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The functional role of mitochondria in neural progenitor cells

A thesis submitted in partial fulfillment of the requirement  
for the degree of Bachelor of Science in Neuroscience from  
The College of William and Mary

by

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Accepted for Honors

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Williamsburg, VA  
May 5, 2020

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## Abstract

Neurodevelopment is a complex process that requires the precise spatiotemporal control of cellular differentiation. As cells differentiate, they undergo metabolic changes that make them increasingly reliant on their mitochondria for energy production. Beyond playing a central role in the bioenergetic support of newborn neurons, functional changes in mitochondria regulate the processes of cellular differentiation. Importantly, mitochondrial function extends beyond energy production. Mitochondria in neural stem cells play an essential role in both producing intermediates for biosynthesis and regulating calcium levels. In this thesis, I review the functional role of mitochondria in stem cell fate decisions, differentiation, and proliferation during neurodevelopment. Due to the challenges of directly measuring mitochondrial activity *in vivo*, I outline how genetically encoded fluorescent probes can be used to indirectly evaluate mitochondrial function. Beyond my review of this field, I engineered three genetically encoded fluorescent probes that can be used to measure both cytosolic and mitochondrial calcium activity in radial glial cells in *Xenopus laevis*. Preliminary imaging indicates the promising potential for these plasmids in the measurement of calcium activity and, by proxy, mitochondrial activity in differentiating stem cells.

## **Introduction**

### Overview

The regulation of the temporal and spatial proliferation of neurons is fundamental to the proper development of the central nervous system. This proliferation is largely dependent on a complicated sequence of stem cell fate decisions to either self-renew or differentiate. Neural stem cell fate decisions are correlated with metabolic changes within the cell, as well as morphological and functional changes in their mitochondria (Khacho et al., 2019). Growing evidence indicates that mitochondria are in fact critical regulators of stem cell fate decisions (Khacho & Slack, 2017; Homem et al., 2014; Khacho et al., 2019; Wei et al., 2018). Disruptions in mitochondrial function during human gestation have been associated with an increased probability of developmental disorders, such as autism spectrum disorders, attention deficit hyperactivity disorder, and dyslexia (Rash et al., 2018; Khacho et al., 2019). Therefore, investigating the functional role of mitochondria in cell fate decisions, differentiation, and proliferation is central to understanding the development of the central nervous system.

Mitochondria undergo important functional changes as cells differentiate. As neural stem cells differentiate into progenitor cells and neurons, they undergo a metabolic shift from glycolysis to oxidative phosphorylation (Khacho et al., 2019). This metabolic shift can only occur with proper mitochondrial structure and function (Khacho et al., 2019; Rash et al., 2018). The metabolic shift was once thought to occur solely to meet the bioenergetic needs of differentiated cells, but it has become clear that mitochondria play a broader role in development (Homem et al., 2014; Khacho et al., 2019). Although mitochondria are central to producing energy via oxidative phosphorylation, they do not solely act as “the powerhouses of the cell.” Mitochondria have a diverse range of functions, including calcium buffering and storage, metabolite production, signaling molecule synthesis, and regulating nuclear gene expression. Through these many functions, mitochondria play a regulatory role in stem cell fate decisions (Khacho & Slack, 2017). *In vitro* studies indicate that interfering with mitochondrial function in undifferentiated

embryonic stem cells reduces their ability to proliferate and differentiate (Mandal et al., 2011; Khacho et al., 2017). Therefore, while functional mitochondrial changes help support the metabolic needs of newborn neurons, they are also central to driving cell fate decisions and neurogenesis.

Much of the research on mitochondria during neural differentiation has focused on their morphology or locations within stem cells, but very few studies have investigated how *functional* changes in mitochondria influence cell fate decisions. In the studies that do examine mitochondrial function, most have been conducted *in vitro* due to the relative accessibility of chemical and genetic manipulations of mitochondrial activity and the ease of measurement of various markers of mitochondrial function. In order to understand how mitochondrial function affects neurogenesis in living animals, however, there is the need to both identify non-invasive methods with which to measure mitochondrial function at a subcellular resolution, and to identify the limitations of these methods.

Mitochondria play many functional roles in differentiating cells, and therefore measuring their function could entail measuring several aspects of mitochondrial activity. One method is to measure the concentration of adenosine triphosphate (ATP) within the cell, or to measure it within the mitochondria. Several ATP biosensors have recently been developed for use *in vivo*. Another commonly-used method for measuring mitochondrial function is measuring mitochondrial calcium concentration. Elevated mitochondrial calcium levels have been shown to stimulate their production of ATP and other biomolecules (McCormack & Denton, 1989; Llorente-Folch et al., 2015). Biomarkers that report ATP concentration and calcium concentrations are useful *in vivo* tools for measuring functional mitochondrial activity, but each have their particular advantages and limitations.

*In vivo* neurodevelopmental studies are often conducted in the African clawed frog (*Xenopus laevis*). *Xenopus* is a tractable animal model for conducting this research because they have clear stages of external development, and the transparency of albino tadpoles make them suitable for imaging fluorescent biomarkers within cells. The optic tectum, in particular, is a widely-studied brain region for measuring the

proliferation and differentiation of neural progenitor cells. Although this system is not as easily manipulable as *in vitro* systems, certain interventions can be used to modify developmental processes. Visual stimulation, for instance, has been shown to modify rates of progenitor cell proliferation and differentiation within the optic tectum, making it a useful tool to for *in vivo* studies (Bestman et al., 2012; Tremblay et al., 2009; Sin et al., 2002; Aizenman & Cline, 2007). Therefore, *Xenopus* tadpoles are a useful system in which to elucidate how mitochondrial function influences processes of neurodevelopment within living animals.

In this thesis, I aim to address how functional changes in mitochondria influence progenitor cell fate decisions during neurodevelopment and how functional mitochondrial activity can be measured in the *Xenopus* optic tectum. To answer these questions, I created several plasmids with genetically encoded calcium indicators (GECIs) which are targeted to the mitochondria or cytosol of tectal progenitor cells. Using these GECIs and time-lapse imaging, mitochondrial calcium activity can be measured as progenitor cells undergo differentiation. My intention in making these markers was to measure how mitochondrial calcium activity in progenitor cells corresponds to their self-renewal or differentiation.

This thesis will consist of two parts. The first part will be a literature review during neurodevelopment. I will discuss the role of progenitor cells in neurodevelopment, and then focus specifically on mitochondrial dynamics in progenitor cells. Because my research focused on measuring calcium activity in progenitor cells as a means of elucidating mitochondrial activity, I will also discuss the broad roles of calcium activity in progenitor cells. Lastly, I will examine current techniques for measuring mitochondrial activity *in vivo* and discuss their strengths and limitations. This first section will serve as the main part of this work. The second part will focus on the research that I conducted prior to the coronavirus pandemic. In this section, I will detail my research methods and analyze my preliminary findings. In the discussion, I will suggest next steps for this research project.



## The role of progenitor cells in neurodevelopment

Stem cells are undifferentiated cells that can divide to either give rise to more stem cells or to differentiated cell types. There are two types of stem cells: pluripotent and multipotent. Pluripotent stem cells are cells that divide indefinitely and can give rise to every other cell type. For instance, embryonic stem cells are a kind of pluripotent stem cell. On the other hand, multipotent stem cells have a limited number of divisions and can only differentiate into a subset of cell types. Multipotent stem cells in the nervous system are called neural progenitor cells (NPCs). NPCs are found in both the developing and adult brain, and they give rise to the neurons and glia of the central nervous system. Since the discovery of neural stem cells, there has been extensive research to understand the internal and external mechanisms that regulate their function.

