015) and the newly acquired transcriptomic data provided by Da Fonte et al. (2017) are excellent tools for revealing and connecting signalling mechanisms regulating RGC neurogenic and steroidogenic gene expression. In turn, studying multifactorial neuronal and steroidal regulation of RGCs provides a better understanding of the mechanisms involved in regulating adult neurogenesis and neuroregeneration in teleosts.

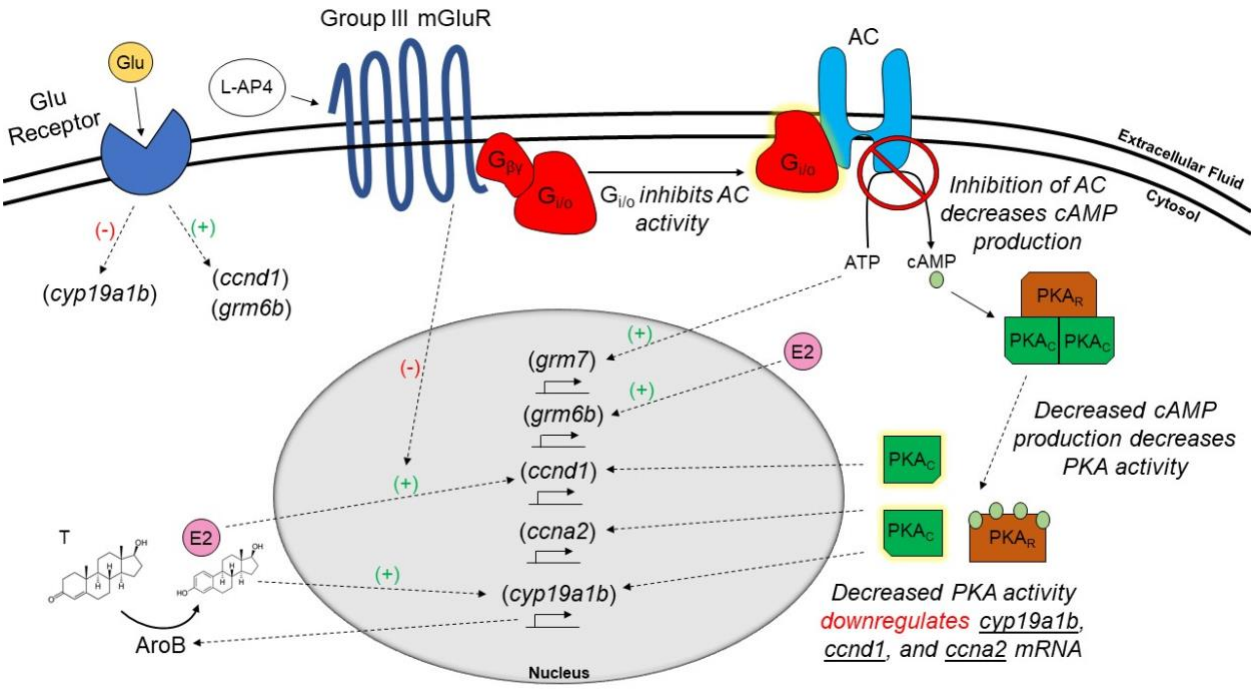

Figure 2.9. Summary of proposed glutamatergic and estrogenic mechanisms regulating *cyp19a1b*, *ccnd1*, *ccna2*, and group III *grm* expression in goldfish RGCs. Activation of group III mGluRs by L-AP4 (10 µM) downregulates *cyp19a1b*, *ccnd1*, and *ccna2* expression via inhibition of cAMP/PKA signalling. Also, cAMP-dependent signalling upregulates *grm7* expression. Also, E2 (1 µM) stimulates *cyp19a1b*, *ccnd1*, and *grm6b* expression. However, E2-induced stimulation of *ccnd1* expression is inhibited by selective activation of group III mGluRs with L-AP4 (10 µM). Glutamate (Glu; 1 mM) increased *ccnd1* and *grm6b* expression but decreased *cyp19a1b* expression.

