EXAMINING THE ROLE OF WNT/β-CATENIN SIGNALING DURING BLOOD-BRAIN BARRIER FORMATION

By

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Under the supervision of Michael R. Taylor

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DEDICATION

To my students past, present, and future without whom none of this would have been possible.

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ABSTRACT

The blood-brain barrier has been studied for 135 years. Over this time, we have learned more about its unique properties and how it forms. While we now know things about properties of the blood-brain barrier its development and role in diseases remains to be elucidated. The most fundamental component of the blood-brain barrier is differentiated brain endothelial cells. These endothelial cells are distinct from peripheral blood vessels in their expression of “barrier properties” such as GLUT1. Differentiated brain endothelial cells serve as a protective layer for the brain against harmful substances in systemic circulation. A signaling pathway known to be involved in blood-brain barrier formation is Wnt/β-catenin signaling; this pathway is well characterized in brain endothelial cells. However, much remains to be explored in other CNS cell types during blood-brain barrier development. Here, I provide evidence that increasing Wnt/β-catenin signaling in radial glial cells disrupts blood-brain barrier development. Additionally, I provide evidence that increasing Wnt/β-catenin signaling in radial glia, but not neurons, causes an inflammatory response within the CNS. Taken together, my results indicate that perturbing Wnt/β-catenin signaling in radial glial cells has profound effects on blood brain barrier development and CNS development. Future studies will determine mechanistically why we observe these phenotypes when we increase Wnt/β-catenin signaling in radial glial cells. This foundational understanding will allow us to further interrogate this pathway in a cell-type specific manner, ultimately allowing us to understand the developmental signals involved in the formation of the blood-brain barrier.

CHAPTER I: INTRODUCTION

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Abstract

The initial work that lead to the field of blood-brain barrier study was published 135 years ago. Since then knowledge of these unique blood vessels within the brain has exploded. For example, we now know these vessels have unique properties when compared to systemic vessels which makes these vessels specialized to prevent harmful substances or cells in systemic circulation from accessing the brain. Despite, this growth the field is still working to elucidate the developmental pathways involved in the development of the blood-brain barrier. Currently, the best understood of the pathways involved in blood-brain barrier formation is Wnt/β-catenin signaling. Recently, scientists and clinicians have begun collaborating to harness the Wnt/β-catenin pathway in the hopes of temporarily disrupting the blood-brain barrier to allow for therapy delivery. However, more work is needed to understand the possible off-target effects of such an intervention as well as further characterizing the Wnt/β-catenin pathway in the blood-brain barrier from a developmental standpoint. My thesis seeks to further characterize the role of Wnt/β-catenin signaling from an endothelial cell nonautonomous point of view to see how that influences both the establishments of the blood-brain barrier and the welfare of my animal models in the hopes of informing future basic and clinical work.

Introduction

History of Blood-Brain Barrier Research

The barrier properties of the brain vasculature were not initially apparent. The first experiments indicating there was something unique about the blood vessels in the brain were done by injecting colored dyes into rodent circulation and noticing that while other tissues had dye leakage into their parenchyma the brain parenchyma remained free of dye (Ehrlich, 1885). Subsequent studies found that injection of dyes into the rodent brain ventricle led to a lack of leakage outside of the central nervous system (Goldmann, 1909, 1913) Together these studies indicated that the brain had vasculature that was unique compared to the rest of the body. The brain vasculature had a barrier that the dye could not pass through, the “blood-brain barrier”. These early methods continue to be used to determine if a certain disease model has a “leaky” blood-brain barrier (Bellavance et al., 2008; Su et al., 2008).

Despite the intrigue of these initial observations, the field was stagnant waiting for new technology and innovation to enable additional understanding of the blood-brain barrier. There have been calls for new tools and innovation to help further our understanding of these unique blood vessels (Saunders et al., 2008). This call for new tools and models has been answered in part by the emergence of zebrafish as a model to study the blood-brain barrier. Zebrafish have been used broadly to answer questions of development because they are a vertebrate that develops rapidly outside of the mother (Driever et al., 1996; Haffter et al., 1996; Patton and Zon, 2001). The use of zebrafish as a genetic system has benefited strongly from the molecular genetics and the genomics revolution and an explosion of novel transgenics are being generated to

study specific cell types during development (Bernardos and Raymond, 2006; Ellett et al., 2011; Lawson and Weinstein, 2002; Le Guyader et al., 2008; Park et al., 2000; Umans et al., 2017). Zebrafish have an enclosed vascular system and the blood vessels within the brain of the fish form a blood-brain barrier (Jeong et al., 2008; Tam et al., 2012; Umans et al., 2017; Umans and Taylor, 2012; Xie et al., 2010). Taken together, these traits make zebrafish an excellent emerging model system in which to study the blood-brain barrier.

The Blood-Brain Barrier Properties

The fundamental component of the blood-brain barrier is the brain endothelial cell. Brain endothelial cells are a differentiated form of endothelial cell and express unique barrier properties. The first barrier property that distinguishes these cells as differentiated brain endothelial cells is the expression of the glucose transporter GLUT1 (Bauer et al., 1995; Dermietzel et al., 1992; Pardridge et al., 1990). In addition to GLUT1, brain endothelial cells express other transporters that allow specific molecules into the brain (Zlokovic, 2008). Additionally, these endothelial cells can be distinguished by being closely bound to one another using tight junctions (Jeong et al., 2008; Nitta et al., 2003; Reese and Karnovsky, 1967; Zhang et al., 2010). In summary, brain endothelial cells express the barrier properties of having specific transporters such as GLUT1 and being bound to one another by tight junctions. Together as a system these specialized endothelial cells allow the brain to get the essential things it needs while being protected from harmful substances in systemic circulation.

Blood-Brain Barrier Development: Wnt/β-catenin signaling

Blood-brain barrier development is a complex multicellular signaling process. Two processes are required during the formation of the blood-brain barrier: new blood vessel formation (angiogenesis) and the acquisition of barrier properties (barriergenesis). It has been established that in the CNS angiogenesis and barriergenesis occur simultaneously (Umans et al., 2017). The signaling pathways involved in these processes are only starting to be elucidated. Currently, the only pathway known to distinguish the blood-brain barrier vasculature from peripheral vasculature is Wnt/β-catenin signaling (Stenman et al., 2008).

Wnt/β-catenin signaling is a complex signaling cascade system that is frequently involved in developmental processes. By intermixing a variety of Wnt ligands, frizzleds, and, coreceptors and coactivators Wnt signaling allows for specific spatiotemporal control of development (Freese et al., 2010; Komiya and Habas, 2008). These proteins at the cell surface transduce the Wnt signal from outside to inside the cell. Briefly, in the presence of Wnt signaling at the membrane β-catenin is left free to translocate to the nucleus and activate transcription of Wnt responsive genes (Freese et al., 2010; Komiya and Habas, 2008). However, in the absence of a Wnt signal β-catenin is bound by a complex of proteins including, Axin1 (Freese et al., 2010; Komiya and Habas, 2008). This leads to β-catenin ’s degradation (Freese et al., 2010; Komiya and Habas, 2008).

Wnt/β-catenin signaling is required for CNS angiogenesis and barriergenesis. Wnt signaling was initially implicated when mouse mutants failed to form brain vasculature and had severe cerebral hemorrhages (Daneman et al., 2009; Liebner et al., 2008). Subsequently, mutants in the orphan G-protein coupled receptor *Gpr124*

were found to exhibit similar phenotypes (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010). In time, it was found that GPR124 functions as an essential coactivator in the Wnt/β-catenin signaling pathway (Posokhova et al., 2015; Zhou and Nathans, 2014; Zhou et al., 2014). These initial mouse studies were subsequently confirmed by experiments with *gpr124* mutant zebrafish (Umans et al., 2017; Vanhollebeke et al., 2015). In mice, endothelial cell specific constitutively active β-catenin was able to rescue brain vasculature in *Gpr124* mutants (Zhou and Nathans, 2014). Taken together these studies show that Wnt/β-catenin signaling is required for blood-brain barrier formation in an endothelial cell specific manner. Additionally, the GPI-anchored MMP inhibitor, Reck, has been identified as a specific coactivator with Gpr124 in this process (Bostaille et al., 2016; Vanhollebeke et al., 2015).

Wnt/β-catenin signaling has effects in the developing brain beyond the differentiation of brain endothelial cells. The role of Wnt/β-catenin signaling is well characterized in neuronal synaptogenesis (Mulligan and Cheyette, 2012). After injury radial glial cells secrete Wnt signals to allow the injured tissue to undergo neurogenesis (Briona et al., 2015). Radial glial cells have also been shown to impact the developing blood-brain barrier (Ma et al., 2013; Matsuoka et al., 2017). Wnt/β-catenin signaling is also emerging as a possible connection between the CNS and the immune system (Marchetti and Pluchino, 2013). In summary, while very important for the blood-brain barrier, Wnt/β-catenin signaling is also involved with multiple cell types in the CNS.

Clinical Significance of the Blood-Brain Barrier

The blood-brain barrier is relevant clinically in two broad ways 1) as a structure that is disrupted in disease states and 2) as an obstacle to therapeutic interventions

(Hawkins and Davis, 2005; Pardridge, 2003). In the case of disease states the disruption of the blood-brain barrier leaves the brain vulnerable to hazards in systemic circulation as well as invasion from peripheral immune cells causing neuroinflammation (Engelhardt and Ransohoff, 2012; Lopes Pinheiro et al., 2016). On the other side, the properties of the blood-brain barrier make it difficult to reach therapeutic levels of many compounds within the brain. This section will not discuss all existing examples of the clinical relevance of the blood-brain barrier but will instead use representative examples of the two ways the blood-brain barrier is significant clinically.

Neurodegenerative diseases and brain cancers are examples of disease states, that have clinically relevant disruption of the blood-brain barrier. Broadly, in neurodegenerative diseases the blood-brain barrier loses its barrier properties leaving the brain more vulnerable to dangers in systemic circulation (Zlokovic, 2008). It remains to be seen whether the vasculature or the neural tissue first show disease phenotypes (Zlokovic, 2008). An additional example of diseases with clinically relevant disruption of the blood-brain barrier is certain brain cancers. Specifically, in Wnt-driven medulloblastomas, the tumor secretes signals resulting in the disruption of the blood-brain barrier (Phoenix et al., 2016). However, brain tumors that disrupt the blood-brain barrier may be more treatable and have improved outcomes as a result of increased exposure to systemically circulating chemotherapeutics, compared to similar tumors that fail to disrupt the blood-brain barrier (Phoenix et al., 2016). Taken together, these examples underscore the importance of understanding how disease states are impacting the blood-brain barrier. Additionally, the examples shed light on the reverse, how the blood-brain barrier is impacting disease states.

Additionally, the protective properties of the blood-brain barrier are frequently an obstacle for therapeutic interventions targeted to the central nervous system (CNS) (Pardridge, 2007). There are two main reasons for this: 1) lack of diffusion/endocytosis making it hard for many drugs to enter and 2) multi-drug resistant transporters continually pumping substances that accumulate back to the systemic side of the blood-brain barrier (Bellavance et al., 2008). Current practice for designing chemotherapeutics targeting the CNS is to follow a modified form of Lipinski's rule of 5 to identify compounds that may be able to enter and stay within the CNS (Benet et al., 2016) . However, Lipinski's rules are extremely limiting in their requirements making it very difficult to develop therapeutics that will not only improve a condition but also be able to reach the CNS at a therapeutic level (Pardridge, 2007). As we continue to learn more about these unique blood vessels and the restrictions it places on drug development, there have been increased attempts to find novel ways to circumvent the blood-brain barrier. I will discuss one current example of this in the next section.

When Clinic and Development Meet

So far, I have discussed both what is known about the blood-brain barrier and the signals that are involved in its development as well as the clinical challenges it poses both in terms of pathology and drug delivery. Here, I will concatenate these two realms by discussing an ongoing effort to spatiotemporally disrupt the blood-brain barrier to circumvent it to enable the treatment of diseases.

There is an effort to better understand the development and maintenance of these vessels in the hopes of improving therapeutics for drug delivery to the CNS (Bellavance et al., 2008; Patel and Patel, 2017). Of interest are the emerging

technologies that seek to modulate Wnt/β-catenin signaling to disrupt the blood-brain barrier (Kahn, 2014). However, more work is needed to determine how to target these interventions and how they may impact non-endothelial cells in the CNS. Continuing to do basic research to understand the developmental processes involved in blood-brain barrier formation can inform the both the functionality *in vivo* and the possible repercussions of these types of treatments (Kahn, 2014).

