THE ROLE OF TRANSCRIPTIONAL COACTIVATOR ZMIZ1
IN NEOCORTICAL DEVELOPMENT

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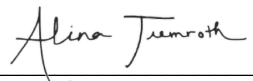

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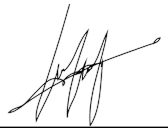

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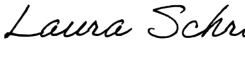

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(Dr. Maria J. Galazo, Cell and Molecular Biology)

The successful generation of cellular diversity and circuit connectivity in the cerebral cortex is fundamental for high cognitive functioning in mammalian species. Disruptions to these processes are implicated in a range of neurodevelopmental disorders such as intellectual disability, autism spectrum disorders, schizophrenia, and other neuropsychiatric differences. Recent research has revealed transcriptional coregulator ZMIZ1 to be involved in a syndromic neurodevelopmental disorder in humans presenting with intellectual disability and other developmental delays. Although these disorders are known to be at least partially caused by disruptions in the cerebral cortex, the role of ZMIZ1 in the developing cortex is unknown. The objective of this study is to identify the potential roles of ZMIZ1 in the development of the neocortex, the portion of the cerebral cortex most responsible for the increased cellular diversity and cognitive function in primates and humans. We employ two Cre driver mouse lines to ablate the *Zmiz1* gene broadly in the neocortex and specifically in corticothalamic projection neurons, which have been shown to upregulate *Zmiz1*, as compared to other projection neurons, during embryonic development. Using these mouse models, in combination with histological analysis, we identify potential roles for ZMIZ1 in the postnatal development of neocortical structure, and in the development of the corpus callosum, as likely mediated by midline closure and midline guidance structures. Together, our findings suggest that *Zmiz1* is required in cortical progenitors for the successful assembly and wiring of the cerebral cortex.

ACKNOWLEDGEMENTS

I would like to start by extending my deepest gratitude to my primary research advisor Dr. Maria Galazo. Her unwavering support and mentorship throughout my undergraduate education has profoundly shaped my scientific mind, myself, and my future aspirations. I have had the absolute pleasure to work with the amazing scientists of the Galazo lab, notably Lee Vaasjo, Rajan K C, Xiao Han, and Abbigail Thurmon. These individuals have contributed immensely to my growth and have generously volunteered their time to train and support me. Lee Vaasjo has been a particularly important mentor for my scientific development and career. His excitement for science and avid review of literature have been critical resources for my education. Importantly, some of the papers he has shared with me have inspired parts of the discussions included in this thesis.

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INTRODUCTION

Neocortical Development

The proper development of the mammalian cerebral cortex is critical for high cognitive functioning, sensory processing, and motor control (Greig et al., 2013). As the largest and most complex region of the mammalian brain, the cerebral cortex contains immense neuronal diversity. The evolution of the neocortex, the largest region of the cerebral cortex, is responsible for much of the increased complexity and overall brain size of many advanced species, including humans (O'Leary et al., 2007). The development of these diverse cellular subtypes and circuits requires intricate and precise developmental mechanisms throughout embryonic and postnatal stages. Disruptions to these processes can lead to a wide range of life-long neuropsychiatric and neurodevelopmental disorders with profound individual and societal impacts. Understanding how neocortical diversity develops will provide critical insight into the neurobiological basis of advanced cognition and how aberrations to this process result in disease.

Laminar Organization of the Mammalian Neocortex

The mammalian neocortex is organized into six layers in the radial dimension, each of which contain specific neuronal subtypes and are histologically differentiable (Figure 1B; Greig et al., 2013). While this traditional six-layer categorization oversimplifies the complexity of neocortical architecture, as there are additional sub-stratifications, this classification strategy is widely accepted due to its histological significance (Lodato et al., 2015). Within these layers, there are two major classes of neurons that inhabit the neocortex: GABAergic neurons and glutamatergic neurons. The

inhibitory GABAergic neurons are critical in modulating the activity of excitatory projection neurons (Lodato and Arlotta, 2015). Projection neurons (PN), which will be the focus of this thesis, account for approximately 80% of cortical neurons (Han and Šestan, 2013). PN are largely excitatory, glutamatergic, and serve as the main inputs and outputs of the cerebral cortex (Han and Šestan, 2013; Lodato and Arlotta, 2015). PN generally possess either intracortical or corticofugal projections. Intracortical PN reside within all layers of the cortex, especially in upper layers 2/3 (Figure 1C). These projections are either associative, projecting within the same hemisphere, or commissural, projecting across the midline into the contralateral hemisphere. In contrast, corticofugal PN primarily reside in deep cortical layers 5 and 6, and project into subcortical regions (Figure 1C; Figure 2B, red; Lodato and Arlotta, 2015). For example, cortico-thalamic projection neurons (CThPN), residing primarily in layer 6 (L6), project from the cortex to the thalamus (Figure 2B, green). As evident in the case CThPN, PN are often classified by their projections and known to reside in specific cortical layers. Other relevant PN in this thesis include the callosal-projection neurons (CPN) of layer 2/3 (Figure 1C; Figure 2).

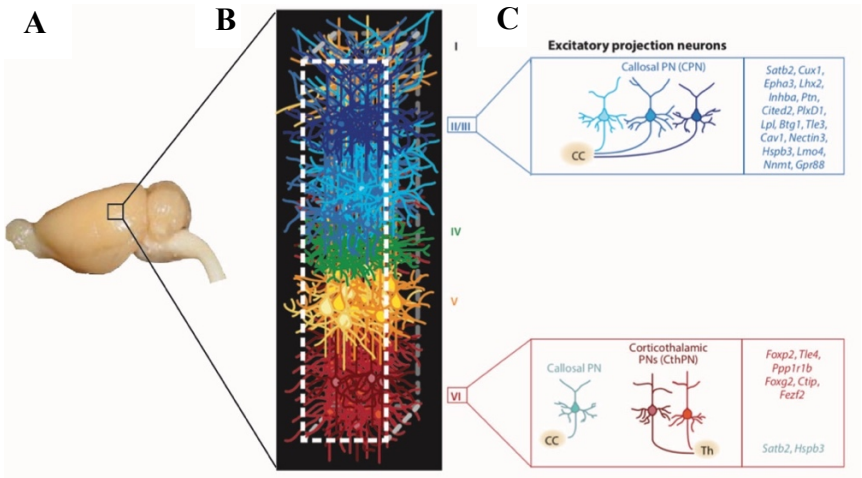

Figure 1: Radial organization of the mouse neocortex. (A) Sagittal view of a whole mouse brain referencing an area of the cerebral cortex. (B) Schematic of distinct PN populations

inhabiting the six neocortical layers. (C) Upper layers 2/3 are highly populated by callosal projection neurons (CPN). Deep layer 6 neuron subtypes include callosal projection neurons (CPN) and corticothalamic projections neurons (CThPN), among others that are not depicted. Known identity controls of these subtypes are depicted in the far-right column and color coded. Figure adapted from Lodato and Arlotta, 2015.

Molecular Logic of Cortical Neuron Differentiation and Migration

The formation of the laminar structure of the neocortex and its diverse cell types requires complex spatiotemporal molecular interactions during embryonic development. During this process, cortical progenitor cells must proliferate and then terminally divide to give rise to neurons in the growing cortex (Figure 2A). Then, newly born neurons must differentiate, migrate to their appropriate cortical layer, and acquire their subtype identity. In mice, the primary model organism used in this thesis, progenitor cells give rise to neurons beginning at embryonic day (E) 11 and ending around E19 (Lodato and Arlotta, 2015). Throughout this period, a new class of PN arises roughly each day. Since the cortex develops from an "inside-out" method, deep layer neurons arise first and upper-layer neurons arise last. Thus, at E11, subplate (SP) neuron generation peaks, followed by L6 CThPN on E12, L5 subcerebral projection neurons (SCPN) on E13, L4 neurons on E14, and L2/3 CPN on E15 (Figure 2A; Greig et al., 2013). This process is tightly regulated by genetic programs that guide the differentiation of progenitors and newly born neurons (Kwan et al., 2012). These programs can be divided into two major categories: (1) molecular guides acting in progenitors and (2) post-mitotic controls of subtype identity in newly born neurons (Grieg et al., 2013).

Post-mitotic controls, often transcription factors (TFs), regulate the differentiation of the newly born neurons into specific neuronal subtypes with unique molecular signatures and connectivity. Precise regulation of these TFs, and other molecular contributors, is critical to the proper development of PN classes and cortical connectivity

(Kwan et al., 2012; Greig et al., 2013; Lodato and Arlotta, 2015). During early embryonic development, many cell populations express similar TFs, but as embryonic and postnatal development continues, TFs become progressively cell-type specific (Grieg et al., 2013). Differentiated PNs will express subtype- and layer-specific TFs (Figure 1C; Han and Šestan, 2013). Many of these TFs can be used as molecular markers for the specific PN subtype (Figure 1C). Elucidating the genetic mechanisms that produce and safeguard functional PN subtypes is an active field of study. As genetic profiling technologies continue to advance, researchers will be able to better classify PN subtypes based on gene expression, greatly enhancing our understanding of neocortical architecture and diversity.

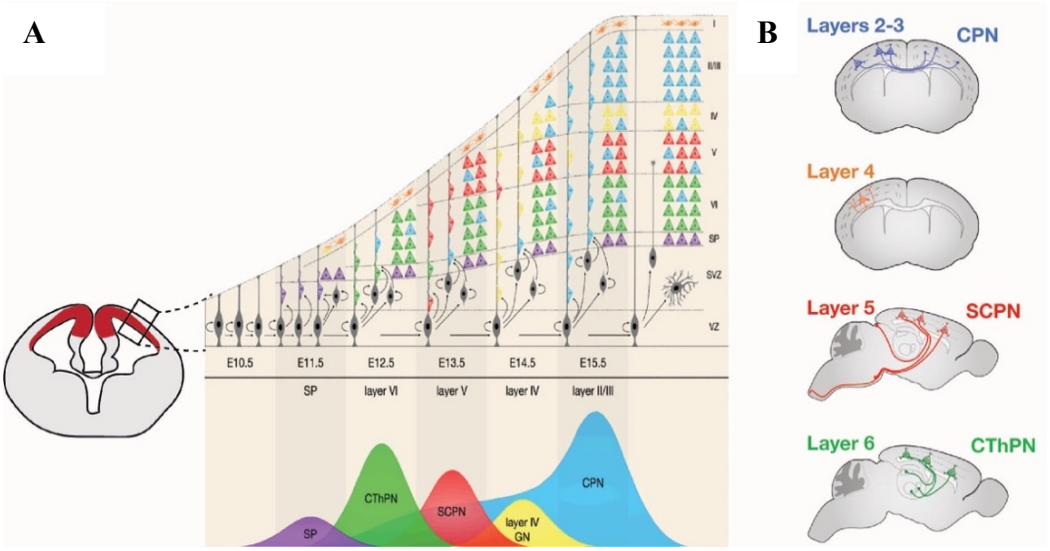

Figure 2: Generation, migration, and differentiation of neocortical cells. (A) Schematic illustrating the terminal division of progenitor cells (grey) in the pallium (dark red region of embryonic brain depicted). Generation of neocortical neurons occurs from an "inside-out" method, in which deep-layer neurons are generation first and upper-layer neurons last. This process begins with subplate neurons (SP; purple) at E11.5. CThPN (green) generation peaks at E13.5; SCPN (red) at E13.5; L4 neurons (yellow) at E14.5; CPN (blue) at E15.5. (B) After migrating to their appropriate cortical layers, PN subtypes continue to acquire their subtype identities and begin to project axons to their targets. L6 CThPN project to the thalamus, L5 SCPN primarily project to the brainstem and spinal cord, L4 neurons project intracortically, and CPN project to the contralateral cortex. Figure adapted from Greig et al., 2013.

Relevant Projection Neuron Subtypes

PN subtypes have distinct molecular, physiological, and functional properties. This thesis will focus on two specific PN subtypes: CThPN and CPN. CThPN play critical roles in sensory processing, decision-making, and working memory (Galazo et al., 2016; Vaasjo et al., 2022). Projecting from the cortex to the thalamus (Figure 2B, green), CThPN are known to modulate the activity of the thalamus and other deep layer cortical neurons, controlling transmission of sensory information in the cortex (Galazo et al., 2016). As the first PN subtype to develop, and possibly the first pyramidal neuron subtype to inhabit the developing cortex, CThPN inhabit the deep layers of the neocortex: the majority in L6, as well as some in L5 (Thomson, 2010; Galazo et al., 2016). Common molecular controls of L6 CThPN identity include transcription factors Tbr1, Tle4, Foxp2, Fezf2 and Fog2 (Figure 1C; Galazo et al., 2016; Hevner et al., 2001; Thomson, 2010). These genetic controls contribute to the differentiation process of CThPN during embryonic development. For example, Tbr1 and Fezf2 are known transcriptional master regulators of CThPN identity during embryonic development and early postnatal stages (Galazo et al., 2016; Hevner et al., 2001; Thomson, 2010). It is unknown, however, whether these same transcriptional programs are responsible for identity maintenance throughout adulthood. While some studies have suggested that similar gene regulatory programs from embryonic development are required throughout life (Deneris and Hobert, 2014), the life-long changes in transcriptional control remain an active field of study. In mice, these embryonic and early postnatal TF programs in CThPN regulate their projections, among other identity characteristics, and these projections reach and innervate the thalamus by E14/E15 (Thomson, 2010).