In embryonic vertebrates, the ventricular zone (VZ) is the primary area from which stem cells proliferate. Newborn neurons must travel long distances from the VZ to reach their final topographic target in the brain. The cells that populate the VZ are called “radial glial cells” (RGCs). Historically, RGCs were considered to mainly play a structural role during neuronal migration by guiding newborn neurons to their proper topographic positions, but it has become increasingly clear that they are also NPCs that can differentiate into glia and neurons (Rash et al., 2018; Bestman et al., 2012; Tremblay et al., 2009). Initially in development, RGCs predominantly undergo symmetric cell division, which produces more neural progenitors and expands the pool of undifferentiated cells. Later on, RGCs undergo asymmetric cell division to give rise to daughter cells that are committed to specific neuronal fates.

After producing daughter cells, other RGCs play an important role in guiding their migration toward the surface of the brain. RGCs have a distinct morphology, with their cell bodies situated in the VZ and with radial processes that terminate as endfeet at the pial surface. This morphology is central to allowing immature neurons, known as neural precursor cells, to crawl along radial processes and arrive at their proper destination. Even so, RGCs do not serve as a passive structural scaffold during development.

RGCs have been found to actively participate in synapse formation amongst newborn neurons and, conversely, feedback mechanisms from neurons can regulate RGC fate decisions (Sharma & Cline, 2010). In the *Xenopus* optic tectum, RGCs have been found to respond structurally and functionally to visual stimulation-mediated neuronal activity (Tremblay et al., 2009; Bestman et al., 2012). Specifically, increased neuronal activity leads to increased rates of neuronal differentiation and decreased proliferation of RGCs. The interaction between RGCs and neurons, therefore, is an important aspect of development.

These findings raise the question of how external neuronal activity can mediate RGC activity, and ultimately about the molecular mechanisms that influence RGC fate decisions. Recent studies conducted in cortical slices and *in vitro* have indicated that mitochondrial structure, function, and location are in fact key regulators of stem cell fate decisions (Rash et al., 2018; Khacho et al., 2017).

#### Mitochondrial structure and function

The numerous functional roles that mitochondria play in the cell, from ATP production to cell signaling, are highly dependent on their unique morphology. Mitochondria are double membrane-bound organelles that have an outer membrane, intermembrane space, a folded inner membrane, and matrix. The cristae, or the folds of the inner membrane, helps increase the surface area of the inner membrane and are essential in the generation of ATP. Neurons have extremely high metabolic needs compared to other cells, and as such require high rates of ATP synthesis.

Mitochondria synthesize ATP through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). First, pyruvate is produced via glycolysis in the cytosol and enters into the mitochondrion, where it is converted to acetyl CoA. Acetyl CoA is then used in a series of biochemical reactions in the mitochondrial matrix, collectively known as the TCA cycle, to produce a small amount of ATP and the reducing agents NADH and FADH<sub>2</sub>. These reducing agents then move onto the electron transport chain (ETC), which is a series of protein complexes situated in the mitochondrial inner

membrane. The protein complexes in the ETC transfer electrons from electron donors to electron receptors through a series of redox reactions, which are used to pump protons out of the mitochondrial matrix. This electrochemical proton gradient is harnessed to power ATP synthase and generate ATP from adenosine diphosphate (ADP). This process is collectively known as OXPHOS. Interestingly, although stem cells mainly rely on glycolytic metabolism to produce ATP, their mitochondria have the capacity to produce ATP via OXPHOS but are suppressed from doing so (Khacho & Slack, 2017). Undifferentiated stem cells have been found to have high levels of proteins that collectively act to suppress OXPHOS by repressing the entry of pyruvate into the mitochondria, the conversion of pyruvate to acetyl-CoA, ATP synthase activity, and glucose oxidation in the mitochondria (Khacho & Slack, 2017). During differentiation, cells exhibit changes in their protein expression that enable the production of ATP through mitochondrial OXPHOS.

Beyond the production of ATP, mitochondria also play an important role in calcium buffering and therefore contribute to calcium homeostasis within the cell. Mitochondrial calcium levels have been shown to be highly dependent on cytosolic calcium concentration (Kirichok et al., 2004; Rizzuto et al., 2000; Jouaville et al., 1999). Additionally, it has been demonstrated that evoked cytosolic and mitochondrial calcium transients cause an increase in both mitochondrial and cytosolic ATP concentrations (Jouaville et al., 1999). Increased intracellular calcium and mitochondrial calcium can both cause higher rates of ATP synthesis, albeit through different pathways (Llorente-Folch et al., 2015). Increases in cytosolic calcium levels stimulate the transport of pyruvate into the mitochondria, while high mitochondrial calcium levels stimulate the TCA cycle and ATP synthase (Llorente-Folch et al., 2015). However, prolonged periods of elevated mitochondrial calcium levels cause mitochondria to release pro-apoptotic proteins that eventually lead to cell death (Pivovarova & Andrews, 2010). The regulation of mitochondrial calcium concentration, therefore, is fundamental to maintaining cell function and viability.

### Functional and morphological mitochondrial changes in progenitor cells

Several studies have investigated the morphological changes of mitochondria during stem cell fate decisions. *In vitro*, morphological differences have been found in mitochondria in pluripotent stem cells, NPCs, and differentiated cells (Khacho, et al. 2016; Kasahara et al., 2013). Pluripotent stem cells have elongated mitochondria, which then fragment as these stem cells commit to NPC fate, and then re-elongate in post-mitotic neurons (Khacho, et al. 2019). A study performed in cell culture by Khacho et al. (2016) found that induced mitochondrial fragmentation in uncommitted stem cells caused them to metabolically resemble NPCs. These cells expressed lower levels of OXPHOS inhibitors and glycolytic enzymes, showing that mitochondrial morphology can cause changes in gene expression. These studies indicate that morphological changes in mitochondria drive their function and are important for supporting metabolic changes in differentiating stem cells.

Mitochondria also undergo active transport to areas in the cell that have high bioenergetic needs. The active transport of mitochondria is an important process in all cells, but it is especially necessary in RGCs and neurons due to their relatively long lengths. Mitochondria are transported through cellular processes on microtubule tracks and are localized to specific areas of the cell. Several studies have found that, during development, the specific areas to which mitochondria are transported changes dynamically. For instance, after neuronal differentiation, mitochondria tend to initially cluster around the nucleus and then, as the neuron matures, localize to presynaptic terminals. The eventual relocation of mitochondria to active synaptic terminals is essential for supporting synaptic activity via both ATP production and regulating calcium levels (Sheng & Cai, 2012). Additionally, as a neuron migrates to its topographic target in the brain, mitochondria tend to localize to the leading edge of the growth cone (Kim et al., 2015). While there has not been much research on mitochondrial localization in neural stem or progenitor cells, these studies in neurons indicate that the dynamic localization of mitochondria within differentiating cells is important for supporting their changing bioenergetic needs and for regulating subcellular dynamics.

Mitochondria are highly dynamic organelles that undergo fission and fusion events in neural stem cells, which is important for ensuring that post-mitotic neurons receive the proper number of mitochondria. Importantly, mitochondrial dynamics are also associated with stem cell proliferation and differentiation. Fission refers to events in which mitochondria divide to produce two or more daughter organelles, while fusion is the process by which independent mitochondria combine into a single organelle. Fission is regulated by the post-translational modification of dynamin-related protein 1 (Drp1) and fusion is regulated by several proteins that mediate outer and inner mitochondrial membrane fusion (Seo et al., 2018). These proteins play a central role in stem cell fate decisions. For instance, Kim et al. (2015) found that preventing mitochondrial fission by knocking out Drp1 function in adult neural stem cells prevented both neuronal differentiation and migration. Kasahara et al. (2013) found that ablating mitochondrial fusion proteins in embryonic stem cells also prevented differentiation. Another study in human induced pluripotent stem cells and their derived cortical neurons found that fusion protein knock-down led to both mitochondrial functional deficits and decreased neuronal differentiation, while their overexpression in NPCs led to increased rates of differentiation (Fang et al., 2016). Ultimately, these studies indicate the importance of mitochondrial dynamics in regulating neuronal differentiation.