Research Problems to Be Addressed

The regulation of the blood-brain barrier in both developmental and disease states remains poorly understood. I have outlined in the previous section what is known about blood-brain barrier regulation from studying it in a developmental context. In the subsequent chapters I will discuss my work to further the understanding of blood-brain barrier regulation with a lens into how perturbing Wnt signaling in non-endothelial cells impacts both the blood-brain barrier and the clinically relevant off-target effects of such interventions. Taken together my work will add insight into how therapeutic techniques seeking to disrupt the blood-brain barrier for drug delivery by modulating Wnt/β-catenin signaling may be of use or harm in a clinical setting. It will also provide new understanding for developmental biologists seeking to better understand the complex signaling system involved in the establishment of the blood-brain barrier.

The first question my thesis addresses is how modulating Wnt/β-catenin signaling in radial glia affects CNS angiogenesis and barriergenesis. To examine this we generated transgenic zebrafish to induce constitutive active β-catenin (Bcat*) in radial glial cells specifically. We also utilized a previously generated inducible constitutively active Axin1 (Axin1*) transgenic line (Knopf et al., 2010). I then crossed

these fish into lines that would allow me to observe the impact on CNS angiogenesis and barriergenesis. After characterizing these initial phenotypes, *in situ* hybridization and qPCR will be done to better understand the impact on Wnt/β-catenin signaling we were observing.

The second question my thesis addresses is if there are any consequences of activating Wnt/β-catenin signaling in radial glia and neurons and the characterization of those consequences. To do this, I used transgenic zebrafish to induce constitutive active β-catenin (Bcat*) in radial glial cells and in neurons in a cell-type specific manner. I then observed the fish as they developed and determined that there were gross abnormalities in the fish with constitutive active β-catenin in their radial glial cells. Looking more closely at these fish, we observed fluorescent punctate that were brighter than the surrounding tissue, and subsequently we discovered them to be mobile in a manner indicating they were immune cells. We then did staining and crosses to determine what type of immune cell these cells were. In doing so, we discovered widespread CNS inflammation in these fish. This led us to try to understand the cause of the mechanism by which that inflammation occurred.

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CHAPTER II: Examining the Consequences of Modulating Wnt/β-catenin Signaling in Radial Glial Cells on CNS Angiogenesis and Barriergenesis

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Abstract

The blood-brain barrier (BBB) is a unique set of blood vessels that protects the brain from hazards in systemic circulation. Two processes are required during the formation of the blood-brain barrier: new blood vessel formation (angiogenesis) and the acquisition of barrier properties (barriergenesis). CNS angiogenesis and barriergenesis require Wnt/β-catenin signaling. Here we determined what role increasing or suppressing Wnt/β-catenin signaling in radial glial cells has on the processes of CNS angiogenesis and barriergenesis. We demonstrate that increased Wnt/β-catenin signaling in radial glial cells causes a loss of CNS angiogenesis and barriergenesis. A reduction in Wnt/β-catenin signaling in radial glial cells does not affect CNS angiogenesis or barriergenesis. In the future, we will determine how Wnt/β-catenin signaling and by VEGF signaling are being impacted by our modulations of Wnt/β-catenin signaling in radial glial cells. Together, this will inform how Wnt/β-catenin signaling in radial glial cells contributes to the overall Wnt/β-catenin signaling niche of the CNS during blood-brain barrier development.

Introduction

The blood-brain barrier (BBB) is the unique set of blood vessels which exists to protect the brain from hazards in systemic circulation. The brain vasculature is distinguished from peripheral vasculature because of the presence of barrier properties in the endothelial cells. The first known marker of brain endothelial cell differentiation is the glucose transporter GLUT1 (Bauer et al., 1995; Dermietzel et al., 1992; Pardridge et al., 1990). Other barrier properties include tight junctions and multi-drug resistant transporters (Jeong et al., 2008; Nitta et al., 2003; Reese and Karnovsky, 1967; Zhang et al., 2010; Zlokovic, 2008). Together these properties protect the brain from harmful substances while allowing it access to the materials in systemic circulation necessary to sustain function.

Blood-brain barrier development is a complex multicellular signaling process. Two processes are required during the formation of the blood-brain barrier: new blood vessel formation (angiogenesis) and the acquisition of barrier properties (barriergenesis). It has been established that in the CNS angiogenesis and barriergenesis occur simultaneously (Umans et al., 2017). The signaling pathways involved in these processes are still being identified. Currently, Wnt/β-catenin signaling is the only pathway distinguishing the blood-brain barrier vasculature from peripheral vasculature (Stenman et al., 2008).

Wnt/β-catenin signaling is required for CNS angiogenesis and barriergenesis. Wnt signaling was initially implicated when mouse mutants failed to form brain vasculature (Daneman et al., 2009; Liebner et al., 2008). Subsequently, mutants in the

orphan G-protein coupled receptor *Gpr124* were found to exhibit similar phenotypes (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010; Umans et al., 2017; Vanhollebeke et al., 2015). In time, it was found that GPR124 functions as an essential coactivator in the Wnt/β-catenin signaling pathway (Posokhova et al., 2015; Zhou and Nathans, 2014; Zhou et al., 2014). These proteins at the cell surface transduce the Wnt signal from outside to inside the cell. In mice endothelial cell specific constitutively active β-catenin was able to rescue brain vasculature in *Gpr124* mutants (Zhou and Nathans, 2014). Taken together these studies show that Wnt/β-catenin signaling is required for CNS angiogenesis and barriergenesis.

Wnt/β-catenin signaling has effects in the developing brain beyond the differentiation of brain endothelial cells. Radial glial cells have been shown to impact the developing blood-brain barrier (Ma et al., 2013; Matsuoka et al., 2017). Additionally, in certain WNT expressing medulloblastomas there is a disruption of brain vasculature (Phoenix et al., 2016). This implies that the level of Wnt signaling occurring within glial cells, specifically radial glial cells could influence the development of the blood-brain barrier.

Here we examine what role increasing or suppressing Wnt/β-catenin signaling in radial glial cells has on the processes of CNS angiogenesis and barriergenesis. To do this we generated a new zebrafish transgenic line and used it in conjunction with previously established lines to modulate Wnt/β-catenin signaling in a cell types specific manner. We then used established reporter lines for CNS angiogenesis (*kdrl*) and barriergenesis (*glut1*) (Beis et al., 2005; Umans et al., 2017). We are continuing to

establish how the activation or suppression of Wnt/β-catenin signaling within the radial glial cells is impacting Wnt signaling in the other cells of the developing CNS.

Results

To determine the effect of modulating Wnt/β-catenin signaling in radial glial cells on CNS angiogenesis and barriergenesis we generated a novel stable transgenic line *gfap:rtTA;cmlc2:EGFP* (Figure 1). This line drives expression of *TRE* responder lines specifically in radial glial cells in a doxycycline-dependent manner and has EGFP expression in the heart for a transgenesis marker (data not shown). The addition of the transgenesis marker in this line assists in screening positive animals and identifying genetically identical siblings in no doxycycline (dox) conditions.

To determine the effect of modulating Wnt/β-catenin signaling in radial glial cells on brain angiogenesis we utilized the *gfap:rtTA;cmlc2:EGFP* in a background that contained *kdrl:mCherry* and either *TRE:Bcat*-EGFP* or *TRE:Axin1*-YFP* (Knopf et al., 2010). This allowed us to generate animals with either increased Wnt signaling in radial glial cells in the case of *TRE:Bcat*-EGFP* and decreased Wnt signaling in radial glial cells in the case of *TRE:Axin1*-YFP*. In observing these animals, we saw defects in angiogenesis in *TRE:Bcat*-EGFP* fish but not in *TRE:Axin1*-YFP* fish (Figure 2, Supplemental Figure 1, and data not shown).

To determine the effect of modulating Wnt/β-catenin signaling in radial glial cells on brain barriergenesis we utilized the *gfap:rtTA;cmlc2:EGFP* into a background that contained *glut1b:mCherry* and either *TRE:Bcat*-EGFP* or *TRE:Axin1*-YFP*. In

observing these animals, we saw significant defects in barriergenesis in *TRE:Bcat*-EGFP* fish (Figure 3).

Discussion

Here, we modulated Wnt/β-catenin signaling in radial glial cells and examined the impact on CNS angiogenesis and barriergenesis. We generated an inducible transgenic line that enabled us to study Wnt/β-catenin specifically in radial glial cells. We then used this transgenic line to drive or suppress Wnt/β-catenin signaling in radial glial cells.

Using our new transgenic line, we were able to modulate Wnt/β-catenin signaling specifically in radial glial cells and observe CNS angiogenesis. Here, our results showed that increasing Wnt/β-catenin signaling in radial glia disrupts CNS angiogenesis. We did not observe CNS angiogenesis phenotypes when we decreased Wnt/β-catenin signaling in radial glial cells. This indicated that increasing Wnt/β-catenin signaling specifically in radial glial cells impacts the normal Wnt/β-catenin signaling process in developing brain endothelial cells because we know that Wnt/β-catenin signaling is required for CNS angiogenesis (Daneman et al., 2009).

Subsequently, we wanted to determine if barriergenesis was disrupted when we modulated Wnt/β-catenin signaling in radial glia. Unsurprisingly, we observed barriergenesis defects when Wnt/β-catenin signaling is increased in radial glial cells. However, barriergenesis occurred normally when Wnt/β-catenin signaling was decreased in radial glial cells. This is consistent with previous work a in a glial cancer model which indicated that tumors with active Wnt/β-catenin signaling had vasculature that lacked blood-brain barrier properties (Phoenix et al., 2016).

Future experiments will determine the global effects of modulating Wnt/β-catenin signaling in radial glial cells. To do this we will examine Wnt and VEGF markers. We hypothesize that when Wnt/β-catenin signaling is increased in radial glial cells it creates a feedback loop causing a global reduction in Wnt/β-catenin signaling within the CNS, which causes the vasculature defects we observe. We also expect that there will be differences in the expression levels of Wnt and VEGF markers in the fish with reduced Wnt/β-catenin signaling in radial glia although we did not observe macro level phenotypes in angiogenesis or barriergenesis.

Materials and Methods

Zebrafish husbandry

AB and TL strains were acquired from the Zebrafish International Resource Center. Embryos and larvae were maintained at 28.5 °C in egg water (0.03% Instant Ocean in reverse osmosis water). For imaging, 0.003% phenylthiourea (PTU) was used to inhibit melanin production. All experiments were performed in accordance with the University of Wisconsin-Madison Institutional Animal Care and Use Committees.

Generation of Transgenic Zebrafish

Vectors were made using Gateway Cloning (Invitrogen) and components of the Tol2Kit (Kwan et al., 2007) unless otherwise specified. All transgenesis was performed by injecting Tol2 mRNA (20 pg) and the appropriate plasmid containing Tol2 inverted repeats (50-100 pg) into fertilized eggs at the single cell stage.

The transgenic line *Tg(gfap:rtTA; cmlc2:EGFP)* was generated from the plasmid *pDestTol2CG2; gfap:rtTA*. This plasmid was constructed by LR reaction using *p5E:gfap*

and *pME:rtTA*, both described previously (Ju et al., 2015), together with *p3E:pA* and the *pDestTol2CG2* destination vector such that positive transgenic drivers could be identified by their green hearts.

Confocal laser scanning microscopy

Zebrafish from 1 to 6 days postfertilization (dpf) were anesthetized in 0.02% Tricaine and immobilized in 1.2% low melting point agarose (Invitrogen, Carlsbad, CA) in glass bottom culture dishes (MatTek, Ashland, MA). Images were taken on a Nikon Eclipse Ti microscope equipped with a Nikon A1R. All images are 2D projections of 3D confocal z stacks generated using either NIS-Elements MIP algorithm.

Quantification

Quantification was done using AngioTool as described previously on cropped images of vasculature (to exclude the eyes and fins) (Zudaire et al., 2011). AngioTool outputs were used to generate graphs and p-values were calculated using unpaired T-tests.

Figures

Figure 1: Representation of the *gfap:rtTa;cmlc2:EGFP* construct:

Cartoon representation of the *gfap:rtTa;cmlc2:EGFP* construct. Construct includes *gfap* promoter driving rtTA and *cmlc2* promoter driving EGFP.