At this same time of embryonic development (E15), the majority of callosal projection neurons (CPN) arise from progenitors and migrate to upper layers 2/3 of the neocortex (Figure 2A, blue). Connecting the two hemispheres of the cortex, CPN are critical in associative information processing (Fame et al., 2011). Disruptions to CPN development and function are implicated in many cognitive disorders such as autism spectrum disorder, highlighting the importance of CPN in cortical functioning (Fame et al., 2011). While many molecular mechanisms control CPN specification, transcription factor Cux1 and chromatin remodeler Satb2 are notable controls of CPN identity. Cux1 is specifically expressed in upper layer CPN and can be used as a molecular marker for CPN (Greig et al., 2013; Rouaux et al., 2013). Chromatin remodeler Satb2 is required for the development of callosal projections in CPN (Alcamo et al., 2008; Greig et al., 2013; Leone et al., 2008).

As evident in the case of CThPN and CPN, genetic profiling has allowed researchers to define cortical neuron subtypes with increasing specificity, based not only on the layer position and projections of a neuron, but on the transcriptome of the neuron throughout development.

Areal Organization of the Cortex

Over the past couple of decades, molecular profiling has revealed gene expression differences of neuronal subtypes across different areas of the cortex (O'Leary et al., 2007). In the tangential direction, as opposed to the six-layer radial dimension, the cortex is divided into areas with distinct gene expression patterns and cortical architecture (Figure 3). This "arealization" is influenced by TFs and other molecular mechanisms

during development and allows for functional specializations of distinct cortical regions (O'Leary et al., 2007). It is important to note that PN subtypes can have distinct features and functions across areas. Notably, PN have different gene expression and axonal projection patterns across cortical areas (Galazo et al., 2016; Han and Šestan, 2013).

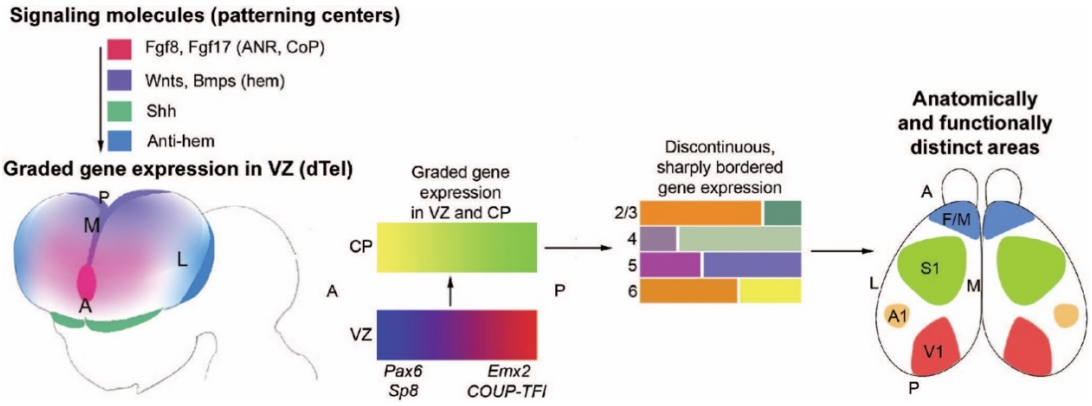

Figure 3: Arealization of the cerebral cortex. Gradient expression of signaling molecules (TFs: Fgf, Wnts, Bmps, Shh, Anti-hem) in the ventricular zone (VZ) of the dorsal telencephalon (dTel; developmental origin of cerebral cortex) during embryonic development creates anatomically and functionally distinct cortical areas in the tangential dimension. In combination with the distinct expression of molecular controls in the radial dimension of the cortical plate (CP), distinct cortical layers and functional areas are produced. Schematic specifically shows the frontal/motor areas (F/M; blue), primary somatosensory area (S1; green), primary auditory area (A1; yellow/orange), primary visual area (V1; red). Figure adapted from O'Leary et al., 2007.

Disease Relevance of the Cerebral Cortex

As the largest and arguably most complex component of the mammalian brain, the successful development of the cerebral cortex requires intricate interactions between various molecular controls with precise spatiotemporal dynamics (Greig et al., 2013; O'Leary et al., 2007). Failure of the neocortex to properly develop can lead to life-long psychiatric and neurological disorders. Disruptions to neuronal positioning and subtype identity are linked to autism, schizophrenia, intellectual disability, and other developmental and language delays (Kwan et al., 2012). These developmental and

neuropsychiatric disorders often result from miswiring of the cortex that impairs cortical communication and, thus, cognitive functioning. Many of these disorders, notably autism and intellectual disability, are specifically linked to disruptions in corpus callosum functioning.

Corpus Callosum Development

Commissural fiber tracts, which connect the two hemispheres of the brain, are integral for interhemispheric communication and high cognitive functioning (Donahoo and Richards 2006; Nishikimi et al., 2013). Widely studied commissures include the corpus callosum, hippocampal commissure, and anterior commissure, among others (Figure 4). Out of these tracts, the corpus callosum (CC) has evolved as the most prominent commissure in primates and humans, and the largest fiber tract in the human central nervous system (Donahoo and Richards, 2009; Mihrshahi, 2006; Nishikimi et al., 2013). Thus, the CC is of notable importance for human cortical communication and wiring.

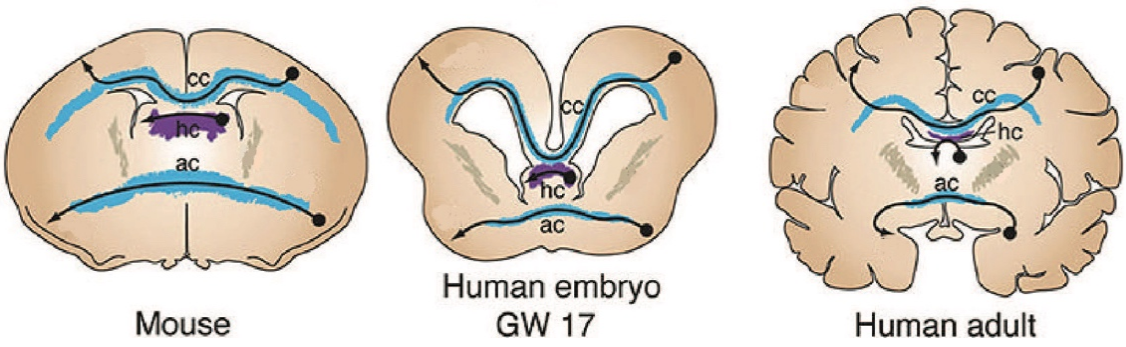

Figure 4: Interhemispheric fiber tracts in mice and humans. The corpus callosum (cc; blue), hippocampal commissure (hc; purple), and anterior commissure (ac; blue) in the mouse, the human embryo during gestation week (GW) 17, and the human adult. In humans, the cc has evolved as the most prominent commissure. Adapted from Suárez et al., 2014.

Development of the Corpus Callosum

The CC largely consists of neocortical projections and, to a lesser extent, cingulate axons (Donahoo and Richards, 2009; Koester and O'Leary, 1994; Suárez et al., 2018). The development of the CC requires the successful completion of the following: (1) patterning and formation of the midline, (2) generation and guidance of callosal neurons and their projections, and (3) innervation of callosal axons with their appropriate target (Donahoo and Richards, 2009; Gobius et al., 2016). The patterning and formation of the midline and its neuronal and glial populations is required to prepare the midline for axon crossing (Donahoo and Richards, 2009; Gobius et al., 2016; Piper et al., 2009; Shu et al., 2003). These neuron and glia populations, which will be discussed later in this introduction, secrete guidance factors and make cellular contacts with callosal projections to guide the developing axons across the midline (Donahoo and Richards, 2009; Shu et al., 2003; Suárez et al., 2014; Tole et al., 2006). This landscape (Figure 5) allows pioneering cingulate axons to cross the midline, which will provide a substrate that neocortical callosal axons use to cross the midline.

The first axons to cross the dorsal midline, and pioneer the CC, arrive at the midline from the cingulate cortex at E14-E15 in mice (Figure 5; Donahoo and Richards, 2009; Koester and O'Leary, 1994; Rash and Richards, 2001). These cingulate pioneer cells provide a scaffold that neocortical axons use to cross the midline about one day later, E15-E17 (Figure 5; Donahoo and Richards, 2009; Koester and O'Leary, 1994; Nishikimi et al., 2013). Both the cingulate neurons and the CPN rely on cell autonomous and non-cell autonomous mechanisms to guide their axons across the midline. Non-cell autonomous factors include guidance cues secreted by midline cell populations that

interact with receptors on crossing axons. The proper expression of receptors on the crossing axons, as well as the ability of the axon to grow and extend, are notable examples of cell autonomous guides. Disruptions to these mechanisms can interfere with CC development and misguide callosal projections (Donahoo and Richards, 2009).

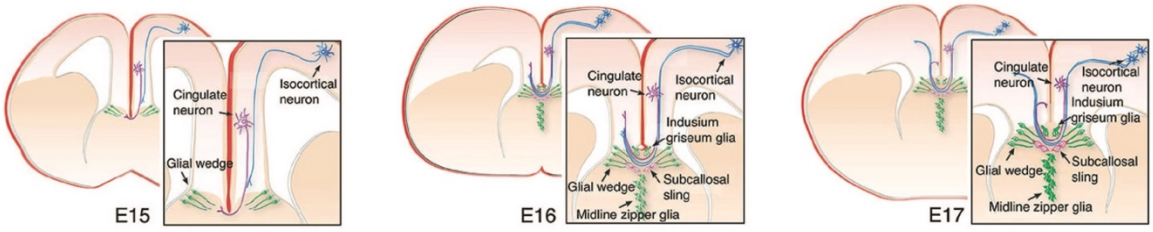

Figure 5: Midline cell populations underlying corpus callosum development. At E15 in mice, cingulate neurons begin to cross the midline, partially guided by guidance factors secreted by the glial wedge (GW). Isocortical neurons, often CPN, follow the cingulate axons and a small population of isocortical neurons cross the midline at E16. At this point, the indusium griseum (IG), subcallosal sling, and midline zipper glia (MZG) are fully detectable by immunohistochemistry. At E17, isocortical neurons continue to cross the midline and extend towards their contralateral targets. Cingulate pioneer axons begin to innervate their targets, primarily in the contralateral cingulate cortex. Midline crossing and the innervation of contralateral targets continues through embryonic development and early post-natal timepoints. Adapted from Suárez et al., 2014.

Both populations of callosal projections, from the cingulate cortex and the neocortex, are thought to respond to similar guidance factors (Donahoo and Richards, 2009; Hatanaka et al., 2009; Piper, Plachez et al., 2009). The axons are guided by attractive and repulsive interactions with extracellular guidance factors in their growth cone, the distal tip of a developing axon. Each growth cone contains receptors that react to specific guidance factors. Binding of a signaling molecule with a receptor on the axon activates secondary cascades inside the cell, either through phosphorylation of coreceptors or through secondary messengers (Donahoo and Richards, 2009). Based on these signaling cascades, the axon will either experience chemoattraction, and grow towards the cue, or chemorepulsion, and grow away from the cue. In the midline

environment, axons will navigate through overlapping guidance cues that carefully guide the axon across the midline and towards its appropriate target in the contralateral cortex (Dickson and Senti, 2002; Donahoo and Richards, 2009). Cell autonomous changes to the gene expression or the cytoskeleton of an axon during this phase may misroute the axon by disrupting its response to guidance cues (Dickson and Senti, 2002; Donahoo and Richards, 2009).

The midline structures that provide these cues are known to be in place by E15, around the time that the cingulate axons first cross the midline and pioneer the CC (Figure 5; Shu et al., 2003; Nishikimi et al., 2013). These structures, and the signaling molecules they secrete, are largely conserved between mice and humans (Nishikimi et al., 2013). Three major guidance populations include the midline zipper glia (MZG), the indusium griseum (IG), and the glial wedge (GW) (Figure 5; Shu et al., 2003; Tole et al., 2006). While these populations are mostly composed of glia, the IG notably contains a significant neuronal population, which may be related to the hippocampal formation but whose function remains elusive (Sanders et al., 2021). These midline glia populations, however, are known to secrete long-range guidance molecules that direct callosal axons (Donahoo and Richards, 2009; Nishikimi et al., 2013). For example, Slits are common attractive cues, while Wnts and Netrins are common repulsive cues to callosal axons (Nishikimi et al., 2013; Tole et al., 2006). MZG assist in the fusion of the ventral forebrain, which is required for callosal crossing (Donahoo and Richards, 2009; Paul et al., 2007). IG glia (IGG) serve as the dorsal boundary of the CC, as they are located dorsal to the CC and secrete Slit2, which is repulsive to callosal axons (Donahoo and Richards, 2009; Tole et al., 2006). The GW, which likely consists of radial glia, also

expresses Slit2 but is located ventral to the CC and thus serves as the ventral boundary of the CC (Donahoo and Richards, 2009). While the proper location of these cell guidance populations is critical for proper axon guidance, the orientation of the cells is also important. One study found that a 180-degree rotation of the GW causes axons to turn away from the midline (Shu and Richards, 2001). The same study also found that misorientation of IGG can misroute callosal projections. These findings highlight the importance of midline glial structures, and their proper development, in the formation of the CC and the successful targeting of callosal axons.