Recent research efforts have begun to elucidate the role of mitochondrial metabolic functions in stem cells, but very few studies have investigated this question specifically in progenitor cells. During differentiation, cells undergo a metabolic shift from glycolysis to mitochondrial OXPHOS to produce larger quantities of ATP (Varum et al., 2011). The metabolic shift supports the physiological needs of differentiated neurons, but that also serves as a regulatory mechanism to drive these stem cell fate decisions (Khacho et al., 2018). NPCs exist in a transitional state between NSCs and fully differentiated cells, and they therefore rely on a combination of aerobic glycolysis and mitochondrial OXPHOS. If differentiating NPCs are prevented from down-regulating the production of enzymes used in aerobic glycolysis, newborn neurons do not survive (Zheng, 2016). Additionally, inhibiting mitochondrial

OXPPOS in self-renewing undifferentiated embryonic stem cells has been found to increase the level of pluripotent transcription factors (Mandal et al., 2011). This points to the importance of the metabolic switch for driving differentiation. Interestingly, this study also found that inhibiting OXPPOS had different outcomes in newly-differentiated stem cells compared to undifferentiated stem cells. It caused differentiated cells to exhibit decreased *Hox* gene expression, which is important for regulating cellular differentiation during normal development (Manal et al., 2011). The effects of attenuating mitochondrial function, therefore, likely has distinct effects in NPCs compared to NSCs.

Lastly, there is still much to learn about mitochondrial calcium activity during neural stem cell differentiation. Some evidence supports the idea that higher mitochondrial calcium levels leads to the induction of Drp1 and mitochondrial fission (Seo et al., 2018). Drp1, as previously discussed, helps regulate neuronal differentiation. It is possible, therefore, that mitochondrial calcium activity might also be important during stem cell fate decisions. More research is needed in this area to further elucidate how mitochondrial calcium levels change as cells differentiate.

While there has been an increased focus on how functional and morphological mitochondrial changes regulate stem cell fate decisions, few studies focus specifically on neural stem cells and even fewer focus on neural progenitor cells. A more explicit focus on NPC mitochondrial activity, particularly *in vivo*, is important for understanding the regulatory mechanisms involved in differentiation and proliferation.

### Calcium activity in progenitor cells

Calcium plays diverse regulatory roles, and is central to orchestrating coordinated cellular behavior during development. Because I specifically focused on using calcium activity as a metric for mitochondrial activity in NPCs, I will spend this section presenting the broad role of calcium in

progenitor cells. Importantly, mitochondria are central to regulating calcium homeostasis and large-scale calcium dynamics within cells.

Progenitor cell populations, particularly RGCs, exhibit waves of calcium activity that propagate across cells. Evidence indicates that these calcium waves play a role in cell proliferation (Weissman et al., 2004; Metea & Newman, 2006). Calcium wave propagation between cells is mediated by extracellular ATP signaling that causes calcium to be released from intracellular stores, such as mitochondria. ATP receptor agonists have been found to abolish coordinated VZ calcium activity across RGCs and ultimately decrease neurogenesis (Weissman et al., 2004). Additionally, the cells that initiate these calcium waves are more likely to be undergoing active DNA synthesis, indicating a connection between increased calcium activity and cell division (Metea & Newman, 2006). *In vivo*, the specific frequency of environmentally-regulated calcium transients have been found to have regulatory effects on certain steps of development, such as growth cone path-finding activity in neuronal migration (Gomez & Spitzer, 1999). Focusing more specifically on RGCs, a study in mouse cortical slices found that propagative calcium waves in RGCs increased during differentiation (Rash et al., 2016). There have been very few studies, however, on how calcium signaling in RGCs regulates neurogenesis, and almost none have addressed this topic *in vivo*.

In the central nervous system, there is rapid and dynamic signaling between neurons and glia, and this signalling is modulated by glial calcium activity. It has been well-established that in the mature mammalian brain, astrocytes, the cell type into which RGCs ultimately differentiate, respond to neurotransmitter release with a corresponding increase in intracellular calcium levels (Tremblay et al., 2009; Haydon, 2001; Hoogland et al., 2009). Tremblay et al. (2009) found that, much like mature mammalian astrocytes, RGCs in *Xenopus* respond to neural synaptic activity with large-scale calcium transients. Additionally, RGCs have been found to undergo rapid structural remodeling in response to neural activity and internal calcium transients (Tremblay et al., 2009). Blocking tectal neuronal activity

with a noncompetitive NMDA receptor antagonist also significantly reduced the number of spontaneous calcium transients in neighboring RGCs (Tremblay et al., 2009). Therefore, while calcium activity within RGCs is central to regulating neurogenesis and neuronal migration, RGC calcium levels are also regulated by neurons.

Beyond this, research also indicates that neuronal activity specifically regulates NPC fate commitments. Deisseroth et al. (2004) found that, in adult hippocampal NPCs, neuronal excitation caused the inhibition of glial fate genes and the simultaneous expression of a transcription factor that regulates neuronal differentiation. Neuronal excitation has been found to influence NPCs by causing calcium influx through L-type calcium channels; blocking L-type calcium currents reduces rates of differentiation (Lepski et al., 2013; Deisseroth et al., 2004). Neural activity-induced calcium activity in NPCs seems to upregulate a gene expression pattern that results in these cells differentiating into neurons rather than glia. This indicates that calcium activity in neural stem cells also seems to influence the type of stem cell fate decisions that are made.

Importantly, calcium transients in radial glia also have been found to regulate the transport and function of mitochondria, both of which are essential for metabolic support (Rash et al., 2018; Mandal et al., 2018). In brain slices, intracellular calcium helps recruit passing mitochondria to radial glial fibers and localizes them to areas with high bioenergetic needs (Rash et al., 2018). Recent research has shown an inverse relationship between calcium concentration and mitochondrial transport, with high local concentrations making it more likely for mitochondria to localize in that area (Rash et al., 2018). Importantly, the failure to properly localize mitochondria can degrade the radial glial scaffold, which can ultimately lead to deficiencies in neuronal migration (Rash et al., 2018). These data indicate that RGC calcium activity is an important regulator of mitochondrial dynamics.

Conversely, mitochondria also regulate calcium dynamics in cells. For instance, disrupting the proton gradient in mitochondria completely eliminates large-amplitude calcium dynamics in RGCs (Rash



et al., 2018). This study, however, used carbonyl cyanide m-chlorophenyl hydrazone, which disrupts OXPHOS but also destroys living cells. In other cell types, mitochondria have been shown to shape cytosolic calcium dynamics by acting as a calcium buffer (Giorgi et al, 2018). Mitochondria likely play this same role in RGCs, although this function has not been rigorously evaluated.

Ultimately, this research indicates the regulatory role of calcium activity in mitochondrial localization, as well as the importance of mitochondrial support in RGC structural dynamics and neurogenesis.

### *In vivo* studies and genetically encoded fluorescent probes

As previously discussed, mitochondria are involved in a wide range of functions, from nuclear gene regulation to metabolism to calcium buffering. Measurements of mitochondrial function, therefore, can be accomplished using a variety of biomarkers which report on the many different aspects of mitochondrial activity.

*In vitro*, several approaches have been developed for measuring various aspects of mitochondrial function, for instance, respiration rate. Tools that measure oxygen levels can be used to measure respiration, although these measurements require specific equipment and generally require isolated cells. Another aspect of mitochondrial function that is often measured is mitochondrial membrane potential ( $\Delta\psi_m$ ).  $\Delta\psi_m$  is the difference between the electrical potential of the cytoplasm and mitochondrial matrix, and is typically 150–180 mV (Brand & Nicholls, 2011). Due to the negative potential of the matrix, cationic vital dyes are the most widely-used indicators to measure  $\Delta\psi_m$ . ATP turnover can also be measured. However, the total amount of cellular ATP does not necessarily report mitochondrial function, since ATP is also used as a signaling molecule in cells (Brand & Nicholls, 2011). The rate of ATP/ADP turnover can therefore be a more accurate metric for measuring mitochondrial function. Other useful metrics to consider include the production of reactive oxygen species, mitochondrial pH levels, calcium

levels, the NADH/NAD<sup>+</sup> redox state, and many others. Ultimately, *in vitro*, performing simultaneous measurements across several of these metrics is considered to be the most informative way to assess mitochondrial function. Although these techniques are useful for *in vitro* experiments, many of these techniques cannot be translated to *in vivo* studies because they both lack specificity and are highly invasive to an intact organism. This has highlighted the need to develop biomarkers that can measure these same metrics of mitochondrial activity.