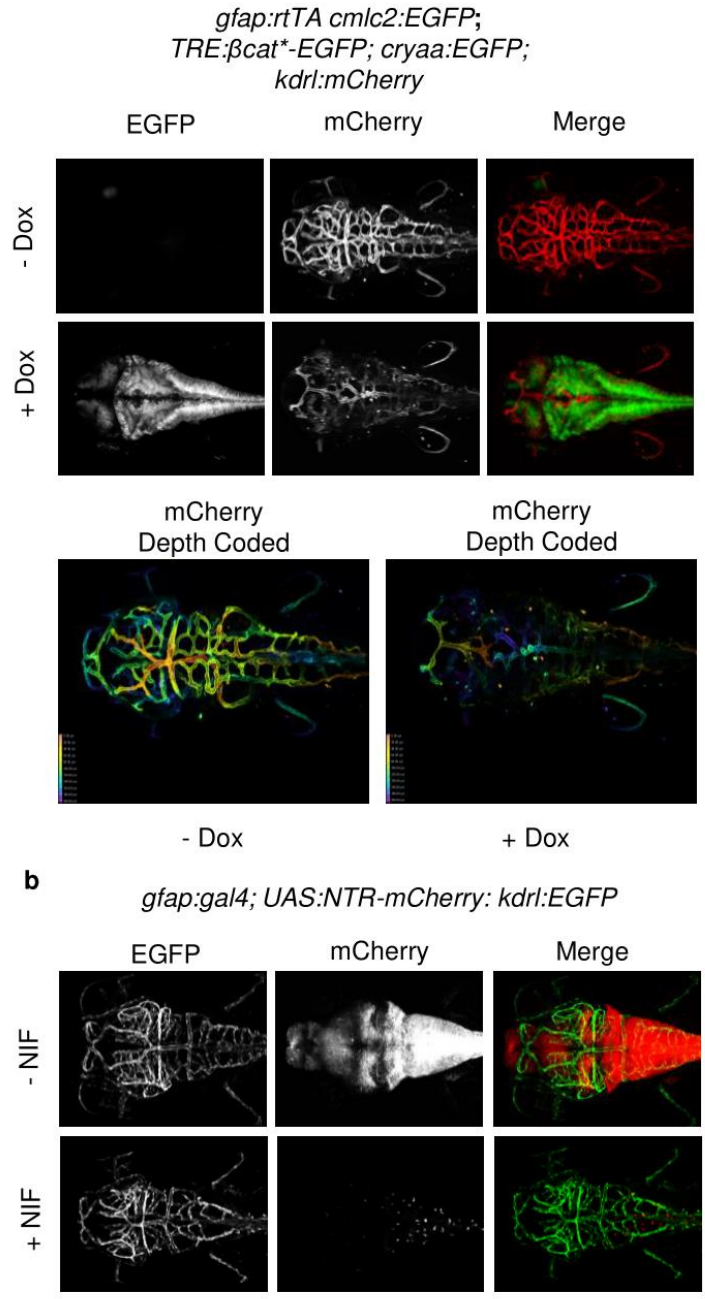


Figure 2:Constitutively active Wnt/β-catenin signaling in radial glial cells disrupts angiogenesis:

Representative images of *gfap:rtTa;cmlc2:EGFP; TRE:β-catenin-EGFP; kdrl:mCherry* animals at 3dpf. Siblings were sorted into treated and untreated groups late 0 dpf.

Animals are sorted visually and imaged at 3dpf. Confocal Z-stacks are taken through the whole brain at 15x magnification. Depth coding is done using Nikon A1R software.

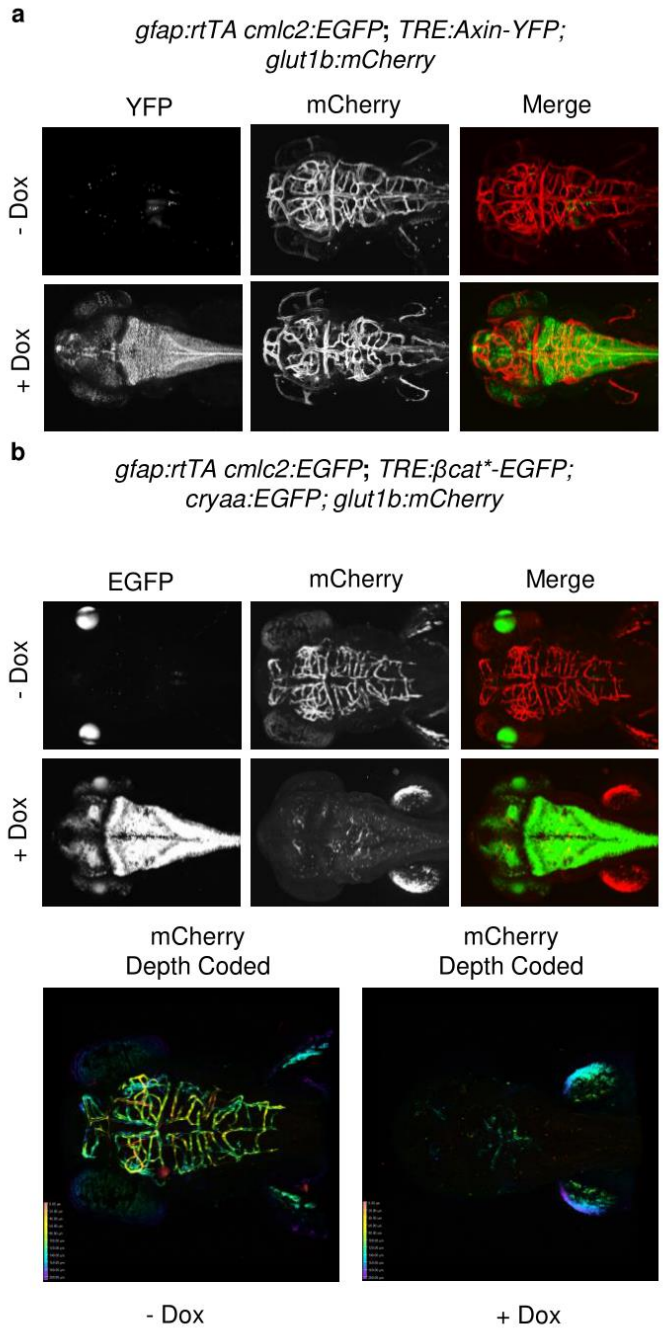
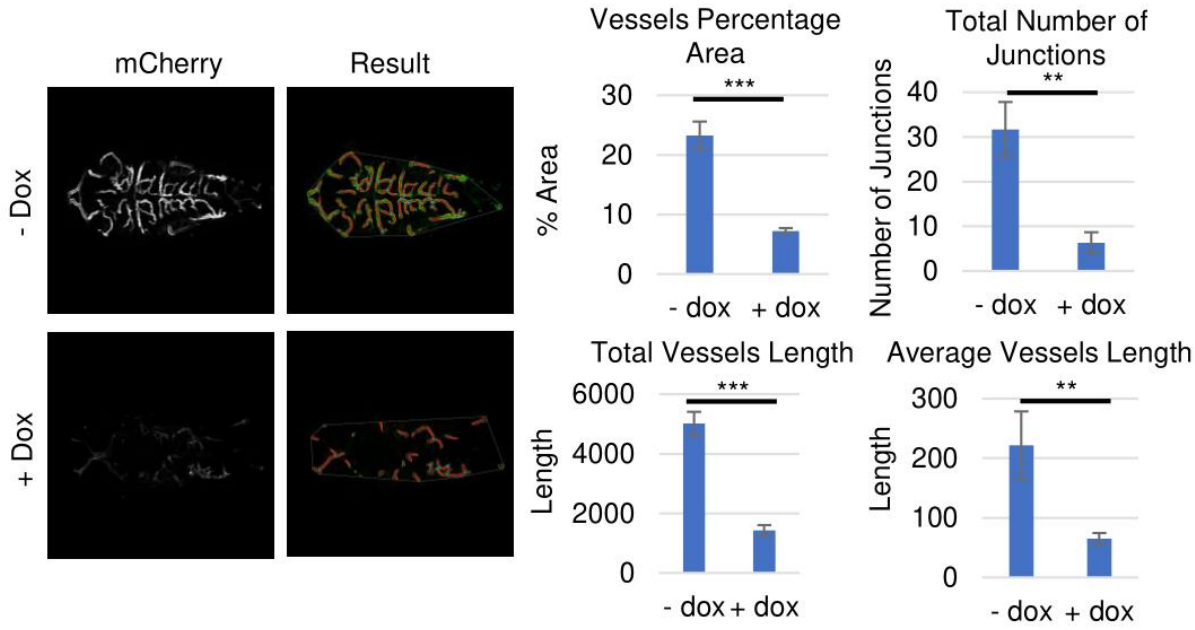


Figure 3: Constitutively Active Wnt/β-cateninsignaling in radial glial cells disrupts barriergenesis:

Representative images of A) *gfap:rtTa;cmlc2:EGFP; TRE:axin1-YFP; glut1b:mCherry* animals at 3dpf. Siblings were sorted into treated and untreated groups late 0 dpf.

Animals are sorted visually and imaged at 3dpf. Confocal Z-stacks are taken through the whole brain at 15x magnification. B) *gfap:rtTa;cmlc2:EGFP; TRE:β-catenin-EGFP; glut1b:mCherry* animals at 3dpf. Siblings were sorted into treated and untreated groups late 0 dpf. Animals are sorted visually and imaged at 3dpf. Confocal Z-stacks are taken through the whole brain at 15x magnification. Depth coding is done using Nikon A1R software.



Supplemental Figure 1: Quantification of Angiogenesis using AngioTool:

Representative images were cropped to remove eye and fin expression of the mCherry in *gfap:rtTA cmlc2:EGFP; TRE:βcat*-EGFP; cryaa:EGFP; kdrl:mCherry* in the presence and absence of dox. These images were run through Angiotool software generating the images labeled as results, where yellow is the outline of the vessel, red is the skeleton representation of the vessel, and blue is a junction. The software quantified the images leading to the results in the graphs ** p<.01 ***p<.001 n=3.

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CHAPTER III: Activated Wnt/β-catenin signaling in Radial Glial Cells, but not Neurons Induces CNS Inflammation

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Abstract

CNS development requires controlled communication across multiple cell types in a spatiotemporal specific manner. One important signaling pathway in CNS development is Wnt/β-catenin. While much is known about Wnt/β-catenin signaling in brain endothelial cells, little is known about how modulation of Wnt/β-catenin signaling in other CNS cell types would influence CNS development. Here, we look at the perturbation of Wnt/β-catenin signaling during development of the CNS in radial glia and neurons. We provide the first evidence that this perturbation of Wnt/β-catenin signaling in radial glia but not neurons causes an inflammatory response. Taken together we provide some of the first evidence that perturbations of Wnt/β-catenin signaling in radial glial cells can lead to inflammation in an apoptotic independent manner.

Introduction

CNS development requires extensive communication across multiple cell types. The complexity and proximity of the cell types in the CNS has made it difficult to fully dissect these developmental pathways in vivo. Of particular interest in the development of the CNS is the Wnt/β-catenin pathway (Briona et al., 2015; Daneman et al., 2009; Freese et al., 2010; Liebner et al., 2008; Mulligan and Cheyette, 2012; Stenman et al., 2008; Zhou et al., 2014). Traditionally, Wnt/β-catenin signaling is thought about in terms of synaptogenesis and angiogenesis within the CNS. The role of Wnt/β-catenin signaling is well characterized in neuron synaptogenesis (Mulligan and Cheyette, 2012). After injury radial glial cells secrete Wnt signals to allow the injured tissue to undergo neurogenesis (Briona et al., 2015). The loss of Wnt/β-catenin signaling in endothelial cells is known to disrupt CNS angiogenesis and barriergenesis (Posokhova et al., 2015; Stenman et al., 2008; Umans et al., 2017; Vanhollebeke et al., 2015). Compensating for the loss of Wnt signaling by introducing the downstream transcriptional activator β-catenin can restore angiogenesis and barriergenesis to the CNS (Cullen et al., 2011). Radial glial cells have also been shown to impact the developing blood-brain barrier (Ma et al., 2013; Matsuoka et al., 2017). It remains to be determined what overexpressing Wnt signaling in non-endothelial cells would do.

Increasing Wnt signaling has been shown to cause CNS phenotypes. For example, models of medulloblastoma have shown that an increase in Wnt signaling within radial glial cells results in a feedback mechanism that effects the signaling being sent into endothelial cells resulting in a loss of angiogenesis and barriergenesis (Phoenix et al., 2016). Additionally, the activation of Wnt signaling has been shown to

be required for the generation of new neurons from radial glial populations following spinal cord injury (Briona et al., 2015). Taken together this suggests that the activation of Wnt/β-catenin signaling in radial glial cell types might cause significant CNS phenotypes. Therefore, additional experiments and caution are necessary prior to attempting to modulate Wnt/β-catenin signaling in a clinical setting.