Neuronal populations in the midline and the CC also play a critical role in guiding callosal axons during embryonic development. Besides the pioneering cingulate axons, transitory glutamatergic and GABAergic neurons in the CC are also known to guide callosal axons, but the mechanisms of these interactions remain unclear (Niquelle et al., 2009). At E15, when pioneering cingulate axons cross the midline, neurons in the subcallosal sling, a transitory neuron population ventral to and parallel to the CC, also begin to migrate and cross the midline (Figure 5; Donahoo and Richards, 2009). Since CC development occurs simultaneously with the migration and formation of the subcallosal sling, it is unclear whether this population helps direct CC development or requires the CC to develop. However, CC agenesis (the failure of the CC to form) is often accompanied by disruptions to the subcallosal sling, suggesting an intimate relationship between the subcallosal sling and CC development (Donahoo and Richards, 2009).

Disruptions to Corpus Callosum Development

While CC dysgenesis can be caused by a myriad of factors, common causes include disruptions to neocortical structure and subtype development, as well as misexpression of guidance factors by the midline cell populations, notably the midline glia (Donahoo and Richards, 2009). Crossing axons, importantly the cingulate pioneers and CPN, require proper neocortical structure to navigate to the midline, though these mechanisms are not fully understood (Donahoo and Richards, 2009). In addition, these neurons must acquire a callosal identity during the cellular differentiation process. For example, expression of chromatin remodeler Satb2 is required for neocortical neurons to adopt a callosal identity and project axons through the CC (Alcamo et al., 2008; Greig et al., 2013; Leone et al., 2008). Misexpression of Satb2, and other identity controls, in CPN can thus lead to a reduced, malformed, or absent corpus callosum. Non-cell autonomous disruptions to callosal axons, such as misexpression of guidance factors by midline glial populations or misorientation of these structures can also misguide axons and cause CC dysgenesis or agenesis (Donahoo and Richards, 2009; Shu and Richards, 2001).

A common result of disruptions to CC development includes the formation of Probst Bundles. Probst Bundles can result when callosal fibers fail to cross the midline and thus remain ipsilateral, forming a “whorl” shape adjacent to the midline (Figure 6; Donahoo and Richards, 2009; Ren et al., 2007). Although the axons appear to be tangled in this “whorl,” these axons have been shown to project ipsilaterally on the rostral-caudal axis in mice and humans (Donahoo and Richards, 2009; Ren et al., 2007). Often, the Probst Bundle is not caused by failure of the axon to extend but either by abnormally structured glial guidance populations, misexpression of guidance factors, or altered sensitivity of callosal neurons to guidance cues (Donahoo and Richards, 2009; Mendes et

al., 2016). In this case, interhemispheric communication is greatly reduced, which can impair high order cognitive functions. Abnormal development of the CC is common in multiple neurosynaptic disorders, including schizophrenia, as well as in autism, intellectual disability, and other neurodevelopmental disorders (Mendes et al., 2006; Nishikimi et al., 2013; Paul et al., 2007).

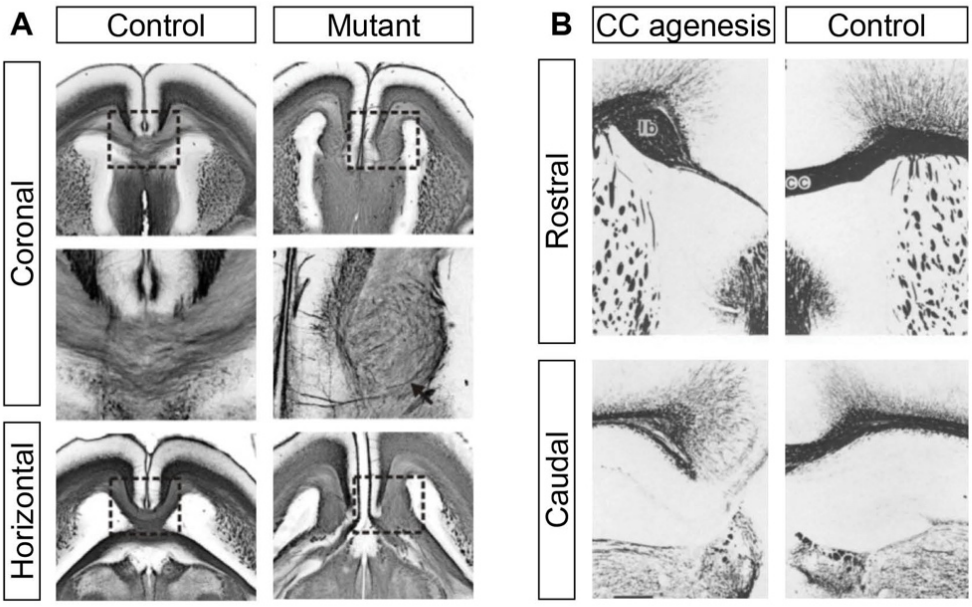

Figure 6: Probst Bundle morphology. (A) Diffusor tensor magnetic resonance imaging (DTMRI) images compare CC morphology in a control mouse and a mutant mouse (Netrin receptor DCC knockout) presenting with Probst bundles. "Whorl" morphology of white matter apparent in mutant (third row, arrow). (B) Silver stained callosal fibers in a mouse presenting with agenesis of CC compared to a control mouse (rostral and caudal coronal sections). Abbreviations: lb, longitudinal Probst Bundle; cc, corpus callosum. Adapted from Ozaki et al., 1987 and Ren et al., 2007.

In summary, the formation of the CC requires the successful guidance of callosal axons by both cell autonomous and non-cell autonomous mechanisms. Midline glia and neuron populations must be properly established in the midline before CPN begin to project their axons. CPN must successfully acquire their subtype identity within the neocortex and respond to guidance cues in the midline environment. Disruptions to the development of the guidance populations, the callosal projections, or the interactions

between the callosal axons and the guidance populations will interfere with CC development.

ZMIZ1 Background

Recent studies have implicated transcriptional coactivator ZMIZ1 in a neurodevelopmental disorder associated with intellectual disability, motor and speech impairments, and other developmental delays (Carapito et al., 2019; Córdova-Fletes et al., 2015; Latchman et al., 2020). In one case report, researchers describe an autosomal dominant inheritance pattern for this syndromic *Zmiz1*-related disorder (Latchman et al., 2020). However, the function of ZMIZ1 in neurodevelopment is unclear.

Cellular Processes Linked to Zmiz1

ZMIZ1 is a transcriptional coactivator involved in chromatin remodeling in the brain (Latchman et al., 2020). Transcriptional co-regulators, such as ZMIZ1, bind to DNA-binding TFs and regulate transcription by altering chromatin structure or regulating transcription complexes (Pinnell et al., 2015; Stallcup and Poulard, 2020). Transcriptional regulation is critical to the development and differentiation of cellular systems. Interestingly, TF co-regulators are extremely gene specific and only target a specific subset of the total genes controlled by any given TF (Stallcup and Poulard, 2020). These genetic subsets are often related to distinct physiological processes, meaning that many TF co-regulators modulate specific physiological phenomena. ZMIZ1 has been shown to interact with chromatin remodeling complexes that regulate the expression of genes involved in synapse and dendrite development in the brain (Carapito et al., 2019;

Latchman et al., 2020; Li et al., 2006; Pinnell et al., 2015). In the cerebral cortex, *Zmiz1* expression is enriched in the deep layers of the neocortex during embryonic development (E18), suggesting a role for *Zmiz1* in the development of the cerebral cortex (Figure 7; Galazo et al., 2016). At E14.5 in the developing neocortex, *Zmiz1* is expressed in apical progenitors, and their daughter basal progenitors and neurons (Telley et al., 2016). A recent study suggests a role for ZMIZ1 in cortical neuron differentiation and migration, yet the role of ZMIZ1 in cortical development remains unestablished. In other biological systems, however, ZMIZ1 is known to be involved in cell migration, cell proliferation, and cell morphology, which may support a role for ZMIZ1 in neuronal migration and differentiation.

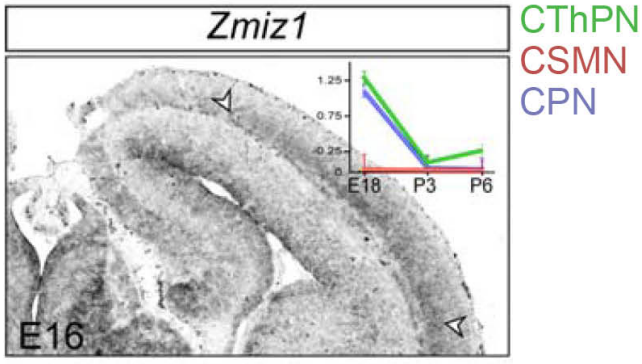

Figure 7: Embryonic and early postnatal expression of *Zmiz1* in the cortical plate. In-situ hybridization and microarray analysis reveal *Zmiz1* expression in layer 6 of the neocortex at E16. Graph indicates *Zmiz1* expression levels at E18, P3, and P6 in corticothalamic projection neurons (CThPN; green), corticospinal motor neurons (CSMN; red), and callosal projection neurons (CPN; blue). *Zmiz1* expression levels in CPN and CThPN peak at E18. Expression in CPN follows the same trends as CThPN between E18 and P3 at slightly reduced levels. After P3, *Zmiz1* expression levels in CPN remain low but CThPN expression increases again. Adapted from Galazo et al., 2016.

A recent study identified a potential role for ZMIZ1 in early post-mitotic positioning of pyramidal neurons in the neocortex (Carapito et al., 2019). In this study, overexpression of *Zmiz1* during late embryonic development, through the introduction of DNA constructs containing common human *Zmiz1* mutations, impaired the migration of

late-born neurons in mice. Instead of migrating to the upper cortical layers, these late-born neurons accumulated in the deep layers of the cortical plate, close to where they arose from progenitor cells in the ventricular zone and subventricular zone (Carapito et al., 2019). While the role of ZMIZ1 in neuronal migration has not been established, ZMIZ1 is known to be involved in the migration of other cell populations, including melanocytes. ZMIZ1 regulates melanocyte migration by contributing to the remodeling of their actin cytoskeleton (Li et al., 2020). Since melanocytes, neurons, and glia all arise from the ectoderm during early gestation (Mort et al., 2015), it is possible that ZMIZ1 may influence the migration of these cell populations through similar mechanisms; however, this hypothesis has not yet been investigated.

ZMIZ1 also has roles in inducing or maintaining cell morphology. In melanocytes, knockdown of ZMIZ1 in-vitro produces irregular cellular morphologies, suggesting the presence of disruptions to apoptosis regulation and the structure of the cytoskeleton (Li et al., 2020). In neurons, in-vivo overexpression of *Zmiz1* results in newly born neurons that develop mostly round morphologies with extended and abnormally oriented processes, instead of the expected pyramidal morphology (Carapito et al., 2019). These findings suggest a role for ZMIZ1 in cellular morphology and polarization.

Besides migration and morphology, ZMIZ1 also has an established role in the proliferation of multiple cell populations. During T-cell development, for example, ZMIZ1 induces pre-T-cell proliferation by inducing several Notch target genes (Wang et al., 2018). Knocking down ZMIZ1 in leukemic cells puts them at a proliferative disadvantage (Pinnell et al., 2015). Additionally, ZMIZ1 promotes proliferation of

melanocytes by regulating apoptosis, repressing apoptotic factors and enhancing anti-apoptotic expression (Li et al., 2020). ZMIZ1 also enhances androgen receptor expression (Li et al., 2021), and the androgen receptor signaling pathway is thought to facilitate neurogenesis by amplifying the neural progenitor cell pool in the developing cortex (Kelava et al., 2022; La Rosa et al., 2021). Since *Zmiz1* expression is enriched in the deep layers of the cortical plate during embryonic development, including during neurogenesis, it is possible that ZMIZ1 could promote the proliferation of cortical progenitor cells. MIZ1, a related protein to ZMIZ1, is thought to promote self-renewal of neural progenitor cells through interactions with transcription factor MYC that control the cell-cycle exit (Kerosuo et al., 2008). ZMIZ1 is known to activate the C-MYC transcription factor in T-cells (Rakowski et al., 2013). Interestingly, C-MYC is thought to be involved in regulating neural progenitor fate in the cerebral cortex, at the stage of neural progenitor proliferation and division (Wang et al., 2020). In the reported experiment, knockdown of C-MYC at E14.5 led to increased generation of upper layer neurons, while over expression at the same timepoint resulted in excessive proliferation of neural progenitor cells (NPC) and a reduction in the overall quantity of cortical neurons (Wang et al., 2020). These studies argue that regulation of MYC activity is critical for NPC proliferation and daughter cell fate. Since this experiment was conducted at E14.5, after most deep-layer neurons are generated, they study cannot report the effect of changes to C-MYC expression levels in the generation of L6 and L5 neurons. Nevertheless, these studies suggest a mechanism by which ZMIZ1 could be involved in regulating NPC proliferation and division, and thus influencing the cellular composition of the cerebral cortex.

Molecular Mechanisms Linked to ZMIZ1 Function

To understand how ZMIZ1 may regulate cellular processes, it is important to review how ZMIZ1 modulates TF function at the molecular level. ZMIZ1 is known to regulate and interact with several key signaling pathways and transcriptional regulation mechanisms: Notch signaling, Smad-signaling, and SUMOylation. These pathways are highly involved in brain development and are often critical in regulating cell fate (Bray, 2006; Hasegawa et al., 2014; Li et al., 2006). However, the mechanistic understanding of how ZMIZ1 regulates TFs remains limited (Li et al., 2006).