Fluorescent vital dyes and genetically encoded fluorescent probes (GEFPs) are two different classes of biomarkers that can be employed to measure mitochondrial function *in vivo*. The issues with fluorescent dyes, however, are that they cannot be precisely localized to organelles, and they are often retained solely in the cytoplasm. Additionally, fluorescent dyes do not exhibit stable expression levels over time, particularly within organelles (Suzuki et al., 2016). Importantly, fluorescent dyes also cannot be targeted to specific types of cells, such as radial glia. The lack of specificity and instability of fluorescent dyes, therefore, makes it difficult to employ them in longer-term *in vivo* experiments.

The advent of GEFPs has significantly expanded the possibility of collecting measurements with a high spatiotemporal resolution during *in vivo* experiments. Various varieties of GEFPs have been developed to measure concentrations of ions, metabolites, and second messenger molecules. Many different design methods have been employed to develop GEFPs, but they most commonly consist of one or more fluorescent proteins (FPs) that are fused to a component that interacts with a relevant analyte and causes a conformational change. This conformational change results in some kind of spectral shift that can be quantified. Unlike vital dyes, localization sequences can be inserted into GEFPs so that they are expressed in specific neuronal populations or organelles. GEFPs can also be continuously produced using the machinery of a cell, making them useful for long-term experiments. GEFPs, therefore, are a robust tool for organellar, *in vivo* measurements and will be the primary focus of the following section.

There are two general classes of GEFPs: intensimetric and ratiometric. Single-FP probes are intensimetric and widely used due to their versatility. Upon binding to their target analyte, the probe undergoes a conformational change that causes a change in fluorescence intensity. The fluorescence intensity of an intensimetric indicator is dependent on the analyte concentration, but is also affected by the concentration of the indicator itself, as well as the path length (*i.e.* the width of the cell) (Park & Palmer, 2014). Therefore, an intensimetric indicator does not necessarily accurately measure the quantitative concentration of the target analyte, but can be useful for showing relative changes over time.

Ratiometric indicators can be a more rigorous tool than intensimetric indicators for measuring dynamic changes on a subcellular resolution. These types of indicators exhibit a shift in their absorption or emission spectra when they bind to their target molecule. The readout is therefore the ratio of two intensities. Although ratiometric indicators can use a single FP, they most commonly employ Förster-type resonance energy transfer (FRET) between two different FPs (Marx, 2017). FRET-based probes consist of a donor FP and an acceptor FP, normally located on either side of a sensor domain. The ability for the donor FP to excite the acceptor FP is highly dependent on the distance between the two. Upon binding the target analyte, the protein undergoes a conformational change that brings the two FPs closer together and allows for excitation of the acceptor FP. The readout of these indicators is the ratio of the intensities of emission spectra for the two FPs. As the analyte binds to the sensor region, the fluorescent intensity of one FP increases while the other decreases.

Compared to intensimetric indicators, ratiometric indicators are generally considered to be able to more accurately report quantitative measurements of an analyte because the absorption or emission spectra ratios are independent of the concentration of the indicator (Park & Palmer, 2015). Ratiometric indicators, therefore, are generally the preferred tool *in vivo*. In application, however, they present several challenges. These indicators tend to have lower sensitivity, or smaller dynamic range, than intensimetric indicators (Park & Palmer, 2014). Additionally, ratiometric indicators must be used with microscopes that

are able to absorb or emit at a larger spectral bandwidth, as well as having the ability to acquire images with two combinations of emission and excitation filters. The broader spectral bandwidths of these indicators also makes them difficult to use simultaneously with other fluorescent indicators. Conversely, because intensimetric indicators have narrower spectral bandwidths, they can usually be co-expressed with other indicators. Lastly, the use of ratiometric indicators tends to decrease imaging rates, making them less suitable for time-lapse imaging or for measuring fast activity within a cell (Suzuki et al., 2016). Due to the limitations of ratiometric indicators, therefore, intensimetric indicators can be a more accessible tool for scientists.

#### Measuring cytosolic and mitochondrial calcium activity

While calcium signaling in RGCs has been relatively well-characterized *in vitro*, the role of mitochondrial calcium dynamics during RGC differentiation has not been widely studied in intact animals. In order to do this kind of research, it is necessary to employ robust, stable, and organelle-specific calcium indicators. Genetically encoded calcium indicators (GECIs) have been extensively developed to address these issues. While there are many different varieties of GECIs, single fluorescent protein (FP)-based GECIs have undergone robust structural and functional optimization to make them useful tools for *in vivo* calcium imaging (Suzuki et al., 2016).

The GCaMP family of genetically-encoded calcium indicators are the most commonly used and have undergone the most extensive optimization. GCaMPs are made with a circularly permuted green fluorescent protein (GFP) fused to calmodulin, a calcium-responsive element, and the M13 peptide. The binding of calcium to calmodulin causes de-protonation of the fluorescent protein, which results in a conformational change that emits a fluorescent signal. The numerous iterations of GCaMPs that have been engineered for different experimental conditions makes this a useful family of biosensors for applications across *in vivo* experiments.

An important consideration when choosing a suitable GECI is the specific cellular environment in which it will be used. Additional challenges arise when using an organelle-targeted GECI. A mitochondrial GECI, for instance, cannot be pH-sensitive and must have a lower calcium affinity than cytosolic GECIs. The mitochondrial matrix is normally ~8.0 pH but can undergo sudden pH fluctuations (Suzuki et al., 2016). Many cytosolic GECIs are pH-sensitive and therefore are not ideal for use in the mitochondrion. The concentration of calcium in organelles can also significantly exceed that of the cytosol and undergo large fluctuations. Depending on the experimental conditions, mitochondria can undergo a 10- to 20-fold change in calcium concentrations (Rizzuto et al., 2012). To address this variability, it is best to employ GECIs that have been engineered to have a lower calcium affinity (Suzuki et al., 2014). These challenges must be taken into account when using a GECI to measure mitochondrial calcium activity.

Organellar CEPIAs (calcium-measuring organelle-entrapped protein indicators) are a group of single-wavelength-excitation GECI that were engineered specifically for use in the endoplasmic reticulum and mitochondria (Suzuki et al., 2014). They were engineered by modifying the calcium-binding domains in cytosolic GECIs to reduce its calcium-binding affinity and respond to organellar calcium concentrations. The CEPIA<sub>mt</sub> variants were created by adding a mitochondrial signal sequence to the CEPIA indicators. More recently, red fluorescent versions of this indicator have been developed (Kanemaru et al., 2020). CEPIAs are particularly useful for mitochondrial calcium imaging because they have a large dynamic range and a high signal to noise ratio (Suzuki et al., 2016). Additionally, because CEPIAs are single-FP-based indicators, they can be used simultaneously for multispectral imaging experiments.

Measuring calcium activity can provide useful insights into mitochondrial function. As discussed, numerous studies have found that increased calcium concentrations in both the mitochondria and the

cytosol stimulate the TCA cycle and OXPHOS. Additionally, GECIs have been widely used for *in vivo* studies and have been optimized for different cellular environments.

Using GECIs as a way to measure mitochondrial activity, however, also presents challenges. Calcium plays multiple roles in cells that do not necessarily relate to metabolism. For instance, RGCs exhibit large-amplitude waves of calcium activity that are important for intercellular signaling, and mitochondria play an important role in buffering cytosolic calcium levels. Even so, evidence indicates that increases in either mitochondrial or cytosolic calcium stimulate mitochondrial ATP production. This link might not be as strong, however, in neural progenitor cells that do not solely rely on OXPHOS for ATP synthesis. Beyond stimulating ATP production, mitochondrial calcium stimulates other aspects of mitochondrial function. The production of TCA cycle intermediates, for instance, is also stimulated by calcium levels, and this is an active process even in stem cells (Khacho & Slack, 2017; Llorente-Folch et al., 2015).