Zebrafish have emerged as an ideal model system to study CNS development using transgenics and in vivo live imaging. There are established promoters that drive expression specifically in certain CNS cells allowing people to interrogate questions in a cell-type specific manner (Bernardos and Raymond, 2006; Park et al., 2000). Therefore, we set out to determine if modulating Wnt/β-catenin signaling in different CNS cell types would cause significant CNS phenotypes in a developmental context. Here we generated three new zebrafish transgenics *gfap:rtTa;TRE:mCherry, huc:rtTa;TRE:EGFP, and TRE:βcat*-EGFP; cryaa:EGFP* (*TRE: βcat*-EGFP*) to interrogate the role of constitutively active Wnt/β-catenin signaling in radial glial cells and neurons respectively. In doing this, we provide the first evidence that activated Wnt/β-catenin signaling in radial glial cells but not neurons induces CNS inflammation.

Results

We generated the *gfap:rtTa;TRE:mCherry* and *huc:rtTa;TRE:EGFP* transgenes by using the tol2 kit. By observing the *gfap:rtTa;TRE:mCherry* we were able to see specific expression in the CNS. The same was true for the *huc:rtTa;TRE:EGFP*. Both transgenes are responsive to doxycycline (dox) (Figure1). Additionally, we generated an inducible *TRE:βcat*-EGFP* line using the tol2 kit. The *TRE:βcat*-EGFP* was cloned from

xenopus *TRE:βcat*-mCherry* (A generous gift from Wilson Clements). We were able to see induction of the *TRE:βcat*-EGFP* in crosses to both the *gfap:rtTa;TRE:mCherry* and the *huc:rtTa;TRE:EGFP* transgenes (Figure 2). And we see dose dependent induction of fluorescent expression (Figure 2). Taken together these transgenics will enable us to induce constitutively active β-catenin specifically in radial glial cells or neurons.

When observing dox-treated *gfap:rtTa;TRE:mCherry TRE:βcat*-EGFP* fish, we noticed many of the fish become curled and experience seizure like activity by 3 days post fertilization (dpf) (Supplemental Figure 1 and data not shown). These phenotypes were not observed in *huc:rtTa; TRE:EGFP TRE:βcat*-EGFP* fish (Supplemental Figure 1). We also observed that *gfap:rtTa;TRE:mCherry TRE:βcat*-EGFP* fish had a significant decrease in survival (Supplemental Figure 1). However, we did not observe premature death in *huc:rtTa;TRE:EGFP TRE:βcat*-EGFP* fish (Supplemental Figure 1). Together, this indicates overactivation of Wnt/β-catenin signaling in radial glial cells has different effects than in neurons.

This led us to take a closer look at the dox treated *gfap:rtTa;TRE:mCherry*; *TRE:βcat*-EGFP* fish. Using confocal microscopy, we observed bright mCherry positive punctate within the CNS. We preformed time lapse imaging to observe these puncta. These time-lapse images revealed that these punctate were moving in a way characteristic of immune cells (Figure 3). This led us to stain with neutral red identify signs of inflammation in *gfap:rtTa;TRE:mCherry*; *TRE:βcat*-EGFP* fish. We observed increased neutral red in *gfap:rtTa;TRE:mCherry; TRE:βcat*-EGFP* fish, including in the spinal cord (Figure 4). This increased inflammation was not present in

huc:rtTa;TRE:EGFP; TRE:βcat-EGFP* (Figure 4). We also did Sudan Black staining to determine if, in addition to microglia/macrophages, neutrophils were present in the CNS of the *gfap:rtTa;TRE:mCherry TRE:βcat*-EGFP* or the huc:rtTa;TRE:EGFP *TRE:βcat*-EGFP* fish. Our staining showed no neutrophils in the CNS of these fish (data not shown) This provided the first evidence that constitutively active Wnt/β-catenin signaling in radial glial cells but not in neurons lead to increased immune cells in the CNS.

To further confirm this inflammatory phenotype, we crossed *gfap:rtTa cmlc2:EGFP* (unpublished); *TRE:βcat*-EGFP* and *huc:rtTa;TRE:EGFP*; *TRE:βcat*-EGFP* into *mfap4:TdTomato* which labels macrophages and microglia. Here, we confirmed an increase of mfap4+ positive cells in the dox induced *gfap:rtTa; TRE:βcat*-EGFP* fish (Figure 5). This was consistent with our neutral red staining. We also confirmed that dox induced *huc:rtTa;TRE:EGFP; TRE:βcat*-EGFP* did not have inflammation (Supplemental Figure 2).We also looked for the presence of neutrophils in either fish type by crossing them to the *mpx:mcherry* background (Figure 5). We observe no difference in neutrophils between treated and untreated *gfap:rtTa; TRE:βcat*-EGFP* fish which was consistent with our Sudan Black staining (Figure 5 and data not shown). Taken together, these results indicated that constitutively active β-catenin in radial glial cells, but not neurons, causes an increase of microglia/macrophage specific inflammation in the CNS.

Discussion

Here, we provide the first evidence that perturbing Wnt/β-catenin signaling in radial glial cells can lead to an inflammatory response. This makes sense given, that we

know Wnt/β-catenin signaling is a very specifically controlled pathway at the cellular level. Our results indicate that increasing Wnt/β-catenin signaling in a cell-type specific manner in radial glial cells resulted in inflammation while doing the same in neurons does not. Here, we also provide new transgenic tools to the zebrafish community for further studying the perturbation of Wnt/β-catenin signaling in a cell-type specific manner. Taken together, this area of research continues to be of interest, and more studies are needed to fully elucidate both the cell autonomous and cell nonautonomous roles of Wnt/β-catenin signaling.

The inflammation we observe in the *gfap:rtTa;TRE:mCherry; TRE:βcat*-EGFP* fish needs to be further explored. Here, we provide the first step, but more work is needed to figure out mechanistically what is causing the inflammation. It is possible that apoptosis is driving the inflammation. It is also possible that Wnt/β-catenin signaling in radial glial cells is regulating immune cells in a way that has not been previously described. Additional studies will be required to further elucidate this mechanism.

The lack of phenotype seen in *huc:rtTa;TRE:EGFP TRE:βcat*-EGFP* fish is perplexing given what we know about the tight regulation of Wnt/β-catenin signaling. It is possible that Wnt/β-catenin signaling expressed cell autonomously in neurons has no effect as our data suggests. It is also possible that we are not driving *TRE:βcat*-EGFP* strongly enough to elicit a phenotype. However, we have induced this transgene at up to 90 µg/mL (data not shown) and still not seen a phenotype in the *huc:rtTa;TRE:EGFP TRE:βcat*-EGFP* fish. It is also possible that the expression of *TRE:βcat*-EGFP* is not

above what endogenous Wnt/β-catenin expression would be in neurons. This will also need to be explored further.

Taken together, our results indicate tight regulation of Wnt/β-catenin signaling in radial glial cells is important for normal CNS development. When perturbed we see inflammation and additional developmental defects which need further characterization. It remains to be determined what mechanism is causing these phenotypes. That said our work gives caution to the idea of broadly modulating Wnt/β-catenin signaling as a treatment strategy because it could lead to CNS inflammation.

Materials and Methods

Zebrafish husbandry

AB and TL strains were acquired from the Zebrafish International Resource Center. Embryos and larvae were maintained at 28.5 °C in egg water (0.03% Instant Ocean in reverse osmosis water). For imaging, 0.003% phenylthiourea (PTU) was used to inhibit melanin production. All experiments were performed in accordance with the University of Wisconsin-Madison Institutional Animal Care and Use Committees.

Generation of Transgenic Zebrafish

Vectors were made using Gateway Cloning (Invitrogen) and components of the Tol2Kit (Kwan et al., 2007) unless otherwise specified. All transgenesis was performed by injecting Tol2 mRNA (20 pg) and the appropriate plasmid containing Tol2 inverted repeats (50-100 pg) into fertilized eggs at the single cell stage.

We used the Tet-On system (Clontech) and the transposon-mediated transgenic approach (Kawakami et al., 2004) to generate inducible transgenic fish that responsive

to Doxycycline (Dox) treatment. The Tet-On fragment was released from the pTet-On vector and inserted into the Tol2 basic vector pT2AL200R150G (a kind gift from Koichi Kawakami) to replace the EGFP fragment. A 3.4 kb zebrafish huc promoter region (Park et al., 2000) was PCR amplified with primer pairs of

5'- AAATTGTGCAAGACTGATGACG-3' and 5'- ATCTAGGTCCTTCGATTTGCAG-3' and used to replace the promoter region within the Tol2 vector, creating vector phuc-Tet-On. To make pTRE-tight-EGFP construct, the EGFP gene was released from pEGFP-N1 vector by BamHI and NotI digestion, inserted into the pTRE-tight vector digested with the same restriction enzymes. The TRE-tight-EGFP fragment was released from pTRE-tight-EGFP by XhoI and ClaI digestion and inserted into the Tol2 vector digested with the same restriction enzymes.

About 30pg of phuc-Tet-On and pTRE-tight-EGFP plasmid DNA were co-injected with about 30 pg of in vitro transcribed Tol2 transposase mRNA into 1-cell-stage embryos. The embryos were raised to adults and screened for germline transmission. Embryos responsive to Dox induction and showing neuronal expression of EGFP were raised to adulthood to establish transgenic lines of Tg(huc-Tet-On; TRE-tight-EGFP).

The gateway system was used to make the pGFAP-Tet-OnAdv construct. The zebrafish GFAP promoter was released from pGFAP-EGFP vector (Bernardos and Raymond, 2006) by XhoI and SalI digestion and inserted into the modified 5' entry clone p5E-MCS from the Multisite Gateway-compatible entry vectors of the Tol2 kit (Kwan, Fujimoto et al. 2007). The Tet-On advanced fragment was released from pTet-On advanced vector (Clontech) with EcoRI and BamHI digestion and inserted into the

middle entry clone pME-MCS digested with the same enzymes. The p5E and pME vector containing the respective promoter and Tet-On advanced sequences were combined with the 3' entry clone p3E-polyA and the destination vector pDestTol2pA2 to create the pDest-GFAP-Tet-OnAdv construct using the LR Clonase II Plus Enzyme mix (Invitrogen). The construct was co-injected with either pTRE-EGFP or pTRE-mCherry together with Tol2 transposase mRNA into 1-cell-stage embryos. Screenings for germline transmission were conducted the same way as mentioned above to establish transgenic lines of Tg(GFAP-Tet-OnAdv; TRE-EGFP) and Tg(GFAP-Tet-OnAdv; TRE-mCherry).

The transgenic line *Tg(TREtight:β-cat*-EGFP, cryaa:EGFP)* was generated from the plasmid *pDestTol2pACryGFP; TREtight: β-cat*-EGFP*. This plasmid was constructed by LR reaction using *p5E:TREtight* (Ju et al., 2015), *pME: β-cat*--EGFP*, *p3E-pA* and *pDestTol2pACryGFP* (a gift from Joachim Berger & Peter Currie; Addgene plasmid # 64022). The plasmid *pME: β-cat*-EGFP* was generated using *pCS2+: β-cat*--mCherry* (kindly provided by Wilson Clements) as a template to PCR amplify *da-cttnb1* and transfer it into *pME-MCS* at KpnI and SalI. EGFP was then amplified from *pME-EGFP* and inserted in-frame at SalI and SacI to make the final construct.

The transgenic line *Tg(mfap4:TDTomato-CAAX)* was generated using the *pDestTol2; mfap4:TdTomato-CAAX* plasmid (generously provided by Anna Huttenlocher with the kind permission of David Tobin)(Walton et al., 2015).

Microscopy

Zebrafish from 1 to 6 days postfertilization (dpf) were anesthetized in 0.02% Tricaine and immobilized in 1.2% low melting point agarose (Invitrogen, Carlsbad, CA) in glass bottom culture dishes (MatTek, Ashland, MA). Images were taken on a Nikon Eclipse Ti microscope equipped with a Nikon A1R. All images are 2D projections of 3D confocal z stacks generated using either NIS-Elements MIP or EDF algorithm. Additional images were taken on a smz18 stereo microscope.

Survival Curve

Zebrafish were observed for survival from day 4 to day 12. Presence or absence of swim bladder was quantified on day 5. Dead animals were identified based on a loss of movement when touched and the absence of a heartbeat. Dead animals were removed. Media was changed every 48 hours.

Neutral Red Staining and Quantification

Zebrafish were incubated in 2.5 µg/ml neutral red (in embryo medium) for 5 hours in the dark. 2 15-minute washes were done prior to imaging. Zebrafish heads were imaged and EDF modules were used to generate images. Quantification was done by segmenting the full z into non-overlapping sections then EDF. Images were blinded and coded and neutral red cells were quantified by hand by multiple individuals. Average counts were calculated and segments from the same original image were added together to get the final number of cells. These counts were then used in t-tests to determine statistical significance.