Notch signaling is a highly conserved signaling pathway important in mammalian development and homeostasis. In the Notch pathway, the binding of a ligand on the surface of a cell with the Notch receptor of a neighboring cell induces proteolytic cleavage of the Notch receptor (Figure 8A). The resulting fragment, the Notch intracellular domain (Nicd), then enters the nucleus of the cell and interacts with co-activators and co-repressors to either activate or repress target genes (Figure 8A; Bray, 2006). Notch is specifically involved in cellular proliferation, differentiation, and apoptosis (Bray, 2006). Many Notch receptors are transmembrane receptors, meaning that Notch signaling is often limited to neighboring cells. ZMIZ1 selectively amplifies NOTCH1, as compared to other Notch proteins, and these two proteins recruit each other to chromatin to regulate transcription (Pinnell et al., 2015). ZMIZ1 is known to regulate NOTCH1 target genes, many of which are involved in regulating neurite out-growth and promoting cell type identity acquisition (Carapito et al., 2019; Pinnell et al., 2015). Notably, NOTCH1 is critical in the identity acquisition of radial glia in the neocortex, which is a significant cell population in midline guidance structures, such as the GW, that

contribute to guiding callosal axons across the midline during embryonic development (Carapito et al., 2019; Donahoo and Richards, 2009).

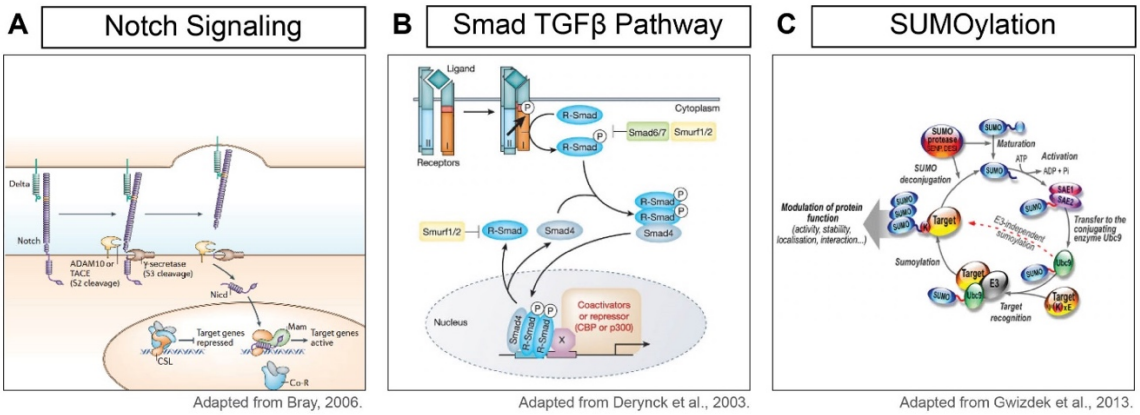

Figure 8: Notch signaling, Smad pathway, and SUMOylation. (A) In Notch signaling, the Delta ligand of one cell binds to the Notch receptor of another cell. This binding recruits ADAM10 or TACE to catalyze the S2 cleavage and γ-secretase to catalyze the S3 cleavage. These cleavage events release the Notch intracellular domain (Nicd). Nicd enters the nucleus to interact with DNA-binding CSL, which recruits co-activators such as Mastermind (Mam) and other TFs, possibly including ZMIZ1, and, in this case, release the co-repressor (Co-R). Target genes are now active. (B) In the Smad TGFβ pathway, the ligand binds to multiple receptors at the cell surface, triggering phosphorylation of receptor-activated Smad proteins (R-Smads). The R-Smads then form a complex with Smad4. This complex can then enter the nuclease, where it can regulate the transcription of target genes. R-Smads and Smad4 cycle between the nucleus and the cytosol. (C) In SUMOylation, SUMO proteins undergo several modifications and form a complex with their target to modify the function of the protein. After SUMO proteins mature and activate, SUMO proteins are transferred to Ubc9, the conjugating enzyme. Ubc9 recognizes substrate proteins and catalyzes a bond between the SUMO and the target.

ZMIZ1 is also critical in regulating the TGFβ/Smad signaling pathway (Li et al., 2006). Smad proteins are substrates of the TGFβ (transforming growth factor β) receptor, a critical signaling pathway for cell proliferation, differentiation, motility, and apoptosis (Li et al., 2006). Notably, TGFβ is known to promote neuronal cell fate in both cortical and hippocampal progenitors in mice (Vogel et al., 2010). This function of TGFβ is dependent on the presence of SMAD4, which is one of the Smad proteins that ZMIZ1 regulates (Figure 8B; Li et al., 2006; Vogel et al., 2010). Smad proteins can influence gene transcription by forming complexes with co-activators and co-repressors in the

nucleus and regulating promoter specificity (Li et al., 2006). SMAD4 forms a complex with R-SMADs (receptor-activated Smad proteins) to regulate transcription in the nucleus (Figure 8B; Derynck and Zhang, 2003). ZMIZ1 interacts with SMAD3/4 to promote this Smad-mediated transcription. Thus, ZMIZ1 may influence cell proliferation and differentiation by regulating Smad protein activity.

ZMIZ1 also regulates transcription using SUMOylation. Through the SUMOylation process, SUMO (small ubiquitin-like modifier) proteins covalently attach to target proteins and make post-translational modifications to their function (Figure 8C; Hasegawa et al., 2014). This process is critical in spatial and temporal gene expression regulation (Hasegawa et al., 2014). In neurodevelopment, SUMOylation is highly involved in neuronal differentiation, as well as in the control of neuronal morphology and synapse development (Gwizdek et al., 2013; Hasegawa et al., 2014). ZMIZ1 specifically colocalizes with SUMO-1 to regulate TFs through SUMOylation (Carapito et al., 2019; Talamillo et al., 2020). SUMO-1 activity begins by mid-embryonic development and is especially active in the cortical plate around E17.5 in mice, after most neocortical neurons have migrated to their appropriate layer in the neocortex but while they are still acquiring their subtype identities (Hasegawa et al., 2014). Overall SUMO activity, however, peaks at E12 and E15-18 in mice, during the development, migration, and differentiation of cortical neurons and glia (Talamillo et al., 2020). Thus, SUMOylation is common throughout neocortical development. However, it is unknown how the involvement of ZMIZ1 in SUMOylation may influence cortical development.

Objective

There is evidence that ZMIZ1 may influence neocortical pyramidal neuron migration and morphology. These findings are supported by the role of ZMIZ1 in cell proliferation, cell migration, and cellular morphology acquisition in melanocytes and T-cells. However, the role of ZMIZ1 in neocortical development is widely understudied. Understanding how disruptions to ZMIZ1 function may result in cortical miswiring, which is often observed in individuals with intellectual disability and developmental delays (common clinical associations with ZMIZ1 developmental disorders), is critical to elucidating the molecular mechanisms that produce functional neocortical circuits and may provide a neurobiological basis to understand ZMIZ1, and related, neurodevelopmental disorders. The objective of this study is to evaluate the role of ZMIZ1 in the development and wiring of neocortical projection neurons. Specifically, this study will evaluate the role of ZMIZ1 in the laminar organization of the neocortex, the wiring of callosal axons in the CC, and the neuron-glia interactions that underlie CC development.

I. Cortical layer organization and projection neuron migration

Rationale

Overexpression of *Zmiz1* in cortical progenitor cells has been shown to disrupt neocortical projection neuron migration (Carapito et al., 2019); however, the role of ZMIZ1 in neuron migration remains unestablished. To understand how ZMIZ1 may be involved in this process, we aim to evaluate if ZMIZ1 is broadly or cell-type specifically required for projection neuron migration. Since *Zmiz1* is known to be significantly upregulated in CThPN, as compared to other PNs, during early- and mid-embryonic development (Galazo et al., 2016), we hypothesize that the presence of *Zmiz1* in CThPN is required for successful cortical neuron migration and layer positioning during embryonic development.

To evaluate this hypothesis, we compared the effects of a CThPN-specific *Zmiz1* deletion with a pan-cortical *Zmiz1* deletion during embryonic development on the subsequent positioning of projection neurons in the early post-natal neocortex.

Mouse Lines

To evaluate the role of ZMIZ1 in the formation of cortical layers, we performed a loss-of-function study with two separate Cre-driver mouse lines: *Emx1*-Cre and *Syt6*-Cre. Global deletion of *Zmiz1* is embryonic lethal in mice at E10.5 (Beliakoff et al., 2008; Li et al., 2006), which immediately precedes neurogenesis (Anthony et al., 2004). Given this constraint, and a need for cell-type specific analysis of *Zmiz1* in the cortex, we employed Cre/loxP mouse driver lines to conditionally knockout *Zmiz1* in cortical progenitor cells and in CThPN with the *Emx1*-Cre and *Syt6*-Cre mouse lines, respectively (Gorski et al.,

2002; Vaasjo et al., 2022). *Emx1*-Cre mice express Cre recombinase starting at E10.5 in cortical progenitor cells, which give rise to excitatory cortical neurons and glia (Gorski et al., 2002). Thus, in *Emx1*-Cre^{+}: *Zmiz1*$^{floxed/floxed}$ (cKO) mice, cortical progenitor cells will develop without *Zmiz1* starting at E10.5, and excitatory cortical neurons and glia will develop without *Zmiz1*. *Syt6*-Cre mice highly express Cre recombinase in L6 CThPN of frontal and medial cortices starting at E15.5 (Vaasjo et al., 2022), after most CThPN are generated from progenitors but before they have established their subtype identity (Greig et al., 2013). Thus, in *Syt6*-Cre^{+}: *Zmiz1*$^{floxed/floxed}$ mice (CThPN KO), CThPN will develop without *Zmiz1* starting at E15.5.

Results

Cortical Depth

To assess the role of *Zmiz1* in the proliferation and structural organization of the neocortex, we first evaluated the effect of *Zmiz1* deletion on the overall depth of the cortical plate. Preliminary analysis of cortical plate depth in the motor and somatosensory cortices at P3 and P7 suggests that *Zmiz1* is likely required in cortical progenitors for the postnatal development of the neocortex.

The cortical depth was measured from pia to white matter, in parallel with the radial organization of neocortical cells, at consistent regions of the motor and somatosensory cortices across the rostral-caudal axis in the cKO (at P3 and P7) and the CThPN KO (at P3) (Figure 9A). At P3, the cKO reveals no significant differences in cortical depth between the control and the mutant (Figure 9C). While the CThPN KO shows a statistically relevant reduction in cortical depth in the medial section of the motor

cortex (Figure 9B, $p < 0.05$), this measurement is the only significant measurement at P3 and only has a $p < 0.05$. Notably, the cortical depth in the medial and caudal somatosensory cortex of the CThPN KO are also reduced but with a p-value of 0.05 and 0.06, respectively (Figure 9B). More samples will be required to verify these findings and address the variation found among samples of the same genotype. It is possible that loss of *Zmiz1* in CThPN is beginning to influence cortical depth at P3 but does not broadly affect cortical size until later timepoints.

At P7, the *Zmiz1* cKO shows significant reductions in cortical depth in the medial motor cortex ($p < 0.005$) and the medial and caudal somatosensory cortices ($p < 0.05$, Figure 9D). Cortical depth of the CThPN KO at P7 has not yet been analyzed. The reduction of the cortical depth in the cKO mutant at P7 but not at P3 suggests *Zmiz1* may be required in cortical progenitors for later postnatal development and proliferation of cells in the neocortex. To further investigate this hypothesis, cortical depth should be measured at later post-natal stages including P14 and P21. This analysis should also be extended to the CThPN KO to determine if a CThPN-specific disruption of *Zmiz1* is sufficient to disrupt neocortical proliferation and overall cortical size.