Therefore, although calcium plays many roles in stem cells, I chose to use cytosolic and mitochondrial calcium activity as proxies to measure mitochondrial function. I created different plasmids that express cytosolic GCaMPs and CEPIA $mt$  that are targeted to radial glial cells. Each plasmid also codes for the constitutive expression of cytosolic RFP to mark the cell.

There are other metrics that could be used to measure functional mitochondrial activity, however, and in the following sections I will review other ways to measure *in vivo* mitochondrial function.

### Measuring cellular ATP levels

Besides calcium buffering, a fundamental role of mitochondria in mature neurons is to produce adenosine triphosphate (ATP) to support the cell's bioenergetic and signaling needs. In neural stem cells, ATP is primarily generated via glycolysis in the cytosol. As previously discussed, the metabolic shift from aerobic glycolysis to mitochondrial OXPHOS is necessary for the survival of newborn neurons and

is a critical regulator of cell fate. Therefore, measuring ATP levels within a differentiating cell would be useful for tracking metabolic changes during stem cell fate decisions.

Although there has been a relatively recent research focus on how the metabolic shift regulates neurodevelopment, other fields have more rigorously examined metabolic shifts in distinct cell types. In cancer biology, specifically, metabolism has been widely studied. First documented in the 1920s, the “Warburg Effect” refers to how rapidly-proliferating cancer cells use aerobic glycolysis, rather than mitochondrial OXPHOS, as their main source of ATP (Warburg, 1925). “Aerobic glycolysis” refers to the fact that this form of energy production occurs in the presence of oxygen, unlike the typical anaerobic glycolysis. More broadly, the Warburg Effect seems to be present in any highly proliferative cell, including stem cells. There is evidence that the same metabolic programs that enable rapid growth and proliferation in self-renewing stem cells during development are in fact hijacked by cancerous cells (Intlekofer & Finley, 2019; Abdel-Haleem et al., 2017). Therefore, using the extensive research from cancer biology about how cellular ATP levels change during the metabolic switch can potentially give useful insights for studies on ATP levels in neural stem cells.

Several well-established methods exist to image and quantify ATP, although few can be used in living animals. Techniques that can image ATP in living animals include using molecular probes, electrophysiological methods, magnetic resonance spectroscopy, ion channel-expressing “sniff-cells,” and firefly luciferase (Lobas et al., 2019; Lalo et al., 2014; Rajendran et al., 2016; Brand & Nicholls, 2011). Many of these methods lack spatial resolution, are invasive, cannot be easily used *in vivo*, need specific imaging equipment, and have off-target effects. Most importantly for cellular-level *in vivo* studies, all of these methods lack subcellular resolution and they cannot target specific neuronal subtypes or subcellular compartments (Lobas et al., 2019). In response to these limitations, genetically-encoded fluorescent ATP biosensors have been developed. These biosensors include ATeam, QUEEN, Perceval, PercevalHR, and iATPSnFR. Unlike GECIs, genetically-encoded ATP biosensors have not been extensively reviewed due

to their relative novelty. The advantages and limitations of each genetically-encoded ATP biosensor will be examined in greater detail below.

*Table 1. Genetically-encoded fluorescent ATP biosensors*

Name	Type	Fluorophore	ATP- responsive element	Reference
Perceval	ratiometric	mVenus	GlnK1	Berg et al., 2009
PercevalHR	ratiometric	mVenus	GlnK1	Tantama et al., 2013
ATeams (GO-ATeam, AT1.03NL, mitoAT1.03, ERAT1.03)	FRET	mseCFP and mVenus	bacterial F <sub>0</sub> F <sub>1</sub> -ATP synthase $\epsilon$ subunit	Imamura et al., 2009; Nakano et al., 2011; Tsuyama et al., 2013; Vishnu et al., 2014
QUEEN	ratiometric	EGFP	bacterial F <sub>0</sub> F <sub>1</sub> -ATP synthase $\epsilon$ subunit	Yaginuma et al., 2014
MaLionR	intensiometric	mApple	bacterial F <sub>0</sub> F <sub>1</sub> -ATP synthase $\epsilon$ subunit	Arai et al., 2018
MaLionG	intensiometric	mCitrine	bacterial F <sub>0</sub> F <sub>1</sub> -ATP synthase $\epsilon$ subunit	Arai et al., 2018
MaLionB	intensiometric	--	bacterial F <sub>0</sub> F <sub>1</sub> -ATP synthase $\epsilon$ subunit	Arai et al., 2018
iATPSnFR	intensiometric	sfGFP	bacterial F <sub>0</sub> F <sub>1</sub> -ATP synthase $\epsilon$ subunit	Lobas et al., 2019

Perceval was one of the first genetically-encoded fluorescent biosensors that was engineered to detect the ADP to ATP ratio in cells. Berg et al. (2009) engineered Perceval by combining a circularly permuted variant of green fluorescent protein with GlnK1, a bacterial regulatory protein which endogenously undergoes a conformational change upon binding Mg-ATP. Perceval is a ratiometric indicator that fluoresces yellow in its unbound state, but the excitation spectra shift upon ATP binding. The sensor also binds ADP, but ADP does not cause a conformational change in the GlnK1 and therefore does not produce the same level of fluorescence response. Because the sensor has an approximately 5-fold



higher affinity for ATP than for ADP, the response level of the biosensor allows the determination of the ATP to ADP ratio. Tantama et al. (2013) optimized Perceval to produce PercevalHR, which is better tuned to respond to the ADP and ATP levels in mammalian neurons. While the measurement of the ATP to ADP ratio rather than the ATP concentration can be a disadvantage, it may be advantageous specifically for analyzing the metabolic switch during neurodevelopment. Research indicates that glycolytic cells have lower cytosolic ATP/ADP ratios compared to cells that mainly use OXPHOS (Maldonado & Lemasters, 2014). This could make Perceval an optimal sensor for examining metabolic changes during differentiation.

To date, the most widely-used ATP probes are a series of FRET-based indicators called ATeams (Imamura et al., 2009). These indicators consist of the  $\epsilon$  subunit of the bacterial  $F_0F_1$ -ATP synthase fused to yellow-fluorescent and cyan-fluorescent proteins. Upon ATP binding, the  $\epsilon$  subunit undergoes a conformational change that causes a corresponding fluorescence spectral change. Additionally, unlike Perceval, these biosensors are not pH-sensitive and are therefore suitable for measuring ATP in various subcellular compartments (Imamura et al., 2009). This family of sensors has undergone relatively extensive optimization and can be targeted to the endoplasmic reticulum and the mitochondria (Imamura et al., 2009; Depaoli et al., 2018; Yoshida et al., 2017).

QUEEN (quantitative evaluator of cellular energy) is a single-FP ratiometric indicator that can detect total ATP levels within a cell (Yaginuma et al., 2014). It is similar to ATeams in that it uses the bacterial  $F_0F_1$ -ATP synthase  $\epsilon$  subunit as its ATP-responsive element, but has not undergone the same level of optimization as ATeams.

Very recently, intensimetric ATP sensors have been developed (Arai et al., 2018; Lobas et al., 2019). These sensors present exciting new research avenues, since their narrow spectral bandwidths make it possible for simultaneous use with other intensimetric GEFs. iATPSnFR was developed by Lobas et al. (2019) using the bacterial  $F_0F_1$ -ATP synthase  $\epsilon$  subunit and superfolder GFP. Importantly, this probe

was found to be responsive to the ATP levels found in cultured neurons and glia, although this has not been verified *in vivo*. iATPSnFR exhibits a modest pH sensitivity, making it potentially problematic for use in subcellular compartments, although this also has not been tested.