Figures

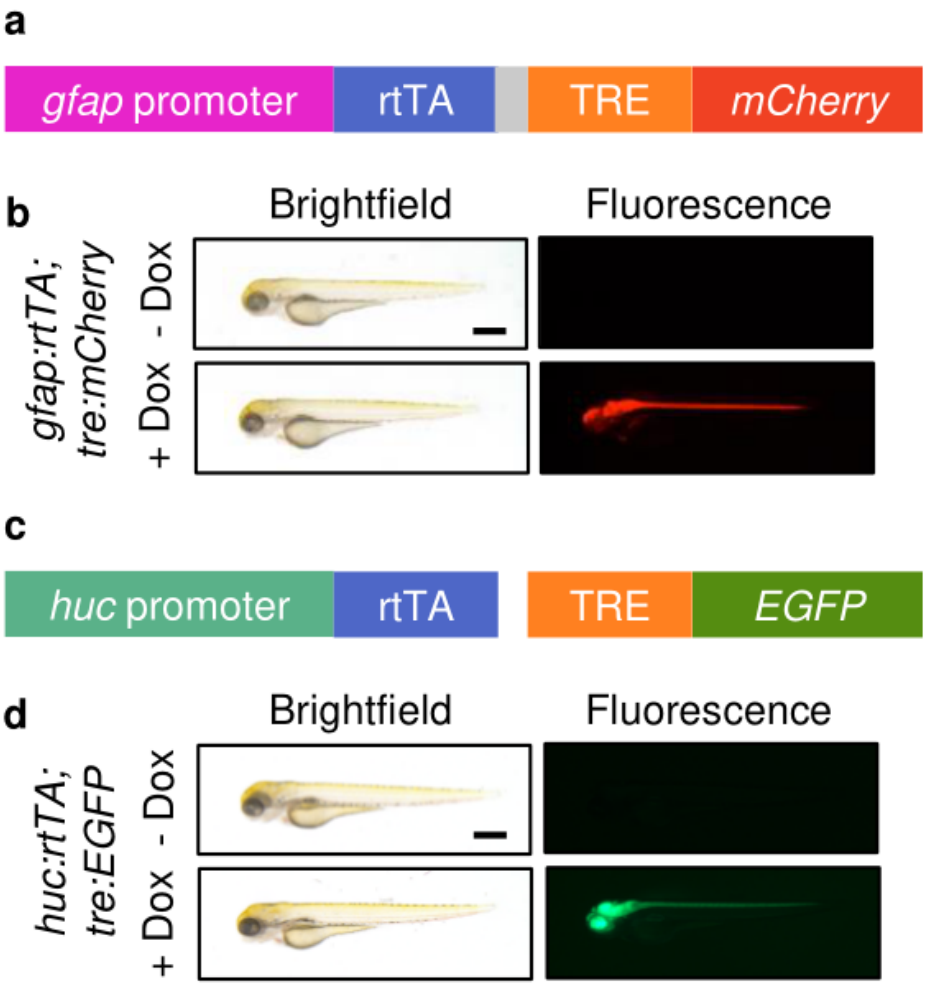


Figure 1: Generation of novel CNS rtta transgenics

A) Graphical depiction of gfap transgenic. B) 3dpf gfap transgenic in the presence and absence of doxycycline (10µg/mL) showing CNS specific expression in the presence of dox. C) Graphical depiction of huc transgenic. D) 3dpf huc transgenic in the presence and absence of doxycycline (10µg/mL) showing CNS specific expression in the presence of dox. Scale bar in upper left image 5 µm.

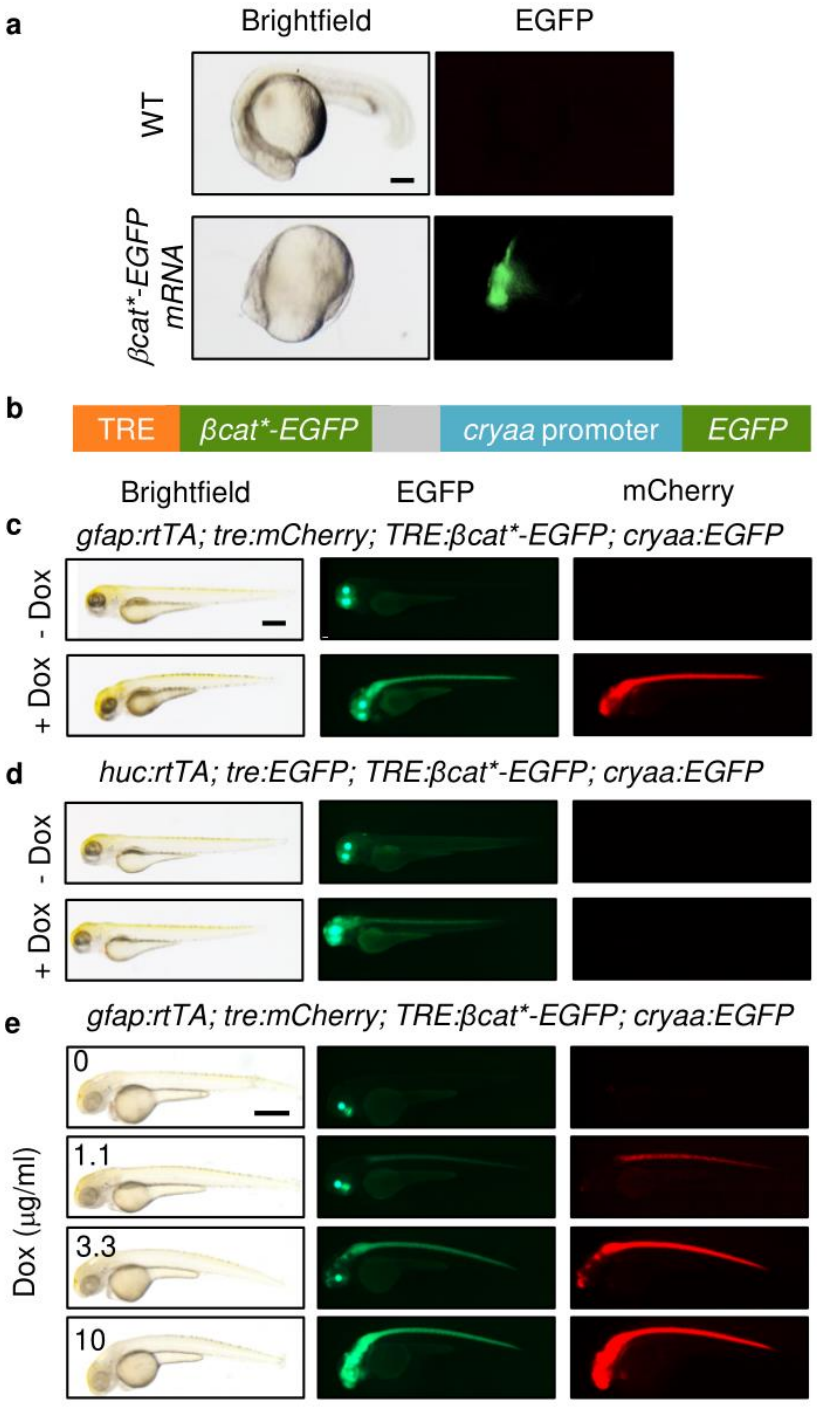


Figure 2: Generation of Inducible Constitutively Active β-catenin-EGFP

A) 1 dpf wild-type and βcat*-EGFP mRNA animals injected at the 1 cell stage indicating the construct and fusion protein are able to be expressed and when expressed

ubiquitously have a deleterious impact on development. Scale bar in upper left image 2 µm B) Graphical depiction of inducible constitutively active β-catenin-EGFP, including cryaa transgenesis marker. C-D) CNS specific induction of βcat*-EGFP using the gfap and huc transgenics respectively. Scale bar in upper left image 2 µm. E) induction of transgenic expression is dose dependent, here this is depicted by a dosage curve of dox in the gfap βcat*-EGFP background. Scale bar in upper left image 5 µm.

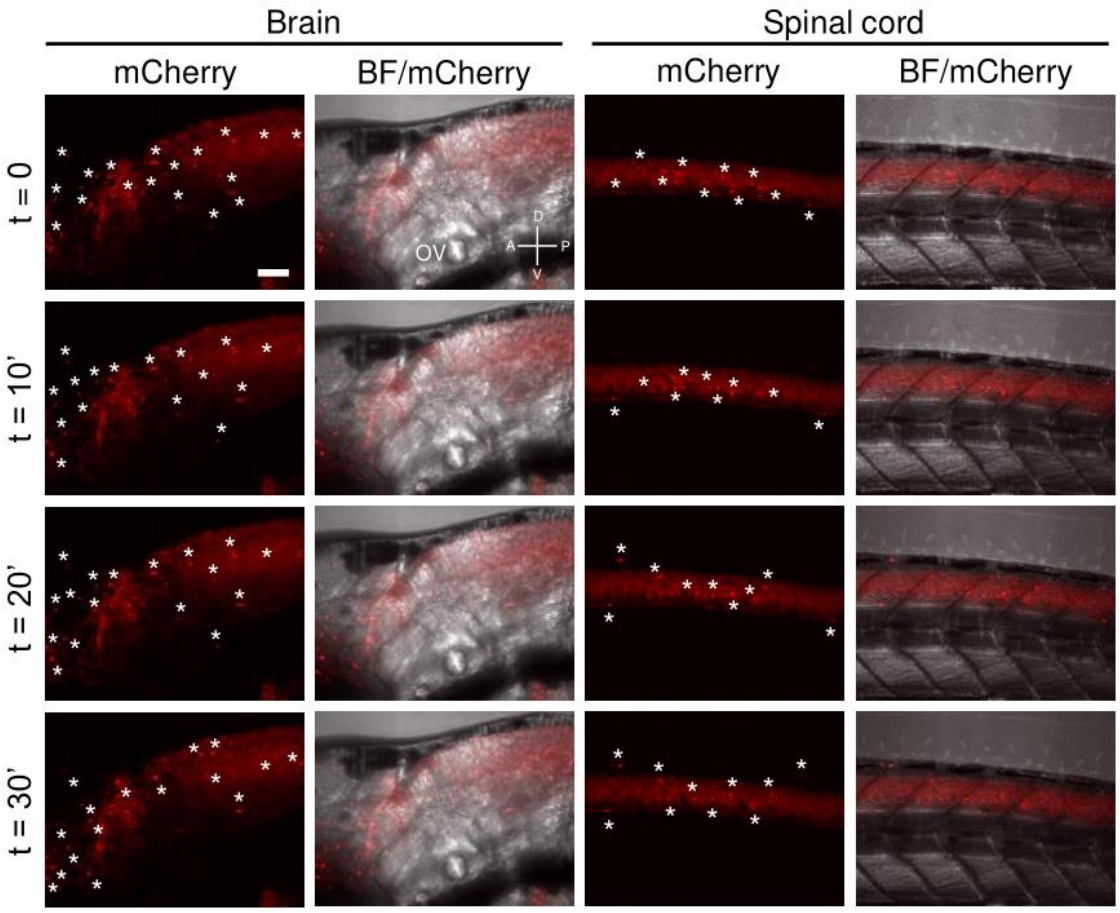


Figure 3: Time lapse images of possible CNS inflammation

Stills of time lapse over 30 min of 4dpf *gfap:rtTA TRE:mCherry; TRE:βcat*-EGFP* head and spine. White asterisk indicates red punctae present. Scale bar 150 µm.