Cortical Layers

To infer the effect *Zmiz1* on neuronal migration and neocortical cellular organization, we evaluated the formation of cortical layers in the *Zmiz1* cKO and CThPN KO. Since ZMIZ1 may be specifically involved in CThPN migration and cortical positioning, we first analyzed the appearance, position, and depth of deep cortical layer 5

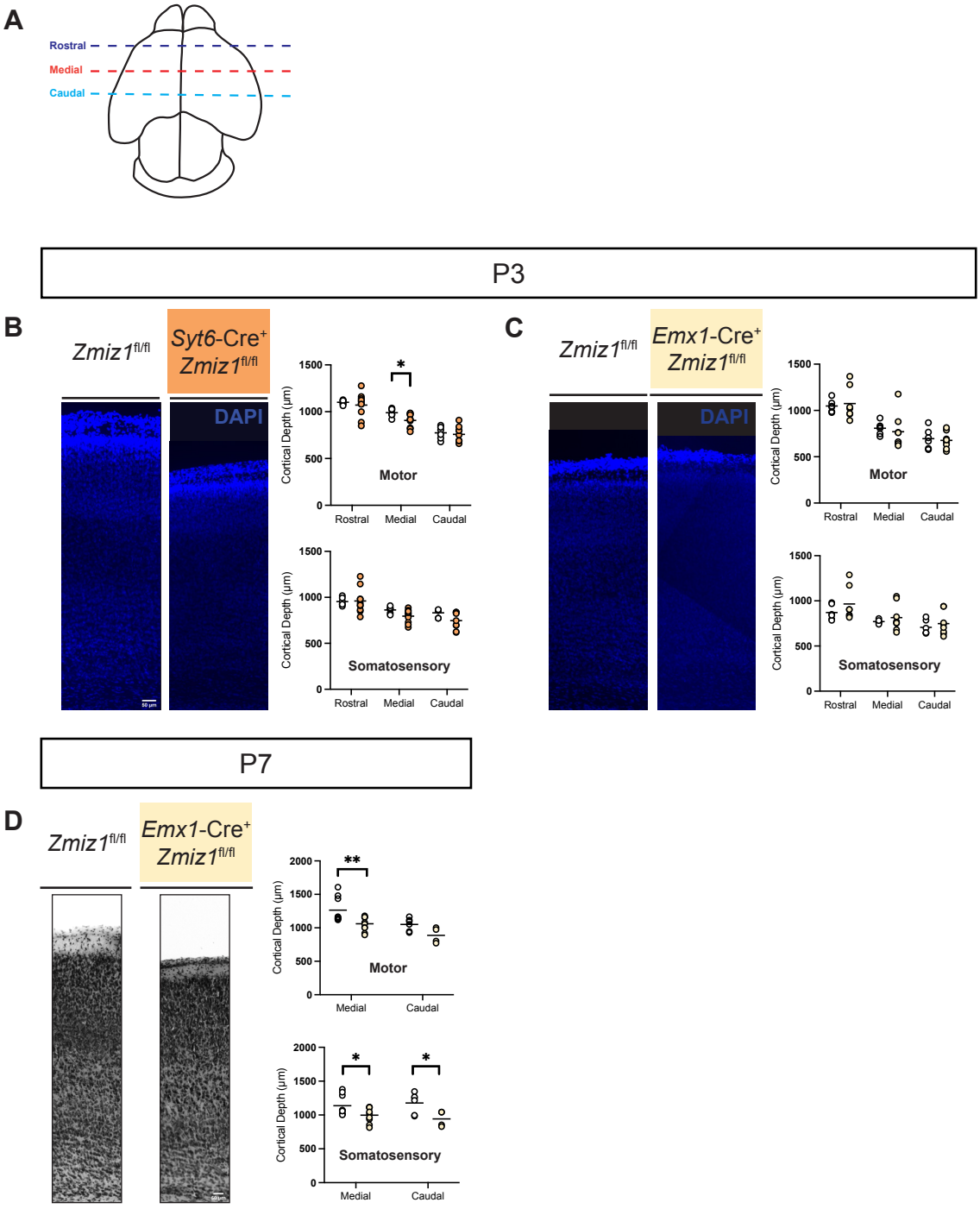

Figure 9: Cortical depth in postnatal mice following conditional knockout of *Zmiz1* with *Syt6*-Cre and *Emx1*-Cre driver mouse lines. (A) Schematic describing general location of "rostral," "medial," and "caudal" sections used in the analysis. (B, C) Cortical depth measurements in the motor and somatosensory cortices at P3 in rostral, medial, and caudal positions in (B) *Syt6*-Cre line and (C) *Emx1*-Cre line. Samples of somatosensory regions used to measure cortical depth with DAPI immunolabeling. Statistical significance calculated using an unpaired two-tailed t-test (* $p < 0.05$, n = 3-5). (D) Cortical depth is significantly reduced in *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ mice at P7 in the medial motor cortex (** $p < 0.01$, n = 3-4) and the medial

and caudal somatosensory cortex (* $p < 0.05$, n = 3-4). Measurements taken from Nissl stained 50 µm coronal sections. Scale bar: 50 µm.

and layer 6, where CThPN reside. To visualize these layers, we immunohistochemically stained P3 brains for Tbr1, a CThPN L6 marker, and Ctip2, a SCPN L5 marker (Grieg et al., 2013; Hevner et al., 2001). We then measured the thickness of these layers in consistent regions of the motor and somatosensory cortices in rostral, medial, and caudal sections (Figure 9A, 10A). These measurements were then normalized to the overall thickness of the cortical plate, resulting in layer to cortical plate ratios that were compared between cKO and CThPN KO (Figure 10B-E).

According to this preliminary analysis, there are no observed significant differences in the relative thickness of L5 and L6 in either the CThPN KO or the cKO (Figure 10B-E). In the *Syt6*-Cre line, the relative depth of L5 and L6 was, on average, higher in the *Zmiz1* KO. However, most of these differences were not statistically significant. The only significant increase was observed in the depth of L6 in the medial motor cortex in *Syt6*-Cre^{+}; $Zmiz1^{fl/fl}$ mice ($p < 0.05$; Figure 10D). Since this result is the only significant measurement in this analysis, this result is not likely indicative of major disruptions to deep layer size in the absence of *Zmiz1* in CThPN at P3. In the *Emx1*-Cre line, there were no significant differences in any of the measurements. The average relative size of L5 and L6 in the *Zmiz1* cKO did tend to be higher in all sections in the motor and somatosensory regions. This trend is the opposite of the *Syt6*-Cre line, in which the *Zmiz1* KO tended to have lower average L5 and L6 depths than the control. While this seemingly opposite effect of the *Zmiz1* deletion in cortical progenitors and CThPN could potentially reveal cell-type specific influences of *Zmiz1*, these observations are not significant and may be a result of expected population variance and a small

sample size. It is important to note that overall cortical depth was observed to be significantly decreased in *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ mice at P7 but not at P3 (Figure 9C, D). If loss of *Zmiz1* in either CThPN or cortical progenitors affects the overall cortical plate size at P7, and not at P3, this cortical layer analysis should be conducted at P7 to determine if *Zmiz1* is indeed dispensable for L5 and L6 layer depth and positioning or if *Zmiz1* acts more postnatally to affect the development of these deep cortical layers.

Discussion

According to this preliminary analysis, loss of *Zmiz1* in cortical progenitors or in CThPN does not significantly affect the relative size of deep cortical layers or the overall cortical depth of early postnatal (P3) mice. However, there is evidence that loss of *Zmiz1* in cortical progenitors results in decreased overall cortical plate thickness at P7. The significant reduction of the cortical plate at P7, but not P3, suggests that *Zmiz1* may have an important role in postnatal development.

While genetic programs during embryonic development are critical in the migration and differentiation of pyramidal neurons in the neocortex, the final positioning and functional connectivity of these neurons is influenced by postnatal electrical signaling and activity-dependent inputs (Jabadoun, 2017; Oberlaender et al., 2012; Wang et al., 2007). At P3, pyramidal neurons are not yet settled in their "final" position and their fate remains semi-plastic. Early life activity that occurs after P3, new sensory and motor inputs and outputs for instance, can influence cortical circuits which in turn influence neuronal positioning and cortical layer organization (De Marco García et al., 2011; Jabadoun, 2017; Wang et al., 2007). The emergence of activity-dependent signals

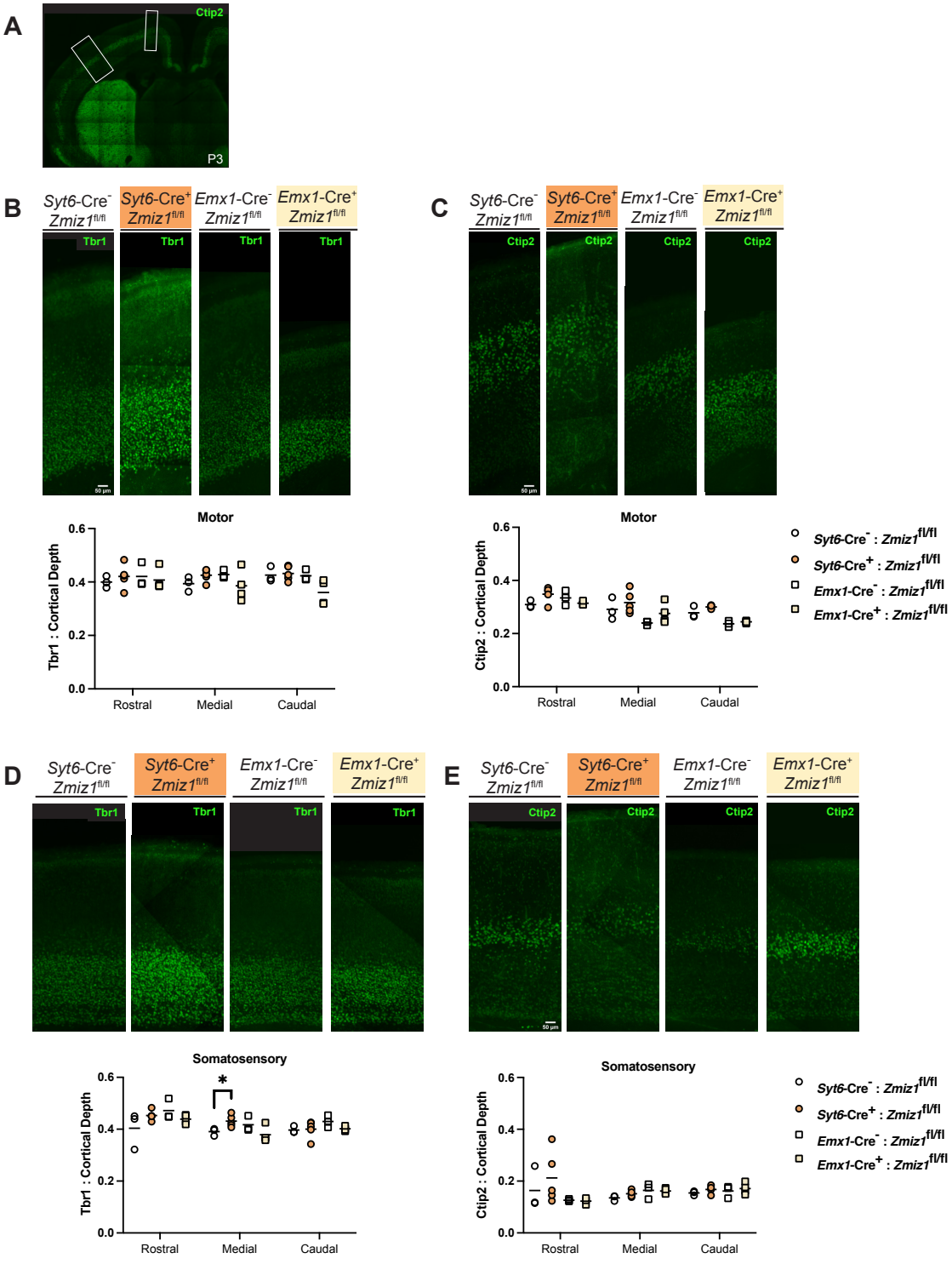

Figure 10: Laminar thickness of deep layers 5 and 6 of the neocortex appears relatively unaffected at early postnatal stages following deletion of *Zmiz1* in cortical progenitors and specifically in CThPN. (A) Medial coronal section of *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ immunostained for Ctip2. Solid boxes indicate the positions of the motor and somatosensory regions for the medial sections analyzed. (B-C) Motor cortex ROIs immunostained with (B) Tbr1 and (C) Ctip2. (D-E)

Somatosensory cortex ROIs immunostained with (D) Tbr1 and (E) Ctip2. (B-E) The depth of the immunostained band was measured and normalized to the overall cortical depth. Data are represented as individual points and the mean. Statistical significance calculated using an unpaired two-tailed t-test (n = 3-5, each point represents the average of 3 repeated measurements). Asterisk indicates statistical significance using a two-tailed unpaired t-test (* $p < 0.05$). Scale bar: 50 μm.

at similar postnatal timepoints as the appearance of significant disruptions to cortical depth and observable cellular organization in *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ mice, P7-P14, raises the possibility that *Zmiz1* could be involved in the modulation of PN in response to extrinsic signaling. Interestingly, the mechanisms by which GABAergic interneurons develop in response to activity-dependent electrical inputs have been found to change after P3 (De Marco García et al., 2011). At P3, neuronal activity begins to regulate the morphological development of interneuron subtypes, instead of laminar positioning. While interneurons develop through semi-distinct mechanisms and originate from different progenitors than excitatory projection neurons, GABAergic interneurons and glutamatergic PN are known to heavily influence each other during postnatal circuit development (Lodato et al., 2011; Miyoshi and Fishell, 2011). These events during critical stages of postnatal development point to the larger theme of the role of developing circuitry in determining PN positioning, connectivity, and function. It is possible that *Zmiz1* could be involved in the transition from intrinsic to extrinsic mechanisms of PN development during these critical postnatal stages.

However, even if this hypothesis were to be true, it is likely that the reductions in cortical size observed at P7 are due to disruptions in cellular proliferation, and not only changes to cellular organization within the neocortex. ZMIZ1 is known to promote the proliferation of T-cells via Notch1 regulation and melanocytes via apoptosis regulation (Li et al., 2020; Wang et al., 2018). Additionally, multiple targets of ZMIZ1, p53 and

TGF beta, are critical regulators of apoptosis (Lee et al., 2007; Li et al., 2006). Thus, it is worthwhile to investigate the role of *Zmiz1* in neuronal proliferation and apoptosis regulation, both embryonically and postnatally. Interestingly, between P3 and P7 in mice, there is known to be a surge in both apoptosis and synchronized glutamate receptor electrical activity (Blanquie et al., 2017), suggesting a relationship between apoptosis and glutamatergic circuit wiring. While this association between *Zmiz1*, apoptosis, cellular proliferation regulation, and circuit wiring is loosely based on literature review, it brings up the likely possibility that *Zmiz1* regulates glutamatergic circuitry development in the postnatal neocortex.