Appendix

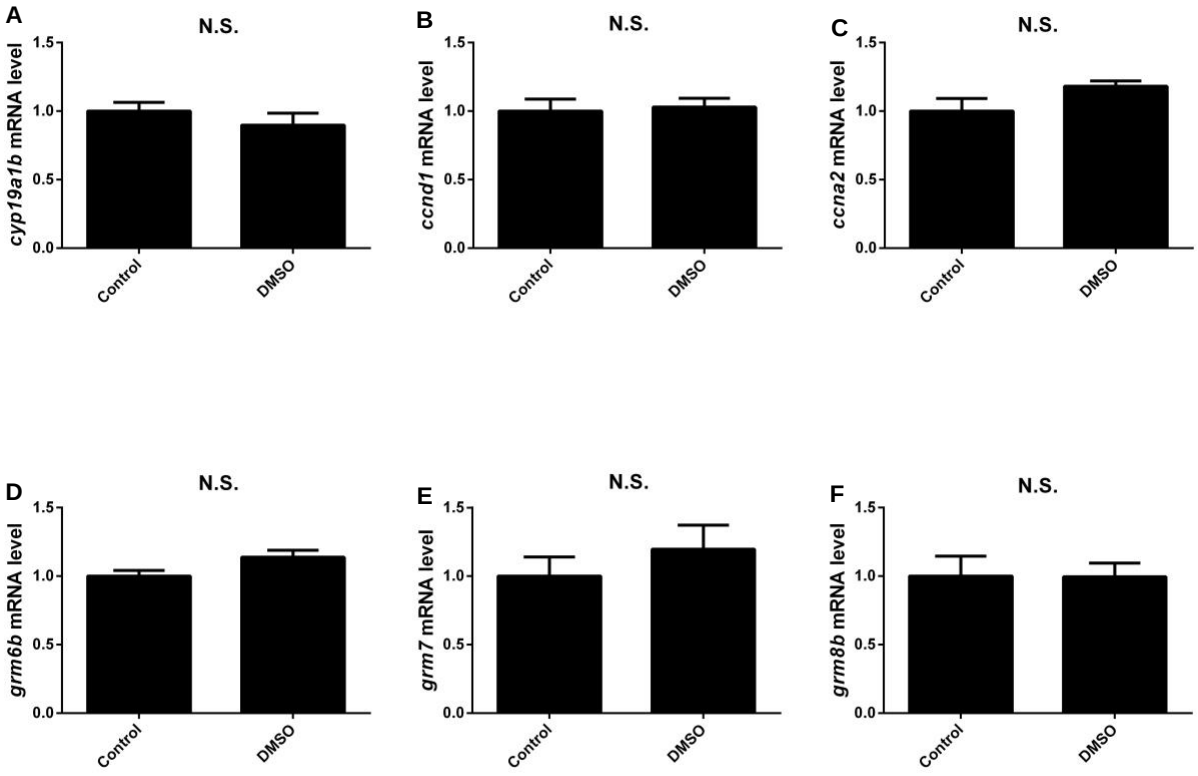

Figure A1. Lack of significant effects of DMSO on *cyp19a1b*, *ccnd1*, *ccna2*, *grm6b*, *grm7*, and *grm8b* mRNA levels in cultured goldfish RGCs. Quantitative real-time PCR analysis showing changes in *cyp19a1b* (**A**), *ccnd1* (**B**), *ccna2* (**C**), *grm6b* (**D**), *grm7* (**E**), and *grm8b* (**F**) mRNA level in primary RGC culture following 48 hr DMSO (0.1%) exposure. Data were normalized to 18S and defined as a fold change relative to control. Bars represent the mean + SEM (n = 4). Data show no statistically significant effect on *cyp19a1b* ($p = 0.384$), *ccnd1* ($p = 0.759$), *ccna2* ($p = 0.111$), *grm6b* ($p = 0.081$), *grm7* ($p = 0.448$), and *grm8b* ($p = 0.940$) mRNA levels.

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