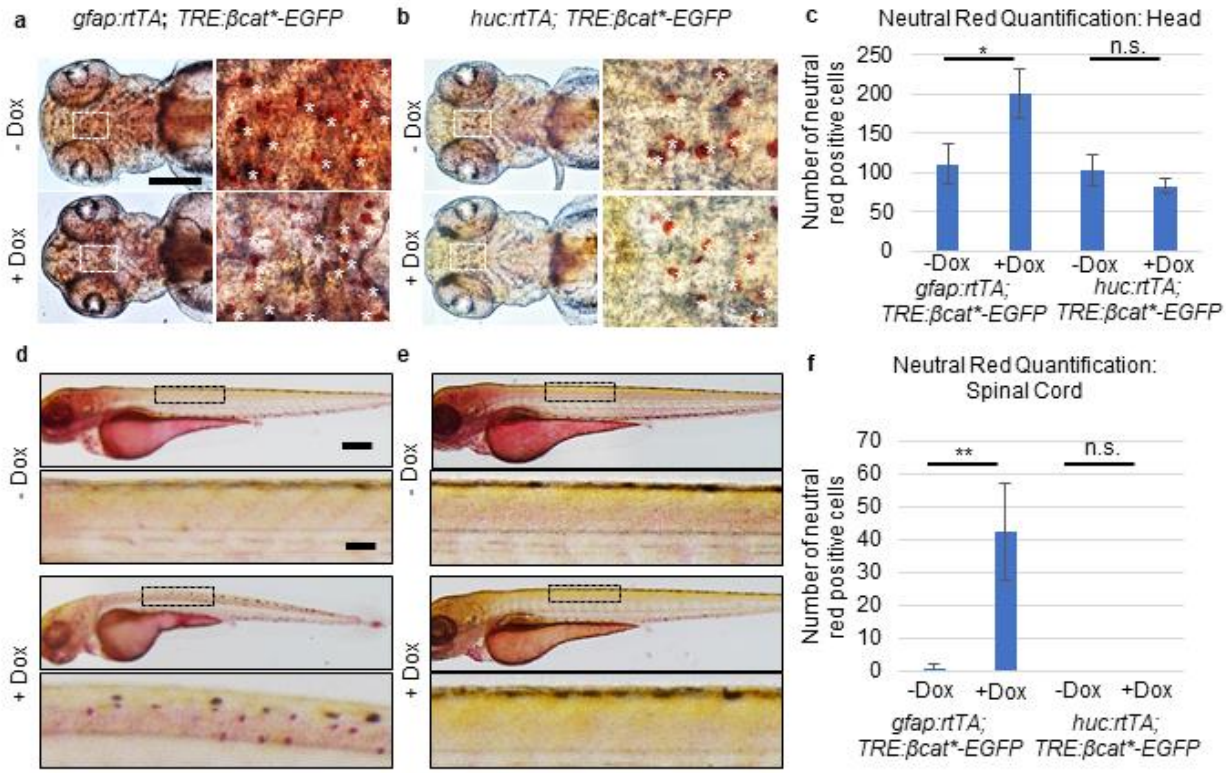


Figure 4: Radial glial specific induction of βcat*-EGFP fish have increased neutral red staining in both the head and spinal cord

A) left heads of neutral red stained gfap βcat*-EGFP animals in the presence and absence of dox 3dpf. Scale bar 150 μm. Right 5x zoom of region boxed in image to the left, white Asterix mark neutral red positive cells. B) left heads of neutral red stained gfap βcat*-EGFP animals in the presence and absence of dox 3dpf. Right 5x zoom of region boxed in image to the left, white Asterix mark neutral red positive cells. C) quantification of neutral red positive cells in the gfap and huc βcat*-EGFP, n=at least 3 for all groups. Single asterix indicated $P<0.05$, n.s. $P>0.05$. D) top image in each pair is a representative image of the trunk and tail of the fish neutral red stained at 3dpf bottom image in a pair is a 5x zoom of the boxed region in the top image. E) top image in each

pair is a representative image of the trunk and tail of the fish neutral red stained at 3dpf bottom image in a pair is a 5x zoom of the boxed region in the top image. F) Quantification of neutral red staining in the spinal cord. Double Asterix indicate $p<0.01$, n.s. $P>0.05$. All quantification was done on n of at least 3 per genotype and condition.

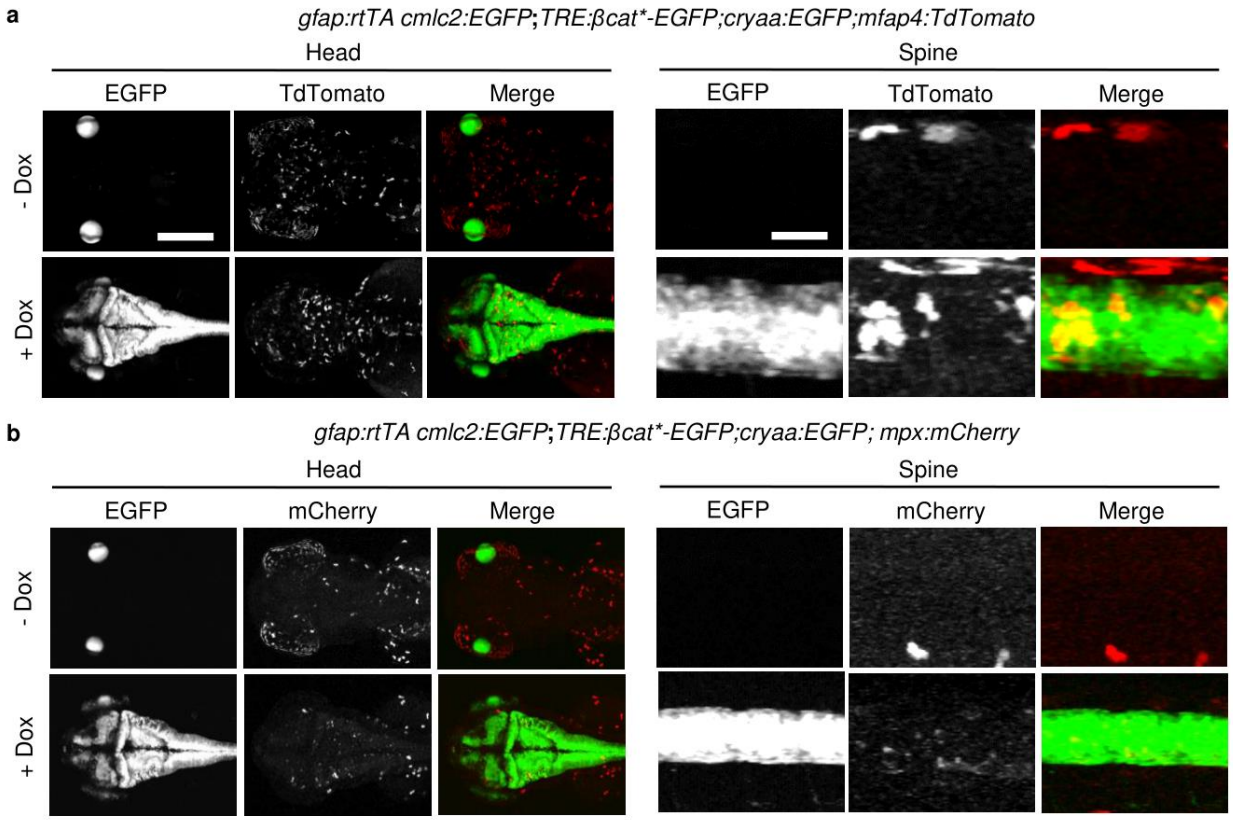
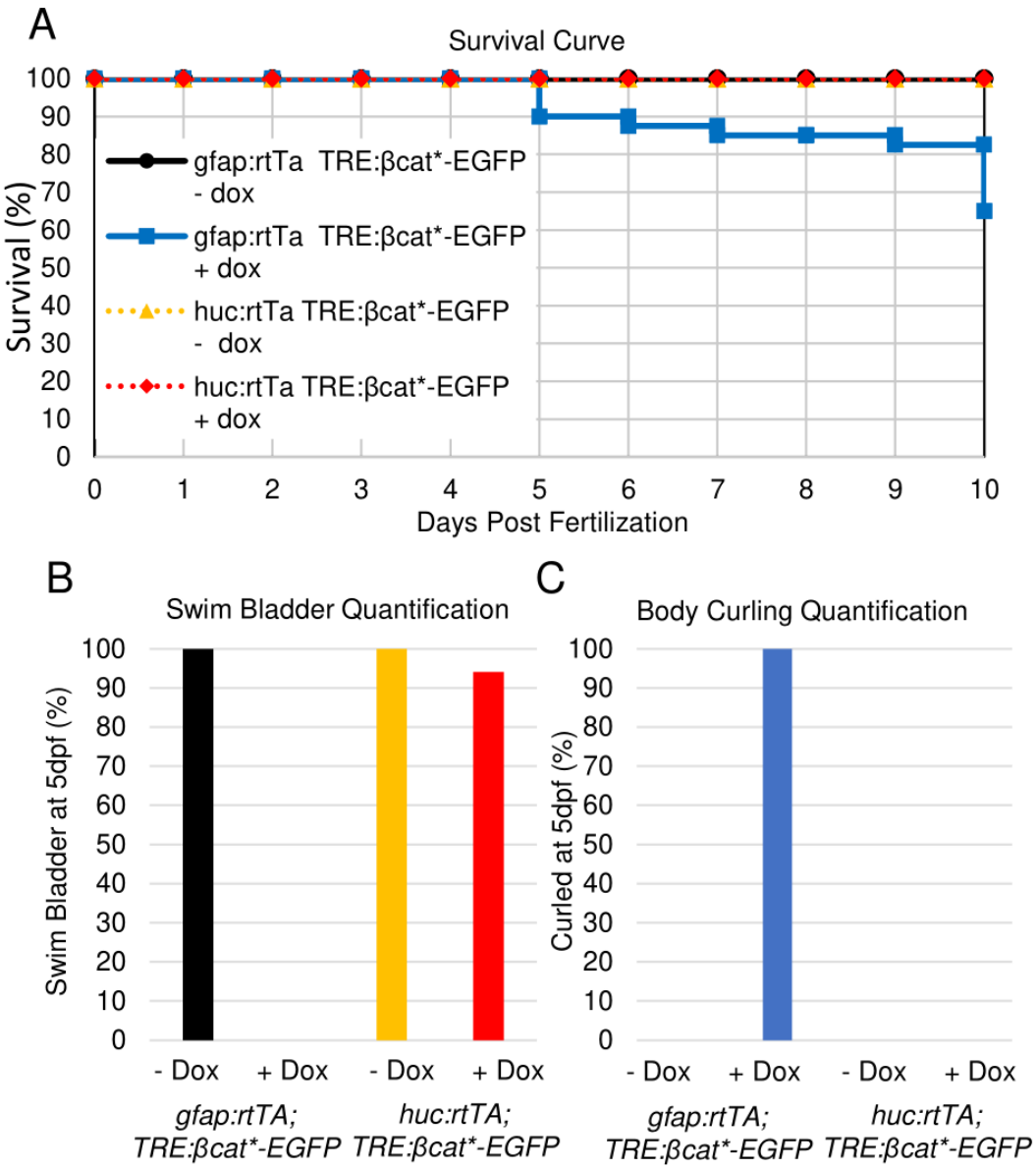


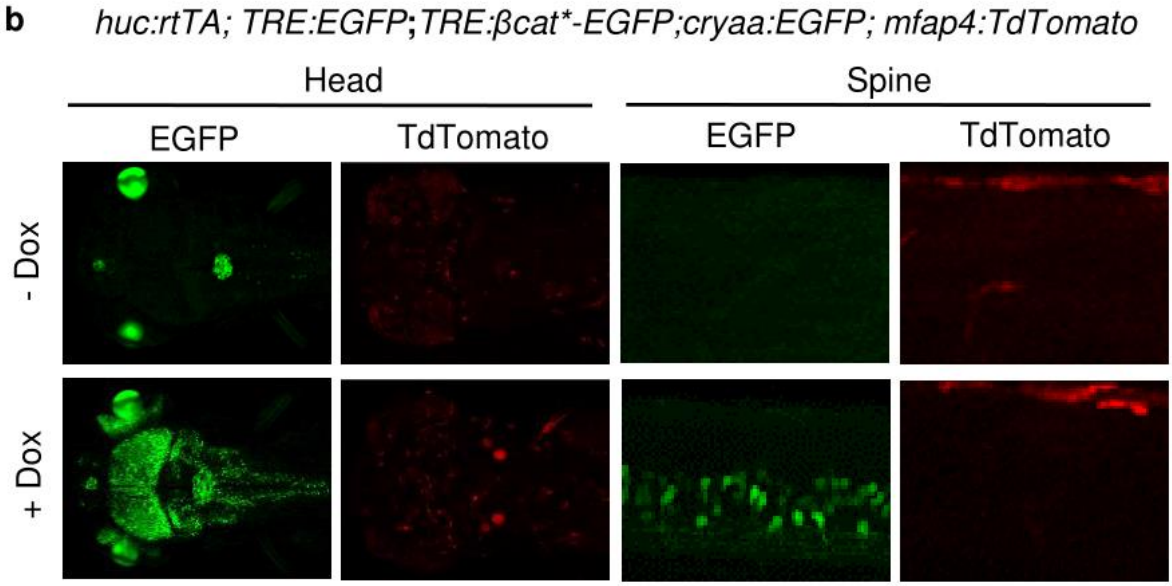
Figure 5: Immune cells present in the CNS of radial glial specific induction of βcat*-EGFP fish are microglia and macrophages, not neutrophils

A) Representative images of *gfap:rtTa; TRE:βcat*-EGFP: mfap4:TdTomato* at 3dpf. Head images are taken at 15x scale bar is 2 µm. Spine images taken at 15x enlarged by 5x to highlight spinal cord, scale bar 0.5 µm. B) Representative images of *gfap:rtTa; TRE:βcat*-EGFP: mpx:mCherry* at 3dpf. Head images are taken at 15x. Spine images taken at 15x enlarged by 5x to highlight spinal cord.