The grossly observed disorganization of cells in neocortical layers of the P7 *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ mice suggest that *Zmiz1* could also be involved in neuritogenesis, which occurs mostly postnatally and is critical in circuit development (Oberlaender et al., 2012). Previous studies have found that in-vitro overexpression of *Zmiz1* in cortical progenitors results in abnormally long cellular processes and disrupts the adoption of pyramidal cell morphology (Carapito et al., 2019). *Zmiz1* has also been linked to melanocyte morphology development (Li et al., 2020), further supporting this potential role in neurons. It is thus worthwhile to investigate the role of *Zmiz1* in neuronal process development in-vitro and in-vivo at various developmental stages. Since significant extension of cellular processes occurs throughout postnatal stages (Gianino et al., 1999; Jabadoun, 2017), the possible role of *Zmiz1* in neuritogenesis may contribute to the postnatal emergence of significant disruptions to the reason that *Zmiz1* may affect neocortical structure more postnatally, rather than strictly embryonically.

In summary, further work is required to determine (1) when *Zmiz1* may be involved in shaping cortical structure, (2) whether ZMIZ1 broadly or cell-type specifically influences cortical layer structure and overall cortical size, (3) the effect of *Zmiz1* in postnatal cell proliferation and/or apoptosis in the neocortex, and (4) how *Zmiz1* may interact with the postnatal environment to affect neocortical circuitry and cellular identity. The morphology of the cortical plate and the laminar structure of cortex must first be described at several developmental timepoints (P7, P14, and P21) to determine if there are significant changes to relative cortical layer size, overall cortical depth, or cellular organization. In addition, it would be worthwhile to quantify overall cellular density, and specifically neuronal cell density, at these same developmental timepoints to determine if cortical size reductions are due to changes in the quantity of cells, the organization of cells, or both. These findings could infer whether *Zmiz1* influences proliferation or apoptosis in the developing neocortex.

These future directions will allow us to elucidate which cell populations require *Zmiz1* for successful migration, cortical layer organization, and proliferation. We can then establish if *Zmiz1* broadly or cell-type specifically influences the structure of the neocortex, and the developmental timeline of these events.

II. Corpus callosum development: callosal projections and midline populations

Introduction

We find that broad cortical deletion of *Zmiz1* during early embryonic development results in corpus callosum (CC) dysgenesis, misrouted callosal fibers, and abnormally positioned midline guidance structures. Given these unexpected observations, we developed the following aims to elucidate possible roles for *Zmiz1* in the development of the CC: (1) examine CC size and morphology across the rostral-caudal axis, (2) visualize callosal wiring lateral to the midline, and (3) investigate the relationship between the abnormal midline guidance structures and the mis-wired callosal axons.

Here, I present a series of experiments to characterize the observed CC dysgenesis in *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ mice. To understand the development and potential causal relationships of this phenotype, we conducted our analysis at several key developmental timepoints: P1, P3, P7, and P14. While most of the results in this chapter are from early post-natal stages (P3 and P7), current research is ongoing to evaluate mid-embryonic stages, specifically E15 when CC fibers first begin to cross the midline and are notably guided by midline neuron and glia populations. Determining when CC and midline guidance structure abnormalities first appear will give critical insight into how *Zmiz1* may influence CC development and how these processes may contribute to the overall wiring of the cerebral cortex.

Results

Loss of Zmiz1 in cortical progenitors disrupts corpus callosum formation and callosal wiring

To investigate the role of *Zmiz1* in CC development, we first characterized callosal fiber crossing across the rostral-caudal axis in cKO mice at P3, P7, and P14. Using cresyl violet staining, we identified partial agenesis of the CC at all three developmental timepoints that varies in severity across the rostral-caudal axis. This phenotype is observed in the homozygous (*Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$) cKO but not in heterozygous (*Emx1*-Cre^{+}; *Zmiz1*$^{fl/wt}$) cKO. In the *Zmiz1*$^{fl/fl}$ cKO, more crossing of callosal axons is observed in rostral than caudal positions (Figure 11A-B). Although the thickness of the CC cannot be accurately determined using Nissl staining, as it does not selectively mark callosal axons, the frequency of crossing appears higher in rostral positions based on observation. The frequency of crossing must be quantified across the rostral-caudal axis to confirm this hypothesis. However, it is consistently observed that the rostral CC extends abnormally ventral into the septum (Figure 11A, D, F; Figure 12J, solid arrows). This observation has been confirmed with L1CAM immunostaining, which labels axons in the central nervous system (Figure 11D; Patzke et al., 2016). To quantify this observation, we determined the angle between the most ventral point of the CC and the vertical orientation of the lateral ventricle at the white matter/ventricle border, as illustrated in Figure 12F. This angle is significantly reduced in *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ mice, suggesting that the observed ventral extension of the CC into the septum is statistically relevant ($p < 0.0001$). At caudal positions, complete agenesis of the CC is observed, in which there is no crossing of CC axons (Figure 11B, C, E). A reduction in the size of the CC and the failure of its fibers to cross the midline is also visible in the horizontal plane. Ventrally, the thickness of the CC (Figure 11J, solid arrow) appears to be reduced, and it is unclear if any of these fibers successfully cross the midline. Tracing

of callosal fibers should be conducted to determine the trajectory of these callosal axons. In the dorsal position, however, there is a clear agenesis of the CC (Figure 11G). These findings suggest that CC agenesis may be area dependent. However, the mechanistic causes of such variations remain unclear.

Partial agenesis of the CC is accompanied by Probst Bundles in the cKO, as referenced in the introduction (Figure 6). Generally, Probst Bundles are the result of disruptions to axon pathfinding and guidance, rather than failure of axon growth or extension (Donahoo and Richards, 2009). Thus, it is likely that these fibers are misguided. These likely mis-wired callosal axons form a "whorl" in the CC dorsolateral to the midline (Figure 11A, E, H; Figure 12D; asterisks). Tracing of these fibers in caudal, horizontal, and sagittal planes will be required to understand the wiring of callosal axons and their relationship to failures in midline crossing.

In addition to the CC, disruptions to the formation of hippocampal commissure (HC) and its fibers are observed. In the horizontal plane, the HC appears to be absent in dorsal sections (Figure 11G, H) and slightly disorganized in ventral positions (Figure 11I, J). When HC fibers fail to cross the midline, a population of cells appear to extend abnormally dorsal and curve around the ipsilateral CC (Figure 11B, C, solid arrows). These cells are likely either granule cells from the hippocampus or the fasciola cinerea (FC), which is closely related to the IG (Sanders et al., 2021). Interestingly, the neuronal population of the IG, which is deformed in the *Zmiz1* cKO (Figure 12B'-E'), is thought to be a subfield of the hippocampal formation (Sanders et al., 2021). This observation may indicate a relationship between the CC and HC in midline crossing; although, this hypothesis must be further studied. In the ventral position, the white matter of the HC

appears more spread out and fails to form a distinct triangular tip towards the CC (Figure 11J). However, this observation needs to be quantified and further investigated across samples. Together, these findings suggest that *Zmiz1* may be involved in the development of multiple commissural fiber tracts and cortical structures, including hippocampal regions.

In summary, the loss of *Zmiz1* in telencephalic progenitors during early embryonic development results in partial agenesis of the corpus callosum (ACC) and mis-wired callosal projections. Partial ACC combined with the abnormal ventral extension of the CC and the observed Probst Bundles suggests that there is severe misguidance of callosal fibers in the absence of *Zmiz1*. It is unknown, however, whether the misrouting of callosal axons is due to intrinsic or extrinsic factors. This question prompts a need to investigate the morphological, genetic, and proteomic qualities of known midline guidance structures to determine if the observed disruptions in the CC are caused by CPN, guidance structures, or a combination of multiple cell populations.

Abnormal midline guidance structures associated with corpus callosum dysgenesis following Zmiz1 deletion in cortical progenitors

The partial ACC observed as a result of the *Zmiz1* deletion in cortical progenitor cells is accompanied by disruptions to midline guidance structure morphology and midline closure. Given the importance of the midline environment in guiding callosal axons, as discussed in the introduction (Figure 5), it is likely that defects in midline cell populations disrupt CC wiring. Defective guidance structures can misroute callosal axons by failing to express required guidance cues, misorienting the signaling gradient, or expressing guidance cues in altered concentrations. However, the *Zmiz1* cKO exhibits

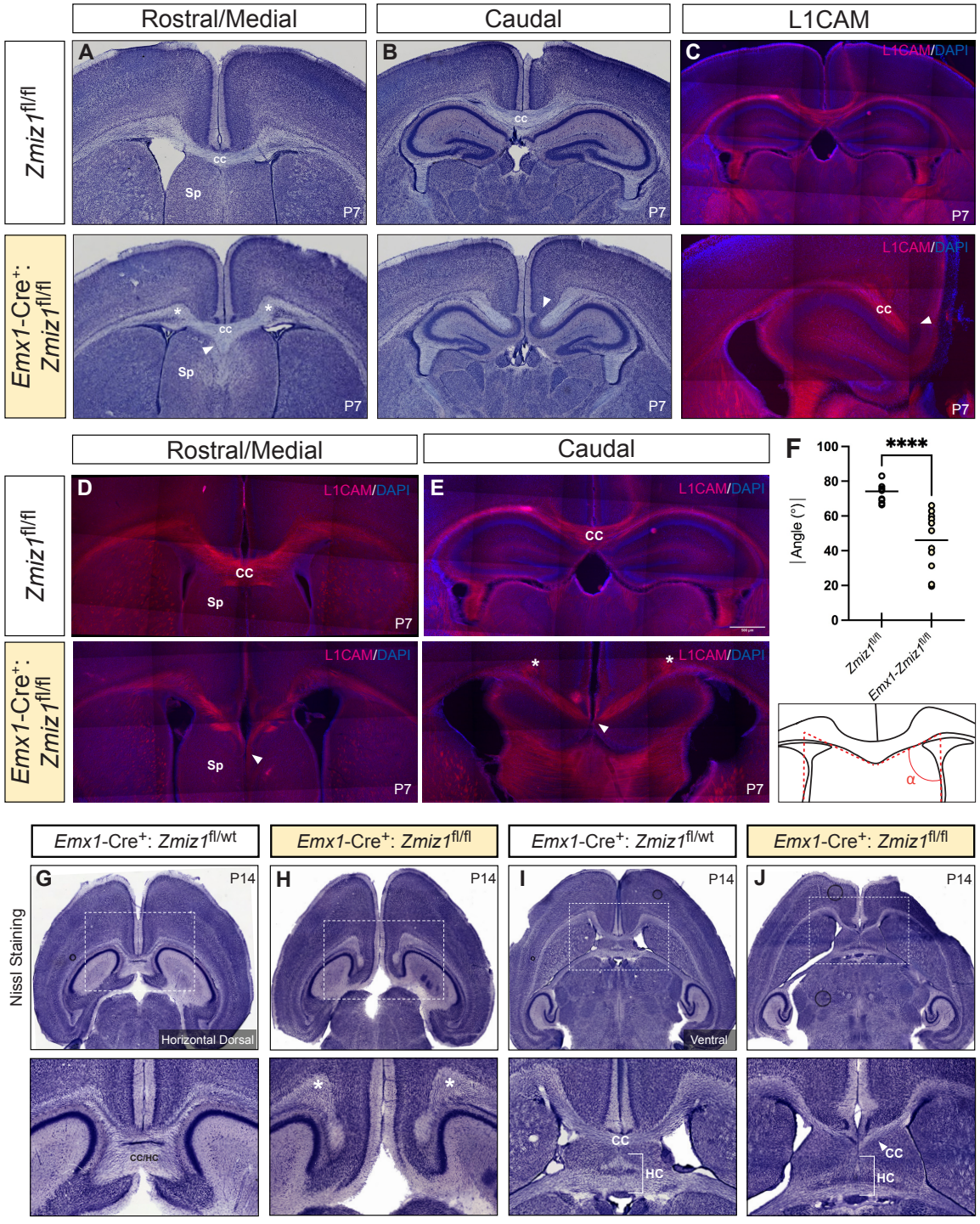

Figure 11: *Zmiz1* is required in cortical progenitors for successful corpus callosum development and callosal wiring. (A-E) P7 Control (*Zmiz1*$^{fl/fl}$) and knockout (*Emx1*-Cre^{+}: *Zmiz1*$^{fl/fl}$) 50 µm coronal sections. (A-B) Nissl staining of P7 sections reveal (A) abnormal CC extension into the septum (solid arrow), Probst Bundles (asterisk), and (B) agenesis of CC. (C, E) Agenesis of CC at caudal positions is confirmed with L1CAM immunohistochemical staining and DAPI counterstain. Caudal CC agenesis is accompanied by an abnormal dorsolateral extension of either the indusium griseum (IG) or fasciola cinerea (FC) (B, C, solid arrow). (D) In some rostral

samples, CC fails to cross the midline and projects abnormally into the septum. (F) The angle (α) of the ventral projection of the CC was quantified as depicted in the schematic in *Zmiz1*$^{fl/fl}$ (control) and *Emx1*-Cre^{+} : *Zmiz1*$^{fl/fl}$ mice. Statistical significance was calculated with an unpaired two-tailed t-test (n = 3-4 with 1-3 serial sections measured per sample). In the knockout (*Emx1*-Cre^{+} : *Zmiz1*$^{fl/fl}$), the measured angle is significantly decreased (**** $p < 0.0001$, t-test), suggesting a significant ventral extension of the CC at P7 as compared with the control. (G-J) Nissl stained 50 μm horizontal sections at P14. The boxed areas are magnified in the images below. (G-H) Agenesis of CC and HC at dorsal positions; accompanied by Probst Bundles (asterisks). (I-J) Partial or complete agenesis of CC (J, arrow) at ventral sections. The HC appears less organized and anatomically distinct in the mutant (J, white bar). Abbreviations: corpus callosum (CC), hippocampal commissure (HC), septum (Sp).

only a partial loss of the CC, which varies in severity among individuals, suggesting that the disruption in CC development is specific to certain cell populations and/or developmental timepoints and does not inhibit the fundamental mechanisms for general callosal crossing. To investigate the changes to the midline environment in the *Zmiz1* cKO, we first examined the morphology of key midline structures.