MaLionR/G/B (monitoring ATP level intensiometric turn-on Green/Red/Blue) indicators are another family of intensiometric ATP sensors that have been developed specifically for multispectral imaging experiments (Arai et al., 2018). These same sensors have been used to study the mitochondria by appending a mitochondrial targeting signal sequence to the N-termini of the various MaLions to create a family of mitoMaLions (Arai et al., 2018). Significantly, this research group was able to simultaneously express a cytosolic MaLion and an mitoMaLion to elucidate rates of OXPHOS vs glycolysis. They compared OXPHOS rates proliferating HeLa cells as compared to non-proliferating murine brown adipocytes and experimentally confirmed the Warburg effect in HeLa cells. This is an exciting use of intensiometric ATP sensors that has vast potential for measuring the metabolic switch in differentiating NPCs.

While ATP sensors are a promising avenue of future research on mitochondrial function during development, they alone are not a sufficient measurement tool. “Mitochondrial function” is typically defined as the ability of mitochondria to synthesize ATP, but, as discussed, mitochondria still play an important role in biosynthesis even in the absence of ATP synthesis. Mitochondria still produce TCA cycle intermediates even in the absence of electron transport chain function (Khacho & Slack, 2017). This is particularly important in stem cells in which the capacity for mitochondria to produce ATP is actively repressed. “Functionality” encompasses many metrics, and solely measuring ATP levels is not necessarily a sufficient indicator of whether mitochondria are functional during development.

Ultimately, measuring mitochondrial calcium activity and ATP levels are two different ways to elucidate functional mitochondrial activity *in vivo*, but neither can directly elucidate mitochondrial activity. Ideally, an experiment would simultaneously employ several measurement techniques. The

advent of intensiometric ATP sensors opens up exciting avenues for future research incorporating both calcium activity and ATP levels to evaluate changes in mitochondrial function.

In my research, I used genetically-encoded calcium indicators as a potential proxy for mitochondrial function. In the following sections, I will detail my plasmid design for studying cytosolic and mitochondrial calcium activity in RGCs in the *Xenopus* optic tectum. Although I did not obtain extensive results, I will discuss future ways these tools can be employed to study mitochondrial activity in RGCs.

## Materials and Methods

### Cloning and Plasmids

Table 2. Plasmids

<b>Construct</b>	<b>Description</b>
pGP-CMV-jGCaMP7b	pGP-CMV-jGCaMP7b was a gift from Douglas Kim & GENIE Project (Addgene plasmid #104484; <a href="http://n2t.net/addgene:104484">http://n2t.net/addgene:104484</a> ; RRID:Addgene_104484)
pGP-CMV-jGCaMP7s	pGP-CMV-jGCaMP7s was a gift from Douglas Kim & GENIE Project (Addgene plasmid #104463; <a href="http://n2t.net/addgene:104463">http://n2t.net/addgene:104463</a> ; RRID:Addgene_104463)
pDONR221	Tol2Kit middle donor vector; gift from Kwan et al.
pME-GCaMP7b	Middle entry plasmid
pME-GCaMP7s	Middle entry plasmid
pDestTol2pA2	Tol2Kit destination vector; gift from Kwan et al.
p5E_sox2bd_fgf-14xgal4-vp16-UAS	Sox2bd and gal4-UAS
pME-CEPIA4mt	Middle entry CEPIA4mt
p3E_2a_tagRFP-T	tagRFP-T and P2A site
pSOX2-GCaMP7b-tagRFPT	Cytosolic GCaMP7b and tagRFPT co-expression targeted to progenitor cells
pSOX2-GCaMP7s-tagRFPT	Cytosolic GCaMP7s and tagRFPT co-expression targeted to progenitor cells
pSOX2-CEPIA4mt-tagRFPT	Mitochondrial green fluorescent calcium indicator and cytosolic tagRFPT co-expression targeted to progenitor cells

Table 3. Primers

<b>Primer</b>	<b>Description</b>
103_calmodulin_F	GCaMP sequencing forward primer
83_gcamp7s_attb1F	forward attB1 PCR primer

84_gcamp7s_attb2R	reverse <i>attB2</i> PCR primer
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*pME-GCaMP7b and pME-GCaMP7s entry plasmid construction*

Middle entry clones with jGCaMP7b and jGCaMP7s inserts were created. The jGCaMP7 sensors are optimized forms of GCaMP6 sensors, and have been engineered for specific kinds of *in vivo* imaging (Dana et al., 2019). jGCaMP7b (Addgene plasmid #104484) has an improved signal to noise ratio and a higher baseline fluorescence than its GCaMP6 counterpart. The high baseline fluorescence is useful for interpreting the calcium activity in sparsely-transfected samples. jGCaMP7s (Addgene plasmid #104463) also has an improved signal to noise ratio while exhibiting higher sensitivity and slower kinetics than GCaMP6s.

Using the Invitrogen Gateway cloning guidelines, forward PCR primer 83\_gcamp7s\_attb1F was designed to add an *attB1* site to the 5' end of the GCaMP inserts, and reverse PCR primer 84\_gcamp7s\_attb2R was designed to add an *attB2* site to the 3' end. *attB* sites are indicated in bold:

FWD: 5' - **GGGGACAAGTTTGTACAAAAAAGCAGGCT**taccggactcagatctcg - 3'

REV: 5' - **GGGGACCACTTTGTACAAGAAAGCTGGGT**acttcgctgtcatcattgtac - 3'

PCR amplification was carried out with *attB*-containing PCR primers and jGCaMP7b and jGCaMP7s templates. The PCR reaction was performed in 32 cycles using Platinum™ II Taq Hot-Start DNA Polymerase and 5X Platinum™ II PCR Buffer (ThermoFisher), with an annealing temperature of 55°C.

After producing and purifying the *attB*-PCR products, a BP recombination reaction was conducted to transfer the two separated GCaMP inserts into the Tol2Kit pDONR221 donor vector to create two middle entry plasmids (pME-GCaMP7b and pME-GCaMP7s). pME-GCaMP7b and pME-GCaMP7s were both restriction digest and sequencing confirmed.

All site-specific recombination-based cloning (Gateway cloning) was carried out using the Tol2 transposon transgenesis constructs (Kwan et al., 2007). The Tol2Kit system facilitates zebrafish transgenesis by using a modular cloning system, making these plasmids amenable for use in zebrafish as well as *Xenopus*.

#### *pSOX2-GCaMP7b-tagRFPT and pSOX2-GCaMP7s-tagRFPT plasmid construction*

The expression plasmid was assembled using a MultiSite Gateway LR recombination reaction. The following entry clones were used in this reaction: p3E\_2a\_tagRFP-T as the 3' element, p5E\_sox2bd\_fgf-14xgal4-vp16-UAS as the 5' element, and pME-GCaMP7b as the middle element. pDestTol2pA2 from the Tol2Kit was used as the destination vector.

The 5E\_sox2bd\_fgf-14xgal4-vp16-UAS plasmid contains 6 repeats of the Sox2 binding domain (Sox2bd) and a minimal fgf4 promoter. It also has a Gal4-UAS system with 14 repeats of the upstream activating sequence (UAS). These elements are flanked by *attR1* and *attL4* recombination sites.

The p3E\_2a\_tagRFP-T plasmid contains a self-cleaving P2A peptide, which is used to create multicistronic expression vectors, and tagRFP-T, a constitutively fluorescent red fluorescent protein. These elements are flanked by *attR2* and *attL3* recombination sites.

The final plasmid, pSOX2-GCaMP7b-tagRFPT, contains a Sox2bd-minimal fgf4 promoter which controls the Gal4 transcriptional activator. Gal4 then binds to the UAS enhancer, which drives the co-expression of GCaMP7b and tagRFP-T.

The sequence of pSOX2-GCaMP7b-tagRFPT was confirmed both by restriction digest and sequencing.