Supplemental Figure 1: Survival Curve and Characterization

A) Survival curve n>15 for each condition. B) Quantification of the percentage of fish in each condition that had swim bladders inflated at 5 dpf n>15 for each condition. C) Quantification of the percentage of fish in each condition that had curled bodies at 5dpf n>15 for each condition.



Supplemental Figure 2: *huc:rtTA; βcat*-EGFP* do not have inflammation in the CNS

Representative images of *gfap:rtTa; TRE:βcat*-EGFP: mfap4:TdTomato* at 3dpf. Head images are taken at 15x. Spine images taken at 15x enlarged by 5x to highlight spinal cord.

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CHAPTER IV: Conclusions

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I would like to thank Anne Broadridge, Audrey Fetsko, Jennifer Gilbert, and Dylan Sebo for their feedback in the writing of this chapter.

Summary

In the prior chapters I have described my work to better understand the developmental signals involved in blood-brain barrier development. Specifically, I describe the developmental consequences of perturbing Wnt/β-catenin signaling in specific CNS cell types. In chapter two I describe the consequences on CNS angiogenesis and barriergenesis of increasing or decreasing Wnt/β-catenin signaling specifically in radial glial cells. Subsequently, in chapter three, I describe the inflammation caused by increasing Wnt/β-catenin signaling in radial glia but not neurons. In this chapter I will summarize my conclusions and propose future directions.

Conclusions

The signals involved in blood-brain barrier formation are still poorly understood. The primary signaling pathway involved in brain endothelial cell differentiation is Wnt/β-catenin signaling (Cullen et al., 2011; Daneman et al., 2009; Umans et al., 2017; Vanhollebeke et al., 2015). This signaling pathway, while partially biochemically characterized, is not well characterized on a cell-type specific level during CNS development. I initially sought to understand how increasing or decreasing Wnt/β-catenin signaling within radial glia would impact CNS angiogenesis and barriergenesis. Briefly, when Wnt/β-catenin signaling is increased specifically in radial glial cells CNS angiogenesis and barriergenesis are disrupted. This is consistent with previous findings from cancer models that Wnt gliomas disrupted the brain vasculature (Phoenix et al., 2016).

I also followed up on additional phenotypes I was noticing when Wnt/β-catenin was increased in radial glial cells. Here, I wanted to determine if there were any consequences for modulating Wnt/B- catenin signaling in radial glia beyond the disruption of brain endothelial cell differentiation. In observing these animals, we saw gross anatomical differences and inflammation. However, animals with Wnt/B- catenin signaling activated in neurons did not show this inflammatory phenotype. We next wanted to determine the cause of the inflammation we saw when Wnt/β-catenin signaling is activated in radial glial cells.

In conclusion, my thesis work indicates that increasing Wnt/β-catenin in radial glial cells has negative consequences in terms of CNS angiogenesis and barriergenesis as well as causing an increase in immune cells within the CNS. My work provides a jumping off point for seeking to answer new questions which I will describe briefly in the following section.

Future Directions

Firstly, from angiogenesis observations I discussed in chapter two we can now ask, why angiogenesis and barriergenesis are disrupted when β-catenin is constitutively activated in radial glial cells. Answering this question requires direct interrogation of several aspects of this pathway including *Wnt* transcripts (*Wnt7a* and Wnt7b),*vegf* transcripts (*vegfaa, vegfab, vegfba,* and *vegfbb*), *Wnt* inhibitors (*wif1, dkk1a, dkk1b,* and *dkk2*) to determine if there is a feedback mechanism similar to the ones seen in Wnt positive gliomas (Phoenix et al., 2016). A global downregulation of Wnt signaling when we activate Wnt/β-catenin signaling in radial glial cells would be consistent with previous

models. However, it is also possible that this feedback mechanism is specific to tumors. This would lead us to assay the radial glia more closely for unexpected phenotype changes at the cellular and protein levels. This in turn will help us better understand how this signaling cascade normally works during development.

In my second research chapter I discussed the characterization of additional phenotypes we observed when increasing Wnt/β-catenin signaling in radial glia. Our recent observation that the inflammation is specific to macrophage/microglia and not neutrophils is puzzling at first glance because neutrophils are thought to be the first immune cells present when inflammation begins. In the future, we hope to test whether the increased number of microglia/macrophages observed is due to a true inflammatory response. To do this, we will assay cytokines (Huising et al., 2004). However, it is possible that we are altering microglia/macrophage cell fate or number by modulating Wnt signaling in the radial glial cells. This would be very novel and would open the door to more closely observing where these microglia/macrophages are coming from and if they are morphologically distinct from the microglia/macrophages normally present in the zebrafish at this stage (Ellett et al., 2011; Herbomel et al., 2001; Wittamer et al., 2011). In both cases this would allow us greater insight into the development of this neuroinflammatory state, which could ultimately help inform the study and treatment of a variety of neurological conditions.

From the onset I hoped that my research would add insight to the complex signaling system involved in the establishment of the blood-brain barrier both with a developmental biology and therapeutic lens. My thesis works provides a basic research

foundation to the role of Wnt/β-catenin signaling in blood-brain barrier development and cautions that modulating this signaling pathway may cause inflammation which clinically could be devastating. This foundation serves as a launch point for new projects to further understand the complex signaling involved in blood-brain barrier development.

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APPENDIX A: Delta Internship Project

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Abstract

Genetics underpins many ethical and legal debates currently facing our nation and the world. However, genetics is an upper level course with multiple prerequisites, meaning most undergraduates will never take a genetics course. To address this gap, the University of Wisconsin-Madison offers “Genetics in the News” a genetics course with no prerequisites for undergraduates of any major and any stage in their education. We wanted to determine if “Genetics in News” was filling this gap by assessing if students learned genetics concepts and if their attitudes toward controversial genetics topics changed. To do this, we administered a paired pre-post test in three different sections of “Genetics in the News”. The test consisted of a concept inventory of genetics topics, an attitude test of controversial genetics topics, and a demographic test. Our results show significant improvement on the concept inventory posttest. Underrepresented students and students from diverse backgrounds including females, racial minorities, first-years, and international students had similar achievement levels to their peers. Students with no prior biology courses had steep gains and only slightly trailed their peers’ posttest scores. Gains on the posttest were similar between STEM and non-STEM majors. Additionally, the attitudinal test indicated students changed their attitudes toward controversial genetics topics. Interestingly, student attitudes did not move unilaterally, indicating the class was not dictating a certain view. Taken together these results indicate “Genetics in the News” helps equip diverse students with the genetics knowledge to enable them to develop their own conclusions about the controversial topics in genetics facing our world today.

Introduction

Genetics is an ever-increasing part of everyday life. With the rise of direct to consumer genetic testing, to the prevalence of GMOs, the general public is continually propositioned by the latest advances in genetics technology. However, most undergraduates will never take a course in genetics as part of their degree requirements. This is because most students taking genetics courses are majoring in some flavor of biology and have already successfully completed biology and chemistry prerequisites before being eligible to take these courses, therefore, there is desire and need to make genetics courses more accessible to students of more diverse majors to prepare them for an increasingly genetic world.

Another consideration is to determine what content from the discipline to expose students to, particularly when thinking about a course that is the stand alone/only exposure to the genetics these students may have. The community has established certain concepts as “core” within the discipline of genetics (McElhinny et al., 2014; Smith et al., 2008). These lists are extensive; therefore, it is important for the instructor(s) to decide if the course is to be a test of all of genetics or is to be more specialized in design.

Additionally, it is important to recognize majors and non-majors approach genetics differently. Non-majors tend to perceive genetics as easier going in and are then surprised by the rigor of the subject (Knight and Smith, 2010). Recognizing this it is important to scaffold appropriately when designing a genetics course for non-majors. Additional considerations for non-majors are intentionally connecting the material to

everyday life (Knight and Smith, 2010). Connecting content to something students can recognize or relate to their own lives has been shown over and over to increase student learning and retention (Knight and Smith, 2010). Importantly majors and non-majors can be equally successful in a genetics course (Knight and Smith, 2010). Taken together this indicates that, while majors and non-majors may approach the subject differently, if the course is properly structured there is no reason to assume non-majors cannot be successful tackling the same content and problems major students would be asked to grapple with.

Another consideration when teaching genetics more broadly is that many of the topics are misunderstood in the general public and or surrounded by vast ethical debate. For example, in the United States the theory of evolution has been frequently tried in court. This can result in students being apprehensive about approaching biology as well as coming in with misconceptions about what evolution is such as thinking a monkey directly evolved into a human (Gregory, 2009). More recent genetic advances such as genetic testing can be controversial (Chokoshvili et al., 2017). With the advances in genetics now being available in the doctor's office, on the grocery store shelves and by ordering kits online, more and more people are being directly impacted by these topics; Therefore it is essential when teaching about these topics to not only anticipate scientific misconceptions and correct them, but to allow space for ethical discussions around these topics.

When selecting evaluation methods, it is important to know what kind of learning you want to capture, as well as what content you wish to evaluate. Concept inventories

have been previously made for a variety of subjects including genetics (Smith et al., 2008). It is important to make sure that a concept inventory is aligned with the Bloom's taxonomy levels of the course objectives (Bloom, 1956; Smith et al., 2008). For our purposes a concept inventory made the most sense as a first step to capture student learning in "Genetics in the News".

This gap between the prevalence of genetics concepts in everyday life and the narrow road to taking a course in genetics led the University of Wisconsin-Madison to develop the course "Genetics in the News". This course has no prerequisites, fulfills a life sciences requirement for graduation, and can be taken at any time during an undergraduate's studies. In brief, the course teaches many core genetics concepts though the lens of the genetics headlines of the last year. Here, I present the first evaluation of core genetics learning concepts for this course and attitudinal data towards controversial topics in genetics covered in this course.

Materials and Methods

Pre/Posttest consisting of 5 coding questions to allow students to be as unidentifiable as possible while still allowing us to pair their pre and posttests. Then 20 multiple choice questions taken from (Pierce, 2017) and paired confidence questions. This was followed by a Likert test consisting of 12 attitudinal questions some written by the research team some taken from (Chokoshvili et al., 2017). This was followed by a demographic test consisting of questions related to gender, ethnicity, seniority, number of previous biology courses, major, international student status, first-generation student status. Full test can be acquired by contacting Annette Dean.

The pretest was administered on the second day of class during class time. The post test was administered after the last concepts being evaluated were taught and was administered on the course website or in person on the last day of class. Students were given 5 points for completing each test that counted towards either their assignment or participation scores. Post tests were not analyzed until final course grades were submitted.

This process was done for three separate sections of the course. Unpaired pre or post tests were removed from the data pool as well as instances where all questions were not answered. Analysis was done using R software and attitudinal data was visualized using the circilize, colorRamps, and colorspace packages (Achim Zeileis, 2009; Keitt, 2012; Team, 2018; Zuguang Gu, 2014).

Results

219 students across the three sections evaluated completed both the pretest and posttest. These test responses were then analyzed as one pool rather than by each section to increase student anonymity as well as increase the number of underrepresented students to the thresholds required for statistical analysis.

Students learned core genetics concepts as measured by our concept inventory. Students had statistically significant improvement on the posttest compared to the pretest (paired t-test, $p=0.0001$, $n=219$, pre mean= 7.69 SD=3.16, post mean= 12.52 SD=3.56) Figure 1. We next wanted to assay whether underrepresented students had similar achievement levels to majority students. Female students learned similarly to male students' pretest t-test $p>0.05$ posttest t-test $p>0.05$ Figure 2. Student of diverse

racial backgrounds learned similarly to their white/Caucasian majority peers pretest t-test $p>0.05$ posttest t-test $p>0.05$ Figure 2. International students learned similarly to their domestic student peers pretest t-test $p>0.05$ posttest t-test $p>0.05$ Figure 2. Taken together, students from underrepresented groups had similar achievement levels on both the pretest and the posttest, indicating that students of different backgrounds in general are learning equally in “Genetics in the News”.