In the development of the CC, three midline populations are particularly significant: the indusium griseum (IG), the midline zipper glia (MZG), and the glial wedge (GW) (Shu et al., 2003; Figure 5). In the *Zmiz1* cKO, the indusium griseum (IG) appears misoriented and disorganized (Figure 12A-I'). The IG, introduced in the introduction, consists of neuronal and glial populations that guide callosal axons during embryonic development (Sanders et al., 2021; Shu et al., 2003). We first identified the IG with immunohistochemical staining for deep layer neuronal markers Tbr1 and Ctip2. Since these markers, notably Tbr1, are found in mature neurons, their presence in the IG would indicate whether mature neurons inhabit the IG in the cKO (Piper, Moldrich et al., 2009). We found that although these neuronal populations populate the IG, which is expected, the Tbr1+ and Ctip2+ cells in the dorsal IG (dIG) appear to be organized in an inverted formation (Figure 12B'-E'). Notably, the inversion of the glial wedge (GW), a related glial guidance structure, can invert the paths of growing axons, and replacement

of the GW-IG midline region with a piece of cortex causes axons to extend ventrally into the septum (Shu and Richards, 2001). It is thus worthwhile to investigate the potential results of the observed misorientation of the IG on the pathfinding of callosal axons. The population of Tbr1+ cells ventral to the CC (Figure 12C, arrow) may represent a population of the ventral IG (vIG). In the cKO, this population appears strikingly reduced and lateral to the abnormal ventral extension of the CC (Figure 12C', open white arrow). It is possible the reduction in this population is implicated in the failure of the ventral border of the CC. Future studies should also investigate the morphology and orientation of the GW and the midline zipper glia (MZG) to further evaluate the influence of *Zmiz1* in establishing the midline environment.

To further examine the development of the IG, we extended our analysis to several positions on the rostral-caudal axis. At rostral positions in the cKO (Figure 12F', G'), there are general disruptions to the cellular organization of the IG and neighboring populations. While the control exhibits a tight organization of Tbr1+ IG cells in distinct vertical columns, the Tbr1+ cells in the *Zmiz1* cKO IG appear more loosely arranged in a wider column that deviates from the straight line of the control (Figure 12G, G'). In addition, the TdTomato+ fibers lateral to the IG appear less distinct in the cKO (Figure 12G, G', open arrows). This lack of definition could be related to the observation that a population of Tbr1+ cells ventrolateral to the IG is largely absent in the cKO (Figure 12G, G', solid arrows). Since this Tbr1+ population appears to border the IG and the lateral TdTomato+ fibers, it is possible that the Tbr1+ population is required for the formation of this midline organization that appears less distinct in the cKO. However, the

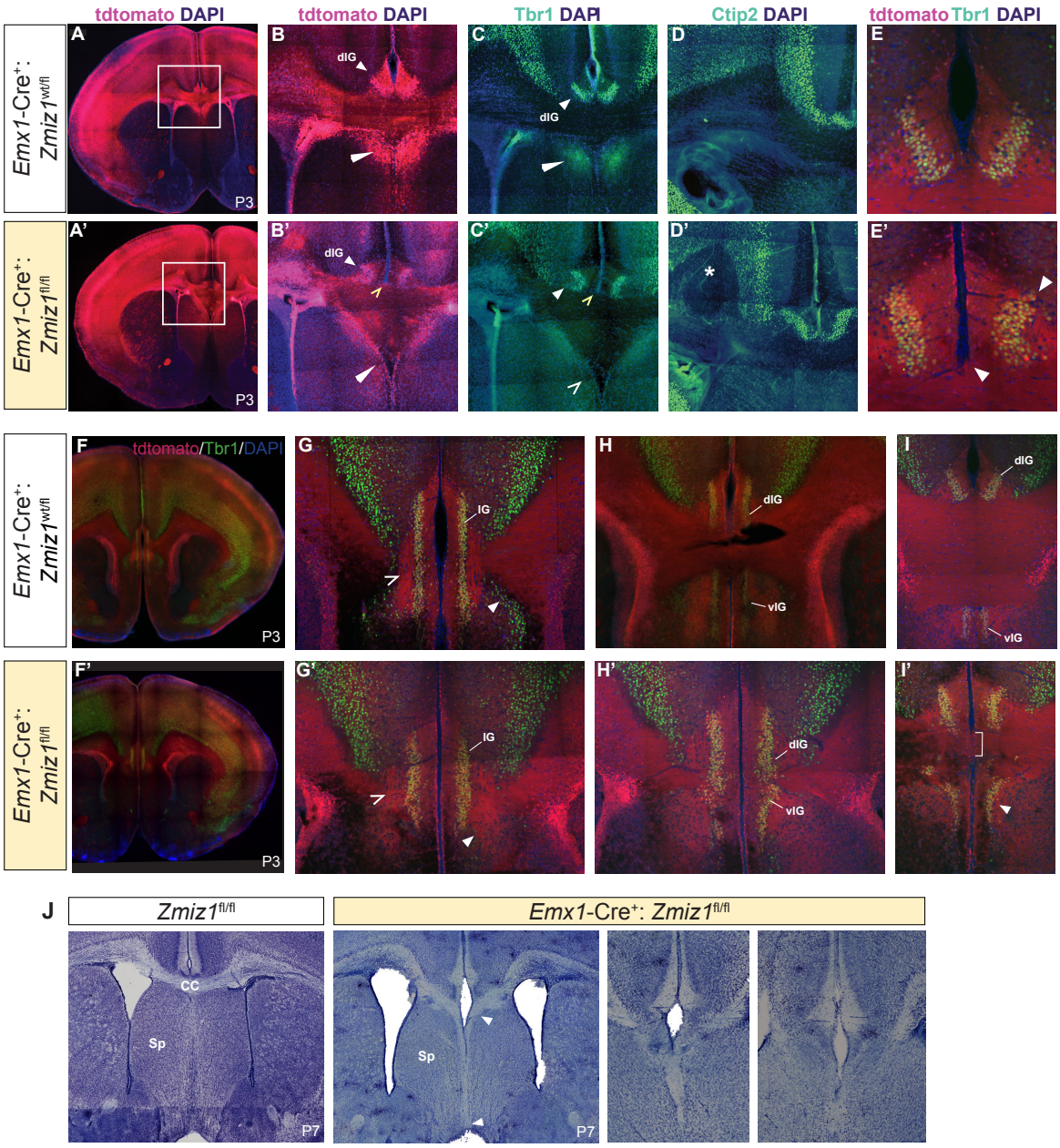

Figure 12: Loss of *Zmiz1* in cortical progenitors is associated with abnormal callosal wiring, midline guidance structures, and midline closure. (A-I') 50 µm coronal sections of *Emx1*-Cre^{+}; *Zmiz1*$^{fl/wt}$ (control, heterozygous knockout) and *Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$ (homozygous knockout) mice at P3. (B-E') are magnified from images (A, A') and similar sections. (A-E') display morphological abnormalities to IG orientation (B'-E', solid white arrows), CC ventral extension into the septum (B', C', bottom arrows), midline closure (B', C', open yellow arrows), and presence of Probst Bundles (D', asterisk) in the mutant. Confocal imaging of Tbr1 immunolabeled sections (F-I') reveal abnormal morphology of the indusium griseum (IG) in rostral sections. In the *Zmiz1*$^{fl/fl}$ mutant, IG cells appear less organized (G', H'), misoriented (I') are mostly absent in ventrolateral positions (G', solid arrow). Tdtomato+ (Cre-reporter) fibers lateral to the IG appear less distinct in their net shape (G', open arrow). Disruptions to the separation of the dorsal IG (dIG) and ventral IG (vIG), as visualized on the coronal plane, are

associated with failure of midline fusion and white matter crossing (H', I'). (J) Severe midline closure defects observed with Nissl staining of P7 mice. Midline defects associated with abnormal ventral extension of the IG into the septum (Sp, solid arrows). Several sections of the same mutant sample are pictured. Abbreviations: corpus callosum (CC), septum (Sp), dorsal indusium griseum (dIG), ventral indusium griseum (vIG).

significance of these differences remains unclear. The disorganization of the IG continues in the caudal direction, notably at the positions where callosal axons begin to cross the midline. When the IG begins to separate on the caudal plane and callosal fibers first cross the midline (Figure 12H, H', I, I'), the ventral IG (vIG) curves away from the midline in the *Zmiz1* cKO and remains curvilinear as CC fibers begin to cross (Figure 12I', solid arrow). In the control, the dIG curves slightly (Figure 12I) but the vIG remains as a well-defined linear column. It is possible that the aberrant separation of the dIG and vIG in the cKO may impede callosal fiber crossing; however, this hypothesis has not been evaluated. A more in-depth analysis of the control, as well as exact section matching will be required to make definitive conclusions about the rostral-caudal transition of the IG and its facilitation of callosal axon crossing. It is important to note that when the CC first appears in the cKO, it is visibly thinner in size than the control (Figure 12I', white bracket). While these two sections are not definitively within 100 μm of each other, the gross anatomy of the sections verify that they are in the same general position on the rostral-caudal axis. To understand how the changes in IG morphology may relate to the observed CC dysgenesis, it would be valuable to visualize the IG in 3 dimensions or through additional planes, such as the sagittal plane. Multi-dimensional visualization would allow for a better understanding of disruptions to IG development on the rostral-caudal and dorsal-ventral axes. It is possible that the varying disruptions to IG

morphology across these axes may relate to the differential severity of CC dysgenesis across the rostral-caudal axis.

Thus far, this analysis has focused on neuronal populations of the midline guidance structures. However, midline glia play a crucial role in guiding callosal axons and establishing the midline environment (Shu et al., 2003). If these populations are also disrupted by the *Zmiz1* deletion, it is likely that they would affect CC development. To determine if *Zmiz1* is involved in the development of these glial populations, we performed a preliminary colocalization analysis to identify cells that may co-express *Zmiz1* and *Gfap*, a molecular marker for mature astrocytes, at P1 (Shu et al., 2003). At this stage, *Gfap* expressing astrocytes do not appear to widely express *Zmiz1* at the rostral midline or the ventral hippocampal commissure (Figure 13B-D'). A colocalization analysis of ZMIZ1 and GFAP at E15 will be necessary to determine if *Zmiz1* is expressed by astrocytes at the time that callosal axons begin to cross the CC. *Zmiz1* has been shown to be expressed in astrocytes, and to bind to MeCP2, which is an established regulator of astrocyte gene expression and is implicated in neurodevelopmental disorders (Yasui et al., 2013). If *Zmiz1* is indeed expressed by astrocytes, these results could elucidate whether ZMIZ1 could be involved in glial-mediated guidance of callosal axon midline crossing.

Cortical midline closure defects associated with Zmiz1 linked CC partial agenesis

Zmiz1 cKO present with cortical midline closure defects and, in severe cases, failure of interhemispheric fusion at the dorsal midline (Figure 12J). While the majority

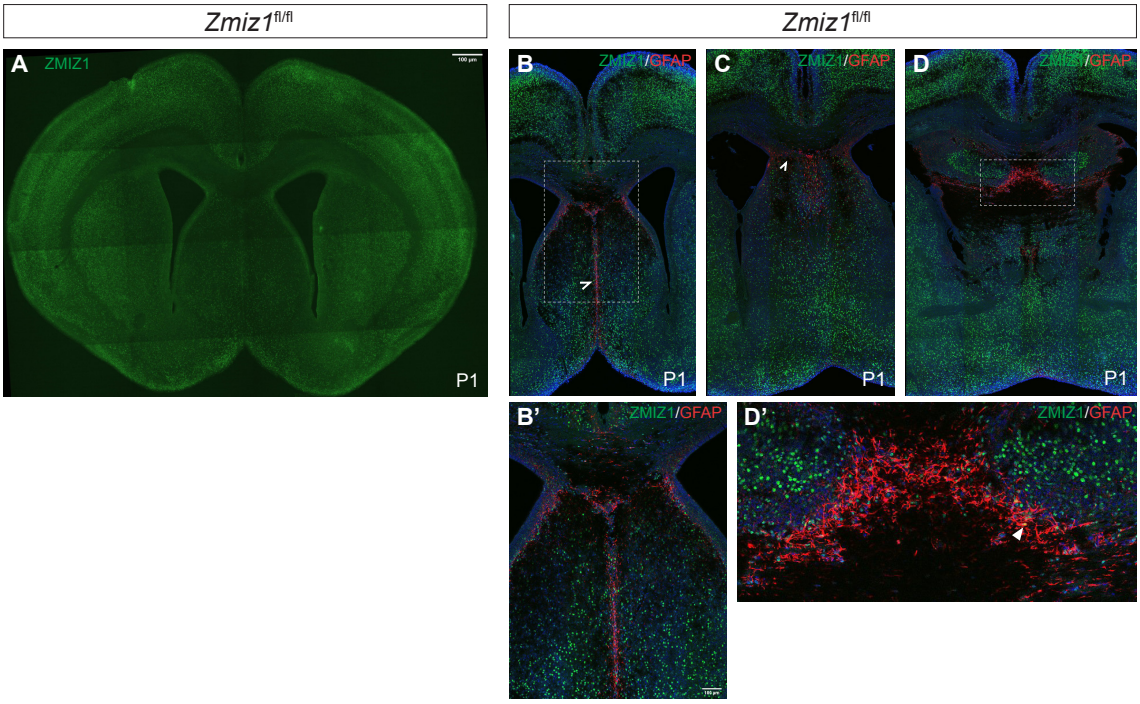

Figure 13: *Zmiz1* and *Gfap* expression at postnatal day one. (A) Immunohistochemical labeling of ZMIZ1 in a rostral section of *Emx1*-Cre^{-}; *Zmiz1*$^{fl/fl}$ (control) at P1. *Zmiz1* expression in both upper and lower cortical layers. (B-D) Immunolabeling of ZMIZ1 and GFAP at P1 in (B, C) rostral and (D) caudal sections. (B, C) GFAP primarily located at the midline ventral to the corpus callosum and on the perimeter of the lateral ventricles. (B) No observed colocalization between ZMIZ1 and GFAP in midline zipper glia (MZG), arrow, at the rostral position. (D) High GFAP levels in (D') ventral hippocampal commissure (vHC). Some cells appear to be colocalized in the vHC (arrow), suggesting *Zmiz1* expressing astrocytes, but overall colocalization appears low. Scale bars: 100 µm.