The same assembly process was used to create pSOX2-GCaMP7s-tagRFPT, except with pME-GCaMP7s as the middle element in the recombination reaction. This plasmid was also sequencing and restriction digest confirmed.

### *pSOX2-CEPIA4mt-tagRFPT plasmid construction*

This plasmid was also assembled using a MultiSite Gateway LR recombination reaction. The following entry clones were used in this reaction: p3E\_2a\_tagRFP-T as the 3' element, p5E\_sox2bd\_fgf-14xgal4-vp16-UAS as the 5' element, and pME-CEPIA4mt as the middle element. pDestTol2pA2 was used as the destination vector.

CEPIA4mt is a calcium-measuring organelle-entrapped protein indicator developed by Suzuki et al. (2014), which has a high calcium binding affinity and includes a mitochondrial targeting signal.

The sequence of the final plasmid product, pSOX2-CEPIA4mt-tagRFPT, was confirmed both by restriction digest and sequencing.

### Animal Use

Tadpoles were obtained from mating *X. laevis* injected with 0.6ml of 1000units/ml human chorionic gonadotropin. Tadpoles were reared in incubators on a 12 hour light/dark cycle at 23°C and staged using the guidelines outlined by Nieuwkoop and Faber (1994). All animal care procedures were carried out in accordance with the guidelines of the IACUC committee at William & Mary.

### Tectal cell transfection

Tectal cells were transfected using the electroporation protocol outlined by Haas et al. (2002). Stage 44–48 tadpoles were anesthetized with 0.02% 3-aminobenzoic acid ethylester (MS-222). The DNA solution was mixed with ~1% Fast Green Dye (Sigma) and loaded into a glass micropipette. The micropipette was inserted into the midbrain ventricle and the solution was pressure injected. Metal electrodes were placed on either side of the brain and three 40V, 1.6 ms pulses at each polarity were

delivered to sparsely transfect tectal cells. After electroporation, the animals were returned to their rearing solution to recover.

Animals were screened 24 hours after electroporation for plasmid expression using a fluorescence microscope. If an animal expressed the plasmid, it was transferred into an individual well on a 6-well plate with Steinberg's buffered solution (58.0 mM NaCl, 0.67 mM KCl, 0.34 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O, 1 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, and 4.62 HEPES).

### Microscopy

Images were acquired using a spinning disk confocal microscope. Tadpoles were anesthetized with 0.02% MS-222 and placed into a custom-sized mold for imaging. Red fluorescent proteins were excited at 561 nm and green fluorescent proteins were excited at 488 nm. Timelapse imaging was conducted only with the 488 nm channel and 1-2 μm Z-steps.



## Results

Fluorescence imaging was conducted to verify the expression of pSOX2-GCaMP7s-tagRFPT in tadpoles (Figure 1A-C). Tectal radial glial cells were sparsely transfected, allowing individual cells to be analyzed. The cell body of radial glia is located in the ventricular zone, with a slender radial process extending outward terminating as an endfoot at the pial surface of the brain. Red fluorescent protein (RFP) generally labels the cytosol of radial glia, while GCaMP7s fluorescence changes in response to cytosolic calcium concentrations.

Figure 1. Radial glia expressing pSOX2-GCaMP7s-tagRFPT

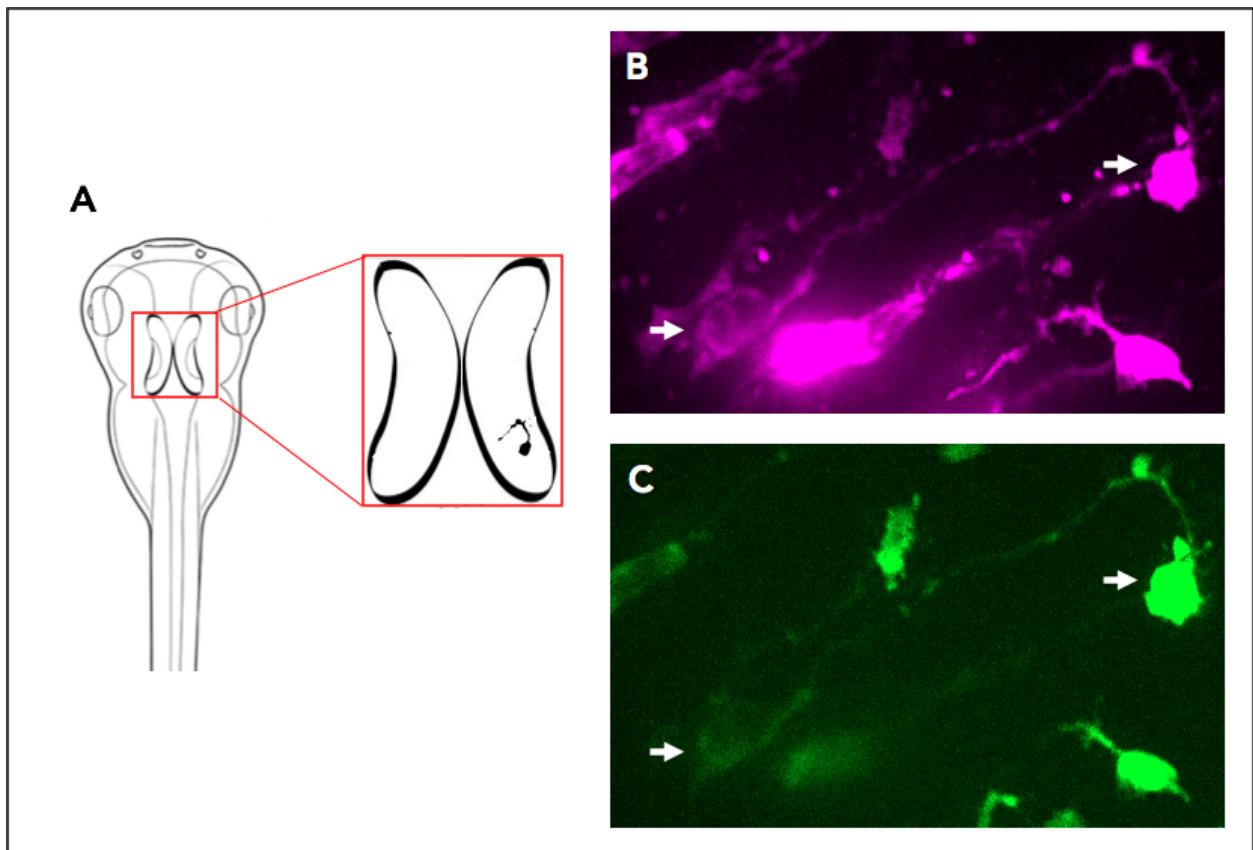


Figure 1A shows the midbrain in a *Xenopus* tadpole (diagram modified from Zahn et al., 2017). The enlarged view shows the location of a radial glial cell in the optic tectum. RFP and GCaMP were co-expressed in radial glial cells in the right optic tectum, although the expression levels of each

fluorescent protein differed along the length of the cells (Figure 1B-C). Specifically, Figure 1B shows that RFP was expressed throughout the cell, although the protein was most concentrated in the endfoot (top right of the image). Figure 1C shows that GCaMP7s exhibited higher fluorescence in the endfoot, and, relative to the RFP, had low levels of fluorescence in the cell body (bottom left of the image). The lower fluorescence of GCaMP could either indicate an uneven distribution of the biomarker or an unequal distribution of calcium in the cell. The endfoot is located in the most superficial part of the tectum, which could partially explain why it appears to be brighter, but would not completely explain the difference in fluorescence levels across the cell. Conclusions cannot be drawn, however, from a single imaging experiment; further tests of the expression of this plasmid must be conducted to evaluate whether this unequal distribution is consistent.