Additionally, we wanted to assay how students if students with different educational backgrounds had different learning gains over the semester. To do this we looked at students with different level of prior biology coursework. As expected, students with prior undergraduate biology courses had a significantly higher pretest score than those with no previous biology course work ANOVA $p<.00001$ Figure 3. On the posttest however, students with no prior biology courses reduced the gap though there was still a significant difference in posttest score between those with prior biology course and those without ANOVA $p<0.05$ Figure 3. This indicates that while students learned in the course, those with no prior biology courses made great gains in their genetics knowledge between the pretest and the posttest.

We next looked at students of different seniority to see if they performed differently on our tests. While students of 2nd year standing had a slightly reduced performance on the pretest compared to other students’ ANOVA $p<0.05$ all students had similar performance on the posttest ANOVA $p>0.05$ Figure 3. This indicates that regardless of seniority students were able to reach the same knowledge level in “Genetics in the News”.

Lastly, we looked at the learning gains of the students broken down by type of major. As expected students in STEM (many of whom were in the biological sciences) had higher pretest and posttest scores, followed by those who were undeclared (many of whom plan to major in biological sciences) and those who are majoring in both a STEM and non-STEM discipline and finally the Non-STEM students ANOVA $p<0.00001$ Figure 3. What is important to emphasize despite the statistical differences in overall starting and ending point is that the gains for each group of students were the same. This emphasizes that the class is enabling students regardless of chosen major discipline to learn at equal rates. Taken together these results indicate that regardless of major discipline “Genetics in the News” is helping students learn at equal rates.

Finally, we wanted to test what if any changes students had in their attitudes towards controversial genetics topics between the start and end of the semester. Here, we saw on several of the questions students’ attitudes were changing, though not in the same direction Figure 4. This indicates that “Genetics in the News” is not leading students to take a particular side on controversial genetics topics.

Discussion

Here we have conducted the first evaluation of the course “Genetics in the News” for both student learning of core genetics concepts and its influence on the attitudes of students towards controversial topics in genetics. We see from our data that not only did the students as a collective group learn genetics concepts but also underrepresented students in general learned similarly to their majority peers. We are excited to have provided the first evidence for the department that “Genetics in the News” is a way to

engage students who likely would not have the opportunity to take a genetics course the opportunity to learn this content. The format for this course likely contributed to students' success. This may be because of the courses extensive use of active learning which has been shown to benefit the learning of all students (Dolan and Collins, 2015; Freeman et al., 2014). Future evaluations of this course could look at how specific activities directly relate to student learning of specific core concepts in the hopes of being able to improve subsequent offerings of the course. Additionally, the test we created could be implemented in other genetics courses, to measure student learning over the course or perhaps students' prior knowledge of some of genetics fundamentals in a more advanced course.

Our data also indicate that the course does lead to appreciable change of opinion on a variety of controversial topics in genetics without biasing the change primarily in one direction or another. As instructors this was a reassuring and affirming result, and as with the genetics content learning results it would be interesting to determine what thinking is taking place that is leading to this change of opinion. One thing that may have contributed were reflective assignments both in class and outside of class that encouraged students to reflect and form opinions about controversial topics discussed in class using the content from class. These types of reflective exercises have been shown to increase student thought and promote new ways of thinking (Ryan, 2013). If these questions were asked in more cohorts over a greater period of time it may be possible to see changes in broader population opinions on these controversial topics.

Our study has a couple of important limitations. First while 219 full tests were completed that still leaves several underrepresented groups at a number small enough that we cannot make statistical conclusions. This would indicate that in order to have sufficient students to meet statistical requirements five to six sections of similar size to the ones we analyzed should be used. We were also limited in terms of who our students are in that they are all UW-Madison students which means our population may be unique when it comes to the opinions or to the positive learning gains we measured. Our concept inventory has not been validated formally so there may be problems in the questions or the categories of core genetics concepts we think they measure which they may not actually measure. Again, this would take additional cohorts of people to take our test as well as some additional analysis on the test itself.

In summary, our study sought to determine if the course “Genetics in the News” successfully taught core genetics concepts and if student’s opinions about controversial topics in genetics changed between the beginning and end of the semester. We did this using a pre/posttest design, with tests consisting of a genetics concept inventory, an attitudinal Likert test, and a demographic form. We found that students learned core genetics concepts, and that student attitudes toward controversial topics in genetics changed, but not in a prescribed direction. Future studies can further validate our, test as well as explore what specific parts of the “Genetics in the News” course are influencing student learning and their opinions towards controversial genetics concepts.

Figures

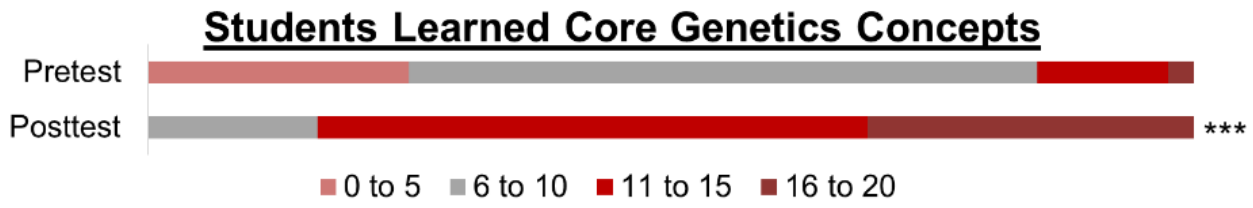

Figure 1: Students learned core genetics concepts. Students learned core concepts in genetics as measured by our concept inventory (20 questions). Colors represent the percentage of students receiving scores within each window of points (p=.0001 paired t-test, n=219, pre mean=7.69 SD=3.16, post mean=12.52 SD=3.56).

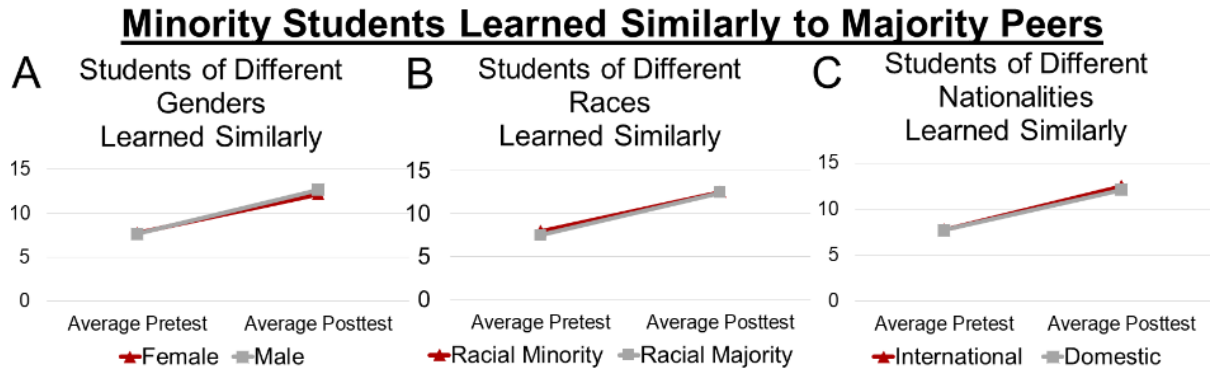

Figure 2: Minority Students Learned Similarly to Majority Peers. Total n=219 **A)** Female n=93 t-test pre p>.05, t-test post p>.05. **B)** Minority n=72 t-test pre p>.05, t-test post p>.05. **C)** International n=28 t-test pre p>.05, t-test post p>.05

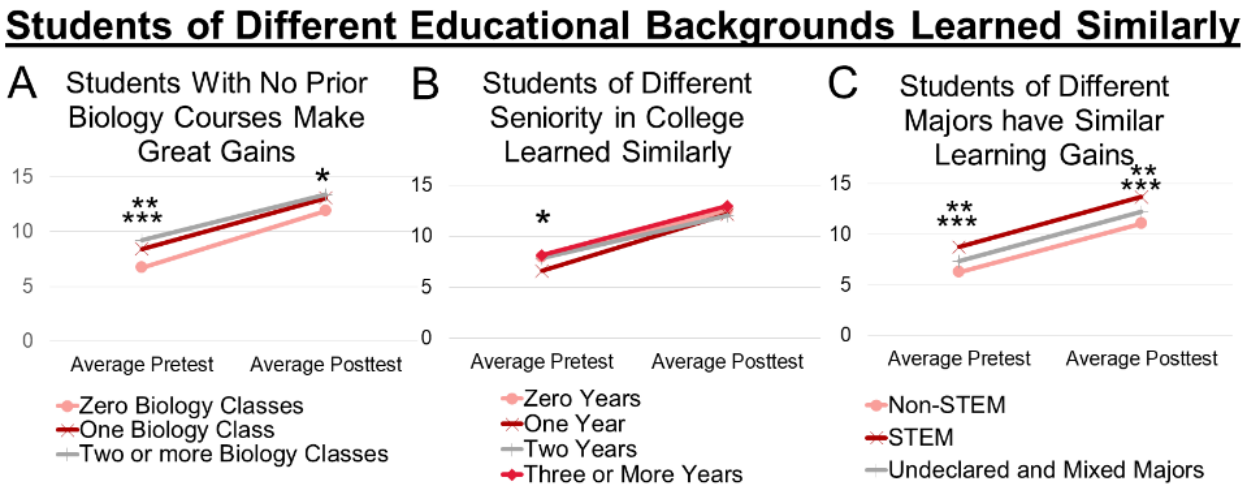

Figure 3: Students of Different Educational Backgrounds Learned Similarly. A) None n=115 One n=67 Two or more n=37 one-way ANOVA pre p<.00001 one-way ANOVA post p<.03 **B)** Zero n=87, One n=59, Two n=35, Three or More n=38 one-way ANOVA pre p<.00001 one-way ANOVA post p<.03 **C)** Non-STEM n=78, STEM n=107, Undeclared and Mixed n=34 one-way ANOVA pre p<.00001 one-way ANOVA post p<.00001.

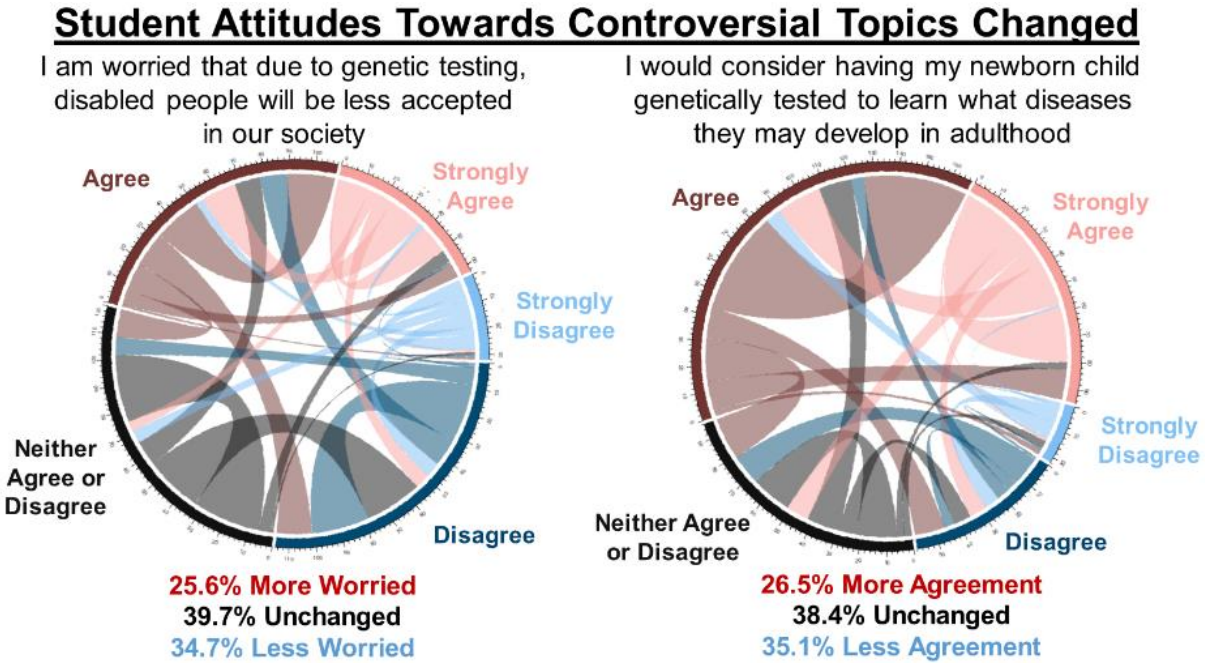

Figure 4: Student Attitudes Toward Controversial Topics Changed. Ribbon origins and colors are based on pre answers, where the ribbon is connected to represents the answer given on the post, and the width of the ribbon represents the number of students following each chord.

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