of samples processed show partial midline closure, some samples collected had completely separated hemispheres, either with thin CC crossing that was damaged in tissue collection or no crossing at all. In the sample with partial closure, the most severe midline closure defects were observed in rostral sections and are closely related to CC agenesis (Figure 12J, Figure 11A, D). In this case, fibers that are expected to form the CC extend towards the dorsal midline but then project ventrally (Figure 12J, Figure 11D), either resulting from or in the failure of the dorsal midline closure. In less severe cases, the midline joins together but fusion of the two hemispheres is visibly reduced as compared to the control (Figure 12I', white bracket). In cases with less severe midline

closure issues, the cellular population bordering the dorsal midline at the point of interhemispheric fusion appears significantly reduced or absent in the *Zmiz1* cKO (Figure 12B', C', yellow open arrows). Further investigation and quantification of the frequency and severity of these defects across samples should be conducted to verify these findings. Nevertheless, these preliminary observations suggest that *Zmiz1* is required in cortical progenitors for successful cortical midline closure.

Discussion

The loss of *Zmiz1* in cortical progenitors results in gross miswiring of callosal projections, abnormal midline guidance structures, and disruptions to midline closure. Many outstanding questions remain regarding the mechanisms behind these disruptions and their effects on cortical function at the molecular, cellular, and circuit scales. Here, I will propose future directions that aim to more intricately characterize the cell-type specific roles of ZMIZ1 in corpus callosum development. If successful, these experiments may provide insight into the intricate relationships between midline populations and the wiring of callosal projections in the cerebral cortex.

Interestingly, loss of *Zmiz1* does not result in complete agenesis of the CC. At many positions in the rostral-caudal axis, many fibers appear to cross the midline, producing an anatomically identifiable CC, while others project aberrantly or fail to even reach the midline. This differential success of callosal fibers of the same position in the rostral-caudal axis to cross the midline suggests that ZMIZ1 has either cell-type specific functions or acts at limited developmental timepoints, or both. Thus, I propose an investigation of the identity differences between the fibers that form the CC, the axons

that project aberrantly within the CC, and the fibers that form Probst Bundles lateral to the midline. Where do these axons originate from and what are their contralateral targets? Do these cells differ in tangential area identity or laminar positioning within the neocortex? Such differences in identity may endow the cells with different axon guidance receptors, expose them to different midline environments based on the timing of crossing, create different subcellular interactions with ZMIZ1, or produce a myriad of other changes in gene expression that could influence axon navigation. To begin this analysis, retrograde and anterograde tracing of the CC and Probst Bundle should be conducted. This tracing could either be accomplished with a retrograde virus in-vivo or with a Dil crystal in fixed tissue. This preliminary analysis should reveal the origin and projection of CC axons and Probst Bundle axons. The laminar and areal identity of these axons could then be hypothesized. Additionally, tracing experiments could evaluate the success of CC axons in reaching their contralateral target. It is possible that callosal axons that successfully cross the midline fail to reach their final target. To infer the role of ZMIZ1 in callosal wiring, it is critical to understand the success of callosal projections in establishing connectivity with their target. While electrophysiological experiments would be necessary to determine the functional connectivity of these cells and circuits, I would prioritize mapping the callosal axons to establish a more comprehensive understanding of how CC connectivity may differ across the brain.

Notably, this study has not controlled for possible sex-differences in the function of ZMIZ1 and the severity of the CC phenotype following *Zmiz1* deletion. Neuroanatomical sex-differences in CC defects in human children with autism spectrum disorder (ASD) have been reported (Nordahl et al., 2015), suggesting that sex-differences

in aberrant CC development are relevant. Future studies should thus take sex into account when evaluating *Zmiz1* function.

Next, we must consider the mechanism by which loss of *Zmiz1* results in misrouted callosal fibers. Based on the gross disruptions to midline guidance structure development and midline closure (Figure 12), I hypothesize that the observed disruptions to callosal axons are at least partially due to aberrant signaling by these guidance structures. To test this hypothesis, I would first determine whether gene expression in the IG, the guidance structure of focus in this thesis, is altered in the *Zmiz1* cKO. If *Zmiz1* is required for the regulation of genes involved with callosal wiring in midline guidance structures, disruptions to the expression of guidance factors may be a cause of CC dysgenesis. A possible experiment could involve isolating the IG through laser capture microdissection (LCM) and then collecting RNA for RNAseq. Differentially expressed genes between the control and the cKO could be identified, and then cross analyzed with a data set of known guidance genes. This procedure could also be used to isolate specific subpopulations of the IG, such as glia, by immunohistochemically staining for the subtype and then using fluorescence activated cell sorting (FACS) to isolate that cell type from the LCM tissue. If repeated for other populations, such as CPN, we could determine (1) if the correct signaling molecules are expressed by the guidance populations and (2) if the crossing axons express the correct receptors to respond to these signals. If the signals and receptors are all expressed at expected levels, it is likely that the orientation and morphological abnormalities of the midline structures or the failure of midline closure mechanisms are primary causes of non-cell autonomous disruptions to callosal projections.

However, to establish a causal relationship between *Zmiz1* expression in midline structures and callosal wiring, a conditional knockout of *Zmiz1* in those populations is required. The *Gfap*-Cre driver line could be employed to knockout *Zmiz1* specifically in mature astrocytes (Chow et al., 2008). If *Zmiz1* expression in astrocytes is required for CC development, this KO will be sufficient to produce CC defects. These results will allow for a better understanding of how the observed disruptions in midline glia, which play an important role in callosal wiring at the midline (discussed in introduction), relate to CC wiring and ZMIZ1 function.

In summary, *Zmiz1* is required in cortical progenitors for successful development of the CC. The mechanisms by which *Zmiz1* influences callosal axon guidance and CC development remain elusive. However, abnormalities in midline guidance structures and midline closure in *Zmiz1* cKO suggest that the observed miswiring of callosal axons is at least partially due to defective extrinsic signaling. Future directions should aim to elucidate the role of ZMIZ1 in interactions between midline structures and callosal axon guidance, including glia-neuron interactions. These findings may elucidate cell-type specific roles of ZMIZ1 and cortical wiring more broadly.

CONCLUSION

Our findings suggest that *Zmiz1* is critical in the development of neocortical circuits. In this thesis, I present a series of histological examinations of two *Zmiz1* conditional knockout mouse models and identify potential roles for ZMIZ1 in the following developmental processes: neocortical laminar structure, overall cortical size, callosal wiring, midline closure, and midline guidance structure-callosal axon interactions. While CC disruptions appear to occur embryonically and are present by P3, ZMIZ1 may affect cortical structure and size in later postnatal stages, starting by P7. Elucidating the role of ZMIZ1 across critical periods of development in postnatal mice will be necessary to understand the influence of ZMIZ1 on cortical wiring. Perhaps ZMIZ1 is involved in mediating extrinsic influences over PN wiring, as suggested by the disrupted midline guidance structures and callosal miswiring in *Zmiz1* cKO, as well as the evidence that ZMIZ1 is more influential in neocortical structure during postnatal stages in which extrinsic electrical signaling emerges. While these hypotheses require additional experimentation and manipulations of *Zmiz1*, they allude to the importance of researching *Zmiz1* to gain a more intricate understanding of how distinct populations of PN are genetically and environmentally fated to adopt their mature connectivity and function.

MATERIALS AND METHODS

Animals

Mice were housed and sacrificed according to the protocols reported in previous papers of the laboratory of Dr. Maria Galazo (Vaasjo et al., 2022). *Syt6*-Cre mice (Tg(Syt6-cre)KI148Gsat/Mmucd; GENSAT) were obtained from the MMRRC and crossed with *Zmiz1* floxed mice (obtained from Dr. Mark Chiang and Dr. Stryder Meadows). *Emx1*-Cre (stock number 005628 from Jackson Labs) were crossed with *Zmiz1* floxed mice. Animals maintained on a 12-hour light and dark cycle with free access to food and water. Both males and females were used in this study, ranging from postnatal day 1 to postnatal day 14. A total of 25 mice were included in the data in this thesis. In Chapter I, the following mice were used: five P3 (*Syt6*-Cre^{+}; *Zmiz1*$^{fl/fl}$), three P3 (*Syt6*-Cre^{-}; *Zmiz1*$^{fl/fl}$), five P3 and three P7 (*Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$), and three P3 and three P7 (*Emx1*-Cre^{-}; *Zmiz1*$^{fl/fl}$). In addition to these mice, Chapter II also included the following mice: one P14 (*Emx1*-Cre^{+}; *Zmiz1*$^{fl/fl}$), and one P3 and P14 (*Emx1*-Cre^{+}; *Zmiz1*$^{wt/fl}$).

Section Preparation

Briefly, mice were anesthetized using isoflurane. Mice were transcardically perfused using cold PBS followed by 4% PFA. Brains were removed and post-fixed overnight at 4°C. The brains were embedded in 4% agarose in PBS and sectioned coronally at 50 µm using a vibratome (Leica VT1000S).

Immunohistochemistry

Sections were washed in PBS and then incubated for 30 minutes to one hour in blocking solution containing 1.5% Triton X-100, 0.025% NaN3, 8% normal goat serum, 0.3%

bovine serum albumin. Then, the sections were incubated in primary antibody and the same blocking solution overnight at 4C. Primary Antibodies used: rat anti-Ctip2 1:250, rabbit anti-Tbr1 1:500, rat anti-L1cam 1:500, chicken anti-Gfap 1:500, rabbit anti-Zmiz1 1:500. Sections were then washed in PBS and incubated in secondary antibody and blocking solution. Secondary antibodies: goat anti-rat Alexa Flour 488, goat anti-rabbit Alexa Flour 488, goat anti-chicken Alexa Flour 555, donkey anti-rabbit Alexa Flour 488 at a concentration of 1:500 for 2 hours at room temperature. Then, sections were rinsed in PBS, mounted on glass slides, and air dried. Mounting media: Fluoromount-G with DAPI.

Antigen Retrieval: For Ctip2 staining, antigen retrieval was performed. Free-floating sections were immersed in citric acid buffer solution and heated in a water bath of 95°C for 5 minutes. The sections were then allowed to cool to room temperature and rinsed with PBS. Subsequent steps were consistent with the standard immunohistochemistry protocol described above.

Cresyl Violet Staining

Sections mounted on gelatin coated Superfrost/Plus slides overnight. The slides were then incubated in a sequence of ethanol solutions for 3 minutes each (70%, 90%, 100%, 100%). Slides were immersed in Xylene for 3 minutes and then rehydrated in sequential ethanol incubations for 3 minutes each (100%, 90%, 70%). Slides were then incubated in Cresyl violet solution containing 0.5% Cresyl violet acetate in distilled water for 2.5 minutes, rinsed with distilled water, and underwent differentiation in a solution

containing 50% ethanol and 0.1% acetic acid. After differentiation, slides were dehydrated in ethanol solutions for 3 minutes each (70%, 90%, 100%, 100%), immersed in xylene for 20 minutes, and cover slipped with Xylene/DPX mounting media.

Image Acquisition

Epifluorescence: Tissue sections were imaged with either the Olympus BX51 or the Leica DMI8 at 10X and 20X magnification. Confocal images were acquired with either Olympus VF30000 or Nikon A1 at 10X or 20X magnification. Image J or Adobe Photoshop were used to pseudo-color monochrome fluorescence images and enhance brightness and contrast.

Brightfield: Tissue sections were imaged with the Olympus BX51 in brightfield at 10X or 20X magnification. Adobe Photoshop was used to enhance brightness and contrast.

Measurements: Distance, Angle

Distance Measurements

Cortical depth measurements in the radial direction were determined using DAPI staining and measured from the white matter border to the pia. Layer thickness was measured from the bottom border of the layer band to the top border of the band, in parallel with the radial organization of the cortex. The Neurolucida “Quick Measure Line” tool was used to measure these distances by calculating the distance between two points. All measurements were repeated three times and averaged to produce the final reported value.

Angle Measurements

Angles were calculated using the Neurolucida "Quick Measure Angle" tool. The angle between three points selected with the cursor was calculated.

Statistical Analyses

Significance between groups was determined using an unpaired two-tailed student's t-test. The sample size used and the degree of significance for each experiment is described in the figure caption of the corresponding figure.

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