*Figure 2. Time-lapse of calcium movement in radial glia expressing pSOX2-GCaMP7s-tagRFPT*

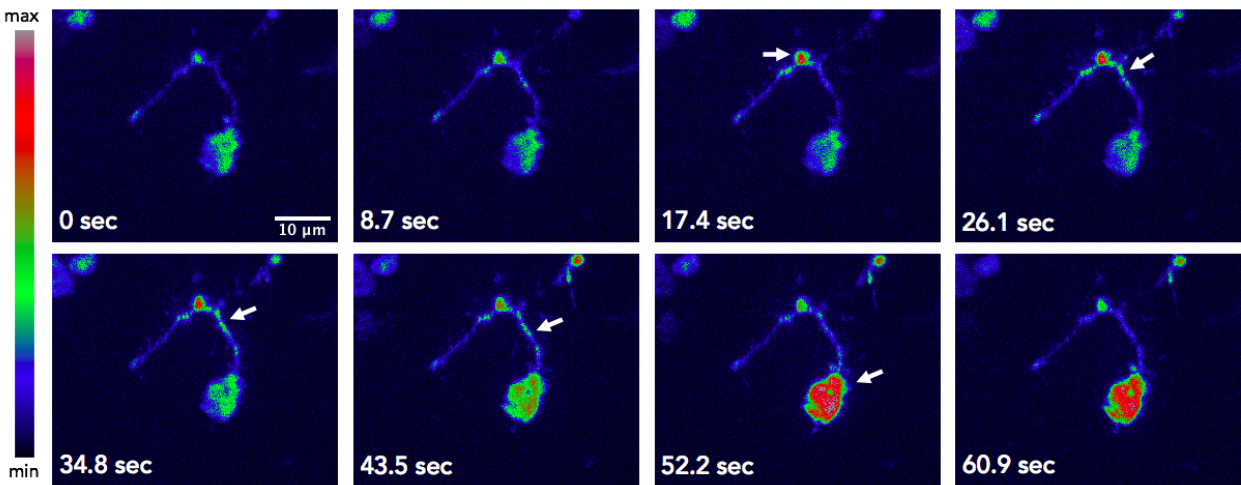


Figure 2 is a time-lapse of the local changes in calcium concentration in a radial glial cell, as indicated with GCaMP7s fluorescence. The time-lapse spans over approximately 1 minute and shows a calcium transient moving through the radial process of the cell and into the endfoot. The cell body did not exhibit a high enough level of fluorescence intensity to be observable in these images, but is situated in the bottom left of each frame. Its location can be seen in Figures 1B-C. In these images, areas of high fluorescence intensity and, by extension, high calcium concentration have been artificially colored to

appear red or green. Areas of low calcium concentration appear blue. At 17.4 seconds, a specific section of the radial process exhibits a sudden increase in concentration (indicated with an arrow). This wave moves down the radial process and terminates in the endfoot.

RGC calcium transients in cortical slice cultures have been found to be mainly bidirectional (Rash et al., 2016). That is, they initiate in the middle of the cell and then propagate anterogradely and retrogradely outwards. The calcium transient in Figure 2 does appear to originate in the middle of the cell and propagate outward, although it is difficult to determine this in the absence of a visible cell body. This could be a potential initial indication of bidirectional calcium transients in RGCs *in vivo*.

## Discussion

The goal of this thesis was to engineer biomarkers to label mitochondrial and cytosolic calcium activity in radial glial cells. My intention was to use these biomarkers to measure mitochondrial calcium activity as a proxy for functional mitochondrial activity, and to see how calcium activity corresponds to differentiation in radial glial cells.

### Next steps

The expression of pSOX2-GCaMP7s-tagRFPT in radial glial cells was experimentally confirmed. The immediate next steps in this research will be to verify whether pSOX2-GCaMP7s-tagRFPT and pSOX2-CEPIA4mt-tagRFPT also can be expressed in RGCs. Additionally, identifying the optimal imaging parameters will be essential to future research. When performing time-lapse measurements, there is a tradeoff between z-dimension resolution and the size of the time intervals. Faster measurements require poorer resolution, and it will be necessary to identify the best balance between resolution and speed for capturing fast calcium dynamics within the cell. Additionally, identifying the optimal biomarker for tracking cytosolic calcium dynamics is important. GCaMP7s has high sensitivity to calcium but slow kinetics relative to GCaMP7b. It will be important to test whether the faster kinetics of GCaMP7b are more suitable for the time scale of calcium transients in RGCs, or whether it is better to use GCaMP7s to maintain a higher sensitivity and larger dynamic range.

There have not been previous studies that have examined mitochondrial calcium in RGCs in live *Xenopus*, so the expected calcium concentrations in this environment are unknown. Therefore, it will be important to verify whether the mitochondrial calcium biomarker, CEPIA4mt, has an appropriate binding affinity to be able to detect the calcium concentrations in RGC mitochondria. There are other varieties of CEPIAmt that have lower binding affinities, allowing them to detect lower calcium concentrations. If the

CEPIA4mt construct does not visibly mark the mitochondria in transfected RGCs, then a different mitochondrial calcium biomarker should be used.

Most of the previous studies that have employed time-lapse imaging to track RGC differentiation have used a 2-photon microscope. The images in this thesis were collected with a spinning disk confocal microscope, which uses lasers to excite the fluorescent proteins. A potential concern is that the laser used in longer time-lapse experiments, the light of which can be seen by the tadpole, might excite visual sensory circuits and affect calcium transients in RGCs. In the optic tectum, visual stimulation has been shown to drive neurogenesis and increase synapse formation in nascent neurons (Bestman et al., 2012; Tremblay et al., 2009; Aizenman & Cline, 2007). Growing evidence indicates that visual activity also evokes calcium transients in RGCs, even though RGCs do not directly receive visual inputs (Bestman et al., 2012; Tremblay et al., 2009). These findings support the relationship between neuronal synaptic activity and radial glial calcium transients. The imaging technique itself, therefore, could possibly cause increased calcium activity in RGCs that might confound measurements.

A potential way to verify whether the imaging itself evokes calcium transients in RGC would be to block excitatory transmission in neurons. Excitatory transmission in the tectum can be blocked with NMDA and AMPA receptor antagonists, such as MK801 and GYKI-54266. RGCs do not express these receptor types and would thus be unaffected, but would no longer receive excitatory inputs from neighboring neurons (Tremblay et al., 2009). To evaluate baseline levels of RGC calcium activity, it would be useful to measure initial calcium transients in a tadpole and compare them to calcium transients when excitatory transmission is inhibited.

### Future directions

As previously discussed, measuring mitochondrial calcium activity can be a useful proxy for mitochondrial function, but, like any *in vivo* method, it cannot paint the full picture. During development,

mitochondrial function goes beyond ATP production; the tricarboxylic acid cycle is important for producing intermediates that are necessary for biosynthesis in the cell (Khaco & Slack, 2017). Therefore, measuring ATP levels alone is not sufficient to understand mitochondrial activity in stem cells. Increases in mitochondrial calcium are useful to measure because they stimulate both the TCA cycle and OXPHOS, and therefore could give insight into many aspects of mitochondrial function that are important during development (Llorente-Folch, 2015). However, additionally measuring mitochondrial ATP levels would give distinct, and useful, information about mitochondrial function. Particularly in progenitor cells, measuring the levels of ATP within the mitochondria would help give insights into the metabolic switch in differentiating cells *in vivo*. This is a metric of mitochondrial function that cannot be directly elucidated by tracking calcium activity.

Ideally, a study would simultaneously track mitochondrial ATP and calcium levels. This would give a more complete characterization of mitochondrial function than either measurement could alone. Studies using the microscope available in our lab are currently limited to red and green channels, but the planned addition of a third channel will expand the possibility to perform multispectral imaging experiments. Newly-developed intensiometric indicators present a promising avenue for tracking mitochondrial function *in vivo*, and for expanding our understanding of how mitochondrial function affects neural progenitor cell differentiation.

Mitochondria have been shown to be central players in the development of the central nervous system. Ultimately, elucidating how mitochondrial function regulates stem cells in the living brain is essential to understanding the complexity of stem cell fate decisions, differentiation, and proliferation during neurodevelopment.

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